

**IDENTIFICATION OF A VACCINE CANDIDATE IN
PROTEIN EXTRACTS FROM *FRANCISELLA TULARENSIS***

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Dedication

To my family: Joseph and Margaret Sikora, and my brother Gregory. Thanks for putting up with me and my antics. You have been a pleasant constant throughout my life. Thanks for supporting me when I decided to go where I wanted. I won't be finished for a while.

To John Cherwonogrodzky (aka Jonny Fever). Your excitement in research has sparked an interest in bacteriology which I have brought to my medical studies and hope to continue throughout my life.

ABSTRACT

Francisella tularensis is one of a small group of bacteria recognized for their virulence and potential for use as biological weapons. In this study we utilize a novel approach to identify an immunologically prominent component of *F. tularensis* that appears to be a promising vaccine candidate. *Francisella* is an intracellular pathogen that infects cells of the reticuloendothelial system. Other bacteria, such as *Brucella* spp. have this part of their life cycle in common. However, while mice injected with *F. tularensis* all die within three weeks of infection, mice injected with *Brucella* spp. survive and produce antibodies to the bacteria which are immunologically reactive not only with *Brucella* spp. but, also with *Francisella*. When we vaccinated mice with a *B. abortus* O-linked polysaccharide (OPS) and then challenged them with 10 LD₅₀ *F. tularensis* LVS, 60% survived. Sera from *Brucella* OPS-primed / *F. tularensis*-challenged mice was used to identify immune reactive proteins from *F. tularensis*. A novel 52 kDa fraction was identified. While vaccination of mice with this partially purified fraction only provided 20% protection to *F. tularensis* challenged mice, both whole cell extracts and a partially purified soluble fraction (>30 kDa) given to *Brucella*-vaccinated mice were 100% protective. The 52 kDa enriched fraction elicited a rudimentary cytokine burst of nitric oxide in a cell culture of J774.1 macrophages. The 52 kDa fraction was degraded by proteinase K and appeared to decrease in size to 36 kDa in the presence of DNAase, suggesting a possible protein and nucleic acid composition. The host response to *F. tularensis* infection is complex, but given the ability of the 52 kDa component to protect

against live vaccine challenge, and its apparent ability to elicit a cytokine burst, this component may have potential use in future vaccine production.

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Abbreviations Used

AcpA	Acid Phosphatase A
BKO	B-cell Knock-Out (mice)
CSM	Chamberlain's Synthetic Media
DNA	Deoxyribonucleic Acid
DND	Department of National Defence
DRES	Defence Research Establishment, Suffield
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked ImmunoSorbent Assay
FPLC	Fast Performance Liquid Chromatography
GCA	Glucose Cysteine Agar
HEPA	High Efficiency Particulate Air (filter)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (buffer)
HlyA	Hemolysin
HRP	Horseradish peroxidase
IFN- γ	Gamma Interferon
IN	Intranasal
IP	Intraperitoneal
J774.1	Murine Macrophage Cell line No. J774.1
KCl	Potassium Chloride
kDa	Kilodaltons
LPS	Lipopolysaccharide
LVS	Live Vaccine Strain
mMEM	Modified Minimum Essential Medium
MW	Molecular Weight
MWCO	Molecular Weight Cut Off (membrane)
NK	Natural Killer (cells)
NO	Nitric Oxide
OPS	O-linked Polysaccharide
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
SDS	Sodium Dodecyl Sulfate
TNF- α	Tumor Necrosis Factor alpha

1.0 Introduction

In 1970, the World Health Organization (WHO group of consultants, 1970) identified *Yersinia pestis*, *Bacillus anthracis*, and *Francisella tularensis*, the causative agents of plague, Anthrax and Tularemia, respectively, as bacterial candidates most likely to be used as biological warfare weapons. Following September 11, 2001 whereby American civilian targets were exposed, infected, and in some cases died from Anthrax, the biological threat has become very real and very near. Although the focus of public concern has been on Anthrax, it should be noted that it takes about 5,000 to 12,000 spores of *Bacillus anthracis* to cause human infection (DIA, 1986), whereas only 1 to 10 cells of *F. tularensis* are needed to cause Tularemia (Cross, *et al.*, 2000). This high virulence alone makes *F. tularensis* a likely organism to be used as a weapon of terror.

