SELECTION OF AN APTAMER AGAINST SURFACE EXPOSED TARGETS ON *YERSINIA PESTIS*

By

PRAVINA P FERNANDEZ

Bachelor of Veterinary Medicine and Animal Husbandry

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Thesis Approved:

Dr. Kenneth D. Clinkenbeard

Thesis Adviser

Dr. Rebecca Morton

Dr. Andrew Mort

Dr. Jean Clarke

Dr. A. Gordon Emslie

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Yersinia pestis, a gram-negative bacterium, causes an infectious disease called plague that affects both humans and animals. Plague is transmitted mainly between rodents and fleas, with humans unintentionally infected by coming in contact with an infected rodent or an infected flea. Although, the presence of plague has been documented since biblical times, the most notable event providing the evidence of plague was during the late 17th century when millions of people died due to Plague, also called Black Death. In the US, the last reported epidemic occurred in 1924-25 in Los Angeles, and recent cases have been reported in endemic areas of Colorado, Arizona, California and New Mexico. The possible use of *Y. pestis* to intentionally cause plague through terrorist attacks, has recently prompted the Center for Disease Control to categorize it as a Class 'A' agent.

While most research on *Y. pestis* focuses on understanding its role in disease pathogenesis and vaccine development, the area of plague diagnostics is still in its infancy with the current diagnoses based on symptomatic observations, bacterial cultures, PCR analysis and/or immunoassays. Drawbacks of these methods include slow detection, expensive methods and lack of sensitivity and specificity.

The goal of this study was to utilize aptamer selection to determine the presence of LOS and F1 antigen on the surface of *Y. pestis* and thus provide a novel diagnostic tool for the enhancement of plague diagnosis.

CHAPTER II

REVIEW OF LITERATURE

History of plague

Plague Pandemics

The first recorded outbreak of an epidemic consistent with plague was observed in 430 B.C. claiming an estimated 300 lives. This epidemic was also believed to claim the life of the great statesman Pericles and contributed to the fall and decline of the Grecian civilization (Drancourt et al. 2002). The first plague pandemic, called the Justinian Plague (A.D. 541-544), originated in Ethiopia and spread to the Middle East and the Mediterranean basin and also affected parts of Mediterranean Europe (Drancourt et al. 2002; Perry et al. 1997).

An outbreak of plague originated in A.D. 1340 from an infection in marmots in the steppes of Central Asia that spread to Europe and resulted in the second plague pandemic that was later called Black Death and had an estimated 30-40% mortality rate in the European population (Drancourt et al. 2002; Perry et al. 1997). In England, during the second pandemic, in England, plague epidemics were noted in 2-5 year cycles that resulted in a recurrent reduction of population continuing into the late 17th century (Perry et al. 1997). The second plague pandemic, according to McGovern et al. 1997, started to decline by the early 18th century for three reasons: the failure of the flea vector *Xenopsylla cheopis* (Hinnebusch et al. 1993) to survive in a cooler European climate, the

emergence of a less virulent strain of *Y. pestis* resulting in a natural immunity in rodents and humans, and iron deficiency of some Europeans that interferred with the iron uptake mechanism of *Y. pestis* (Buetler et al. 2001).

The third (and current) plague pandemic started in the Yunnan province of China in 1855 and spread along the coast of southern China into Hong Kong, India, Africa, Europe, Hawaii and North and South America (Perry et al. 1997). However, while still an ongoing problem, the mortality rate and dissemination is much lower than the previous pandemics because of effective public health measures and use of antibiotics after 1950 (Feldmann et al. 2002; Echenberg M. 2002).

Anthropological Studies on Past Plague Epidemics

The use of molecular biology applications makes it possible to detect microbial genomic fragments in ancient human remains and aids in making a fair and accurate diagnosis of ancient diseases (Drancourt et al. 2002).

In 1998, Drancourt et al. detected the presence of *Y. pestis* in 400-year-old human skeletal remains from an excavation site in France. DNA from the dental pulp was extracted and amplified by polymerase chain reaction (PCR) using primers specific for *Y. pestis rpoB* gene encoding for the β-subunit of RNA polymerase, and virulence specific *pla* gene encoding for plasminogen activator (Drancourt et al. 1998). In another study, DNA was extracted from dentine of medieval plague victims at a burial site in France. PCR amplification carried out with two primers specific for the *rpoB* and *pla* gene respectively, and one nonspecific primer for part of the 16s rRNA gene, was unsuccessful. This led the authors to hypothesize that *Y. pestis* was not the cause of Black Death (Gilbert et al. 2004). The DNA extracted by Pusch et al. 2004 from a 17th century

skeletal remain in Germany was analyzed by PCR using primers specific to the *caf1* gene which encodes for the F1 antigen and *Y. pestis* specific amplicons were detected (Chanteau et al. 2000). Dental DNA isolated from two human skeletal remains from the 6th century in Upper Bavaria were analyzed to detect *Y. pestis* specific *pla* fragment showed a positive result, thus confirming evidence of plague afflicting areas of southern Germany during the first pandemic (Wiechmann et al. 2005).

Further studies with remains of rodents and ectoparasites might allow better understanding of the role of these agents in past plague epidemics (Drancourt et al. 2002).

Bacteriologic and Biochemical Properties of *Y. pestis*

The genus *Yersinia* belongs to the family *Enterobacteriaceae* and has 11 species, of which 3 are animal pathogens: *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* (Sulakvelidze et al. 2000). *Yersinia enterocolitica* is frequently isolated from soil, water, animals and a variety of foods and causes a range of intestinal and extraintestinal infections from mild gastroenteritis to mesenteric lymphadenitis and septicemia (Sulakvelidze et al. 2000). Of the 18 serogroups in *Y. enterocolitica*, those that predominate in human illness are O:3, O:8, O:9, O:5, 27 (Weagant et al. 1998; Aleksic et al. 1984).

Yersinia pseudotuberculosis strains isolated from humans and sylvatic animals (Tsubokura et al. 1988) have almost identical biochemical properties with the exception of differences in fermentation of various sugars including mellobiose, raffinose and salicin (Weagant et al. 1998). *Yersinia pseudotuberculosis* infections result in gastroenteritis and a limiting mesenteric lymphadenitis. Most common symptoms include abdominal pain and fever with absence of diarrhea (Nuorti et al. 2004).

Yersinia pestis is a gram-negative, non-sporeforming, non-motile, coccobacillus that exhibits bipolar staining with common hematologic stains (Perry et al. 1997). The organism can grow from 4 - 40ºC with an optimum growth temperature of 28-30ºC and optimum growth pH of 7.2 to 7.6. However, it can survive extremes of pH from 5.0-9.6 (Perry et al. 1997). It has a cell wall typical of enteric bacteria, and its lipopolysaccharide is characterized as rough, with the absence of O-antigen (Vinogradov et al. 2002). It was determined by Brubaker et al. 1991 that *Y. pestis* is an obligate parasite with a nutritional requirement for L-isoleucine, L-valine, L-methionine, L-phenylalanine and glycine at all temperatures, and biotin, pantothenate, glutamic acid and thiamine at 37ºC.

Achtman et al. 1999 established that *Y. pestis* evolved from *Y. pseudotuberculosis* about 1500-20,000 years ago, and subsequent microevolution resulted in the generation of three biotypes of *Y. pestis* differentiated by ability to convert nitrate to nitrite and ferment glycerol. Biotype Antiqua is positive for both characteristics, biotype Orientalis does not ferment glycerol due to a 93 base pair (bp) deletion in the *glpD* gene encoding glycerol-3-phosphate dehydrogenase (Motin et al. 2002) and biotype Mediaevalis does not convert nitrate to nitrite (Perry et al. 1997). Analysis by rRNA ribotyping indicates that a link exists between the ribotypes and the biovars of *Y. pestis* (Guiyoule et al. 1994) with ribotypes F and G found in biovars Antiqua and Orientalis and ribotype O common to biovars Mediavalis and Antiqua.

The first pandemic was thought to be caused by Antiqua ribotype O, whereas a mutant of this strain, Mediavalis ribotype O, caused the second pandemic, and Orientalis ribotype B initiated the third pandemic (Guyiole et al. 1994; Perry et al 1997). Based on

the mutation profile of the three isolated biovars of *Y. pestis,* it was observed that biovars Mediavalis and Orientalis arose from biovar Antiqua (Tong et al. 2005). Zhou et al. 2004 proposed a new biovar called Microtus that is able to ferment glycerol positive and is arabinose and nitrate negative.

Genetic analysis of *Y. pestis*

In 2001, the genome of *Y. pestis* biovar Orientalis strain CO92 was sequenced (Parkhill et al. 2001). The isolate was from a fatal case of human pneumonic plague in Colorado that occurred as a result of exposure to an infected cat (Doll et al. 1994). *Yersinia pestis* CO92 possesses a 4.65 Mb chromosome that is almost identical in size to the 4.60 Mb chromosome of the *Y. pestis* KIM10+ strain biovar Mediavalis sequenced in 2002. Both of these strains possess three plasmids, pMT1 or the pFra that are approximately 96.2 Kb and 100.9 Kb respectively, the 70.3 Kb pYV or the pCD1, and the 9.6 Kb pPST or the pPCP1 respectively (Parkhill et al. 2001; Deng et al. 2002; Wren et al. 2003). The 100 Kb pFra plasmid and the 9.6 Kb pPST plasmid are unique to *Y. pestis* (Brubaker et al. 1991; Sodeinde et al. 1992).

The *Y. pestis* KIM10+ strain shares a 95% sequence homology with the CO92 strain, but it is smaller in size by approximately 50 Kb with fewer insertion sequence (IS) elements and an absence of an integrated prophage that is believed to play a role in phage replication (Parkhill et al. 2001; Deng et al. 2002). More than 90% of the *Y. pestis* KIM10+ open reading frames (ORF) match the ORFs of *Y. pestis* CO92 while the rest of the un-matched ORFs generally encode for hypothetical proteins (Deng et al. 2002). Another difference between the CO92 and the KIM10+ strain of *Y. pestis* is the additional rRNA operon carried on the KIM10+ strain (Deng et al. 2002). An interesting feature of

the CO92 strain is the difference in the guanine-cytosine (GC) bias, with an increase in the guanidine bases on the leading strand of the replication fork (Parkhill et al. 2001; Lobry et al. 1996). It is considered that this bias could either be the result of acquiring DNA through prophages or by inversions or translocations of blocks of DNA (Parkhill et al. 2001). PCR analysis confirmed the presence of the translocation as well as the direction of the orientation of the DNA blocks, with a high proportion of inverse orientations that could have occurred during the evolution of *Y. pestis* (Parkhill et al. 2001). In 2000, Eisen et al. noted certain conserved genes or proteins that were present equidistant from the origin of replication of closely related species and identified an X-shaped structure called X-alignment in a scatter-plot of conserved genes. These were thought to be due to large chromosomal inversions around the origin of replication. Comparing the genome of *Y. pestis* with *E. coli* K-12, Deng et al. 2002 noted that a distance of approximately 400 Kb from the origin of replication appears conserved in both species, with a few inversions between the two replichores. This similarity on the backbone genome of the two species indicates that they might have been separated from a common ancestor about 500 million years before.

Pathogenicity islands are 5-150 Kb regions that lie scattered within the backbone sequence of a genome, but unlike the conserved regions of the backbone that have 'house-keeping' activities, these islands show atypical GC content acquired through lateral transfer and commonly encode for virulence genes (Hacker et al. 1997). A 102 Kb *pgm* locus within *Y. pestis* was identified by Buchrieser et al. 1998 as a pathogenicity island that is involved in uptake of iron and that shares homology to the high pathogenicity island (HPI) of *Y. enterocolitica*. In some cases, a number of genes within

these islands were believed to have been acquired from insect pathogens through horizontal transfer (Parkhill et al. 2001). For example, homologous genes of the insecticidal toxin complexes (tcs) from *Photorhabdus luminescens* that destroy the mid-gut epithelium were identified in *Y. pestis* (Waterfield et al. 2001; Parkhill et al. 2001). *Y. pseudotuberculosis* also possesses many of the genes similar to other insect pathogens and this soil and water dwelling organism might have obtained the genes prior to the divergence of *Y. pestis* (Wren et al. 2003; Hinnebusch et al. 2004).

In addition to the two known systems, the *psa* gene for the pH6 antigen and the *caf* gene for the F1 antigen, 8 additional systems were identified in *Y. pestis* that may be involved in formation of fimbriae and adhesins. Five of these systems are bordered by transposases and integrases that indicate a horizontal acquisition (Parkhill et al. 2001). *Escherichia coli* and *Salmonella typhimurium* also show similar highly redundant genes containing stop codons and frameshift mutations that are related to fimbrial production (Townsend et al. 2001).

Yersinia pestis CO92 genome contains a total of 149 pseudogenes, of which 51 are caused by disruption of the genome by IS elements. IS elements form about 3.7% of the entire genome and are present in much higher number in *Y. pestis* than other bacterial species (Parkhill et al. 2001). In 2004, Song et al. isolated a human avirulent strain of *Y. pestis* 91001 that possessed large chromosomal deletions and pseudogenes, believed to be the cause of avirulence, and an additional 21 Kb plasmid pCRY that contained a cryptic Type IV secretory system. Based on the analysis of the distribution of pseudogenes in *Y. pestis* within 11 natural foci of plague in China, it was noted that within the same focus group, *Y. pestis* isolates showed equal mutational profiles that allowed it to adapt to a

certain lifestyle and thus shape its genome accordingly (Tong et al. 2005; Zhou et al. 2004). Within the pCD1 plasmid that mostly encodes for the Type III Secretory System (TTSS) and the Low Calcium Response (LCR) genes, IS elements were observed that might indicate it acquired these genes from *Y. pseudotuberculosis* and *Y. enterocolitica* (Perry et al. 1998). Pseudogenes to the invasin YadA and lipoprotein YlpA were also observed within the pCD1 plasmid (Hu et al. 1998). About 26% of pseudogenes in *Y. pestis* were related to production of surface associated proteins, with five of those present in the LPS genes (Skurnik et al. 2000; Prior et al. 2001). The pMT plasmid integrates into the chromosome at multiple sites with a high frequency, indicative of homology between the IS100 sequences (Du et al. 1995).

Sequencing the genome of *Y. pestis* has revealed the emergence of a pathogenic species that has changed its genetic makeup by selectively expanding its genome through horizontal transfer of its plasmids and chromosomal genes followed by stages of genome reduction, enabling it to survive in a specialized niche in a flea vector and mammalian hosts with a resultant highly virulent system that is in contrast to its 'non-fatal' ancestral species (Parkhill et al. 2001).

Virulence factors of *Y. pestis*

Fraction 1 (F1) antigen

The 100 Kb plasmid pFra encodes for the capsular F1 antigen and the *Yersinia* murine toxin (YMT) (Du et al. 2002). The pFra varies in size from a smaller 60 Kb to a larger 200 Kb plasmid. Based on studies done on plasmid content in various *Y. pestis* strains around the USSR and several countries in Asia, Africa and South America, some

‘cryptic’ plasmids were found in addition to the three usual plasmids (Filippov et al. 1990; Anisimov et al. 2004).

The pMT1 of *Y. pestis* strain CO92 is observed to be smaller in size as compared to the pFra of *Y. pestis* strain KIM10+ due to the loss of certain IS100 elements; however both plasmids show a greater than 50% homology to the pHCM2 plasmid of *S. enterica* serovar Typhi (Prentice et al. 2001). Within the plasmid pFra, there are 115 putative regions that could code for protein, of which only 7% show a significant (at least 25% amino acids) homology to hypothetical proteins (Lindler et al. 1998). By attempting to cure the pFra plasmid within the *Y. pestis* M231 strain, Protsenko et al. 1991 noted that although the plasmid was lost, it still retained the ability to produce small amounts of F1 antigen phenotypically, indicating that the plasmid was integrated into the chromosome.

Galyov et al. 1990 cloned and sequenced the *Y. pestis caf1* gene noting that it had a putative ribosome binding site 5 bp upstream from the ATG codon and the presence of a signal sequence that resembles a prokaryotic consensus signal sequence. Along with the signal sequence, the *caf1* gene encoded for a 170 amino acid F1 antigen with a molecular weight of 17.6 kDa (Von Heijne et al. 1986). Cleavage of the signal sequence resulted in a secreted F1 antigen of 15.5 kDa that is characterized by the presence of more than 50% β-sheet with the C-terminal region of the protein being highly immunogenic (Galyov et al. 1990). By analyzing the protein mass of F1 antigen by MALDI-TOF, Tito et al. 2001 also observed higher molecular masses of about 114 and 226 kDa that might correlate to 7-mer and 14-mer sub units of an F1 antigen forming a helical structure with each turn containing 7 sub-units. Scanning electron microscopy

established the presence of the capsular F1 antigen on the surface of *Y. pestis* expressed at 37ºC, with large amounts of soluble F1 antigen also released from the bacterial surface into the surrounding medium (Chen et al. 1977).

Three gene products required for the formation of the F1 antigen include a transcription unit called *caf1R* on the *caf* operon that is 1.6 Kb upstream from the *caf1M* gene (Karlyshev et al. 1992). The *caf1R* encodes for a 37 kDa protein (Wu et al. 1991) and it stimulates the transcription of *caf1M* (Karlyshev et al. 1992). By sequencing the genome of the pMT1 plasmid, Lindler et al. 1998 noted a 40 bp region in the *caf1R* operon that showed a high homology to the *afrR* locus in *E. coli*, coding for the AfrR protein that belongs to the AraC family that regulates transcription. The *caf1M* is an ORF that encodes for a protein, 258 amino acid residues long with a molecular weight of about 287 kDa and shares homology with the PapD protein of uropathogenic *E. coli* that functions as a periplasmic transport protein (Lindberg et al. 1989; Galyov et al. 1991). The *caf1A* gene is required for the assembly of capsule (Karlyshev et al. 1992).

Molecular modeling of the F1 antigen revealed that it shares a high homology to the interleukin-1 (IL-1) family of cytokines (Zav'yalov et al. 1995). However, Krakaeur et al. 1998 noted that the F1 antigen neither elicited any IL-1 activity nor stimulated human peripheral blood mononuclear cells (PBMC) to produce other anti inflammatory cytokines like IL-4 and IL-10. Sodhi et al. 2004 revealed that recombinant F1 antigen (rF1) protein of *Y. pestis* induced activation of macrophages *in vitro* as is evident by up regulation of TLR5, increased production of nitrous oxide (NO), TNF-α, IL-1, IFN-γ and IFN-γ dependent chemokines.

Mitogen activated protein kinases (MAPK) are responsible for signal transduction events controlling cell growth, death and differentiation (Robinson et al. 1997). The JNK or stress activated protein kinase; a sub-group of the MAPK is activated by various stimuli including T-cell co-stimulation, stress and cytokines. Sharma et al. 2005 have established that in the presence of rF1, the JNK subgroup of MAPK, is activated by phosphorylation. In the presence of JNK inhibitor SP600125 as well as other pharmacological inhibitors such as wortmannin, genistein and H7, rF1-induced activation of murine macrophages was decreased in a dose dependant manner indicating that the kinase cascade is required for macrophage activation (Sharma et al. 2005). F1 antigen was observed by Du et al. 2002 to block the uptake of *Y. pestis* by phagocytosis, by acting together with the Type III secretory system to inhibit phagocytic adhesion receptor interactions. However, some virulent strains of *Y. pestis* have been isolated such as the Java 9 from Indonesia, that lack *caf* gene encoding for F1 antigen or the pFra plasmid, thus indicating F1 antigen might not have a major role in virulence (Welkos et al. 1995).

The role of F1 antigen as an immunogen has resulted in using this protein in the production of vaccines (Simpson et al. 1990). Sharma et al. 2005 noted that while *Y. pestis* has an overall immunosuppressive effect, the F1 antigen induces the expression of IgG1. Of all *Yersinia* antigens, mice showed highest immune response titres ranging from 1:2000 to 1:250,000 to F1 antigen after an aerosol challenge of *Y. pestis* CO92 and post-challenge treatment with ofloxacin and streptomycin (Benner et al. 1999). IgG1 subclass of immunoglobulin was predominantly noticed in mice injected intramuscularly with a F1 antigen/V-antigen fusion protein that indicates a Th2 immune response stimulated by the release of IL-4 (Williamson et al.1999; Snapper et al. 1988).

F1 antigen is an important antigenic determinant in *Y. pestis* commonly used as a target in diagnostic tests to detect plague because of its stable nature, high concentrations and specificity to *Y. pestis* (MacIntyre et al. 2004). The passive hemagglutination test to detect anti-F1 antibody is used most commonly around the world as a primary diagnostic test (MacIntyre et al. 2004). An inexpensive and sensitive fibre optic biosensor was developed by Anderson et al. 1996 for fluoroimmunoassays that could accurately detect up to 100 ng/ml of F1 antigen. Chanteau et al. 2003 developed a rapid diagnostic dipstick test using F1 antigen specific antibodies could detect up to 500 picograms/mL of F1 antigen and showed high specificity in people suspected with plague in Madagascar.

Murine toxin

In 1966, Kadis et al. observed that the effect of Yersinia murine toxin (YMT) on mammalian cells was mainly on the mitochondria in which it inhibited mitochondrial respiration in the electron transfer chain at the $NADH_2$-coenzyme Q reductase complex. In order to distinguish YMT from total proteins in *Y. pestis*, Montie et al. 1964 noted that metabolic inhibitors like tryptophan analogs were repressed by the production of the YMT in *Y. pestis* cells. High concentrations of heavy metals like silver or mercury ions interact with sulfhydryl groups and tryptophan residues on YMT resulting in its detoxification (Montie et al. 1973). Brown et al. 1977 was critical of this conclusion noting that the toxin concentration of YMT in the mitochondrial respiration experiments was 1000 times above normal mean lethal dose (LD_{50}) and that the concentration of the toxin frequently exceeded the mitochondrial protein used in the study. In contrast, Brown

et al. 1977 observed that the YMT interferred with the β-adrenergic receptor agonists of catecholamines.