F. tularensis can be readily grown on agar or in broth culture medium, harvested, and disseminated. Within a human host, its incubation period can range from one to 14 days (Dennis, *et al.*, 2001). Once infected into a population, it may confound primary care physicians who may struggle to identify the unusual agent causing severe “flu-like” symptoms which can be associated with a high rate of morbidity and mortality. A covert attack on a population would require enhanced epidemiological skills of primary care physicians to investigate and correctly identify potentially rare, or unusual disease causing organisms (Christopher, *et al.*, 1997). While response to late diagnosis can be aggressive antibiotic therapy, these treatments are not always given in time to cure infected individuals. It is because of this fact that *F. tularensis* is listed high on threat lists of biological weapons by committees

and countries around the world (The Biological and Toxins Weapons Convention, Geneva, Switzerland, 1971 to present).

A key element for defense against use of *F. tularensis* as a bioterrorist weapon is access to an effective and safe vaccine. Unfortunately, the only available vaccine to Tularemia has recently been suspended. In the following study, a vaccine candidate for *F. tularensis* is successfully identified. Our production technique and assays describing the composition and activities of the component identified are described. A description of the discovery and significance of Tularemia, where it is found, the bacterium and its infection into humans, methods of detection, treatment and research towards finding a suitable vaccine are described. Although much has been accomplished, there is much more to do and it is hoped that the novel approaches identified in this work will assist the scientific community in developing new measures counteracting the bioterrorist threat.

1.1 Discovery of *Francisella tularensis*

In the early portion of the 20th century, various researchers described a small bacterium that caused a plague-like disease in rodents. George McCoy, a physician in the Tulare County of California, first described the disease in detail (McCoy and Chapin, 1912a and 1912b). At this time, the agent became known as *Bacterium tularense*. Shortly after, Edward Francis described the same disease in humans and labeled the collection of symptoms Tularemia. He suggested that insect vectors were responsible for transmission of the disease between rodents and humans (Francis, 1921) and was able to successfully isolate the organism from serum.

Francis himself was infected with *F. tularensis* three times and eventually died from Tularemia (Tarnvik, *et al.*, 1997). The bacterium eventually was named in his honor.

A disease similar to Tularemia also was described in Japan by Ohara (Ohara, 1926). Ohara infected his wife and was able to recover an organism similar to *F. tularensis* from a dissected lymph node, a clear demonstration of Koch's postulates. Francis later confirmed the identity of Ohara's plague-like disease organism as *F. tularensis* (Francis and Moore, 1926).

1.2 Significance of Tularemia

While worldwide prevalence of Tularemia is decreasing, it is still an important pathogen in some regions. The number of reported cases of Tularemia in the United States in 1994 totaled 96 (Koo, *et al.* 1994). In Japan, the incidence of Tularemia peaked in the two decades following World War II (Ohara, *et al.*, 1996) with a total of 1372 cases being reported since 1924. A large proportion (93%) of the cases in Japan resulted from infection of persons handling diseased rabbits; few cases were the result of tick bites. In Sweden, over five thousand cases have been reported throughout the past seventy years (Tarnvik, *et al.*, 1996). Many of these cases have been linked to a cycling population of voles. Because *F. tularensis* can persist in cold water for months at a time without losing its ability to infect host organisms, it poses a concern in colder climates. In south western Canada, a recent survey showed that antibodies to *F. tularensis* can be detected in serum collected from rural dogs and cats, indicating that the bacterium is present in the wild and is a potential threat to humans (Leighton, *et al.*, 2001).

1.3 Epidemiology and Taxonomy of Tularemia

F. tularensis primarily is distributed throughout the northern hemisphere. Cases have been reported in Japan, Russia, Sweden, Spain and the United States (Ohara, *et al.*, 1996; Tarnvik, *et al.*, 1996). Its distribution across continents has resulted in further classification of the species into subspecies *tularensis* and *palaeartica*, which are distributed mainly into North America and Eurasia, respectively (Olsufiev, *et al.*, 1959). *F. tularensis* biovar *tularensis* is also classified as Jellison Type A, whereas biovar *palaeartica* is classified as Jellison Type B. This classification was done to describe differences in virulence and natural reservoirs of the disease. The North American *F. tularensis* biovar *tularensis* is more prevalent worldwide. This biovar is highly virulent to mammals and most commonly infects lagomorphs (rabbits and hares). Other natural hosts include various rodents (voles, squirrels, muskrats, beavers) and arthropods (Cross and Penn, 2000). In North America, a common mode of transmission to humans is via ticks (Jellison, *et al.*, 1974) such as the common wood tick, *Demacentor andersoni* (Cross and Penn, 2000). Handling of infected animals can lead to infection. Infection with *F. tularensis* biovar *tularensis* is associated with a 5% mortality (Doan-Wiggins, 1991). *F. tularensis* biovar *palaeartica* is found mainly in Asia and Europe. This bacterium is lower in virulence than the North American strain but, in addition to being isolated from rabbits, it also has been found in water (Jellison, 1974) and can be easily aerosolized (Dennis, *et al.*, 2001), making it a potentially dangerous bioweapon. Current live vaccine protocols for Tularemia have been developed using attenuated *F. tularensis* biovar *palaeartica*.

In addition to biovars *tularensis* and *palaeartica*, *F. tularensis* biovar *novicida* and the separate species, *F. philomiragia* also have been identified (Forsman, *et al.*, 1994). Both have low virulence for mammals as well as a different cellular morphology than *F. tularensis* biovar *tularensis* but are linked to the genus *Francisella* by similarities in their genetic code. *F. tularensis* biovar *novicida* has only been successfully recovered from a Utah Water source (Larson *et al.* 1955). Based on 16S RNA sequencing, *F. tularensis* appears to be closely related to a number of water-borne bacteria such as *Piscirickettsia*, *Cycloclasticus* and *Caedibacter* (Titball, *et al.*, 2003). In terms of human pathogens, the most closely related organisms were *Coxiella burnetti* and *Legionella* spp.

1.4 Microbiology and Virulence Factors of *Francisella tularensis*

F. tularensis, the causative agent of Tularemia, is a Gram-negative, rod-shaped bacterium measuring 0.2 x 0.7 μm (Eigelsbach and McGann, 1984; Saslaw, *et al.*, 1961). It is a nonmotile, obligate aerobe. Culture requirements for *Francisella* are more stringent than for other bacteria. It is a slow growing bacterium and successful culture of the organism often requires glucose-cysteine agar (GCA) with thiamine or cystine heart agar, but peptone cystine broth and a chemically defined medium also have been used (Chamberlain, 1965). Small, gray colonies surrounded by a green zone of discoloration are typically produced after 2-3 days of growth on GCA. The double membrane of *F. tularensis* has a high lipid content (50-70%) and contains a large fraction of saturated C20 to C26 fatty acids (Hood, 1977; Eigelsbach and McGann, 1984). *F. tularensis* is capable of producing human disease with transmission of as

little as 10 organisms (Cross and Penn, 2000). As a result, extreme caution is required in the laboratory, often requiring biohazard level three protocols.

The capsule of *F. tularensis* is thought to be a requirement for virulence. Hood (1977) discovered that loss of virulence was associated with loss of the capsule. However, Cherwonogrodzky *et al.* (1994) observed that culture of an avirulent strain of *F. tularensis* on synthetic medium allowed the bacteria to regain virulence. Carbohydrates present in the capsule appear to be antigenic and responsible for imparting humoral immunity in vaccinated individuals (Tarnvik, 1989) while cell surface proteins are thought to be responsible for conferring long-term cellular immunity. The role of carbohydrates and cell surface proteins in the pathogenesis of *F. tularensis* infection has not been thoroughly investigated.

Recently, some novel research has investigated the apparent lack of hemolytic activity possessed by *F. tularensis*. Lai *et al.* (2003) discovered that *F. novicida* produces a hemolysin, defined as novilysin. They proved, through solid and liquid culture assays, that *F. novicida* produces a hemolysin of fairly low activity. However, novilysin was only identified in *F. novicida*, not *F. tularensis*, indicating the need for further research in this area. Moreover, this novilysin cross reacts with *E. coli* hemolysin HlyA. The same research group identified homology between the *F. tularensis* acid phosphatase A (AcpA) and a *Pseudomonas aeruginosa* hemolytic phospholipase C. The homology between *Francisella* and other pathogenic organisms suggests that there is a great deal of cross reactivity between seemingly distant organisms. Also, it does not preclude the sharing of similar virulence factors amongst seemingly dissimilar organisms. If hemolysin is common and cross reactive with both

Francisella and *Eschericia*, there may be similarities with other virulence determinants.

Francisella appears to contain within its cellular machinery components which are similar, but in low activity, to other organisms. It may be possible to protect a host against *Francisella* challenge by infecting it with a similar, but related organism.