YMT from *Y. pestis* strain M23NP was isolated and purified by Seguin et al. 1987 with high pressure liquid chromatography (HPLC) and they observed that two components, YMT A and B had molecular weights of 240 kDa and 120 kDa respectively, and both had an iso-electric point of 3.8. YMT is highly toxic in mice and rats, but non toxic in all other mammals (Brubaker et al. 1991). However, an in-frame deletion of *ymt* in *Y. pestis* KIM did not lower mouse virulence markedly (Hinnebusch et al. 2000).

YMT is expressed at 26ºC in the flea vector and it was observed that in the absence of an active YMT, the ability of *Y. pestis* to block flea proventriculus was reduced (Hinnebusch et al. 2002). The *ymt* gene that encodes for the YMT is believed to have been acquired through several unsuccessful transposition attempts through horizontal transfer. Lindler et al. observed in 1998 that this might be due to the presence of incomplete transposons and ORFs flanking the *ymt* gene, remnants of genes that shared homology to *S. typhimurium*, *Shigella sonnei* and *Pseudomonas syringae* ORFs and homology of DNA sequences to other plasmids.

YMT belongs to the Phospholipase D (PLD) super family (Waite et al. 1999) and possesses two conserved motifs of histidine and lysine (HXKX_4DX_6G (G/S) (Rudolph et al. 1999). The catalysis of YMT results in the synthesis and breakdown of an intermediate phosphoenzyme molecule (Rudolph et al. 1999). The PLD identified in YMT is similar to the human PLD1 and its functions include hydrolysis of glycerophosphatides and ‘transphosphatidylation’ reaction wherein there is a transfer of the phosphatidyl group from phosphatidylcholine to various alcohols, ethanolamine and

serine (Rudolph et al. 1999; Yang et al. 1967). PLD is observed to help *Y. pestis* survive in the flea gut by protecting it from cytotoxic digestion and spheroplast formation by the blood plasma (Hinnebusch et al. 2002).

The evolution of *Y. pestis* from *Y. pseudotuberculosis* denotes a change from an enteric pathogen to one that requires an arthropod vector for transmission, and Hinnebusch et al. 2002 considers that the acquisition of the *ymt* gene might be involved in this process.

Lipopolysaccharide (LPS) of *Y. pestis*

LPS is an integral part of the outer membrane of gram-negative bacteria and is composed of Lipid A that is linked to the core polysaccharide and the O-antigen (Prior et al. 2001). LPS from *Y. pestis* contains the Lipid A bound to the core polysaccharide that is made up of 3-deoxy-D-mannoctulosonic acid (KDO) and sugars like heptose and N-acetylglucosamine, but lacks the repeating sugar subunits of the O-antigen, resulting in a rough phenotype (Prior et al. 2001; Chart et al. 1995; Vinogradov et al. 2002). The absence of a functional O-antigen in *Y. pestis* LPS is due to the presence of multiple mutations within the group of the *ddhB*, *gmd*, *fcl* and *ushA* genes (Skurnik et al. 2000). This inactive gene cluster in *Y. pestis*, lies between the *hemH* and the *gsk* genes, similar in location to the other *Yersinia* species (Prior et al. 2001), with the most homology to the *Y. pseudotuberculosis* serotype O:1b (Skurnik et al. 2000). It was observed by Kukkonen et al. 2004 that the absence of O-antigen in *Y. pestis* enabled a plasminogen-mediated proteolysis, adhesion and invasion and also protected it from recognition by complement and subsequent killing in mammalian serum (Brubaker et al. 2005).The genes that encode

for the Lipid A are scattered around the *Y. pestis* genome while the genes that encode for the O-antigen are located in clusters within the genome (Skurnik et al. 2004).

LPS isolated from *Y. pestis* EV76 was grown at 26ºC, isolated by the hot phenol water method (Westphal et al. 1962) and analyzed by Darveau et al. 1983. It appeared reddish brown when visualized by the silver staining procedure by Tsai and Frasch (Tsai et al. 1982) and appeared grey when isolated from bacteria grown at 37ºC. Knirel et al. 2005 and Prior et al. 2001 noted that LPS isolated from some *Y. pestis* strains grown at 37ºC migrated faster than at 25ºC on a SDS-PAGE gel that may indicate smaller LPS molecules at 37ºC.

Butler et al. 1977 observed LPS from *Y. pestis* to be mitogenic for spleen cells, with a lowered mitogenicity in the presence of polymyxin B. This was thought to be due to either the attraction of the cationic polymixin molecules to the negatively charged phosphate residues in LPS, or due to inhibition by lymphocytes to polymixin B (Butler et al. 1977). LPS of *Y. pestis* and *Y. pseudotuberculosis* seemed more resistant to bacterial cationic peptides as compared to *Y. enterocolitica* LPS (Bengoechea et al. 1998). LPS from *Y. pestis* stimulates TNF-α and IL-6 to a lower level than *E. coli* LPS, thus avoiding the cellular host defense mechanisms (Prior et al. 2001).

Lipid A is a polar molecule consisting of a β-(1,6) linked D-glucosamine disaccharide with about 4-6 saturated fatty acid molecules and two phosphate groups (Skurnik et al. 2004). The hydroxyl groups of the disaccharide are acylated by dodecanoic, hexadecenoic and 3-hydroxytetradecanoic acids (Dalla et al. 1985). Lipid A of *Y. pseudotuberculosis* and *Y. pestis* share a uniform fatty acid composition, with a predominance of 3-hydroxytetradecanoic acid and a low level of dodecanoic acid (Frolov

et al. 1989). A change in the fatty acid composition of Lipid A from 26ºC to 37ºC was observed, with tri-, tetra-, penta-, and hexa-acyl lipid A at 26ºC and tri- and tetra-acyl lipid A at 37ºC (Kawahara et al. 2002). It was speculated by Rebeil et al. 2004 that the increased acylation at 26ºC would enable *Y. pestis* to survive in the flea vector and also in the external environment. Knirel et al. 2005 noted that LPS isolated from *Y. pestis* strains in Eurasia and Madagascar have shown intraspecies variations in oligosaccharide components and acylation of Lipid A at 25ºC and 37ºC. At 6ºC, Knirel et al. 2005, isolated 2 forms of LPS from *Y. pestis* KIM218 strain, LPS 6A and LPS 6B. Lipid A from LPS 6A had 4-6 acyl groups that had 4-amino-4-deoxyarabinose (Ara4N) instead of the phosphate groups on its terminal position with the core polysaccharide possessing a galactose molecule and D-glycero-D-talo-octulosonic acid (Ko). LPS 6B lacked Ara4N and possessed different tetra acylated fatty acids with a lower percent of N-acetylglucosamine and both Ko and KDO phosophorylated with phosphoethanolamine (Knirel et al. 2005).

Additional research needs to be carried out to understand the relationship between LPS genetics and structure as well as differences in biosynthesis of LPS within strains of *Y. pestis* as well as LPS of other pathogenic *Yersinia* (Skurnik et al. 2004).

pH6 antigen

In 1961, Ben-Efraim et al. isolated a pH6 antigen from *Y. pestis* that was synthesized at temperatures and pH similar to the mammalian body and had molecular masses ranging from 15 kDa to 75 kDa at 37ºC with aggregates formed by the 15 kDa sub unit (Lindler et al. 1990). The pH6 antigen is expressed on the surface of *Y. pestis*

when the bacteria are grown between pH 5.0 and 6.7 and between 35ºC and 41°C (Perry et al. 1997). The *psaA* gene is a structural gene that encodes for the psaA protein of the pH6 antigen and electron microscopy analysis showed the presence of flexible 'fibrillar' organelles made up of individual linear strands, several bundles of strands or thin aggregates of pH6 antigen (Lindler et al. 1993). The *psaE* gene is located upstream of the structural *psaA* gene and is responsible for maximal production of the pH6 antigen with a homology of about 44% to MyfA, a major sub unit for the fibrillar structure of *Y. enterocolitica* (Lindler et al. 1990; Iriatre et al. 1994). Mice were injected with wild type and mutant *Y. pestis* by Lindler et al. 1990 by a retro bulbar injection and he noted that the mutant strain of *Y. pestis* lacking the *psaE* gene required a 200 fold greater LD_{50} as compared to the wild type strain.The pH6 antigen has also been reported to bind to several human IgG subclasses by acting as a bacterial Fc receptor in order to prevent detection by other specific immune complexes (Zav'yalov et al. 1996). Though not required for adherence to cells, the main role of the pH6 antigen appears to be antiphagocytic (Huang et al. 2004). Its expression is induced in the presence of and within human macrophages (Lindler et al. 1993; Huang et al. 2004). Makoveichuk et al. 2003 determined that the pH6 antigen interacted with lipoproteins like apoB within blood plasma and also within macrophages and speculated that expression of pH6 antigen could prevent interaction of bacteria with the host cells.

Pesticin

Bacteriocins are toxins released by bacteria that kill closely related species. For example colicins produced by *E. coli* may kill only *E. coli* and other members of the

Enterobacteriaceae family (Braun et al. 1994). Most colicins act by forming pores within the cytoplasmic membrane by ultimately disrupting the transmembrane potential within the cell (Braun et al. 1994; Pilsl et al. 1996). However, a few bacteriocins such as colicin M and pesticin do not form pores in the cell membrane. Colicin M acts by inhibiting the activity of the lipid carriers to take up additional peptidoglycan precursors and thus interferes with peptidoglycan synthesis (Harkness et al. 1989) whereas pesticin, a toxin produced by pPCP1 plasmid of *Y. pestis* has N-acetylglucosaminidase activity and acts by cleaving the β-(1,4) linkage between N-acetyl glucosamine and N-acetyl muramic acid of the peptidoglycan layer (Ferber et al. 1979; Sodiende et al. 1988). Dinitrophenol inhibits the interaction of colicins with cell membranes by interfering with oxidative phosphorylation; however pesticin was not affected by the action of dinitrophenol (Elgat et al. 1969). Pesticin, like colicin M, can also convert sensitive bacterial cells into an osmotically stable spheroplast (Hall et al. 1978).Within the pPCP1 plasmid, the *pst* gene was found to encode pesticin with a molecular weight of 40 kDa, while *pim* encoded the Pesticin Immunity Determinant (PIM) with molecular wt of 16 kDa (Pilsl et al.1996). According to Sodeinde et al. 1988, all bacteriocins and bacteriocin related immunity genes are expressed on plasmids as a protective mechanism to ensure a selective advantage over non-bacteriocin carrying seggregants, analogous to using antibiotics to maintain selective pressure within bacterial cultures. Fetherston et al. 1995 sequenced the pesticin receptor gene (*psn*) in *Y. pestis* and found it to be identical to the FyuA receptor in *Y. enterocolitica*. The pesticin receptor increases the sensitivity of *Y. pestis* to pesticin and the siderophore Yersiniabactin (Ybt), thus enabling pesticin to gain access into the

bacterial cell and hydrolyze murein (Pilsl et al. 1996; Fetherston et al. 1995; Rakin et al. 1994).

Plasminogen activator

The plasminogen activator is a unique surface molecule that provides *Y. pestis* with a combination of proteolytic, adhesive and invasive function (Lahteenmaki et al. 2003). The *pla* gene encodes for two proteins, α-Pla and β-Pla, from a precursor pre-Pla, and it possesses 69% and 59% homologies to the gene *E* of *S. typhimurium* and *ompT* of *E. coli* respectively, whereas the protein homology based on the amino acid alignment was 71% for Pla and Protein E and 47.5% between Pla and OmpT (Sodiende et al. 1989). However, both Protein E and OmpT do not show any plasminogen or coagulase activity (Sodeinde et al. 1989).

Plasmin is a serine protease that functions by cleaving fibrin clots and noncollagenous proteins in the extracellular matrix and basement membrane, and also by activating pro-collagenases, resulting in the dissemination of *Y. pestis* into the internal tissues (Mignatti et al. 1993; Lahteenmaki et al. 1998). The α-2 anti plasmin inactivates plasmin; however, in the presence of the plasminogen activator, plasmin is activated resulting in a brief proteolytic activity that is contained within a small area by adhesion of *Y. pestis* to laminin and heparan sulfate in the basement membrane (Lahteenmaki et al. 1998). Phage display studies carried out by Benedeck et al. 2005 to characterize laminin-binding motifs on Pla showed that amino acid sequence WSLLTPA on Pla interacted with laminin more significantly at 37°C than at 26°C.

The plasminogen activator decreased the LD_{50} of bacteria for mice via a subcutaneous route by a thousand fold indicating that the plasminogen activator is required for the dissemination of bacteria from the peripheral sites of infection (Welkos et al. 1997). It was observed by Sodiende et al. 1992 that the clot-forming ability of *Y. pestis pla* gene product was due to a pro-coagulant, fibrinogen-proteolysis mechanism with the absence of crosslinks normally seen in a fibrin clot (Hinnebusch et al. 1998).

V-antigen

When grown at 37ºC under aerated conditions *Y. pestis* produces the V-antigen (vwa^+), with a molecular weight of 38.5 kDa (Straley et al. 1981). A temperature shift from 26ºC to 37ºC in the presence of low calcium results in a shut down of synthesis of rRNA and tRNA, but a constant synthesis of mRNA, release of V-antigen and other physiological alterations in the bacteria that will ultimately result in apoptosis (Charnetzky et al. 1982). At 37ºC in the presence of exogenous ATP and 2.5 mM Mg^{+2}, *Y. pestis* (vwa^+) grown on a synthetic calciumdeficient media released significant amounts of V-antigen (Zahorchak et al. 1982).

It was determined by Portnoy et al. 1983 that the 70 Kb pVY019/pCD1 plasmid encodes for the V-antigen, and its production is regulated by the Lcr phenotype (Straley et al. 1986). It was shown by Yother et al. 1986 that the pCD1 plasmid contained operons *trtA* and *trtB* that are transcriptionally activated at 37ºC, *trtB* being responsible for the synthesis of the V-antigen. The *lcrF* operon lies adjacent to the *trtB* and acts as a positive regulator, as any mutation within the *lcrF* results in a decreased activity for *trtA* and *trtB* (Yother et al. 1986). Mutational analysis of the *trtB* operon showed it to be influenced mainly by *lcrF* and hence, for easy understanding, *trtB* was renamed *lcrF* (Yother et al.

1986). The *lcrGVH* operon encodes for the V-antigen and is also transcriptionally regulated at 37ºC (Price et al. 1989). The gene *lcrV* that is a part of the *lcrGVH* operon was cloned into *E. coli* and a purified recombinant V-antigen was isolated that is able to stimulate serum antibody and T-cell responses similar to native V-antigen (Leary et al. 1995).

In 1994, Motin et al. created a fusion peptide (PAV) with Staphylococcal P-antigen and V-antigen of *Yersinia* and upon inoculating into mice suppression of TNF-α and IFN-γ was noted. However, this suppression was not limited only to *Yersinia,* and hence it could serve as a generalized anti inflammatory agent (Nakajima et al. 1995). A mutant rLcrV lacking 30 amino acids in position 271-300 was observed to lack the ability to release IL-10 and inhibit cytokines, compared to the wild type (Overheim et al. 2005).

It is thought that LcrV might be important for targeting Yersinia outer proteins (Yops) into eukaryotic cells, by forming a complex with LcrG that acts as a negative regulator for Yops secretion in the absence of LcrV (Nilles et al. 1998; Matson et al. 2001). It was hypothetized by Fields et al. 1999 that the V-antigen will associate with the Ysc proteins involved in Yops secretion at the surface of *Y. pestis,* acting as a link between the eukaryotic cell and the bacterium.

Type III Secretory system and *Y. pestis*

The 70 Kb pCD1 virulence plasmid is present in all three pathogenic *Yersiniae* and encodes for the TTSS and the Low Calcium Virulence response (LCVR) (Portnoy et al. 1984; Geimski et al. 1980; Hu et al. 1998). The pCD1 of *Y. pestis* and *Y. pseudotuberculosis* have a GC content of 46-47% as compared to the pCD1 of *Y. enterocolitica* that has a GC content of 47-48.5% (Brubaker et al. 2005). The TTSS of

pCD1 of the three pathogenic *Yersiniae* secrete a varied assembly of proteins called Yops along with Ysc proteins that are needed for introducing them into eukaryotic cells (Cornelis et al. 2002). Initial studies on Yops in *Y. pseudotuberculosis* showed that they were expressed at 37ºC but were thought to be degraded rather than inserted into the bacterial outer membrane (Bolin et al. 1988). In 1990, Michiels et al. established that Yops were not secreted proteins, but bound to the outer membrane with some Yops being weakly soluble. Most of the *yop* genes, *YopB, YopD, YopE, YopH, YopM,* and *LcrV* appear to be almost identical in *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* (Cornelis et al. 1998). However, Neyt et al. 1997 observed in certain low virulence strains of *Y. enterocolitica,* a transposon Tn2501 encoding for arsenic resistance inserted into the pCD1 plasmid and this was thought to be the only important difference of the pCD1 plasmid among the three pathogenic *Yersiniae* (Cornelis et al. 1998).

Yops can be further divided into two distinct groups of proteins with a few Yops forming effectors that are delivered to eukaryotic cells by extracellular *Yersinia* adhering to the cell surface, whereas other Yops (translocator Yops) form a delivery apparatus (Cornelis et al. 1998). The secretion of Yops takes place by the mechanism called TTSS (Michiels et al. 1991; Cornelis et al. 1998) that has also been developed in other gram-negative bacteria as a multi-protein highly refined system that can effectively disable mammalian cells (Cornelis et al. 2000). In *Y. enterocolitica,* VirA and VirC are involved in Yops transport across the bacterial membrane, whereas VirB has a regulatory function (Michiels et al.1991). VirF, a transcriptional regulator for the entire yop virulon, is sensitive to temperature changes of *Yersiniae*, but is not affected by calcium responses

(Cornelis et al. 1989) and, it encodes for a 30 kDa protein with its carboxy terminal resembling transcriptional regulators of the Arabinose operon (Wallace et al. 1980).

Yersinia pseudotuberculosis produces proteins YopH and YopE that are able to inhibit phagocytosis (Rosqvist et al. 1988). When Bliska et al. 1991, introduced YopH expressed by the *yop2b* gene into eukaryotic cells, it was observed that the cells showed multiple dephosphorylation events that interfered with regulatory and signal transduction pathways of eukaryotic cells. Using catalytically inactive protein tyrosine phosphatases (PTPases) probes, Black et al. 1997 identified focal adhesion kinase p130 (Cas) on human epithelial cells as the main target of YopH. YopE is a GTPase activating protein that belongs to the Rho family of GTPases and mediates a cytotoxic response on a confluent layer of HeLa cells and on mouse macrophages (Rosqvist et al. 1990). This family of GTPases hydrolyzes ATP resulting in the switching-off of the actin polymerization (Viboud et al. 2001) that takes place when YopE is translocated by YopD into the cytoplasm of the target cell (Rosqvist et al. 1994; Cornelis et al. 1998). YopB forms a pore in the target cell membrane of effector proteins and enables translocation of effector Yops (Hakansson et al. 1996) in eukaryotic cells. The *lcrGVH* operon ends with the *yopD* gene and the entire operon encodes for *lcrGVH-yopBD* gene (Francis et al. 1998). This is based on the fact that the rho-independent transcription terminator was found 13 bp downstream of the *yopD* gene with no additional ORFs present. YopD functions as a negative regulator of Yop production and is also translocated into the cytosol of eukaryotic HeLa cells (Francis et al. 1998). The YopB protein has 2 hydrophobic regions in the central portion, whereas YopD has just one transmembrane region indicating that YopsB and D are present within the outer membrane of pathogenic

Yersiniae (Bliska et al. 1991; Hakansson et al. 1993). Via confocal microscopy, Black et al. 1997 also identified integrin receptors that serve as a transmembrane bridge on the cells with invasins on *Y. pestis* and *Y. pseudotuberculosis* (Persson et al. 1997). YadA is an adhesin that is encoded in *Y. pseudotuberculosis* and *Y. enterocolitica*, but in *Y. pestis,* the *yadA* gene has a frameshift mutation, that inactivates the protein production (El Tahir et al. 2001). YopP in *Y. enterocolitica* induces apoptosis in mouse monocyte-macrophage cell line J774A by causing membrane blebbing and nuclear and cytoplasmic shrinkage (Mills et al. 1997). Syc proteins were isolated by Wattiau et al. 1994 and they act as chaperones to YopE, YopH, YopD and YopB. SycH and SycD possess a conserved leucine rich motif in the C-terminal region that might be associated with protein binding (Wattiau et al. 1994). YscB functions as a chaperone to YopN in *Y. pestis* by presenting it in a specific conformation that would enable it to block Yop secretion in the presence of calcium (Jackson et al. 1998). YopJ in *Y. pestis* shows a 94% homology to a similar protein in *Y. enterocolitica*, however, the chaperone to YopJ is encoded by an ORF upstream of the YopJ encoding operon and shares the homology to an operon located upstream of the operon encoding the Avr protein in *P. syringae* (Alfano et al. 1997). Yop J acts by blocking the release of TNF-α in the macrophage and IL-8 in the epithelial and endothelial cells and NF-κB, that is a transcription factor activated at the beginning of inflammation thus inhibiting immune response (Cornelis et al. 1998; Schesser et al. 1998).