1.5 The infection and host responses

Tularemia can classically cause a cluster of six different clinical manifestations: ulceroglandular, glandular, oculoglandular, oropharyngeal, typhoidal and pneumonic disease (Evans *et al.*, 1985; Cross and Penn, 2000). Fever and lymphadenopathy are features common to most infections. While the host incubation period for infection averages 3 to 5 days, it can range from 1 to 14 days and extend up to 21 days (Sanders and Hahn, 1968).

Ulceroglandular disease is found in up to 80% of clinical situations and is usually the result of tick bites, animal bites or handling of infected carcasses. An ulcer typically forms at the site of initial infection and eventually progresses to form a black eschar. Tender lymphadenopathy in the region draining the infection site occurs with this type of infection. Often, the instigating organism can be cultured from a lymph node aspirate or biopsy (Ohara, 1926). Glandular disease occurs with the absence of a cutaneous lesion, but requires the presence of infected lymph nodes. Oculoglandular disease is often the result of the patient inoculating the conjunctiva with contaminated digits. Conjunctivitis with preauricular lymphadenopathy and periorbital edema is a feature of this type of infection. Oropharyngeal Tularemia occurs with ingestion of contaminated substances (Jansen, *et al.*, 2003). An

exudative pharyngitis and cervical lymphadenopathy is usually present which may progress to ulceration of the oropharynx. Typhoidal Tularemia is a systemic form of the disease. The patient appears toxic and may exhibit septic shock. Significant outcomes may include rhabdomyolysis, disseminated intravascular coagulopathy and secondary renal failure. The pneumonic form of the disease can occur from hematologic spread from a distant infection site, or from inhalation of vegetative cells. Tularemic pneumonia, a true colonization of the lungs, presents with a triad of ovoid opacities, pleural effusions and hilar adenopathy (Choi, 2002) and should be considered when a community acquired pulmonary infection does not respond well to conventional β -lactam antibiotics.

1.6 Identification of *Francisella tularensis*

Diagnosis of *F. tularensis* infection is difficult for investigators. The organism is difficult to culture from blood and requires special media supplemented with cysteine to grow. The danger of aerosolization is a constant hazard to laboratory workers (Shapiro, *et al.*, 2002) as during the initial plating procedure, the organism can be confused for a *Haemophilus* species. Both laboratory personnel as well as pathology staff are in danger of exposure, since *Harmophilus* is routinely handled in a Level 1, or benchtop, environment. *Francisella*, when identified, requires the use of a BioSafety cabinet and a Level 2 work environment. Regardless, current protocols using serum agglutination tests indicate that a single titre greater than 1:160 or a four-fold rise in serum titre is indicative of *F. tularensis* infection (Evans, *et al.*, 1985). Often obtaining a detailed history to ascertain exposure to the organism and a low

threshold of suspicion is necessary to facilitate accurate diagnosis.

Grunow, *et al.* (2000) were successful in developing environmental detection systems for *F. tularensis*. These systems use either PCR, targeting sequences of the 17 kDa TULA gene, or ELISA using monoclonal antibodies. The systems are capable of detecting between 10^2 and 10^4 bacteria/mL of serum. Highly specific detection systems, such as these, are powerful tools in the early and rapid detection of *Francisella* in both the environment and in human patients.

1.7 Methods for treating Tularemia

Diagnosis and treatment of *F. tularensis* can be difficult. The bacterium is classified as an intracellular pathogen because of its ability to reside within host cells. Because of this property, choice of antibiotics is limited. The current antibiotic of choice is streptomycin (Enderlin, *et al.*, 1994), but gentamicin, tetracycline and chloramphenicol can be used as alternatives. Streptomycin has the best cure rate with few relapses, whereas gentamicin and the other aminoglycosides have been shown to have poor bioavailability for intracellular pathogens and abscesses. Fluoroquinolones, such as ciprofloxacin and levofloxacin, also have been used with some success. Cephalosporins are a poor choice of antibiotic and have resulted in therapeutic failures (Choi, 2002).

1.8 Immune Modulation - Vaccine development

The intracellular nature of Tularemia has challenged scientists in creating a safe, and effective vaccine. In the disease process, the bacterium invades macrophages and epithelioid cells (Cross and Penn, 2000). Often the liver and spleen are the first organs targeted (Conlan, *et al.*, 2003). Unless respiratory inoculation took place, the lungs are colonized after the reticuloendothelial system. An immune response is elicited and granulomas are formed. Humoral immunity develops towards carbohydrate elements of the bacterium, but this is not sufficient to clear the disease. Cell-mediated immunity, utilizing a T-cell response against surface antigens, is necessary to cure the host (Tarnvik, 1989). Once the host is cured, it is thought that immunity is sufficient for a lifetime of protection against Tularemia, although examples to the contrary are present.

Current vaccine protocols utilize a live vaccine strain (LVS) developed from *F. tularensis* biovar *palaeartica* from Russia. This LVS lacks a sufficient cell coat, or capsule, to protect itself from host defenses. A low-grade infection of *F. tularensis* results from LVS administration and sufficient cell-mediated immunity to protect the host is elicited. While it is thought that cell surface components are necessary for imparting long-term immunity, the exact role of T-cells and B-cells for protection against *F. tularensis* has not been fully explored.

The intracellular, parasitic nature of *F. tularensis*, and host response to this infection, has been the focus of recent research in this area. Hood (1977) and Skrodzki (1968) have suggested that toxic components of *F. tularensis* differ from those of *Salmonella* and *Shigella*, which are often of the classical two-part toxin class of protein toxins. Their research indicates

that toxic components from *F. tularensis* possess very little toxicity and are thermolabile (Skrodzki, 1968). Contemporary research has so far been unsuccessful in isolating a specific virulence factor which explains the rather high lethality of *F. tularensis*.

Further work by Sandstrom *et al.* (1992) has shown that the lipopolysaccharide (LPS) classical endotoxin component of *F. tulatensis* LVS, which is analogous to the endotoxins from other gram negative bacteria such as *Eschericia coli*, possesses very little toxicity. LPS from *F. tularensis* LVS failed to stimulate lymphocytes in primed individuals, had no lethal activity in galactosamine-sensitized (LPS sensitive) mice and failed to activate the Limulus amoebocyte lysate assay for LPS (Sandstrom *et al.*, 1992). It does not act as a pyrogen (Guss, 1970) and is 1000 times less lethal than LPS from *Escherichia coli*. LPS from *F. tularensis* LVS does not induce tumor necrosis factor alpha or nitric oxide in animal cells (Ancuta, *et al.*, 1996). In some pathogen/host model systems, it has been suggested that LPS modulates humoral immunity, triggering the production of antibodies in infected individuals. However, for *F. tularensis*, components other than the LPS appear necessary for the production of the cellular immunity which eventually clears the host of infection.

In a comparison between high and low-virulence strains of *F. tularensis*, investigators determined that immunization with LPS from *F. tularensis* LVS did not provide adequate protection against challenge from a highly virulent strain (Fulop, *et al.*, 2001). However, LPS and *F. tularensis* LVS in combination did protect against the high virulence strain. This protection was abolished when CD4+ and CD8+ cells were removed, indicating, once again, that a cell-mediated process is required for development of an immune response to infection

and that cellular components are necessary for this immunity. The authors suggest that other antigens, in addition to LPS, are required in the development of a successful vaccine for virulent *F. tularensis*.

The host immune response to *F. tularensis* infection is an interesting one. Lai *et al.* (2001) suggest, from work completed with J774.A1 murine macrophages, that colonization of these macrophages results in apoptosis. The host response of programmed cell death to infection may be a protective measure to expose the bacterium to a less hospitable environment, a mechanism that would specifically target intracellular pathogens such as *Francisella* (Kaufmann, 1999). Once within the intracellular environment, *F. tularensis* appears to possess a mechanism to counter the destructive effects of the polymorphonuclear neutrophils (PMNs). Reilly *et al.* (1996) characterized a novel acid phosphatase (AcpA) from *F. tularensis*, which suppresses the respiratory burst from PMNs. This AcpA was recovered both from cell lysates and from cell culture medium. It had an activity greater than any other acid phosphatase purified, at that time and appeared to be able to hydrolyze neutrophil-surface components responsible for triggering the respiratory burst associated with apoptosis (Reilly, *et al.*, 1996).

Mounting of an immune response to intracellular pathogens is a complex task. The cellular immunity, that is a requirement for long-term protection against intracellular pathogens, such as *F. tularensis*, is based on T-cells, B-cells and Natural Killer (NK) cells. Cytokines produced, such as Interferon Gamma (IFN- γ), tumor necrosis factor alpha (TNF- α) and nitric oxide (NO) serve as messengers between these immune components. Intraperitoneal (IP) and

intranasal (IN) injections of *F. tularensis* have an LD₅₀ approaching a single organism, in a susceptible murine line such as BALB/cByJ (Fortier, *et al.*, 1991), making mice a good *in vivo* model for studying the immune response to infection.