Six effector proteins, YopE, YopH, YpkA/YopO, YopM, YopP/YopJ, and YopT, are known to be translocated across the eukaryotic membrane by a directional process (Cornelis et al. 1998). Ysc injectisome is a peptidoglycan spanning protein pump

along with a stiff needle like structure protruding out of the bacterium that serves as a hollow conduit allowing the effector proteins to translocate across the two membranes and peptidoglycan layer in one step (Cornelis et al. 2002). The essential part of the pump is an ATPase that resembles α and β sub units of the F_1F_0 proton translocase (Cornelis et al. 2002). The YscC at the distal portion of the injectisome is a monomer that belongs to the family of secretins (Cornelis et al. 2002) and the YscF with a molecular weight of 6 kDa, polymerizes to form the terminal portion of the needle (Hoiczyk et al. 2001). Based on the fact that YscF is a surface protein in *Y. pestis,* Matson et al. 2005 reported that mice inoculated with YscF intra venously showed a high serum titer after being exposed to wild type *Y. pestis.* The level of protection offered by YscF was believed to be comparable to F1 antigen and V-antigen. YscP acts as a 'molecular ruler' controlling the length of the needle that assists in translocation and in *Y. pestis* it is about 41±8nm (Journet et al. 2003).

Iron transport system and regulation in *Y. pestis*

The Ferric uptake repressor (Fur) protein is a negative transcription regulator that binds to DNA when complexed with ferrous iron or any other divalent metal ion (Neilands et al. 1990). Fur-regulated genes possess operator regions or iron-binding boxes called *fur* boxes that are 21 bp regions that bind to the Fur ferrous complex. However, in the presence of cytoplasmic iron, the repression is relieved, resulting in the formation of apo-Fur (Neilands et al. 1994). The sequence of the *fur* regulatory system in *Y. pestis* shares a significant homology to the 1.9 Kb *E. coli fur* regulatory system (Staggs et al. 1991; Schaffer et al. 1985).

Pathogenic *Yersiniae* possess high molecular weight iron repressible proteins (Irp) on the outer membrane that are induced under low iron conditions at 37ºC in *Y. pestis* (Perry el al. 1979). Pigmentation^{+} isolates are known to possess a siderophore independent mechanism for iron acquisition enabling them to grow in an iron deficient media (Sikkema et al. 1989). These pigmentation^{+} colonies develop the pigmentation phenotype that are brown in color and grow on hemin, whereas pigmentation^{-} colonies are avirulent, unable to absorb pigments and appear white on hemin containing media (Jackson et al. 1959). Pigmentation^{+} strains also possess proteins IrpA-IrpE and a pigmentation specific Peptide F (Sikkema et al 1989). IrpA is expressed by both pigmentation and non pigmentation phenotypes, whereas IrpB-IrpE is expressed by only pigmentation^{+} cells (Sikkema et al. 1989). It was observed that in the presence of iron chelators, the pigmentation^{-} phenotype from *Y. pestis* cannot take up additional hemin at 37ºC once the iron stores within it are utilized (Brubaker et al. 1991). Pigmentation^{-} mutants often arise via spontaneous deletion of the 102 Kb pigmentation (*pgm*) locus, that is known to encode for a 7 Kb hemin storage (*hms*) locus required for the Hms^{+} phenotype and the yersiniabactin (Ybt) iron transport system (Perry et al. 1997).

The Ybt transport system is a siderophore-dependant system in pathogenic *Yersiniae* (Wake et al. 1975; Bearden et al. 1998). A 22 Kb *hms* locus has been identified by Bearden et al. 1997 that encodes for the Ybt in *Y. pestis.* A pigmentation-independent iron transport system, *yfe*, was located on the *Y. pestis* chromosome that required an ATP-binding cassette and a *ybt*, *yfe* double mutant showed a complete loss of virulence in mice after intra-venous inoculations, whereas only the the *yfe* mutant showed a 100-fold loss in virulence via the sub-cutaneous route (Bearden et al. 1997).

Pathogenesis of *Yersinia pestis*

Flea vectors and their role in transmission of plague

Yersinia pestis is cycled from an infected to an uninfected host through the flea vector *X. cheopis* (Hinnebusch et al. 1993) and is termed a dangerous pathogen because of its ability to cause an acute and fatal infection in its mammalian host including humans.

Within a flea, in normal circumstances, the proventriculus that lies anterior to the mid gut is closed, but opens up like a valve during feeding, letting the blood meal into the mid gut, where digestion takes place by liquefaction and hemolysis of the blood meal, followed by defecation (Hinnebusch et al. 2005). After ingesting blood from an infected host, *Y. pestis* accumulates and multiplies in the proventriculus of the flea and partially 'blocks' the proventriculus that causes the flea to regurgitate the infected blood meal into an uninfected host (Hinnebusch et al. 1996; Hinnebusch et al. 2005).

In 1998, Hinnebusch et al. observed that fleas had a shorter lifespan and a decreased tendency to block the proventriculus when maintained at temperatures greater than 30ºC. It was also observed that only pigmentation$^+$ bacteria with an intact *hms* locus were capable of causing blockage in fleas by promoting hydrophobicity in the flea mid-gut by the aggregation of blood cell debris and bacterial mass (Hinnebusch et al. 1996; Titball et al. 2003). During the first week of infection in a flea with pigmentation$^+$ and pigmentation$^-$ mutants, the mid gut appears dense and agglutinated with reddish brown erythrocytic remnants and bacterial aggregates forming a biofilm like matrix (Jarrett et al. 2004). The chromosomal *hmsT* and *hmsP* genes are responsible for biofilm production. HmsT belongs to the family that synthesizes cyclic-di-GMP that acts as an effector of

extra-cellular polysaccharides while HmsP possesses phosphodiesterase activity that hydrolyzes cyclic-di-GMP (Kirillina et al. 2004; Hinnebusch et al. 2005). It is believed that by regulating cyclic-di-GMP production, the *hms* locus will control biofilm formation in *Y. pestis*. After the first week, the pigmentation^{+} mutant spreads to the proventriculus whereas the pigmentation^{-} mutant remains within the mid-gut and is eventually flushed out of the flea (Hinnebusch et al.1996).

A quantitative PCR method was described by Hinnebusch et al. 1998 that estimated approximately 10^5 *Y. pestis* organisms per flea would ensure blockage in a lab colony of *X. cheopis* with an average time of 21 days for the development of infection in fleas after ingestion of an infected blood meal. This assay was considered to be a better indicator of plague detection as compared to previously used mouse inoculation assays (Engelthaler et al.1999).

YMT was observed by Hinnesbusch et al. 2002 to be required for survival of *Y. pestis* in flea mid gut, by protecting it from spheroplast formation and bacterial lysis. By analyzing *Y. pestis* isolates in certain Brazilian strains displaying atypical plasmid content in the flea vector, de Almeida et al. 2003 also observed that certain strains carrying plasmids pFra and pYV were essential for transmission in rodent. Further research to characterize the biochemical role of the PLD and the HMS proteins within the flea vector as well as molecular and genetic changes of *Y. pestis* encountered within the flea need to be carried out (Hinnebusch et al. 2005).

Geographic distribution and epidemiology

Current enzootic plague foci in North America include Southwestern and Pacific coastal regions of the U.S. with other stable enzootic foci also seen in Africa,

Asia, and South America but not in Western Europe (Perry et al. 1997). In Hawaii and Australia, epizootics of plague died out soon after a few human epidemics as it was not able to establish an infection in an enzootic host (Perry et al. 1997).

The epidemiology of plague cases during 1980 was observed by Butler et al. 1989, and they noted that the cases seen in Tanzania and Madagascar appear to contain the most active plague foci (Chanteau et al. 2000). In Madagascar, rats are the main epizootic hosts responsible; the universal flea *X. cheopis* is the indoor vector of plague in urban, as well as in rural zones, whereas the endemic flea *Synopsyllus fonquerniei* is the outdoor and rural vector in Madagascar (Chanteau et al. 2000). The first case of plague reported in the U.S. was in wild rodents in the Berkerly Hills of California in 1908 (Cully et al. 2000).

Disease pathogenesis

In terms of disease pathogenesis, the major difference between *Y. pestis* and *Y. pseudotuberculosis* is the ability of *Y. pestis* to spread by inoculation of a flea from the site of injection (Welkos et al. 1995) and cause virulence in mammalian hosts. Mice exposed to *Y. pestis* strain CO92 by sub cutaneous inoculation and aerosol challenge showed a LD_{50} of 1.9 and $2x10^4$ colony forming units respectively (Welkos et al. 1995).

After inoculation of a vulnerable host by an infected flea vector (Hinnebusch et al. 1997), the bacteria produce an acute lymphadenitis characterized by an inflammation of the lymph node termed as ‘bubos’ packed with bacteria in the extra-cellular portions of the lymph node resulting in peripheral inflammation, hemorrhage and necrosis (Hinnebusch et al. 1997). Inguinal and femoral lymph nodes are most commonly affected resulting in buboes (Butler et al. 1989). White rats and guinea pigs develop a papule at

the site of inoculation, followed by an enlargement of the regional lymph node as an indicator to disease by bubonic plague, in comparison to mice that do not show any bubo formation (Sebbane et al. 2005). When rats were inoculated with approximately 500 *Y. pestis* bacteria intra-dermally, Sebbane et al. 2005 noted that the left inguinal lymph node appeared necrotic and enlarged with an edematous hemorrhagic capsule that resembled an infected human lymph node. They also observed the lymph node that was proximal to the site of inoculation was infected initially, followed by blood, spleen and other tissues. If the bubonic form of plague at this stage is unchecked, it can develop into a septicemic form that is then spread into the liver, lungs and spleen, where they start multiplying and accumulate with about 10^6 cells per gram of tissue (Brubaker et al. 1991).

Pneumonic plague is characterized by cough, chest pain and bloody sputum with a short incubation period of 1 to 3 days and a high morbidity and high fatality rate of 100% (Perry et al. 1997; Ratsitorahina et al. 2000). It was reported by Gani et al. 2004 that humans are potentially at a high risk of contracting pneumonic plague in non-endemic regions by aerosolization of the bacteria or by importing the organism via people traveling from endemic areas or through infected flea vectors. Though the estimated transmission rate of a secondary case of pneumonic plague is about 1.3 cases per case of primary bubonic plague, Gani et al. 2004 observed that with attentive public health measures and antimicrobial therapy, the risk of transmitting this disease is greatly reduced.

Host Susceptibility

Over 200 mammals in 73 genera have been reported with plague and rodents such as mice, rats, gerbils, ground squirrels, marmots and prairie dogs are characterized

as important enzootic hosts (Perry et al. 1997). Guinea pigs with infected flea bites will develop a red papule around the site of inoculation that develops into a fatal septicemia in 2-3 weeks (Perry et al.1997). Ground squirrels carrying *O. montanus* are frequently associated with human plague (Perry et al. 1997). Cats are most susceptible to plague and when Gasper et al. 1993 fed 16 healthy cats with an infected mouse that had died of plague he observed that 38% developed the illness with swelling of lymph nodes similar to humans. Carnivores such as domestic dogs, domestic ferrets, Siberian polecats, black bears, badgers, coyotes, raccoons and skunks are highly resistant to plague (Perry et al.1997).

Antibiotic treatment against plague

In an event of an outbreak of *Y. pestis,* historically tetracycline, streptomycin and chloramphenicol have been established as antibiotics of choice (Perry et al. 1997). However, Smith et al. 1995 tested the sensitivity of 78 *Y. pestis* strains to 14 antimicrobial agents and determined that most of the strains were susceptible to ceftriaxone and ciprofloxacin, while being more resistant to the three traditional drugs of tetracycline, streptomycin and chloramphenicol. Frean et al. 1996 then tested additional antimicrobial drugs in *Y. pestis* strains isolated from 1982-1991 from humans infected with plague in parts of South Africa. He noted that injectable drugs like cefotaxime and oral drugs like levofloxacin and ofloxacin could inhibit *Y. pestis* with their minimum inhibitory concentration less than 0.03-0.06 µg/ml. Byrne et al. 1998 created a streptomycin model and noted that mice treated initially with streptomycin for 2-2.5 days after infection and re-dosed every six hours for 5 days, survived the infection. However, mice treated with streptomycin and re-dosed for just 3 days did not survive, possibly due

to the establishment of pneumonic plague. He also observed that mice treated with antibiotics like streptomycin, ciprofloxacin, ofloxacin and ceftriaxone during early stages of infection showed 100% survival rate. In contrast, mice treated with the same antibiotics only during the later stages of infection had a 50-60% survival rate.

Emergence of drug resistant strains interferes with antibiotic treatment and this has been reported in Madasgascar, where a *Y. pestis* strain 17/95 resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, spectinomycin, sulfonamides, tetracycline and minocycline was detected (Galimand et al. 1997). The resistant gene was carried on a 150 kb plasmid pIPI202, and this plasmid could be transferred from *Y. pestis* 17/95 to *E. coli* K802N at frequencies of $1x10^{-2}$, and re-transferred back to *Y. pestis* with frequency of $1x10^{-4}$ and *E. coli* with a frequency of $5.7x10^{-5}$. In 2001, Guiyole et al. reported the emergence of another *Y. pestis* strain 16/95 from a case of bubonic plague in Madagascar that carried a streptomycin resistance gene in a self-transferable plasmid, 40 kb pIP1023. They also observed that, even though both the plasmids carrying resistance genes in *Y. pestis* were found in the same geographical area but different districts, they were not related in terms of ribotypes (Guiyole et al. 1997), size of plasmid and types of streptomycin resistance. Hinnebusch et al. 2002 noted that the flea mid gut environment seemed an ideal place for *Y. pestis* to acquire resistance genes and speculated that the resistant genes observed in the cases reported in Madagascar could have been transmitted within the flea. He also experimentally observed that in 3 days, the resistant plasmid could be transferred from *E. coli* to *Y. pestis* within the flea mid gut with a frequency of 10^{-3} and that it took only 4 weeks for more than 90% of *Y. pestis* to acquire the antibiotic resistant plasmid.

Detection of *Y. pestis*

Detection methods for *Y. pestis* include isolation and identification by growth on selective media and biochemical assays (Perry et al. 1997), PCR analysis (Norkina et al. 1994, Trebesius et al. 1997, Williams et al. 1984) and antigen detection using Immunogold dipstick method (Chanteau et al. 2000). Laboratory tests carried out to determine the presence of *Y. pestis* are very specific but lack sensitivity especially in endemic areas because of contamination of samples while being transported to the laboratory, antibiotic treatment of patients prior to analysis giving a false negative result, and loss of F1 antigen in the surrounding agar medium (Chanteau et al. 2003).

Anderson et al. 1998 developed a fiber optic biosensor as a sandwich fluoroimmunoassay. In this case, a fluorescently-labeled rabbit anti-plague IgG was bound to a probe with silane and a hetero-bi-functional cross-linker, N-succinimidyl- 4-maleimidobutyrate, and then incubated with F1 antigen for an hour. The decrease in signal meant that F1 antigen is covalently bound to the antibodies on comparing to a standard. The biosensor could effectively differentiate between positive and negative samples.

To determine the presence of F1 positive *Y. pestis,* Albizo et al. 1968 demonstrated a technique of adding plague antiserum to blood agar and noted precipitin around the F1 positive colonies. Soergel et al. 1982 developed a radio-immunoassay and could detect less than 2 ng of F1 antigen in *Y.pestis* in clinical samples using antibody-coated beads and radiolabeled immunoglobulin. Neubauer et al. 2000 developed PCR assays to correctly identifty *Y. pestis* specific genes of 16s rRNA, V antigen, F1 antigen and plasminogen activator to serve as a rapid diagnostic agent by combining all the PCR

cycling parameters into one sample. However, Rahalison et al. 2000 noted that when comparing PCR amplification of *caf1* gene with F1 antigen ELISA assay and growth on culture media of samples that had been isolated from human cases of plague in Madagascar, the test was highly specific (about 96%) but not very sensitive.

In order to detect F1 antigen of *Y. pestis* from urine, bubo aspirate and serum of suspected plague patients, Chanteau et al. 2000 developed an immunogold dipstick method using antibodies specific to F1 antigen. On evaluation of this method, it was found that it had a higher specificity to *Y. pestis* in bubo aspirates than in serum and urine. Chanteau et al. 2003 developed a Rapid Diagnostic Test (RDT) that consisted of a F1 antigen dipstick with two antibodies, an IgG1 and a κ chain isotype that are specific to F1 antigen and interact with two different epitopes on F1 antigen. These two antibodies are conjugated to gold particles and Mab G6-18 that is immobilized on a nitrocellulose membrane. In the presence of up to 0.5 ng/mL F1 antigen, it was observed that two pink bands are visible; the upper band indicating the presence of the control, while the lower band detects the presence of F1 antigen. The RDT showed 100% sensitivity on freshly isolated strains of *Y. pestis,* with 100% specificity. A specificity of 100% was observed on comparing *Y. pestis* containing samples to other Yersinia species as well as other *Enterobacteriae*. However some of the drawbacks included the kit being sensitive to areas of high humidity, difficulty in determining a weakly positive band and misinterpreting of the two bands.

Due to the lack of a standardized ELISA kit to determine F1 antigen, in 2004, Steptloesser et al. evaluated a capture kit similar to an ELISA test to quantify F1 antigen from clinical samples. This kit was able to detect upto 4 ng/mL of F1 antigen with 90%

sensitivity from sera and bubo aspirates rapidly, within 2 hours. However to diagnose pneumonic plague and cases with no bubo formation, first bacterial isolation and identification would need to be carried out, and in the case of F1 negative strains, PCR testing would be necessary.

Aptamer selection

Introduction to Aptamer selection

A method for rapidly selecting ideal binding sequences from a pool of random sequences of oligonucleotides was developed by Tuerk et al. 1990 and termed Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Gp43/T4 DNA polymerase was used as a target and after four rounds the pool complexity, was reduced to two sequences of which one was identical to the operator and the other showed four differences in the randomized domain (Tuerk et al. 1990; Gold et al. 1995). This process differs from the *in vitro* evolution by Kramer et al. 1974 in that, one does not only differentiate or develop mutant species, but also during SELEX any molecule that binds to a target is acquired and amplified (Tuerk et al. 1990). Schneider et al. 1992 tested the accuracy of SELEX in predicting a high affinity binding site using the R17 coat protein of bacteriophage as a target as its interaction with RNA had already been characterized. Using mathematical analysis and computer simulation, Irvine et al. 1991 predicted that some variations of RNA, DNA and peptide molecules had the greatest impact and developed strategies and guidelines for enhanced effectiveness. In order to select for high affinity binders, it was suggested by Irvine et al. 1991 that target concentration should be higher in initial rounds and should decrease in the later rounds in order to improve the stringency of the selection. A rule of thumb that is followed for predetermining the

number of rounds is to consider ten fold enrichment for each round of SELEX, thus ensuring capture of rare high affinity sequences (Gold et al. 1995). Binding affinity and specificity for RNA and DNA aptamers show no difference. However, when 'winning' RNA and DNA oligonucleotides with the same sequence are tested, they do not bind well to the target (Gold et al. 1995). A key step in SELEX is 'partitioning' of oligonucleotides with affinity for target from those without significant affinity (Fitzwater et al. 1996). In a normal solution, aptamers are unstructured however; upon associating with their ligands they fold into molecular architectures wherein the ligand becomes an inherent part of the nucleic acid structure (Hermann et al. 2000).

Aptamers to specific targets

Aptamers to protein kinase C

According to Nishizuka et al. 1992, hydrolysis of inositol phospholipids by phospholipase C results in an improved and lengthened period of inactivation of protein kinase C (PKC) ultimately resulting in cell differentiation and activation. Conrad et al. 1994 isolated RNA aptamers PA6 (generalist) and PA10 (specific) that could select and bind to protein kinase C and thus inhibit and disrupt intracellular protein-protein interactions that determine signal transduction pathways. The aptamers isolated were used to detect the isozyme of PKC in rat brain extract; PA6 could detect both isoforms of PKC, while PA10 could detect only the βII isozyme (Conrad et al. 1996).

Thrombin aptamer

Bock et al. 1992 isolated DNA aptamers to thrombin with binding affinities of 25-200 nM. The tertiary structure of the aptamer is sequence specific and thermodynamically and kinetically stable indicating that the physical characteristics of

the aptamer are conserved on binding to thrombin (Wang et al. 1993). 1 and 2-D NMR spectra showed potassium ion responsible for the stability of the G-quartets in the aptamer (Macaya et al. 1993). By observing the Nuclear Overhauser Effect Spectroscopy, it was noted that the TT loops link the G-quartets on one side, thus spanning the narrow groove, while a TGT loop will cover the wide groove at the other end (Macaya et al. 1993).

Latham et al. 1994 used a modified 5-(1-pentynyl)-2'-deoxyuridine instead of thymidine and created a synthetic pool that could bind to thrombin with a Kd of 400 nM. The site of interaction of DNA aptamers is the exosite of the thrombin, an anion rich region with Lys 21 and Lys 65 present within the binding site and a wide cluster of hydrophobic residues (Paborsky et al. 1993). Defibrotide is a single strand polydeoxyribonucleotide molecule that prevents veno-occlusive diseases due to complications associated with high doses of chemotherapy and stem cell transplantation (Linker et al. 2002). The DNA aptamers for thrombin were compared in their binding efficiency to a defibrotide derived aptamer and it was shown that even though twice the concentration of defibrotide-derived aptamer was required for inhibiting platelet aggregation, it was 10 times less potent. This indicates that the aptamers selected for thrombin can be used as a tool *in vivo* to control thrombin-mediated interactions (Bracht et al. 1994). Li et al. 1994 tested the efficacy of the thrombin aptamer in inhibiting clot bound thrombin and platelet aggregation in an *ex vivo* whole artery angioplasty model by comparing it to a synthetic thrombin inhibitor PPACK and heparin. It was shown that thrombin aptamer significantly reduced the accumulation of fibrinopeptide (FPA) as

compared to heparin and PPACK and also possessed the ability to prevent platelet aggregation in a high shear rate of 64%.

Potyrailo et al. 1998 built the first aptamer-based sensor by attaching the fluorescently-labeled thrombin aptamer to a glass slide by a linker and flowing thrombin on a slide. Binding was detected by evanescent wave induced fluorescence anisotropy with a Kd of 47 nM. Bovine thrombin shares 85% homology to human thrombin as seen with a monoclonal antibody generated against bovine thrombin that also bound to human thrombin (Moriyama et al. 2001). Liu et al. 2003 isolated two RNA aptamers against bovine thrombin that did not interact with human thrombin, with a Kd of 165 nM and 240 nM respectively. These aptamers could inhibit bovine thrombin that catalyzed bovine fibrin clot formation *in vitro* specifically and their secondary structures were characterized by the formation of a typical stem loop with distinctive 'head loops' in the randomized region.

Aptamer binding to Cyanocobalamin

Lorsch et al. 1994 isolated an RNA aptamer specific to cyanocobalamin, wherein binding depended on only high concentration (1M) of Lithium Chloride (LiCl). LiCl was chosen as a buffer, because it is highly cationic and is thought to neutralize the anionic phosphates on the RNA aptamers at higher concentrations. Substitution of LiCl with other salts like NaCl and KCl did not enhance binding. Analysis of the crystal structure of a vitamin B_{12}-RNA aptamer complex reveals that the aptamer creates a large surface binding site that rests with one face of its ligand and thus closely resembling typical protein-ligand complexes. Hydrophobic packing, direct RNA-ligand hydrogen bonding and electrostatic interactions help in binding. Also, bases and riboses in the

vitamin B_{12} aptamer assemble to create a hydrophobic surface with many bumps and pockets that complement the ligand (Sussman et al. 2000).

Aptamers to Adenosine

Adenosine triphosphate (ATP) is an important substrate in many reactions and adenosine is a component of many biological cofactors (Huizenga et al. 1995). Sassanfar et al. 1993 isolated RNA aptamers to ATP that showed Kd of range from 0.7-8 μM depending on salt concentrations. DNA aptamers to ATP were isolated by Huizenga et al. 1995 wherein differences observed were, the RNA version of the DNA aptamer and vice versa did not bind to ATP (Deickmann et al. 1997). RNA aptamer to ATP formed a 11 nucleotide (nt) loop at the ATP binding site, while in the DNA aptamer, the binding site was in a cleft between two stems on top of a G-quartet (Huizenga et al. 1995).

Deickmann et al. 1996 observed by 3-D Nuclear Magnetic Resonance (NMR) the conserved structure of the RNA aptamer to adenosine monophosphate (AMP). The RNA aptamer with its conserved loops and a bulged G formed a novel structure around the AMP; the backbone of the loop nucleotides resembling the Greek letter zeta (ζ). The class II cAMP aptamer was able to discriminate between cAMP and its analogs 5'- and 3'-AMP, that differ only by the hydrolyzed cyclic phosphodiester moiety. Additionally, fragments of cAMP such as the nucleoside moiety (adenosine) and the base moiety (adenine) are also recognized by the aptamer with nearly-equal affinity (Koizumi et al. 2000).

Aptamers to Arginine and Citrulline

L-citrulline and L-arginine differ in two ways. The urea group in L-citrulline and the guanidine group in L-arginine are neutral. The C=O bond in L-citrulline is a

hydrogen bond acceptor, while the C=N-H group in L-arginine is a hydrogen bond donor (Famulok et al. 1994). In 1994, Famulok et al. isolated a binding motif for L-citrulline and then changed this motif for recognition of L-arginine. Using glycine derivatized epoxy agarose as their matrix, wherein the amino acid has the potential to remain in its zwitterion state; they separated bound and unbound RNA molecules by affinity chromatography for seven rounds. The DNA of one of the citrulline binding sequences Cit-16 was mutagenized by 30% and used to select for L-arginine, obtaining an aptamer with a Kd of 56 µM (Famulok et al. 1994).

Burgstaller et al. 1995 carried out structural analysis of the aptamers to L-arginine and L-citrulline and observed that G29-U13 in L-citrulline and G30-C13 at L-arginine are protected in the presence of ligands and any alterations at these positions result in interference with binding to target. In 1996 Geiger et al. went a step further and isolated RNA aptamers that differentiated between enantiomers D and L-arginine by a Kd of 12,000.

Aptamers to Flavin and Nicotinamide

Lauhon et al. 1995 selected RNA aptamers that bind to flavin and nicotinamide cofactors that have RNA components in their structure. The aptamers however could not differentiate between the oxidized and reduced form of riboflavin and required the presence of a monovalent cation like K+. Nicotinamide possesses a proper glycosidic linkage and hence it resembles ribonucleosides. The aptamer clones could also discriminate NAD from NADH by one order of magnitude (Lauhon et al. 1995).

Aptamer selection using L-selectin as a target

Selectins are a family of three proteins, L-, E- and P- selectin that mediate adhesive interactions between leukocytes and endothelium and between leukocytes and platelets. Each selectin molecule has a carbohydrate recognition domain at the amino terminal, an epidermal growth factor-like motif, short consensus repeats similar to complementary regulatory proteins, a transmembrane domain and a short cytoplasmic tail (Rosen et al. 1994).

Hicke et al. 1996 first claimed to have demonstrated *in vivo* use of an aptamer directed against a cell surface receptor by isolating DNA aptamers with high specificity to L-selectin with nanomolar affinity. This binding was dependant on the presence of divalent cation such as calcium. Ringquist et al. 1998 characterized the univalent and bivalent L-selectin aptamer binding to human leukocytes and observed that bivalent (produced by the dimerization of the univalent aptamers) showed an improved affinity and a slower rate of dissociation. Flow cytometry was used in this study to detect the aptamer ligand binding. Romig et al. 1999 using affinity chromatography selected for DNA aptamers for a human -Ig fusion protein target.

Aptamers to aminoglycoside antibiotics

A 109 bp RNA aptamer (J6) to tobramycin aminoglycoside antibiotic was selected by Wang et al. 1996. Binding was determined by constructs labeled with fluorescein and rhodamine. NMR analysis of the tobramycin-aptamer interaction showed that tobramycin binds to the major groove of the RNA aptamer at its stem loop junction (Jiang et al. 1997). Hamasaki et al. 1998 simplified the J6 aptamer with a truncation by 19 nt from the 5’ end to a 39 nt called J6f1, that had terminal GC base pairing. J6f1 could

bind to tobramycin with a Kd of 5.1 nM. However, by surface plasmon resonance it was determined that binding of RNA aptamers to tobramycin showed lack of selectivity leading to the conclusion that a new approach would have to be selected to design RNA targeting drugs (Verhelst et al. 2004).

RNA aptamers that could bind to neomycin and kanamycin B aminoglycoside with a nanomolar affinity were also detected (Jiang et al. 1999, Kwon et al. 2001).

Aptamers to theophylline and caffeine

Jenison et al. 1994 selected 38 nt RNA aptamers that could differentiate between theophylline and caffeine by presence of one methyl group with the RNA aptamer binding affinity to theophylline 10,000 times greater than caffeine. The RNA aptamer forms a binding pocket that locks the theophylline through hydrogen bonds and stacking interactions, in addition to 1-2-3 stacking motifs and a 'base zipper' (Zimmermann et al. 1997), accounting for biased recognition by the RNA aptamer (Hermann et al. 2000).

DNA aptamers to cellobiose

Yang et al. 1998 selected DNA aptamers that bind to cellobiose a disaccharide, that is made up of a simple, uniform repeat of up to 250 β(1,4)- glucose linkages. The dissociation constant was $6x10^{-7}$ M^{-1}. This low Kd value is consistent with other protein binding affinities of carbohydrates wherein the association constants for lectin-carbohydrate interactions vary from 10^3 to 10^5 M^{-1} and association constants of anticarbohydrate antibodies are reported to be 10^2 to 10^5 M^{-1} (Yang et al. 1998, Goldstein et al. 1986, Pavliak et al. 1993). The low affinities of aptamers associated with carbohydrate molecules is believed to be due to inability of the sugar molecules to form

charged interactions with potential aptamer species and the absence of planar ring structures that would favor binding to the nucleic acids (Yang et al. 1998).

Aptamer selection using biotin as a target

Biotin lacks the planar aromatic rings and charged functional groups that are common to most targets binding to aptamers. Additionally it possesses two aliphatic head groups that are present at right angles, making it difficult for biotin to insert itself between the bases of the nucleotides (Wilson et al. 1998). As this unusual structure does not present a problem for a streptavidin-biotin binding, Wilson et al. 1998, created a model of the way aptamer bound to biotin and compared it to the biotin streptavidin binding. A pseudoknot was the most likely structure present to bind to biotin, wherein a single base substitution within the pseudoknot would inhibit binding, while the restoration of the single base helped in reconstituting binding. The dissociation constant observed was lower than the streptavidin biotin binding of 10^{-15} M with a half-life of 120 days (Green et al. 1975). Structural analysis of the aptamer-biotin interaction revealed that at the proximal portion of the fatty acid group of biotin, the aptamer binds less tightly than to its head group (Nix et al. 2000).

Applications of aptamers

A two site binding assay is a common diagnostic format that involves sandwiching the analyte between two ligands, wherein one is used as a capture and the other as a probe (Jayasena et al. 1999). An RNA aptamer to vesicular endothelial growth factor (VEGF) was selected in this manner. This aptamer was labeled with fluorescein at its 5' end and bound to VEGF in a diagnostic format with antifluorescein F_{ab} fragment coupled with alkaline phosphatase as a capture probe (Drolet et al. 1996). Lin et al. 1995

isolated a DNA aptamer to human neutrophil elastase (HNE) that was then labeled with fluorescein molecules and the interaction of the DNA ligand with HNE was detected by flow cytometry (Davis et al. 1996). When aptamers were linked to fluorophores, such as fluorescein and phycoerythrin, they were able to maintain their binding properties and could thus be used in diagnostic applications (Jayasena et al. 1999).

There is a need for rapid detection and analysis of analytes in an emergency medical situation or on a battle field (Jayasena et al. 1999). Biosensors must fulfill this need by meeting the following criteria *(a)* the ability to initiate a binding event without adding additional reagents; *(b)* the ability to detect and quantify the target within the preferred concentration range and the time period; and *(c)* the ability produce the same result repeatedly (Jayasena et al. 1999). Potyrailo et al. 1998 isolated a DNA aptamer specific for human thrombin that was used to detect binding of the target protein by evanescent wave-induced fluorescence anisotropy. Here, the 5' end of the aptamer was labeled with fluorescein and its 3' end was modified with an alkyl amine attached to a glass surface. The advantage of this sensor is that it does not require its target to be labeled and therefore, can be extended to *in vivo* measurements (Jayasena et al. 1999).

Capillary electrophoresis is used as a separation technique in aptamer selection using the conventional capillary format. Highly efficient and reproducible separations take place in an environment whereby molecular interactions may selectively bind to the aptamer without being inhibited by adverse buffer conditions (Heegaard et al. 2003). German et al. 1998 first used capillary electrophoresis as a method to isolate DNA aptamers to IgE. Wiegand et al. 1996 fluorescently labeled the DNA probe and utilized it in an affinity probe capillary electrophoresis format, wherein the free and the complexed

aptamers were detected by well separated peaks (Jayasena et al. 1999). Mendonsa et al. 2004 isolated DNA aptamers to IgE with a dissociation constant of 29 nM after four rounds of selection using capillary electrophoresis as a separation method.

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CHAPTER III

SELECTION OF AN APTAMER AGAINST SURFACE EXPOSED TARGETS ON *YERSINIA PESTIS*

Abstract

Yersinia pestis, the causative agent of plague, is one of the CDC 'category A' agents of priority for biodefense interests. Lack of effective diagnostic tools makes the development of a rapid, highly sensitive, specific and feasible diagnostic approach for identification of *Y. pestis* a high priority. In our study, we have attempted to select aptamers against surface-exposed F1 antigen and rough Lipo-oligosaccharide (LOS) of *Y. pestis* by the process of Systematic Evolution of Ligand by Exponential Enrichment (SELEX). LOS was isolated by the hot-phenol method, identified by SDS-PAGE, purified by size exclusion chromatography and characterized by mass spectrometry and NMR. The Lipid A portion of the LOS was found to be unfavorable for aptamer binding. The core polysaccharide (CPS) of the LPS separated from the Lipid A by mild acid hydrolysis was also unstable for aptamer binding due to its high flexibility and inability to form charged interactions with potential aptamer species. Isolation of F1 antigen was by salt precipitation, purification by chromatography and characterization by Western Blot, SDS-PAGE and mass spectrometry. The purified F1 antigen was incubated with an aptamer pool of 10^{15} oligonucleotides in a 1:10 mole ratio. Bound oligonucleotide-target complexes were separated from unbound species by electrodialysis. PCR amplification of

the bound pool and subsequent strand separation was carried out for 8 rounds of SELEX, the recovered pool cloned and sequenced with 39 clones showing a consensus motif – GTGAG—GTTG--. However, binding assays carried out to determine kinetics of F1 antigen- aptamer interactions were unsuccessful. It is thought that the round 8 pool is not yet simplified to be highly specific for F1 antigen and hence additional four rounds of SELEX are being carried out. The aptamer thus selected might be used in *in vitro* diagnostic assays to detect F1 antigen of *Yersinia pestis*.

Introduction

Yersinia pestis, the etiologic agent of plague is a nonspore forming, nonmotile, gram-negative bacteria that belongs to the class *Enterobacteriaceae* (Perry et al. 1997). In human beings, the bacterium manifests itself as an acute and sometimes fatal infection of which there are three forms (Perry et al. 1997). The bubonic form is transmitted by the flea vector *Xenopsylla cheopis* between infected and uninfected hosts and is characterized by severe lymphadenitis termed bubos mostly on regions of the groin, neck and underarms. The septicemic form is characterized by fever, malaise, chills, tachycardia and hypotension. The pneumonic form causes severe respiratory distress with a high rate of morbidity and mortality (Hinnebusch et al. 1993; Hinnebusch et al. 1997; Hull et al. 1987; Perry et al. 1997). Although rodents are highly susceptible to the bubonic form of plague (Perry et al. 1997), humans are believed to be more susceptible to an aerosol infection resulting in pneumonic plague, especially in non-endemic areas (Gani et al. 2004). This fact highlights the potential use of *Y. pestis* as a bioterrorism agent (Inglesby et al. 2000).

The World Health Organization has predicted that 50 kg of *Y. pestis* released in the atmosphere over a city inhabited by 5 million people might cause 150,000 cases of pneumonic plague and approximately 36,000 deaths (Navas et al. 2002). In such an environment, *Y. pestis* would remain viable for about one hour, and attempts to flee the affected regions by infected individuals would only result in spreading the infection over a larger area (Riedel et al. 2005).

Diagnostic assays to detect the presence of *Y. pestis* in clinical samples include staining, isolation and identification using selective media and biochemical assays (Perry et al. 1997), enzyme immunoassays (Williams et al. 1986) and PCR analysis (Norkina et al. 1994; Tsukano et al. 1996). However, most of these assays are time-consuming, lack sensitivity when tested in endemic areas due to contamination, and after a shortage of resources to set up enzyme or PCR assays (Chanteau et al. 2003). Recent research in *Y. pestis* diagnostics in biological samples include development of a Rapid Diagnostic Test (RDT) (Chanteau et al. 2003), a partial dipstick method using IgG antibodies specific for F1 antigen (Thullier et al. 2003) and a capture kit identical to an ELISA assay (Splettsloesser et al. 2004). Although these tests show reasonable sensitivity and specificity, problems encountered were inability to detect *Y. pestis* in cases of pneumonic plague or cases that lacked bubo aspirates, low range of detection and failure of early detection of *Y. pestis* infection in clinical samples (Splettstoesser et al. 2004).

The outer membrane of most rram-negative bacteria contains structures that are believed to play a role in adherence, invasion and evasion of cell defense systems (Abath et al. 1992). In *Y. pestis*, outer membrane structures include the plasmid encoded Yersinia outer proteins (Yops), plasminogen activator, F1 antigen, V-antigen and the

chromosomally encoded LPS (Abath et al. 1991). The properties of these structures along with their roles in virulence are discussed in Table 1.

In order to select a biological target that will be ideal for aptamer binding, three major criteria need to be considered. The target should be expressed on the surface of the microorganism; it should be present in high copy numbers and should be very specific to the particular organism of interest. We have considered F1 antigen and LPS of *Y. pestis* as ideal targets over other surface expressed structures based on the reasons given below:

a) Specificity of target to the organism of interest - LPS in *Y. pestis* is of the rough phenotype lacking the repeating sugar unit of the O-antigen and termed henceforth as lipooligosaccharide (LOS) in our paper. This lack of O-antigen differentiates *Y. pestis* LOS from its two closest members in the *Yersiniae* family, *Y. pseudotuberculosis* and *Y. enterocolitica* (Prior et al. 2001; Skurnik et al. 2003). While the lipid A moiety of all pathogenic *Yersiniae* are almost identical to other Enterobactericeae, the core polysaccharide analysis shows the carbohydrate backbone of *Y. pestis* and *Y. enterocolitica* is somewhat different (Aussel et al. 2000; Bruneteau et al. 2003). F1 antigen is a capsular protein encoded by the *caf1* structural gene within *caf* operon in 100 kDa pFra plasmid of *Y. pestis* (Perry et al. 1997). The pFra plasmid shows common ancestry with pHCM2 plasmid of *Salmonella enterica* serovar Typhi (Prentice et al. 2001). BLAST searches of the four *caf* genes responsible for F1 antigen production and expression show homology to several known, unknown and hypothetical proteins in *E. coli* and *S. typhi* (Hu et al. 1998). However, to date, an absolute sequence homology has not been established, indicating F1 antigen to be a unique protein specific to *Y. pestis*.

b) Surface exposure of target - The Lipid A moiety of LPS of most gram-negative bacteria is embedded within the outer layer of the outer membrane, while the core polysaccharide and O-antigen are expressed on the surface (Raetz et al. 2002). The structural feature holds true for *Y. pestis* LOS as well, with the exception of absence of O-antigen. F1 antigen is a capsular protein expressed on the surface of *Y. pestis* at 37°C (Du et al. 2001). Scanning electron microscopy revealed that a granular layer of F1 antigen is not only expressed on the surface of *Y. pestis* but also in the supernatant, when grown in broth (Chen et al. 1977).

c) High copy number of target for aptamer binding - In order to recognize the target efficiently it is important to maintain a proper ratio of aptamer:target. There is an average of 10^6 molecules of LPS on the surface of a single gram-negative bacterium including *Y. pestis* (Raetz et al. 2002). F1 antigen exists as aggregates of about 300 kDa on the *Y. pestis* cell surface in normal physiological solution and as a monomer of about 15.5 kDa in the presence of SDS (Galyov et al. 1990; Tito et al. 2001).

In our study, we have attempted to select an aptamer against LOS and F1 antigen on *Y. pestis* using electrodialysis as a novel partitioning technique. The aptamer thus selected can be used in future *in vitro* diagnostic assays.

Materials and Methods

Extraction and Characterization of LOS of *Y. pestis* for aptamer selection

Hot phenol water method to extract LOS of *Y. pestis* - A purified target to be presented to the aptamer pool is an important consideration when carrying out SELEX. Hence, in order to isolate crude LOS from the outer membrane of *Y. pestis,* a modified hot phenol water method (Galanos et al. 1969) was carried out. Stock solution of *Y. pestis* strain KIM D27 biovar Mediaevalis, lacking the 70 kb pVY plasmid, was grown on blood agar medium by overnight incubation at 27°C. A single colony was selected and grown in brain heart infusion (BHI) broth at 27°C for 18 hours to an OD_{600} of 1.2-1.3. *Yersinia pestis* cells were pelleted by centrifugation at 9500 x g at room temperature (RT), and the pellet washed thrice in distilled, deionized (dd) water. Washed cells were then resuspended in dd water to obtain a thick slurry and sonicated with a Branson sonifier 450 in 30 second pulses for a total of 1.5 minutes, three times to rupture cell particles. Lysozyme from chicken egg white (Sigma, L-6876) in concentrations of 0.2% (w/v) was added to the mixture, allowed to incubate overnight at RT and resonicated as above. Micrococcal nuclease (Sigma, N-3755) that hydrolyzes 5'-phosphodiester bonds of DNA molecules in 40 U amounts and 0.1% (w/v) RNAase A (Sigma, R-6513) were then added, and the mix was allowed to incubate at 4°C for 48 hours. Equal volumes of hot molten phenol at 65°C was added to the bacterial slurry, incubated for 30 minutes and allowed to cool on ice to separate the aqueous and the phenol phase. This process was repeated twice to obtain the aqueous phase. The pooled aqueous phase was then dialyzed extensively against water for 4-5 days using a 25 kDa dialysis membrane (SpectraPor®).

The dialyzed sample was ultracentrifuged using a 55.1 Ti rotor at 100,000 x g for 2 hours at 4°C and the pellet resuspended in minimal amounts of dd water.