There have been a number of *in vivo* studies investigating the role of cellular and humoral immunity, in the form of T-cells and B-cells, and cytokines in controlling and clearing *Francisella* infection. It has been observed that mice lacking mature T-cells (nu/nu athymic mutant) can partially control infection, but ultimately succumb thus demonstrating the requirement of T-cells for long term protection. Studies using IFN- γ deficient mice indicate that this component is necessary for initial defense against *Francisella* (Elkins, *et al.*, 1996). Further research implicates the requirement of functioning B-cells for defense (Dreisbach, *et al.*, 2000). Interestingly, exposure to *F. tularensis* LVS LPS was insufficient to upregulate IFN- γ and activate B-cells. Using B-cell deficient mice (BKO), Culkin *et al.* (1997) demonstrated the requirement of functioning B-cells for protection against secondary *F. tularensis* infection. Successful clearance of *F. tularensis* from a host confers long-term protection. In BKO mice, primary infection with the organism was successful, but long term protection was absent. Measurements of the cytokines IFN- γ and NO were similar in both BKO and native mice, suggesting that primary clearance of the pathogen is required for induction of long term immunity. The results from this experiment with BKO mice suggest a role for B-cells in long-term protection against intracellular pathogens. The B-cell arm of the immune system may be responsible for T-cell priming (Bosio, *et al.*, 2001) and regulation of the T-cell response to *F. tularensis*. It appears that the host response to intracellular colonization with *F.*

tularensis is a complex process.

The role that antibodies play in the protection against intracellular pathogens is somewhat controversial. Recently, it has been shown that humoral immunity does play a role in protecting against such organisms as *Ehrlichia*, *Salmonella*, *Mycobacterium*, *Legionella*, *Brucella* and *Rickettsia* (Winslow, *et al.*, 2000). Both antibody and cellular responses are required to suppress and clear infection. With commonalities being shared amongst bacteria, this observation is applicable to our own research. The elicitation of an antibody response surely is part of the key to the development of a successful vaccine for *Francisella*.

Subcellular vaccines are preferred over live vaccines because of the ease of quality control and 'cleanliness' of preparation. However, subcellular vaccines have so far been ineffective for Tularemia because they elicit only a humoral response. However, some subcellular components are respectable T-cell mediators. Carbohydrates present in the capsule appear to be antigenic and responsible for imparting humoral immunity in vaccinated individuals (Tarnvik, 1989) while cell surface proteins are thought to be responsible for conferring long-term cellular immunity. Requirement for development of a cell-mediated immune response, coupled with the need for the development of humoral immunity has presented a significant challenge to the creation of a vaccine for *F. tularensis*.

With knowledge that LPS from *F. tularensis*, by itself, is ineffective in conferring long-term immunity to infection, there has been a move in the research community to characterize proteins from *Francisella* with the intention that these proteins be used as subcellular vaccines. It is thought that proteins exposed to the host immune system might be

key in this development. As a result, researchers have focused their efforts on identifying and characterizing immunologically significant proteins obtained from the organism.

Early work identified membrane polypeptides that could be recognized by T-cells from individuals that had recovered from *F. tularensis* infection (Sjostedt, *et al.*, 1990). T-cell induction did not occur in individuals not exposed to *F. tularensis*. A 17-kilodalton (kDa) T-cell stimulating protein was soon identified and denoted TUL4 (Sjostedt, *et al.*, 1991). TUL4 protein is a lipoprotein observed in Western blots utilizing serum from *F. tularensis* exposed individuals and has been used to develop a hand-held device for the early detection of *F. tularensis* (Grunow, *et al.*, 2000).

A 23 kDa protein was identified by comparing *F. tularensis* grown in liquid Chamberlain medium to *F. tularensis* grown in the intracellular environment of murine J774.1 cells (Golovliov, *et al.*, 1997). This protein appeared necessary for growth in the macrophage intracellular environment. Other proteins have been identified by comparison of the live vaccine strain to wild-type strains of *F. tularensis* using two-dimensional gel electrophoresis (Hernychova, *et al.*, 2001); these include a DNAK heat shock protein 70 (73.6 kDa), GroEL (56.7 kDa), acid phosphatase (56.1 kDa) and a 23 kDa protein. These proteins were all present in wild-type strains of the organism. Using serum from *F. tularensis* exposed individuals, immunoreactive proteins also were identified (Havlasova, *et al.*, 2002). While many proteins were immunoreactive, the TUL4 lipoprotein (17 kDa), a 60 kDa chaparonin, the DNAK chaparonin, a 10 kDa chaparonin and the 23 kDa protein were most notable. These proteins were not detected with serum obtained from subjects not exposed to *Francisella*.