Quantitation of crude LOS of *Y. pestis* by Purpald assay - Equal volumes of 3-Deoxy-D-*manno*-oct-2-ulosonic acid (KDO) standards of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mM and sodium periodate (Sigma-Aldrich, S-1878) in concentrations of 32 mM and 64 mM were added to a 96-well flat-bottomed plate and incubated at RT for 25 minutes. This was followed by adding 136 mM Purpald reagent (Sigma-Aldrich, 162892) in 2N sodium hydroxide, incubating for 25 minutes and measuring the $OD_{550.}$

Mild Acid Hydrolysis to separate Core Polysaccharide (CPS) from Lipid A of *Y. pestis* LOS - Glacial acetic acid (GAA) was added to the crude LOS solution to final concentration of 1.5%. The mixture was boiled for 2 hours, cooled and pelleted at 16,500 x g for 15 minutes. The supernatant with the CPS was collected for further use while the pellet containing the Lipid A was discarded.

Size Exclusion Chromatography to purify crude CPS of *Y. pestis* - Size exclusion chromatography was carried out after equilibration with degassed 50 mM ammonium acetate on a HW50 column packed with Toyopearl matrix of size 50 µm at a flow rate of 1 mL/min. Fractions were collected, pooled and concentrated on a vacuum concentrator (Eppendorf).

Characterization of CPS of *Y. pestis* LOS by Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) analysis - CPS of *Y. pestis* was further characterized by subjecting it to MS (Voyager) and ^{1}H NMR analysis.

Isolation of F1 antigen from *Y. pestis* by modified Miller's method (Miller et al. 1998) - Two strains of *Y. pestis,* D27 and KIM6 (lacking pH6 antigen) were used in this study. *Yersinia pestis* was grown in BHI broth at 37°C for 2 days to an OD_{600} of approximately 1.3 to generate F1 antigen on the surface of *Y. pestis*. The bacterial pellet obtained by centrifuging the broth culture at 14,000 x g for 45 minutes at 4°C was resuspended in phosphate buffer saline (PBS) pH 7.2 and incubated with gentle rolling at room temperature for 30 minutes. This suspension was then centrifuged at 14,000 x g for 30 minutes and the supernatant was saturated with 50% ammonium sulfate (Sigma-Aldrich) and allowed to incubate for 1 hour. The saturated suspension was centrifuged at 14,000 x g for 30 minutes at 4°C; the pellet was resuspended in a minimal volume of PBS and dialyzed against PBS overnight at 4°C. The dialyzed crude extract of F1 antigen was centrifuged at 27,000 x g for 30 minutes to remove insoluble material and the supernatant filtered through a 0.2 µm cellulose acetate filter (Nalgene® Labware).

Purification of crude F1 antigen by High Pressure Liquid Chromatography (HPLC) - Crude F1 antigen was purified by HPLC on a HW55 column using Toyopearl beads at the rate of 2 mL/min after equilibration in PBS. The fractions obtained were collected, pooled and desalted.

Determination of concentration of purified F1 antigen of *Y. pestis*

Purified F1 antigen was quantified by spectrophotometrically measuring its $OD_{260/280}$ using the ND-1000 Nanodrop® and by bicinchoninic acid (BCA) assay and color change measured at 562 nm.

Characterization of F1 antigen by SDS-PAGE and Western Blot - The molecular weight of purified F1 antigen was determined by electrophoretically separating it on a 15% SDS-PAGE gel and staining with silver stain. A western blot analysis was carried out by using mouse specific IgG1 anti-*Y. pestis* capsular F1 antigen (QED Bioscience Inc.) and detected by antimouse whole cell IgG conjugated to alkaline phosphatase using BCIP/NBT for colorimetric analysis on a semi- dry transfer apparatus.

Confirmation of F1 antigen as a target by Peptide Mass Fingerprinting (PMF) - PMF was carried out by trypsinizing F1 antigen protein overnight to generate peptides that were subjected to MALDI-TOF analysis on Voyager and analyzed by MS-Fit and Mascot programs.

Optimization assays for electrodialysis - Optimization assays were carried out to determine the effectiveness of electrodialysis. This was done first by end-labeling ^{33}P ATP to the 78 bp complex oligonucleotide pool. In this process, 10 pMoles of oligonucleotides were incubated with Optikinase (Perkin-Elmer) and γ^{33}P ATP (Perkin Elmer) for 10 minutes at 37°C. The enzymatic reaction was stopped by 0.5 M EDTA. The 5' end labeled oligonucleotide pool was separated from ATP by using a Sephadex G-25 column (Amersham). The radiolabeled oligonucleotide pool was placed in a 100 kDa dialysis bag with 10 mM Tris and 192 mM Glycine at 10 mA for two hours.

Additionally, to determine the effective retention of F1 antigen within dialysis membrane while F1 antigen in tris - glycine buffer was placed within dialysis membrane and electrodialyzed at 10mA for 1 hour at RT. The solution within the dialysis membrane containing the F1 antigen was collected, precipitated by trichloroacetic acid, electrophoresed on a 12.5% SDS-PAGE gel and silver stained.

SELEX methodology -Each round of SELEX was carried out by incubating 10^{14}-10^{15} molecules of ssDNA;

CATGTACTGTACCCTCGCACTGTG- N_{30}- CTTGACTTCGCTGGACTCACTACG

with forward primer, CATGTACTGTACCCTCGCACTGTG and biotinylated reverse primer 7B-CGTAGTGAGTCCAGCGAACTGAAG that is a part of the oligonucleotide pool with F1 antigen in 20 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 50 mM KCl and 100 mM NaCl (HMKN) in a 10:1 ratio for a period of 2 hours at RT. The DNA bound to F1 antigen was separated from the unbound by electrodialysis and amplified using two-step Polymerase Chain Reaction (PCR) by initial heating for Immolase (Bioline, USA) enzyme activation at 95°C for 7 minutes, denaturation at 95°C for 5 seconds, annealing at 67°C for 5 seconds and repeating process 29 times. An aliquot of the PCR product was separated on a 4.5% agarose gel by electrophoresis to confirm the presence of 78 bp DNA. The rest of dsDNA was strand separated by incubating dsDNA with strepavidin coated Dynal® beads (Dynal Biotech) at RT for 30 minutes as per manufacturer's instructions. The solution was heated to 95°C for 5 minutes to separate the two DNA strands and flash cooled with 100% ethanol. Separation of the single stranded biotinylated product using magnetic bead was carried out, and the product measured spectrophotometrically using standard of 33 for ssDNA on the Nanodrop spectrophotometer.

Cloning and sequencing of Round 4 - Round 4 pool from SELEX B (see results) was PCR amplified with nonbiotinylated reverse primer and cloned by the TOPO-TA cloning kit (Invitrogen) using the pCRII® TOPO vector with blue-white screening. White colonies were selected and plasmid purified by Qiagen plasmid Mini kit and sequenced.

SELEX C – Rounds 1-8 using F1 antigen of *Y. pestis* as target with 78 bp oligonucleotide sequences - Each SELEX round was carried out by ssDNA that is a part of the aptamer pool with F1 antigen in HMKN buffer in a 10:1 ratio for an hour at RT. The DNA bound to protein was separated from the unbound by electrodialysis and amplified by PCR. An aliquot of the PCR product was separated on a 15% native PAGE gel by electrophoresis, stained with Sybr Gold® and image taken on VersaDoc gel documentation system (Bio-rad) to confirm the presence of 78 bp DNA. The remaining dsDNA was strand separated by heating to 95°C for 5 minutes to separate the two DNA strands and flash cooling with 100% ethanol. The solution was then incubated with ice cold streptavidin-coated Dynal beads for 5 minutes. The streptavidin-biotinylated negative sense ssDNA complex was separated from positive sense ssDNA using a magnetic column and the product measured spectrophotometrically using standard 33 on the Nanodrop.

Cloning reaction of Round 4 and Round 8 of SELEX D - Round 4 and Round 8 pool were PCR amplified using nonbiotinylated Primer 2, cloned by the TOPO-TA cloning kit using the pCRII® TOPO vector and sequenced. Round 8 pool was electrophoresed on a 15% native PAGE gel and gel purified twice to eliminate low molecular weight products that were inadvertently cloned previously.

Binding assays - To determine the specificity and sensitivity of the interactions, a binding assay was carried out. The oligonucleotide pools of Round 8 and Round 0 were radiolabeled with ^{33}P ATP and amplified by PCR using primers of unequal concentrations and lengths with and electrophoresed on a 20% denaturing urea gel. The 78 bp oligonucleotide band was excised and gel purified by isobutanol extraction and

ethanol precipitation. F1 antigen in concentrations of 2.5 µg, 400 ng, 77.5 ng and 40 ng were immobilized on a nitrocellulose membrane using the Microsample Filtration Manifold (Schleider and Schuell) for 10 minutes at RT. The membrane was blocked in PBS blocking buffer (Invitrogen) at RT for one hour, air dried and blotted with approximately 70,000 counts per minute (cpm) of Round 0 or Round 8 ssDNA for one hour at RT and washed in PBS twice for 10 minutes duration. An autoradiograph was taken after 24 hours and Round 8 and Round 0 bound to the membrane immobilized F1 antigen was quantitated by liquid scintillation analysis.

Results and Discussion

Purification and Characterization of LOS of *Y. pestis*

Crude *Y. pestis* LOS was isolated by the hot phenol water method and its molecular weight and purity in terms of lack of protein contamination were determined by electrophoresing a 12.5% SDS-PAGE gel containing LOS extract (Figure 1). The gel was stained with silver stain (Owl Separation Systems), and a low molecular weight band less than 6 kDa was observed at the dye front of the gel. This band is indicative of the rough phenotype of *Y. pestis* LOS, with Lipid A and core polysaccharide but absence of repeating sugar units of the O-antigen (Prior et al. 2001). Coomassie Blue staining showed no protein contamination within the crude LOS extract (results not shown). In order to quantify the crude LOS extract, a Purpald assay was carried out. This assay detects the presence of KDO that connects the core polysaccharide to the Lipid A. The main principle of the Purpald assay is the oxidation of the unsubstituted vicinal glycol in KDO and heptose by sodium periodate, releasing formaldehyde that reacts with the Purpald reagent, giving it a distinctive purple color that can be measured

spectrophotometrically (Lee et al. 1999). Based on the Purpald assay, concentrations of LOS were found to be approximately 6 µg/mL.

The presence of negatively charged phosphate residues on Lipid A as well as its hydrophobic nature (Schromm et al. 1998; Raetz et al. 2002) made it easier to repel the α-phosphate residue on the deoxyribosyl phosphate backbone of oligonucleotides. This resulted in difficulty in selecting an aptamer for LOS. Therefore, one approach was to separate the CPS from the Lipid A by hydrolyzing the KDO residue connecting them by mild acid hydrolysis, in order that the CPS could be used as an aptamer selection target. Crude CPS was purified by size exclusion chromatography (Figure 2) with peaks noted at 25-30 minutes. Further characterization was carried out by MS (Figure 3) and NMR analysis (Figure 4). Prior et al. 2001 carried out a MS study on LPS of *Y. pestis* and noted that the m/z signal was closer to 3000; however, this was in the presence of a Lipid A and CPS, but not an O-antigen. Kawahara et al. 2002 studied changes in Lipid A composition of *Y. pestis* strain Yreka isolated at 27°C and 37°C by MALDI-TOF analysis. He detected major peaks concurrent with Lipid A at an m/z of 1178, 1404, 1586 and 1823 at 27°C and 1081, 1307 and 1726 at 37°C respectively. Further comparison was also carried out by observing electron spray ionization (ESI)-MS of *Y. pestis* CPS by Vinogradov et al. 2002. In our study, we observed major peaks between m/z of 700-1500 that are indicative of the combinations of CPS sugars lacking the Lipid A and the Ko-Kdo moiety. Knirel et al. 2005 observed by ESI-MS that the *Y. pestis* core sugars, $GlcNac_1Glc_1Gal_1Hep_3Kdo_1$ showed a m/z of 1354 while the core lacking GlcNac had a m/z of 1138.38.

Vinogradov et al. 2002 carried out a ^{1}H and ^{13}C NMR analyses on CPS of *Y. pestis* and observed that the chemical shift of the Ko residue was identical to the α-Ko of *Burkholderia cepacia* in the range of 3.7-4.5 ppm while the rotational nuclear overhauser effect spectroscopy (ROESY) for Glc-L, D-Hep_3 was identical to *Klebsiella pneumoniae* with a range of 3.5- 4.0 ppm. ^{1}H NMR analysis was carried out in our study and we observed two sets of peaks between 1.8-2.3 ppm and 4.3 ppm. As further analyses using ^{13}C NMR was not carried out, we assume these peaks represent chemical shifts from α-Kdo, Heptose, Hexose and N-acetyl glucosamine sugars.

LOS is not considered a good target for selection

Carbohydrate moieties have been shown to have a low affinity to aptamer molecules because of lack of charged interactions, planar ring structures (Yang et al. 1998) and constant movements about the β-glycosidic linkages connecting the sugars residues (Rajyaguru et al. 1997). These constant movements interfere with the induced-fit binding that is common with aptamer binding (Jayasena et al. 1999). Furthermore, studies carried out in our laboratory by Snider. et al. 2005 determined that LPS from *E. coli* O157 did not interact with an oligonucleotide pool with high specificity and sensitivity. Aptamer selection using core polysaccharide and O-polysaccharide of *Francisella tularensis* subspecies *tularensis* LPS as a target immobilized on nitrocellulose showed a high affinity to both, purified core polysaccharide and O-polysaccharide. However, no binding was observed to whole *F. tularensis* cells. This might have been due to the recognition of core polysaccharide by the aptamer Ft1 that is not exposed above the outer membrane in whole bacterial cells (Thirumalapura et al. 2005, personal communication). Hence, CPS of *Y. pestis* LOS was not considered a suitable target for aptamer selection.

Selection and Characterization of F1 antigen of *Y. pestis*

Capsular F1 antigen is expressed on the surface of *Y. pestis* at 37°C or when the bacteria come in contact with the eukaryotic host. Crude F1 antigen was obtained by concentration with ammonium sulfate and further purification carried out by HPLC (Figure 5). F1 antigen exists as a multimer in physiological solutions and hence is eluted in the void volume at fractions 30-40 (Miller et al. 1998). Both the BCA assay and the spectrophotometric analysis estimated the concentration of F1 antigen to be approximately 40µg/mL. Analysis of F1 antigen by SDS-PAGE and silver staining (Figure 6a) revealed that on isolating the monomeric 15.5 kDa F1 antigen from *Y. pestis* KIM6, a band of 14 kDa was also observed. That band was later determined to be surface expressed virulence factor, pH6 antigen, with a molecular weight of 15 kDa (Lindler et al. 1990). A mutant strain of *Y. pestis* KIM6 lacking the pH6 antigen gene was later used to isolate F1 antigen and has replaced *Y. pestis* D27 in this study. Western blot analysis (Figure 6b) carried out to confirm the presence of F1 antigen showed a specific band at 15.5 kDa, by a colorimetric analysis. The masses of the peptides generated from F1 antigen showed a greater than 95% match to hypothetical peptide masses seen in the database (Figure 7).

Electrodialysis as a novel partitioning technique to separate aptamers bound to F1 antigen from those unbound

Although partitioning is an important step in SELEX (Fitzwater et al. 1996), separation strategies for selecting bound oligonucleotides are varied and not 100% effective. In our study, we have considered a new method of selecting bound oligonucleotides by a process of electrodialysis (Figure 8). In this system, a complex

sample containing oligonucleotides bound to target and unbound oligonucleotides is placed in a 100 kDa dialysis membrane, within a chamber with an ionic buffer, and an electric current is passed through it. The force of the electric current will drive the unbound molecules out of the membrane, while the oligonucleotides bound to large multimeric F1 antigen are trapped within the dialysis membrane. The bound oligonucleotides are then recovered and subjected to further analysis. A pre-SELEX partitioning run was carried out by introducing the radiolabeled ssDNA complex to a dialysis membrane and electrodialyzing it for an hour. Representative samples (Figure 9) were collected at regular intervals from within the dialysis membrane, dialysis tube, the upper and lower chamber and membrane cap separating the two chambers; radioactivity was measured in counts per minute on the scintillation counter. It was observed that within 1 hour, almost all the oligonucleotides were driven from within the dialysis medium into the surrounding buffer. Additionally, we were also able to retain F1 antigen in the dialysis membrane during electrodialysis (Figure 10). These observations led us to conclude that electrodialysis is an efficient way of partitioning bound and unbound oligonucleotides.

Systematic Evolution of Ligand by Exponential Enrichment (SELEX) using purified F1 antigen as a target

A total of 8 rounds of SELEX (Table 2) were carried out against the F1 antigen of *Y. pestis*. After the first four rounds of SELEX called SELEX A, excessive smearing was noted on the 4.5% agarose gels after electrophoresing PCR product of Rounds 3 and 4 to confirm the presence of the 78 bp oligonucleotide (results not shown). Hence, the process of selection was repeated for another 4 rounds termed SELEX B to determine the

source of the problem. As the problem persisted, it was initially thought that smearing within agarose gels during electrophoresis could be the result of either excessive concentration of oligonucleotides, interference of F1 antigen during electrophoresis or excess polymerase. Ten fold dilutions of PCR product of Round 4 SELEX partially reduced the smears. F1 antigen was also assumed to be carried over from each round of SELEX and hence accumulated protein could interfere with electrophoresis. However, trichloroacetic acid (TCA) precipitation, SDS-PAGE analysis and silver staining did not show a sufficient concentration of F1 antigen to cause interference during electrophoresis (results not shown). Clones of SELEX B were sequenced and nineteen sequences (Figure 11) were analyzed manually and by using the Pretty and Pile-up algorithms on SeqWeb (Figures 12a and 12b). While no homologous motifs were detected within these clones, reduction in pool complexity was observed by the multiple sequence dendrogram. However, due to inability to acquire PCR product without intense smearing, the data collected after repeating four rounds of SELEX could not be validated and hence the process had to be repeated for the third time.

By running aliquots of PCR product of Round 2, 3 and 4 of SELEX C on a 15% native PAGE gel, smearing was noted below and in the wells of the gels and through the lanes, indicating accumulation of DNA possibly due to protein contamination and or coagulation. In order to further analyze the source of contamination, the PCR products of all the four rounds of SELEX were run on a 15% SDS-PAGE gel and silver stained (Figure 13). The resultant product obtained showed a low molecular weight band below 10 kDa that was identical to the molecular weight of streptavidin protein (Figure 14), and subsequently it was discovered that heating Dynal beads coated with streptavidin

probably resulted in a leaching of streptavidin along with ssDNA that was carried over into the next round of SELEX. Hence the process of strand separation was changed, by first heating the PCR product containing dsDNA to 95°C and flash cooling it with 100% ethanol to maintain single stranded products. Streptavidin-coated Dynal beads were then added to the separated DNA product. Biotinylated negative sense DNA strands complexed to the streptavidin coated Dynal beads were separated from the non biotinylated positive sense strands by a magnetic column. Using this altered methodology of strand separation, SELEX rounds 1-8 called SELEX D were repeated once again.

For Round 1, at a 1:10 ratio of oligonucleotide to target F1 antigen, 7.2 nMoles of oligonucleotides would require a concentration of 0.72 nMoles of F1 antigen in SELEX. As the volume required was very large, a ten fold dilution of ssDNA was used in selection thus requiring a lower mole amount of F1 antigen. This parameter was followed for subsequent rounds of SELEX as well. Analysis of PCR product on a 15% native PAGE gel of Round 5 SELEX D indicated an accumulation of oligonucleotides within the well of the gel. A probable reason might have been excessive amounts of oligonucleotides, and hence it was decided to repeat SELEX round 3 and gel purify the band of interest. This was carried out by excising the 78 bp band, eluting it against minimal amounts of Diethylpyrocarbonate (DEPC) water overnight at RT and ethanol precipitating the supernatant. The recovered DNA was then measured spectrophotometrically to determine the $A_{260/280}$ ratio. Approximately 1.2 µg of dsDNA was recovered from the gel purified Round 3 SELEX pool that was strand separated and used in succeeding rounds of SELEX. Due to contamination of the stock primer solutions, PCR product from the negative controls of Round 7 SELEX D that lack the

oligonucleotide product, show a positive 78 bp band. These were assumed to have been misamplified PCR product or primer-dimers. In addition to Round 3, gel purification of 78 bp oligonucleotide pool was also carried out in Rounds 6, 7 and 8 by using the Qiaex® II Agarose Gel Extraction method. Cloning and sequencing of rounds 4 and 8 were carried out and 59 sequences (Figure 15) of Round 4 (repeat) and 20 sequences from Round 8 were analyzed manually by visual analysis on MS-Word as well as on the Pile up algorithm from SeqWeb. From the 50 sequences obtained from the Round 8 pool, only 20 were of the 30 bp length that could be analyzed further (data not shown). The length of the rest of the sequences ranged from 2 bp to 10 bp, indicating there might be a misstep during PCR amplification, due to secondary structure folding. No primer-dimers were noted by sequence analysis. Gel purification and subsequent recloning of Round 8 pool revealed that of 63 sequences analyzed from Round 8 pool, 39 sequences showed homologous sequences (Figure 16).

Binding Assays

Immobilization of F1 antigen within the nitrocellulose membrane did not result in any binding with Round 8, as determined by autoradiography and quantitative measurement on a scintillation counter (Figure 17a and b). Since, the maximum amount of F1 antigen immobilized was 2.5 µg, it might not be in adequate amounts for binding to occur, and immobilization of aggregated F1 antigen multimers might result in covering the epitopes that are available for aptamer binding. As sequencing of Round 8 showed the presence of two motifs, it might indicate that by our process of electrodialysis, we have not succeeded in isolating a strong 'binder' to our target, but have isolated a group of poor binding species. Additional rounds of selection will have to be carried out to

effectively partition out the strong and weak binding aptamer species. Further plans include characterization of potential aptamer species by sequence analysis and binding assays. The aptamer will also be tested to determine its specificity for *Y. pestis* and using closely related *Y. pseudotuberculosis* and *E.coli.*

Summary and Conclusions

Due to its ability to cause fatal primary pneumonic plague infections, *Y. pestis* has been considered as a bioterrorism agent by the Center for Disease Control and Prevention (Rotz et al. 2002). The widespread devastation that is predicted to be caused by primary pneumonic plague will be further complicated by lack of rapid and suitable diagnostic tests (Riedel et al. 2005). Aptamer biotechnology is thought to overcome some of the limitations that are observed when utilizing antibodies as diagnostic agents (Jayasena et al. 1999).

Aptamer selection is an iterative process of binding, partitioning, amplification and strand separation called SELEX (Tuerk et al. 1990). This process will result in the reduction of the complex aptamer pool with approximately 10^9 or 10^{13} molecules to a small family of rare molecules that are highly specific to the target of interest (Jayasena et al. 1999). Once the pool complexity is sufficiently reduced, assessment of the pool is carried out by cloning, sequencing, sequence analysis and binding assays.

Factors important to consider when selecting microbial cellular targets for diagnostic applications are external presentation on surface of organism, high copy number and uniqueness. In addition it is necessary to have highly purified target molecules for SELEX. Several targets on the surface of *Y. pestis* were considered including translocator Yops, V-antigen, F1 antigen, LOS, plasminogen activator and pH6

antigen. LOS and F1 antigen of *Y. pestis* were initially selected as suitable targets for aptamer selection because of the above mentioned criteria. The LOS of *Y. pestis* is present on the outer membrane, its CPS moiety is unique to *Y. pestis*, it lacks the O-antigen that is common to its closest related species in the *Yersiniae* family, and there are on an average more than a million molecules of LOS are present per bacterium (Raetz et al. 2002; Bruneteau et al. 2003; Skurnik et al. 2003). However, based on previous experience while working on aptamer selection in our laboratory utilizing LPS of *E. coli* as a target, it was observed that the presence of negatively charged phosphate groups attached to the glucosamine dissacharide on the Lipid A moiety and the hydrophobic characteristics of the acyl side chains may make it unsuitable for aptamer interaction (Snider T, personal communication).

The core polysaccharide (CPS) of *Y. pestis* is a heptameric sugar molecule made up of N-acetylglucosamine, heptose sugars, glucose and 3-deoxy-D-mannoctulosonic acid (KDO) (Vinogradov et al. 2002). The CPS was considered as a target in aptamer selection in place of intact LOS because of its hydrophilic nature and presence of several hydrogen binding regions. The CPS was separated from the Lipid A of LOS by mild acid hydrolysis and further characterized by MS and ^{1}H NMR analysis. Although CPS has certain positive attributes such as hydrophilicity, hydroxyl-hydrogen binding sites, upon further consideration we decided that CPS of *Y. pestis* was not a favorable target for aptamer selection as we had initially thought. Our concern was that the rotational freedom of the glycosidic bonds present between the sugar residues makes it unfavorable for the induced fit binding of the aptamer to the CPS. Additionally, the hydroxyl groups

present in the sugar molecules were thought to be less avid for forming hydrogen bond interactions with potential aptamer species (Rajyaguru et al. 1997; Yang et al. 1998).

As LOS of *Y. pestis* was determined unsuitable for aptamer interaction; we decided to consider F1 antigen of *Y. pestis* as a target for aptamer selection. F1 antigen is a multimeric capsular protein expressed on the surface of *Y. pestis* at 37°C. It is encoded by the *caf* genes present on the pFra plasmid unique to *Y. pestis* (Perry et al. 1997). We isolated, purified and characterized F1 antigen as a target by ammonium sulfate precipitation, HPLC and peptide mass fingerprinting respectively.

The first step of SELEX is an affinity binding process that entails incubating the purified target with the complex aptamer pool under suitable binding conditions. Our affinity binding process involved incubating target F1 antigen with a 78 bp oligonucleotide pool made of a 30 bp random sequence flanked by 24 bp of primer binding sites in a 1: 10 molar ratio in HMKN buffer for 1 hour at room temperature. Partitioning of bound aptamer species to target from unbound is a very important step in selection (Fitzwater et al. 1996). In the past, partitioning was carried out by affinity chromatography, nitrocellulose membrane filtration, centrifugation and capillary electrophoresis. We separated oligonucleotides bound to F1 antigen from unbound oligonucleotides by a novel process of electrodialysis. Two-step PCR amplification of the target bound aptamer pool was carried out with a non-biotinylated forward primer and a biotinylated reverse primer. The PCR amplified biotinylated dsDNA was strand separated first by incubating with streptavidin coated Dynal beads. The non-biotinylated positive sense strand was then separated from the biotinylated negative sense strand by heat denaturation at 95°C and flash cooling in ice cold ethanol bath. The biotinylated negative

sense strand complexed to streptavidin-coated Dynal beads was immobilized on a magnetic column and archived, while the positive sense strand collected, quantified spectrophotometrically and used in subsequent rounds of SELEX.

After four rounds of SELEX termed SELEX A, we observed that the 78 bp PCR amplified bound aptamer pool showed intense smearing when electrophoresed on a 4.5% agarose gel. The selection process was repeated again for four rounds and termed SELEX B, with similar results. As results for both SELEX A and B could not be validated, a third selection process was carried out termed SELEX C. Smearing was observed after only two rounds of SELEX C. A probable cause was determined as F1 antigen carry over and coagulation or contamination with PCR products such as excess polymerase enzyme. To confirm our theory, we electrophoresed the PCR products of four rounds of SELEX C on a 15% SDS-PAGE gel. We observed low molecular weight bands (<15 kDa) in Rounds 2, 3 and 4 of SELEX. We did not observed these bands in Round 1 PCR product that had not yet undergone strand separation or in our 78 bp oligonucleotide positive control that lacked the F1 antigen and had not undergone the selection process. The negative control, that lacked the 78 bp oligonucleotide product did not show these low molecular weight bands as well. By extensively troubleshooting our methodology, we found that these low molecular weight bands were similar to a subunit of commercially available streptavidin. We determined that due to erroneous strand separation process, wherein incubating PCR product of bound aptamer pool with streptavidin coated Dynal beads and denaturation by heating resulted in leaching of streptavidin. This leached streptavidin was carried over in subsequent rounds of SELEX, possibly resulting in interference of oligonucleotide migration under electrophoresis.

The process of strand separation was altered to a more appropriate process of first denaturing the positive and negative sense strands of the PCR product of bound aptamer pool. This was then followed by flash cooling in ethanol ice bath and immobilizing the negative sense strand bound to streptavidin coated Dynal beads on a magnetic column. Eight rounds of SELEX termed SELEX D were thus successfully carried out using F1 antigen as a target and electrodialysis as a partitioning method. However, we did note that in Rounds 7 and 8, we were amplifying low molecular weight PCR products ~50 bp in addition to our 78 bp oligonucleotide. This was initially thought to be primer dimers, but on sequence analysis was assumed to be mis-amplified PCR product. The cause of this product appearing in our PCR reactions in Rounds 7 and 8 is not exactly clear.

After a certain number of SELEX rounds are carried out to reduce the pool complexity, an assessment of the reduced pool is carried out by cloning, sequencing, sequence analysis and binding assays to determine kinetics of interaction. Rounds 4 and 8 from SELEX D were cloned and sequenced. Sequence analysis of Round 4 clones showed a reduction in pool complexity as observed by multiple sequence alignment dendrogram. Out of 63 clones of Round 8 that were sequenced, 39 clones showed the presence of consensus motifs within each sequence. In some sequences, variations of one or two bp in consensus motifs were also noted.

As we had observed sufficient reduction in Round 8 aptamer pool complexity, we decided to determine the specificity of Round 8 interaction for F1 antigen by carrying out a binding assay. Purified F1 antigen in concentrations of up to 2.5 µg were immobilized on a nitrocellulose membrane, blocked, air dried and incubated with

radiolabeled Round 8 and Round 0. An autoradiograph taken 24 hours later did not show any significant interaction between Round 8 and F1 antigen. Round 8 and Round 0 did show binding to single stranded DNA binding protein that was used as a positive control. Non specific binding to the nitrocellulose membrane as well as to bovine serum albumin (BSA) that was used as a negative control was not observed. In contrast to Round 8 aptamer pool, up to 80 ng of immobilized F1 antigen on nitrocellulose membrane could be detected after probing with primary anti-F1 antibody. Although consensus motifs exist between the cloned sequences of Round 8, we concluded that an unsuccessful binding assay may be due to lack of specificity of Round 8 to F1 antigen. It was determined that additional rounds of selection would be necessary to ensure a specific aptamer species that would recognize F1 antigen.

Our future plan includes carrying out 4 additional rounds of SELEX to increase the specificity of aptamer pool to F1 antigen. This will be followed by assessing Round 12 by cloning, sequence analysis and binding assay. We anticipate that additional rounds of SELEX will reduce the pool complexity significantly from Round 8 so as to obtain strong binding species with greater consensus. The aptamer we select will thus be used as an *in vitro* probe to detect the presence of F1 antigen of *Y. pestis* in clinical samples.

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Table 1: Outer membrane structures of *Y. pestis*

Surface exposed targets on *Y. pestis*	Properties	Role in virulence	Homology to other bacterial species	References
pH6 antigen	Fimbrillar structure on surface of *Y. pestis* that is encoded by the *psa* locus with three operons *psaA*, a structural determinant and *psaE*, a transcriptional activator and *psaB*, a chaperone *psaA* expression depends on *psaE* activity, pH below 6.7 and temperature above 34ºC.	Interferes with phagocytosis Possible role in adhesion	MyfA of *Y. enterocolitica* shares a 44% homology to *Y. pestis* pH6 antigen in *Y. pseudotuberculosis* shares homology to pH6 antigen in *Y. pestis*	Iriarte et al. 1993 Lindler et al. 1993 Price et al. 1995 Yang et al. 1996 Huang et al. 2004
V antigen	It is encoded by the *lcrGVH* operon on the pCD1 plasmid under low calcium conditions at 37ºC. Immunogold studies showed approx 40-80 LcrV clusters on the surface of each *Y. pestis* cell	Protective antigen Helps in secretion of Yops directly into eukaryotic cells	LcrV of *Y. enterocolitica* and *Y. pseudotuberculosis* PcrV of *Pseudomonas aeruginosa*	Zahorchek et al. 1982 Price et al. 1989 Hill et al. 1997 Fields et al. 1999 Pettersson et al. 1999
F1 antigen	Capsular protein encoded by the *caf* operon on the 100 Kb pFra plasmid at 37ºC *Y. pestis* expressing F1 antigen probed with F1 specific IgG and imaged with Immunogold microscopy revealed aggregates of F1 antigen surrounding *Y. pestis* surface	Protective antigen Stimulate the immune response	None	Du et al. 2002 Simpson et al. 1990
Yersinia outer proteins (Yops)	Translocator *yops* encoded by 70 Kb pCD1 plasmid under low calcium conditions or in presence of eukaryotic cells	Needle like delivery apparatus formed on *Y. pestis* surface by Ysc proteins that assists in translocating effector Yops into mammalian cells	Yop proteins also homologous to *Y. pseudotuberculosis* and *Y. enterocolitica* *S. typhimurium* Prg proteins Esc proteins in *E. coli*, Mxi proteins *in Shigella flexneri are* homologous to Ysc proteins	Cornelis et al. 1998 Hu et al. 1998 Kubori et al. 1998 Sukhan et al. 2001 Blocker et al. 2001 Gauthier et al. 2003
Plasminogen activator (Pla)	Expressed by the *pla* gene on the 9.5 Kb pPCP1 plasmid in *Y. pestis* Based on topology models comparing Pla and OmpT it was observed that Pla forms a β- barrel structures that protrude about from the lipid bilayer of the outer membrane and the outer loops of the Pla situated above the core region of LPS	Converts plasminogen into plasmin that possesses proteolytic activity resulting in dissemination of the bacteria from the site of inoculation. Demonstrates adhesive actions by mediating *Y. pestis* binding to basement membrane proteins like heparan sulfate proteoglycan and laminin. Inactivation of complement proteins C3, C3b and C4b further assisting in the spread of infection	OmpT in *E. coli* shares a 50% homology to Pla protein PgtE of *S. enterica* serovar *typhi* shares a 75% homology to Pla peptide.	Kukkonen et al. 2004 Lahteenmaki et al 2001 Sodiende et al. 1992 Sodiende et al. 1989 Sodiende et al. 2001
LPS	Rough phenotype, lacking O-antigen due to mutations in the O-antigen gene cluster. While the Lipid A is present within the outer membrane, core polysaccharide is surface exposed.	Lipid A moeity is an endotoxic substance. Absence of O-antigen is believed to enhance the effect of the plasminogen activator and assist in disseminaton of *Y. pestis*	*Y. pseudotuberculosis* serotype O:1b	Skurnik et al. 2000 Prior et al. 2001 Kukkonen et al. 2004

Table 2- SELEX rounds carried out by incubating F1 antigen and 78 bp oligonucleotide pool having sequence CATGTACTGTACCCTCGCACTGTG- N30- CTTGACTTCGCTGGACTCACTACG in a 1:10 ratio in HMKN binding buffer at RT. Separation of bound and unbound oligonucleotides was carried out by electrodialysis.

SELEX Rounds	Concentration of oligonucleotides	Conc. of F1 antigen
A		
1	1 nM	100 pMoles
2	200 pMoles	20 pMoles
3	10 nMoles	1nMole
4	617.5 pMoles	70 pMoles
B		
1	10 nMoles	1 nMole
2	340 pMoles	34 pMoles
3	533 pMoles	53 pMoles
4	560 pMoles	56 pMoles
C		
1	1 nMole	100 pMoles
2	720 pMoles	72 pMoles
3	264 pMoles	26.4 pMoles
4	2.3 nMoles	230 pMoles
1 (repeat)	1 nMole	100 pMoles
2 (repeat)	700 pMoles	70 pMoles
3 (repeat)	350 pMoles	35 pMoles
4 (repeat)	450 pMoles	45 pMoles
5 (repeat)	200 pMoles	20 pMoles
4 (repeat 2)	27.6 pMoles	2.76 pMoles
5 (repeat 2)	220 pMoles	22 pMoles
6	336 pMoles	33.6 pMoles
7	14.3 pMoles	1.43 pMoles
8	34.4 pMoles	3.42 pMoles

Fig. 1- 5, 10, 15 and 20 µl of rough LOS fractions on a 15% SDS-PAGE gel, stained with silver stain.

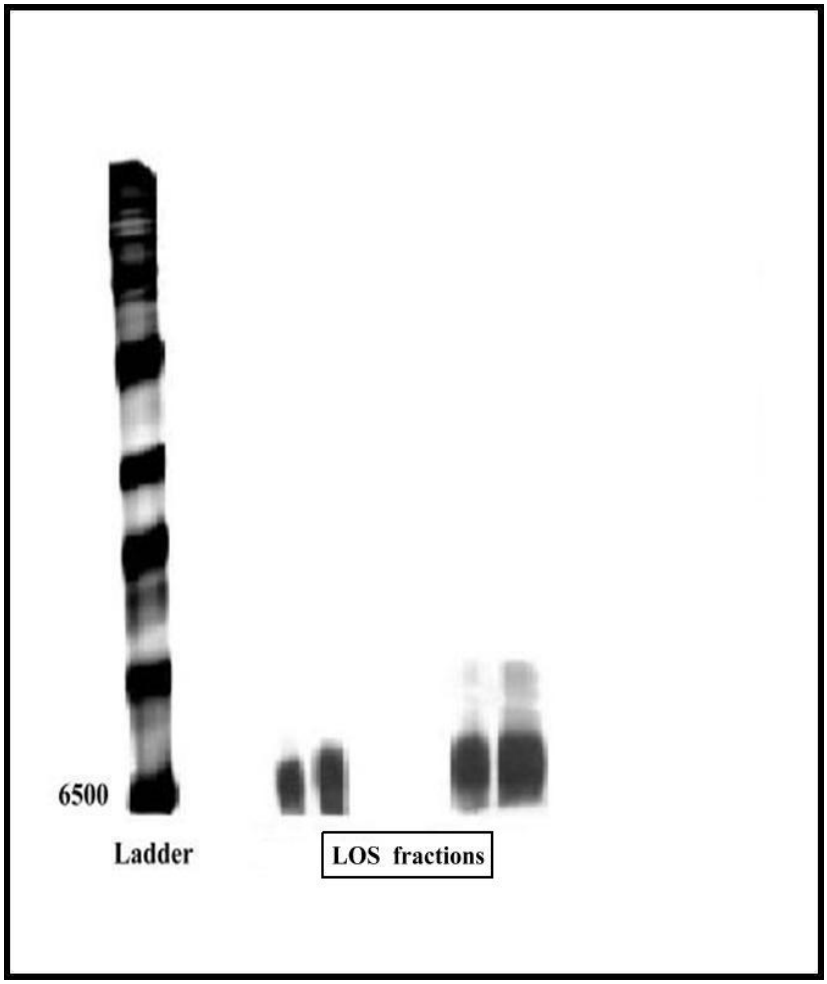


Fig. 2- Size exclusion chromatography reveals that fractions of CPS are eluted out 25-30 minutes after injection of sample into the column. The large peak on the right is indicative of ammonium acetate.

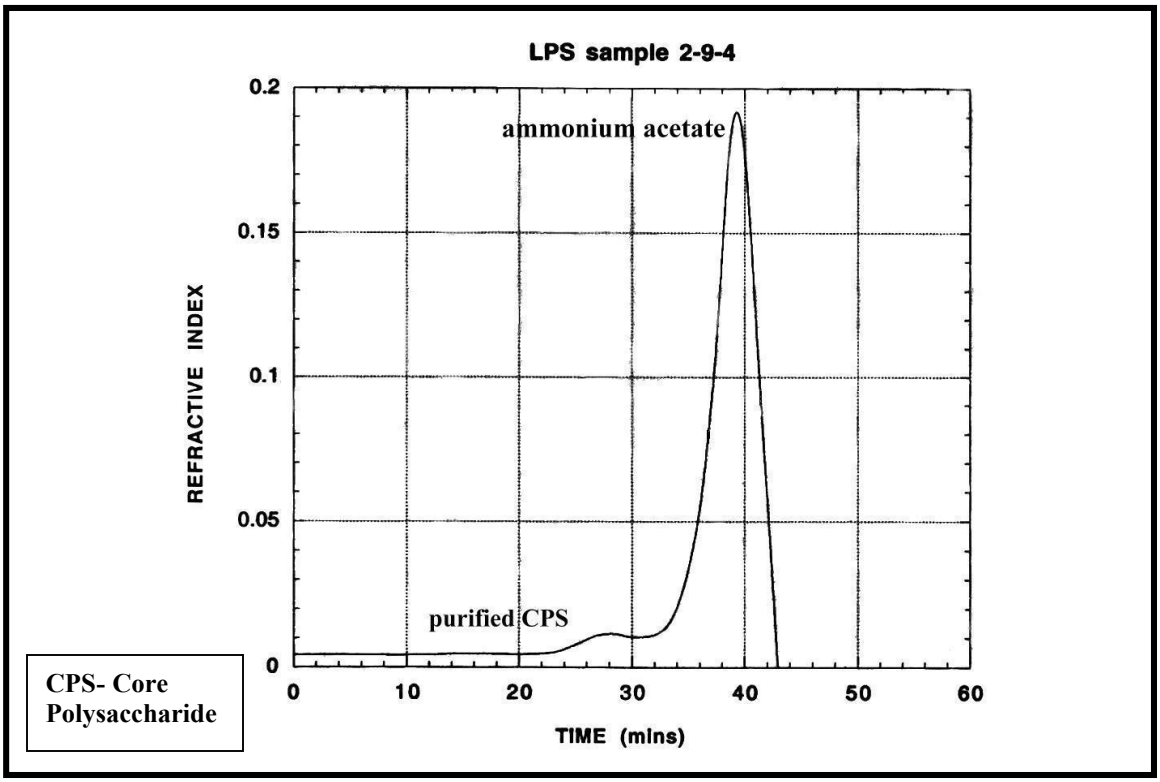


Fig. 3- Purified CPS samples subjected to MS indicating combination of residues of N-acetylglucosamine, N-acetylgalactosamine, Heptose, Hexose, KDO in m/z values comparable to results obtained by Knirel et al. 2005