Over the past 3-4 years, there has been a thrust in research to map immunoreactive proteins. One group has undertaken 2D gel electrophoresis to compare serum from unexposed individuals (naive) to that of Tularemia survivors with the intention of identifying immunologically active components. Havlasova *et al.* (2002) was successful in identifying 22 components by mass spectrophotometric analysis. The group identified the 17 kDa TUL4 protein (Sjostedt *et al.*, 1991) that has since been incorporated into a handheld immunochromatographic assay (Grunow *et al.*, 2000). A chaperonin protein with a molecular weight of 60 kDa was also identified (CPN60) which appears in other pathogenic bacteria such as *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Clostridium difficile*. This apparent cross reactivity to a protein thought to be responsible for adherence is part of the basis for our work presented. We hope to demonstrate that an immune response to a single organism, such as *Francisella*, may provide a level of protection against other organisms. Similarly, defense against a broad group of organisms, for example the gram negative pathogens, may provide a base level of protection against a rare, but pathogenic member of that particular class. As such, the cross reactivity between *Francisella* and *Brucella* is the basis of our identification of an immunologically reactive component to *F. tularensis*.

The genome of *F. tularensis* strain Schu-S4 has been published and is rapidly becoming a powerful tool in the analysis of the intracellular bacterium (Prior *et al.*, 2001). Identified within the genome were a large number of open reading frames (1804) with the current identification of only 124 proteins (Hubalek *et al.*, 2003). These proteins were identified by 2D gel electrophoresis and time-of-flight mass spectrometry. There were

numerous proteins identified within the 50 kDa molecular weight range with homology to such bacterium as *Salmonella typhimurium*, *Clostridium symbiosum*, *Vibrio cholerae* and *Borrelia burgdorferi*. Previous researchers had identified the 10 kDa chaperonin, the 17 kDa TUL4 and the 60 kDa chaperonin as being immunologically active (Havlasova, *et al.*, 2002). The 60 kDa chaperonin protein (Accession No.P94798) is identified strongly with serum from *F. tularensis* survivors.

Previous work has documented the cross-reactivity between *Brucella* spp. and *Francisella* spp. (Francis, 1926). In agglutination studies, this cross-reactivity has ranged from 23 to 32% in specimens collected from volunteers previously infected with *Francisella* or *Brucella* (Behan, *et al.*, 1982). Like *Francisella*, *Brucella* is an intracellular pathogen that infects the reticuloendothelial system. However, unlike Tularemia, Brucellosis can manifest as a subacute, or dormant, disease. Successful clearance of infection is thought to be due to immunity directed towards the LPS (Montaraz, *et al.*, 1986). The polysaccharide components necessary for successful clearance have been extracted and incorporated into a subcellular vaccine that is useful in protecting against *Brucella*. Moreover, specific moieties present in the *Brucella* polysaccharide are shared amongst other pathogenic bacteria, such as *Escherichia coli* O157:H7, *Salmonella godesburg* and *Pseudomonas maltophilia* 555. This knowledge of cross-reactivity between *Francisella* spp. and *Brucella* spp., coupled with the observation that successful clearance of *Brucella* is due to antibodies directed towards surface antigens such as LPS formed the basis for our current research model.

1.9 Using *Brucella* immunized mice to identify *Francisella* components

We hypothesize that immunologically significant proteins, with potential vaccine or industrial use, can be identified using sera from mice that have naturally, and successfully cleared infection.

In this study we utilize a novel approach to identify immunologically significant components to *F. tularensis*. Normally, mice injected with *F. tularensis* die before immune sera can be recovered. A group of *Francisella* sensitive mice were immunized with a novel *B. abortus* OPS vaccine and, after immunization, these mice were challenged with *F. tularensis* LVS. Surviving mice were used to recover sera with antibodies directed against cell surface components from *F. tularensis* LVS. These antibodies were used to identify immunologically significant proteins with potential for use as a vaccine for *F. tularensis*. Cell surface components characterized then, were tested for their ability to protect unimmunized mice against *F. tularensis* LVS challenge.

2.0 Materials and methods

2.1 Bacterial Cultures and Growth

A stock culture of *F. tularensis* live vaccine strain (LVS) was acquired as a freeze-dried experimental vaccine (Lot #11, Code Number: NDBR 101, 2.4×10^9 colony forming units per ml when rehydrated) from the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, Maryland, USA.