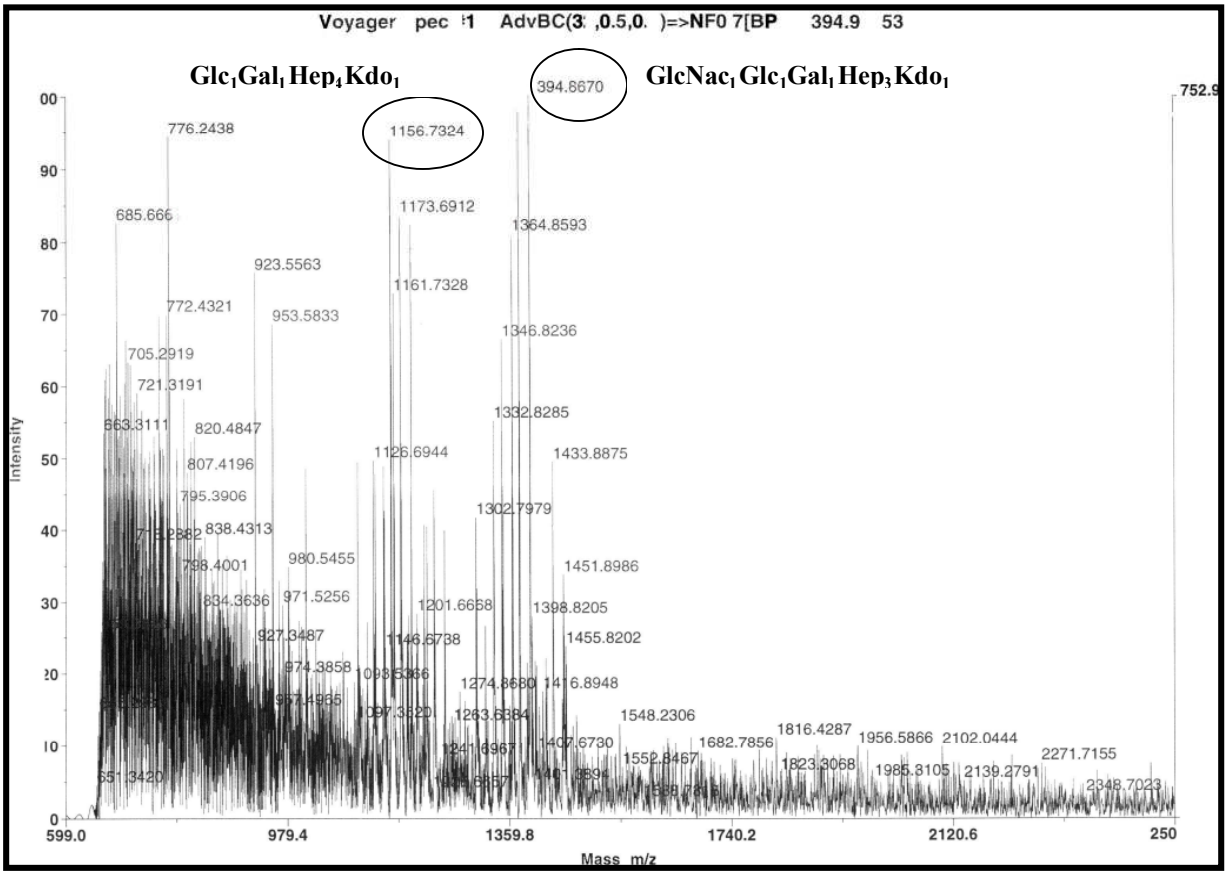


Fig. 4- ^{1}H NMR analysis of CPS of *Y. pestis* LOS (Referenced to Vinogradov et al. 2002)

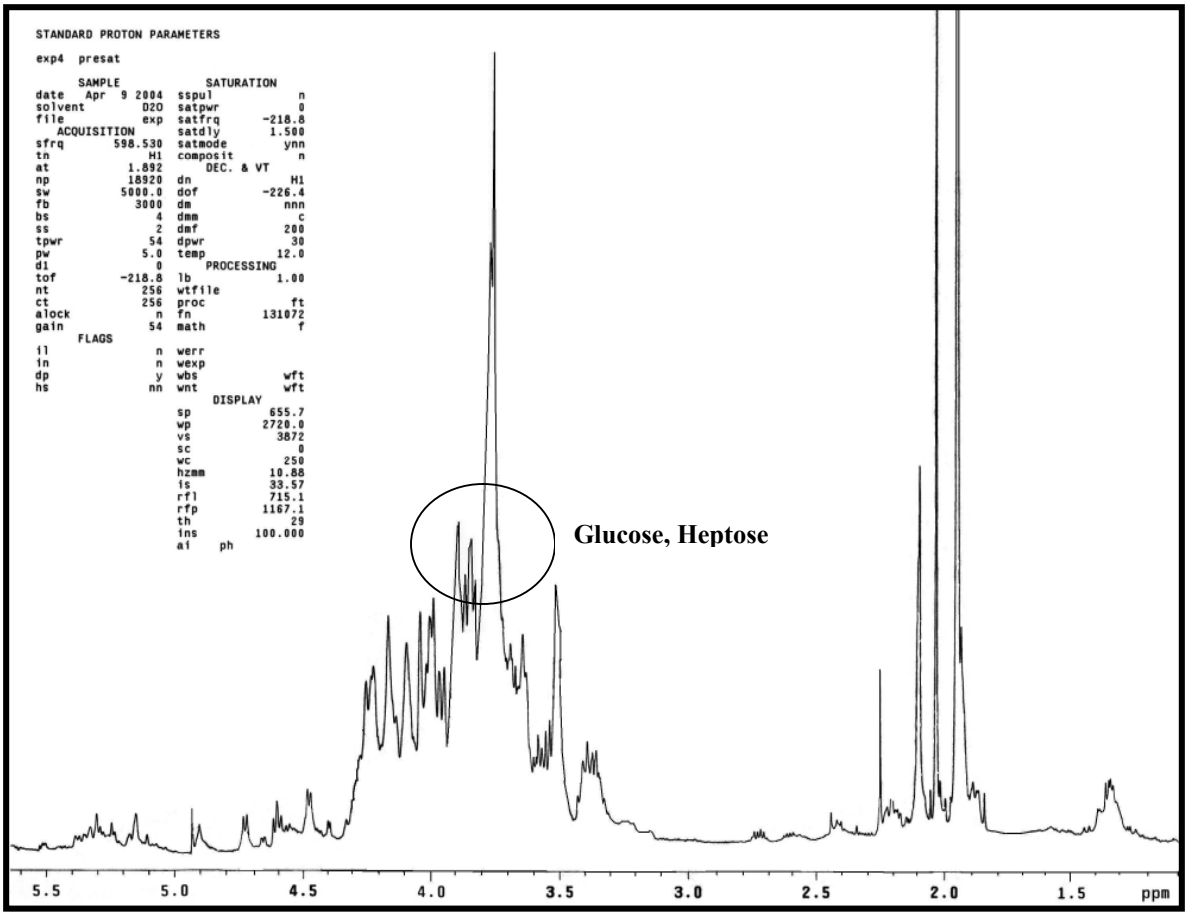


Fig. 5- Fractions of multimeric crude F1 antigen purified by HPLC obtained in void volume (first peak at 25 minutes) *(courtesy R. Kovi)*

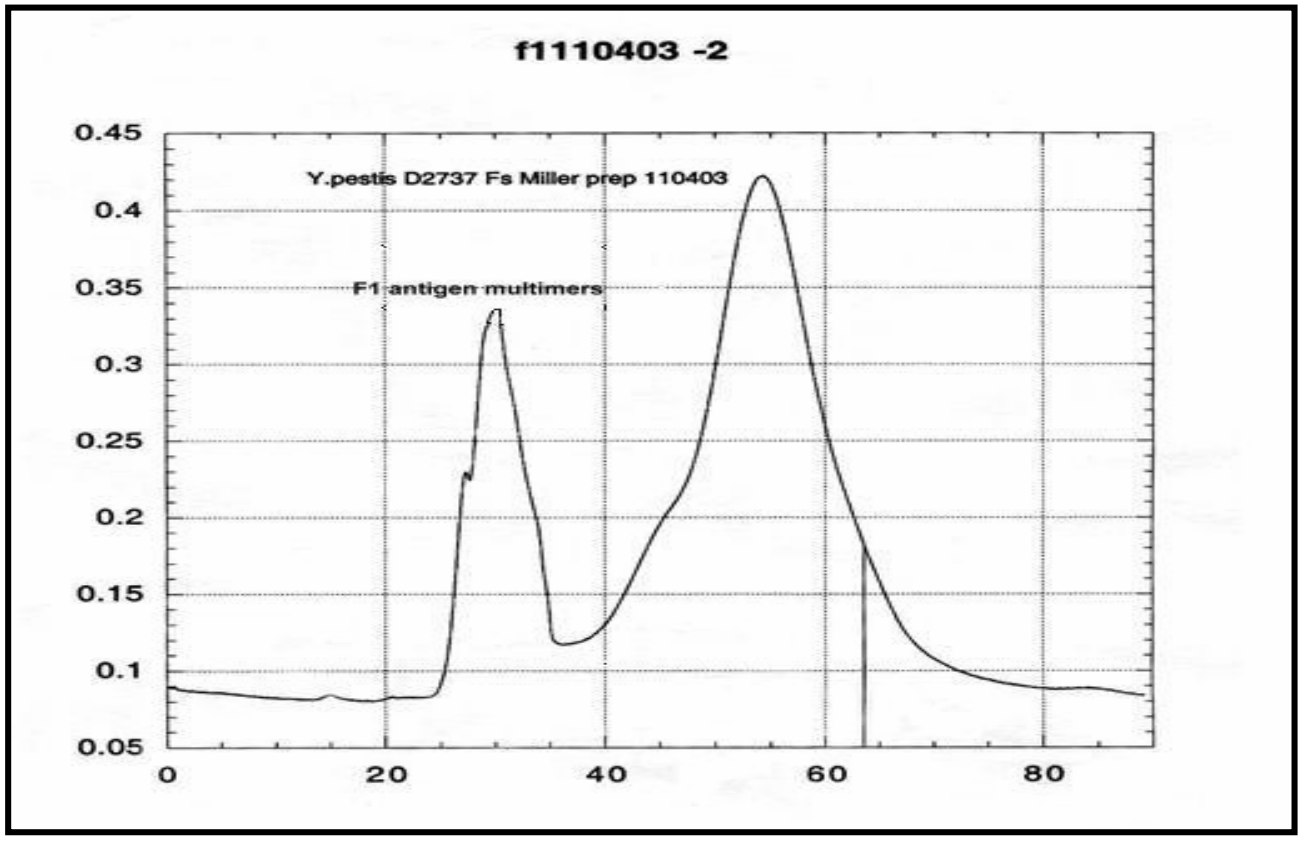
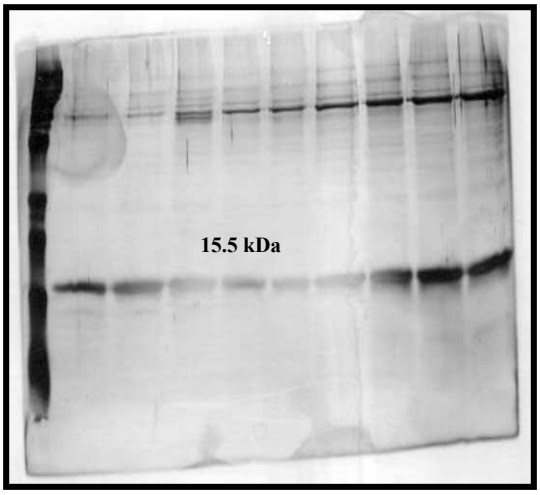


Fig. 6a- 15% SDS-PAGE gel of F1 antigen of *Y. pestis* KIM6, M_r 15.5 kDa

Fig. 6b- Western blot of 15.5 kDa F1 antigen of *Y. pestis (courtesy R. Kovi)*



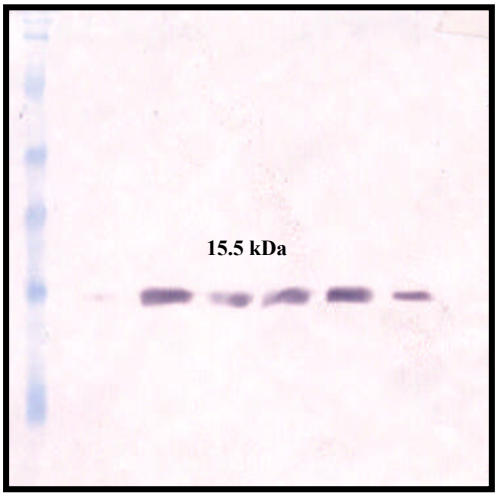


Fig. 7- PMF of F1 antigen reveals greater than 95% homology to known peptides in the database, indicating with high confidence that the target is purified F1 antigen *(courtesy R. Kovi)*

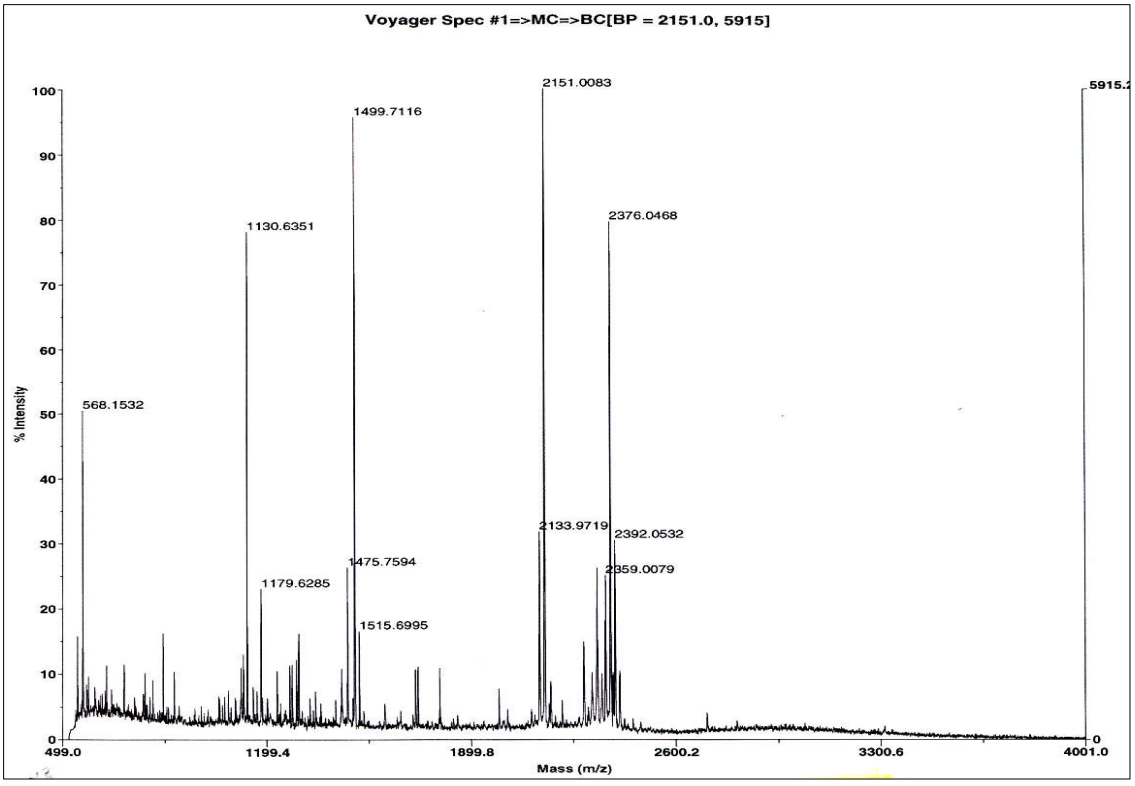


Fig. 8- Schematic depiction of electrodialysis technique with F1 antigen and bound and unbound oligonucleotides

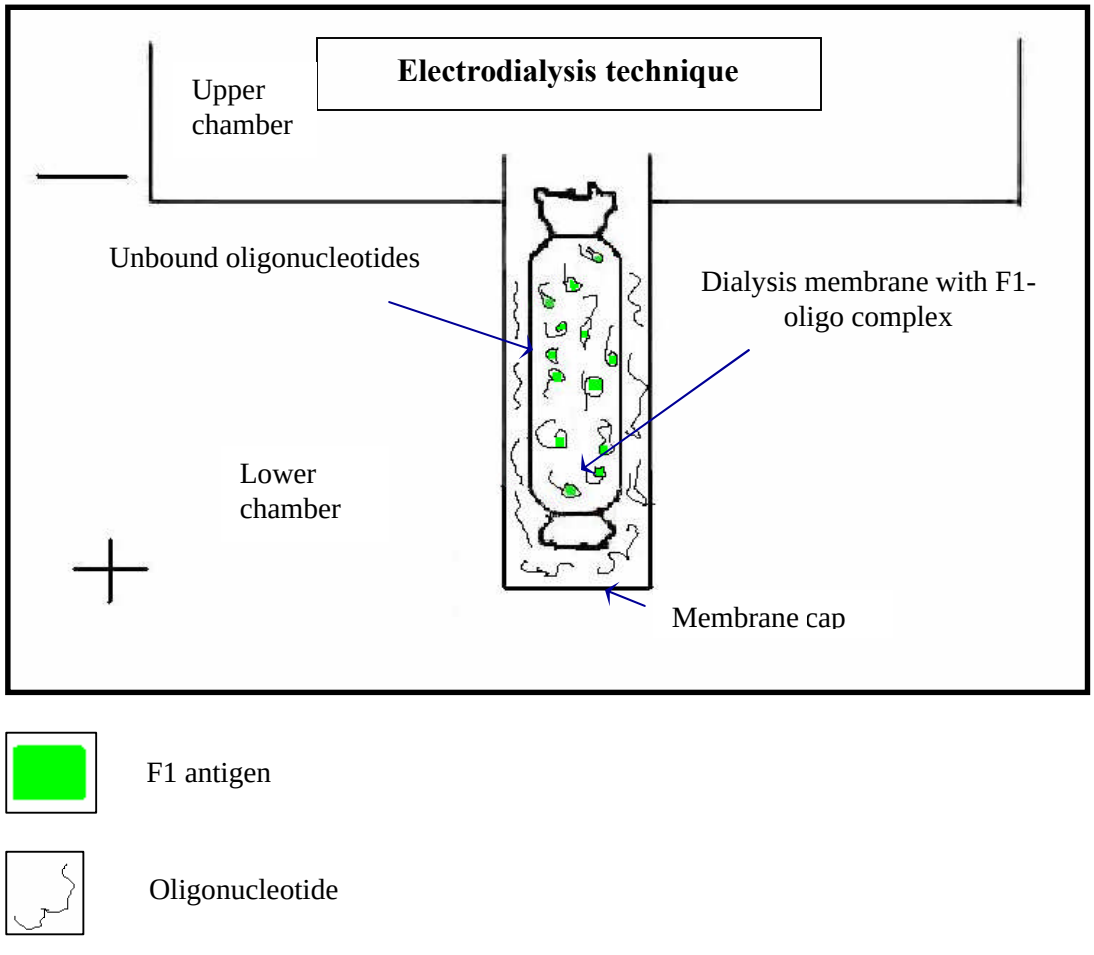


Fig. 9- 1 µl volumes of samples within the dialysis membrane, dialysis tube, upper and lower chambers and membrane cap were collected at 0, 30, 60, 90 and 120 minute intervals and measured on a scintillation counter

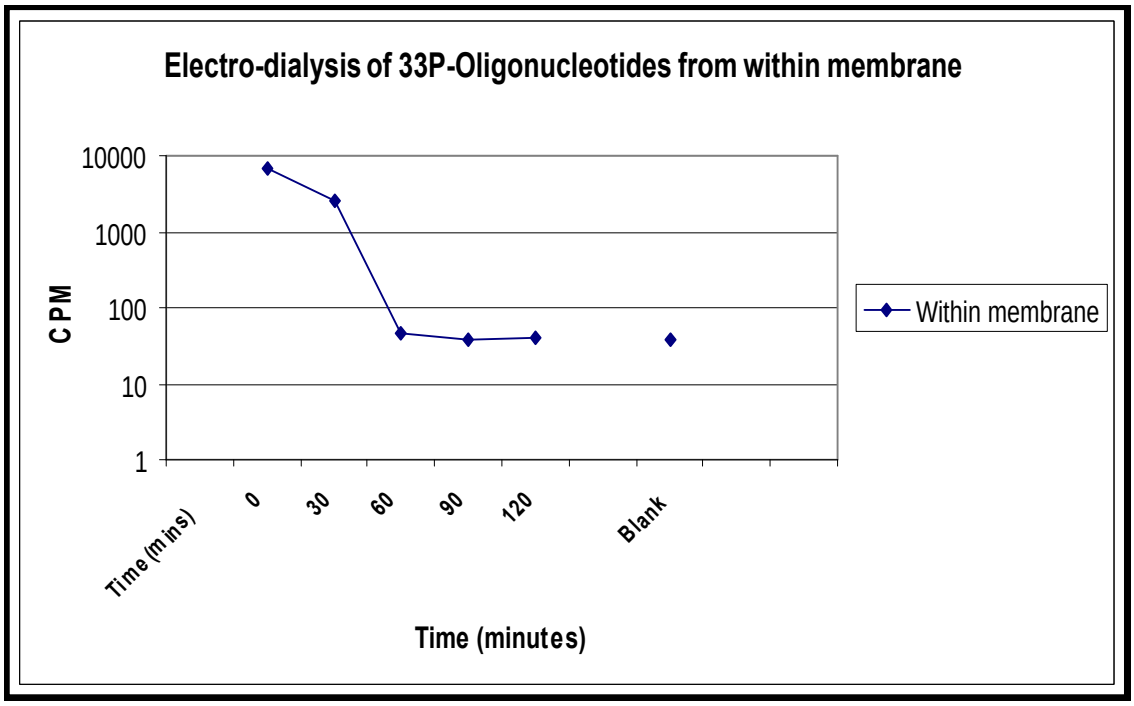


Fig. 10- TCA precipitation of F1 antigen within the dialysis membrane after electrodialysis and electrophoresing on SDS-PAGE gel

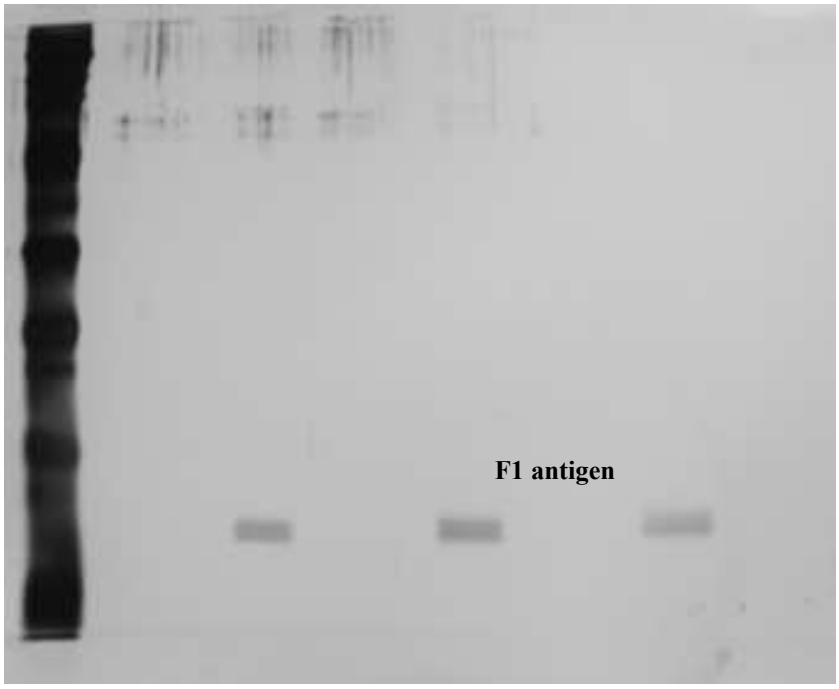
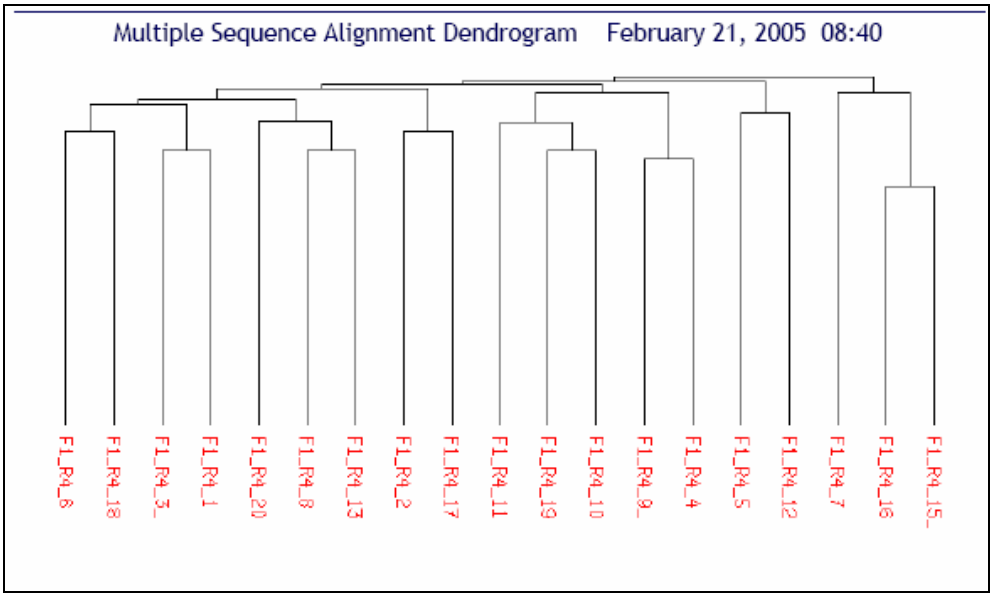


Fig. 11- Sequences of Round 4 clones isolated after cloning using pCR® TOPO II vector and chemically competent *E. coli* cells

Fig. 12a- Dendrogram indicating homology of Round 4 sequences

Below **Fig. 12b**- Sequence alignment by Pretty software indicating consensus regions




```
F1_R4_15_  ~~CCCTAGGT AACGTGCGAT GGCTTGGGAG GG~~~~~~~~~ ~~~
 F1_R4_16  ~~CTTTGTGT TAATTGCCAT TGAATGGTAT GG~~~~~~~~~ ~~~
  F1_R4_7  ~~~~~~~~~GC TACAGGGGAT GGCGTGACGT CCTAGGC~~~ ~~~
   F1_R4_12 ~~~~~~~~ACG TACCTGGTTA AACGACCGTT AGGGTAG~~~ ~~~
    F1_R4_5  ~~~~~~~~~~ GGGGTGCTTA AAGAAAAGAT GGGCCACTTT C~~
    F1_R4_4  ~~~~~~TCTG CGCCGGTAAA TACTTGTTAT CGCGGC~~~~ ~~~
   F1_R4_9_  ~~~~~CGCGC TGCTGGTAGC TAATTTATGT AGTG~~~~~~ ~~~
   F1_R4_10  ~~~~TTTGGA GGCTAGTGGC AGCGGACTTG TTGGCTTGA~ ~~~
   F1_R4_19  ~~~~~~~~GG CGCATGTTCC AGCTGAGACA GCGGTTGG~~ ~~~
   F1_R4_11  ~~~~~~~~GG CGGTTGTTCG TGCACCCGGT AACGGTGT~~ ~~~
   F1_R4_17  ~~TGTCTGCA TACCAGGGTG CGTAAGTGGA GC~~~~~~~~ ~~~
    F1_R4_2  TAGAAGTGTA TACTAGTGTA TTGAGCTGGC ~~~~~~~~~~ ~~~
   F1_R4_13  ~~~~~~GGTA ACGATAGCAG AGTCTGAGGC GGTG~~~~~~ ~~~
    F1_R4_8  ~~~~~~~GAA GACATGCCTG CTCTTTAGGC GCTGCCTTGA ~~~
   F1_R4_20  ~~~~~~~~~~ ~~~AAGGGAA CGATTAAGGT GTTGATTGGA TGT
    F1_R4_1  ~~~~~~~~~G TTCAAGGGTG ACCTTATGGC CGCGATGTC~ ~~~
   F1_R4_3_  ~~~~~~~~~~ ~~~TAGGTTC ACCATGGGTC TGATATGGCC GTG
   F1_R4_18  ~TGGTTTGGG GTCTGTGCCG GCCTTGTGTT GCTTGAC~~~ ~~~
    F1_R4_6  ~~~~~~~~~~ ~~~AGTGTCC ACTTAGTGTA GCCTAAGCAT GAG
```

Fig. 13- SDS- PAGE of Rounds 1-4 of SELEX after silver staining. Lane 1 (far right)- Molecular marker, Lane 2- PCR product of 1 nM oligonucleotides in the absence of F1 antigen, Lane 3- PCR product of Round 1 SELEX, Lane 4- PCR product of Round 2 SELEX, Lane 5- PCR product of Round 3 SELEX, Lane 6- PCR product of Round 4 SELEX (note the presence of low molecular weight bands), Lane 7- F1 antigen complex with primary monoclonal anti F1 antibody and protein G, Lane 8- F1 antigen, Lane 9- Amplification of PCR product without oligonucleotides.

9 8 7 6 5 4 3 2 1

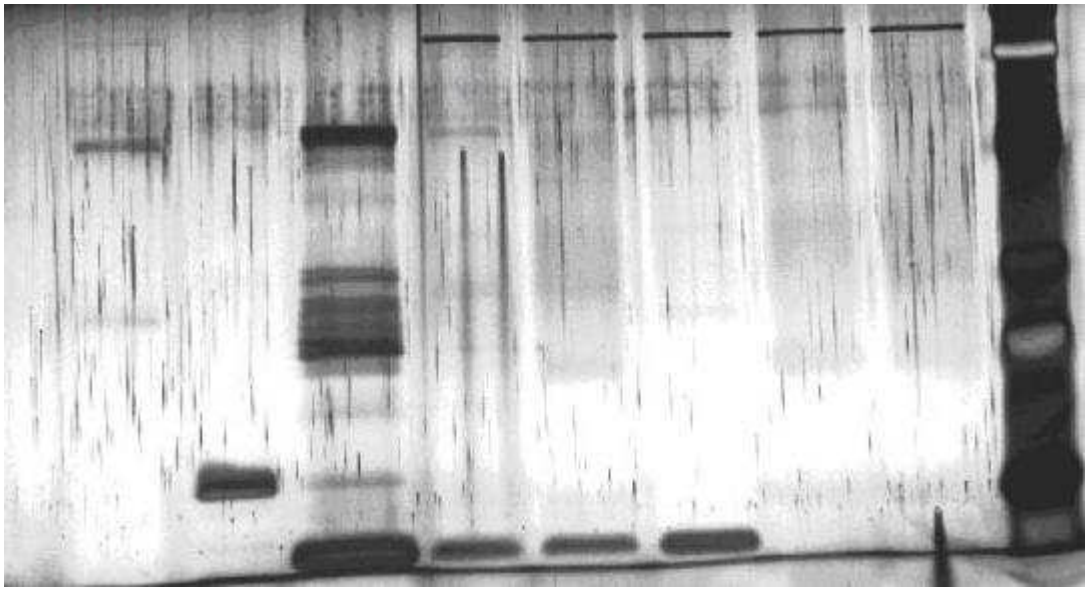


Fig. 14- PCR product of Round 3 SELEX that was amplified after incubating with streptavidin coated Dynal beads on compared to ten fold dilutions of streptavidin coated Dynal beads.

Lane 1 (far left)- molecular marker, Lane 2- PCR product of Round 3 SELEX amplified of streptavidin beads, Lanes 3-5- Ten fold dilutions of PCR product of Round 3, Lane 6- 15.5 kDa product of streptavidin, Lane 7- Streptavidin coated Dynal beads, Lanes 8-10- Ten fold dilutions of streptavidin coated Dynal beads.

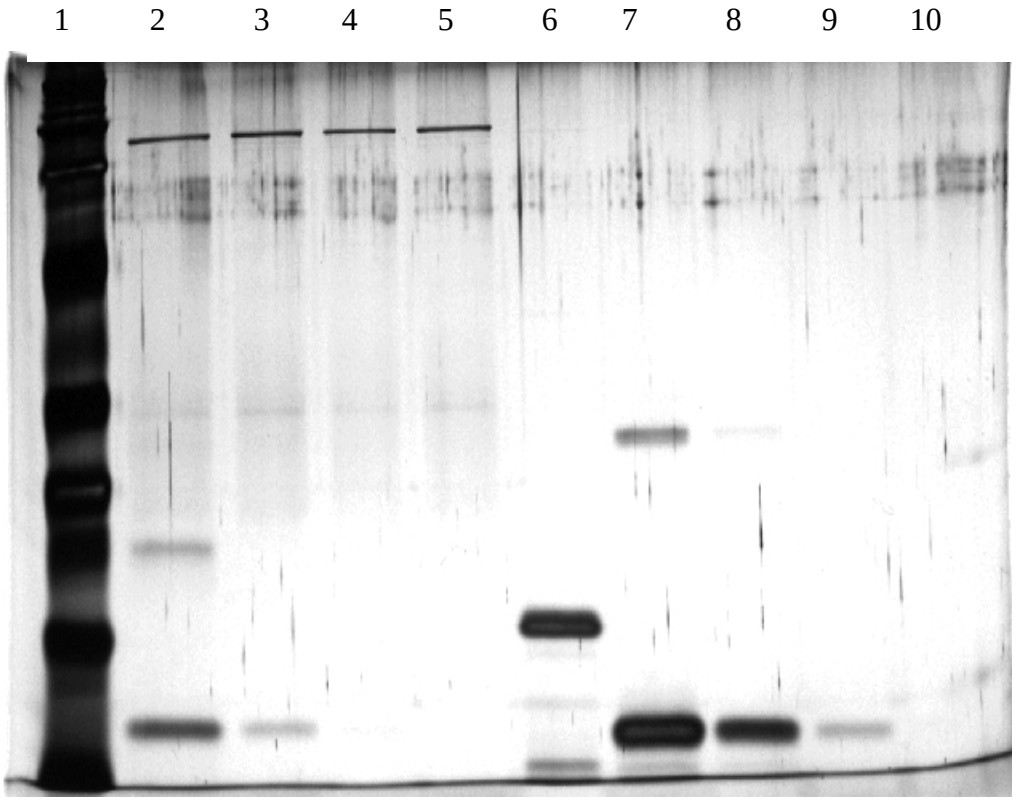


Fig. 15- Analysis of Round 4 sequences by multiple sequence alignment dendrogram showing a reduction in pool complexity

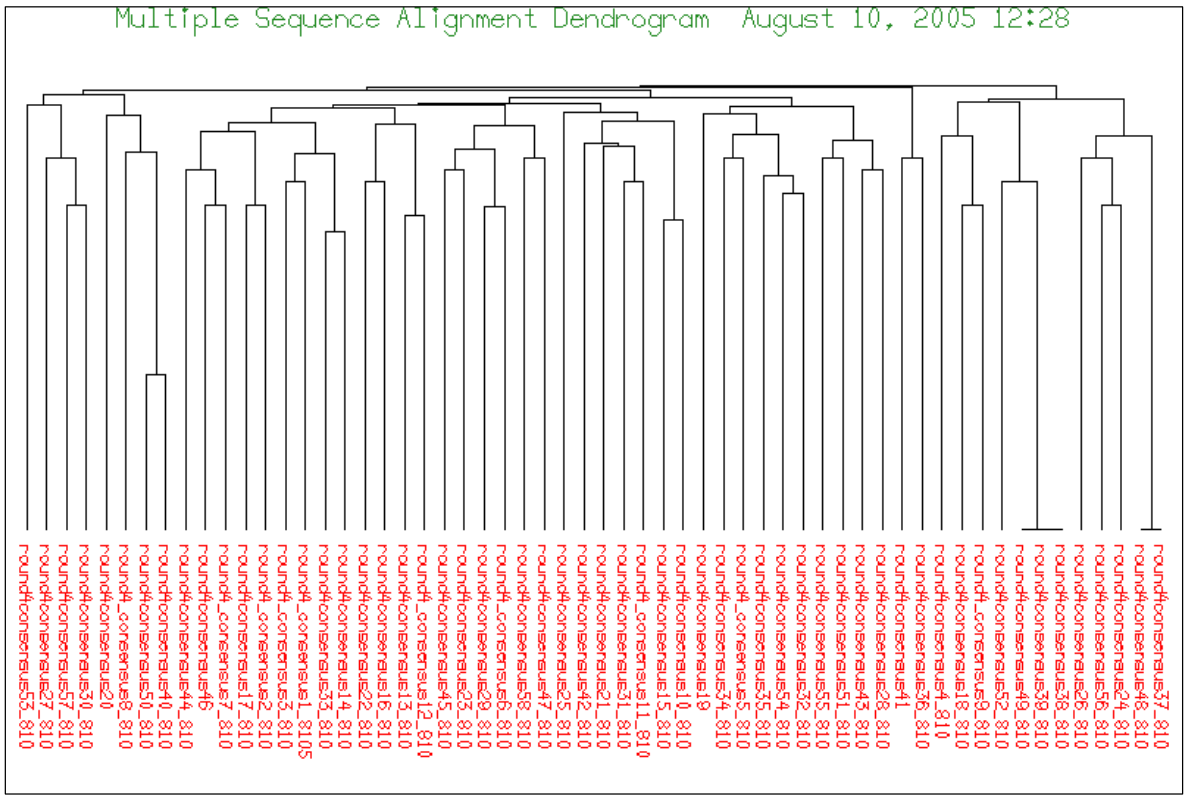
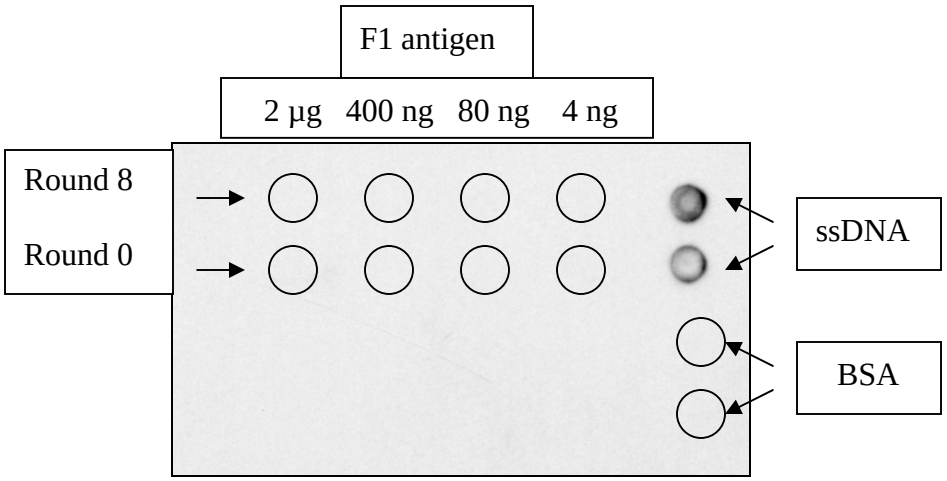
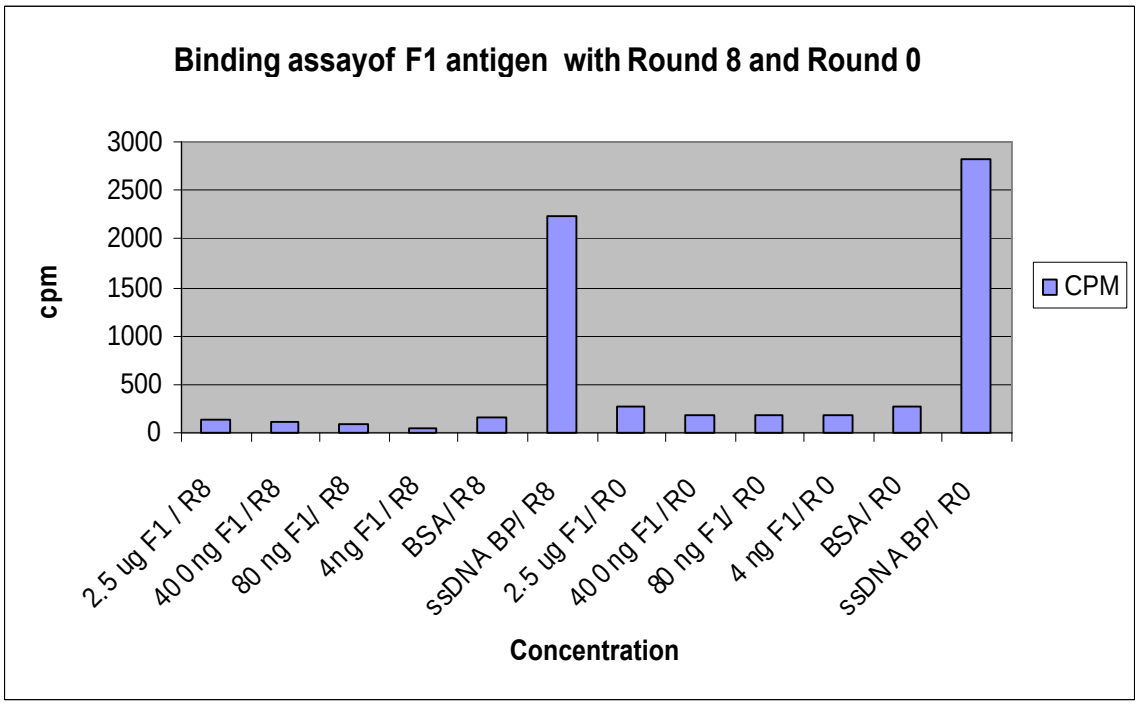


Fig. 16- Round 8 sequences showing homologous sequences indicative of potential aptamer sequences.

Fig. 17a- Autoradiograph obtained 24 hours after incubating F1 antigen immobilized on nitrocellulose membrane with radiolabeled Round 8 and Round 0 pool.

Fig. 17b- Quantitation of binding assay





VITA

Pravina P Fernandez

Candidate for the Degree of

Master of Science

Thesis: SELECTION OF AN APTAMER AGAINST SURFACE EXPOSED TARGETS ON *YERSINIA PESTIS*

Major Field: Veterinary Biomedical Sciences

Biographical:

Personal Data: Born in Bombay, India on June 10, 1979, daughter of Peter A Fernandez and Leonora P Fernandez.

Education: Graduated as a Bachelor's in Veterinary Science and Animal Husbandry at the Bombay Veterinary College, Bombay, India in Sept, 2002.

Graduated with a M.S. in Veterinary Biomedical Sciences at Oklahoma State University, Stillwater, OK in May, 2006.

Experience: Worked as a part- time veterinarian at the animal clinic run by Dr SV Shah, Bombay, India. Volunteer at the Bombay Zoo and Society of Prevention to Cruelty to Animals (SPCA)

Professional Memberships: ASM (2006).

Name: Pravina P Fernandez Date of Degree: May, 2006

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: SELECTION OF AN APTAMER AGAINST SURFACE EXPOSED TARGETS ON *YERSINIA PESTIS*

Pages in Study: 121 Candidate for the Degree of Master of Sciences

Major Field: Veterinary Biomedical Sciences

Scope and Method of Study: *Yersinia pestis* is a CDC 'category A' bioterrorism agent. This study attempts to select an aptamer to detect the presence of LOS and F1 antigen on *Y. pestis*. Aptamer selection by Systematic Evolution of Ligand by Exponential Enrichment is an iterative process of binding, partitioning, amplification and strand separation.

Findings and Conclusions: The Lipid A as well as the core polysaccharide of LOS was found unfavorable for aptamer binding. Purified F1 antigen was considered a suitable target for aptamer selection. Our partitioning method using electrodialysis and eight rounds of selection resulted in thirty nine clones from Round 8 with a consensus motif –GTGAG—GTTG--. However, binding assays were unsuccessful due to non-specificity of Round 8 to F1 antigen, and and hence additional four rounds of SELEX are being carried out. The aptamer thus selected might be used in *in vitro* diagnostic assays to detect F1 antigen of *Yersinia pestis*.

ADVISER'S APPROVAL: Dr Kenneth D. Clinkenbeard