Brucella abortus strain 30 was acquired from the Animal Diseases Research Institute - Nepean, (ADRI-Nepean) Ontario. Growth was maintained on *Brucella* agar (Merck Frosst Chemicals, Canada) with 1.5 ppm crystal violet incubated as for *F. tularensis* LVS. Five-percent phenol-killed *B. abortus* 1119-3 was acquired from Dr. Janet Payeur, United States Department of Agriculture (USDA), Ames, Iowa. This was the source for O-polysaccharide vaccine, which was purified by a method previously described (Lord, *et al.*, 1998).

Staphylococcus aureus ATCC 25923 was obtained from Fisher Scientific (Ottawa, Bactrol Discs Set A), *Escherichia coli* O157:H7, *Salmonella godesburg* and *Pseudomonas maltophilia* 555 were obtained from Dr. Douglas Griffith (NRC, Ottawa).

F. tularensis LVS was grown using a synthetically defined medium as described by Chamberlain (1965) (appendix B). Chamberlain's synthetic medium (CSM) was used for all *F. tularensis* LVS growth experiments. *F. tularensis* was grown either on agar or in broth synthetic media at 37°C, 90% humidity and 5% CO₂. Broth cultures were grown in a rotating shaking incubator set at 125 rotations per minute. Agar cultures were maintained at the conditions specified in an incubator. A single vial of stock culture was used to inoculate 10 ml

peptone-cysteine broth tubes. These were incubated for 48h until the culture possessed an opaque blue appearance when illuminated with incident light. These were added to 500 mL CSM base in 1 L sterile flasks then incubated as described until early logarithmic or late stationary phase, depending on requirements. The absorbance of the liquid culture was measured at 600nm and recorded.

F. tularensis LVS was subcultured by transferring 5 mL bacterial suspension into 500 mL pre-warmed sterile CSM. Agar plates were created by preparing a double strength solution of CSM and adding it to 4% agar (Difco, Detroit Mich., USA). *Staphylococcus aureus* ATCC 25923, *Escherichia coli* O157:H7, *Salmonella godesburg* and *Pseudomonas maltophilia* 555 were grown in Luria Broth consisting of: 10 g/L Bacto-peptone, 5 g/L Bacto yeast extract (Sigma Chemicals, St. Louis, Minn., USA) and 10 g/L sodium chloride all set to a pH of 7.4. These cultures were incubated, centrifuged and sonicated as noted for LVS in this section.

Prior to infection of subject mice, *F. tularensis* LVS (10 LD₅₀ is equal to 1 X 10⁵ cells) was diluted in pre-warmed PBS (phosphate buffered saline). Intranasal inoculations were completed with 0.01 mL delivered to a single nostril of each anaesthetized mouse. Intraperitoneal injections were completed with an injection of 0.1 mL (10 LD₅₀ is equal to 1 X 10⁶ cells). Saline without phosphate was found to decrease the virulence of the bacterium.

2.2 Preparation of bacterial products

To protect against aerosolization, all sonications were completed within a Biosafety Cabinet under Level 2 Biological safety conditions. Bacterial cultures were sonicated following centrifugation (10,000 x g, 4°C, 30 min). The cell product pellet was resuspended in sterile PBS and stored at -70°C until used. A Soniprep 15 and a Process Timer (MSE™) was used to prepare sonicated bacterial products. Cellular suspensions of the pellet returned following centrifugation were cooled at 4°C during sonication. The amplitude of the 10 mm probe was adjusted manually to 10 μm. The sequence of sonication was 5 cycles of 15 seconds of pulses with 1 minute rest between cycles. *Staphylococcus aureus* ATCC 25923, *E. coli* O157:H7, *S. godesburg* and *P. maltophilia* 555 were centrifuged (10,000 x g, 4°C, 30 min) and the pellet suspended in 25 mL sterile PBS (22°C). A sequence of sonication of 5 cycles of 15 seconds, with one minute rest between cycles, was performed for each bacterial sample. The disrupted cell suspension was centrifuged (10,000 x g, 30 minutes) and the supernatant removed. Sodium azide was added to a final concentration of 0.1% prior to storage at 4°C.

The disrupted cellular components following sonification were separated from unbroken cells or cell debris by centrifugation at 10,000 x g, 4°C for 30 min. The supernatant was decanted and stored. This stored material, free of debris and cells, was dialyzed with a Spectrapor 10,000 molecular weight (MW) cutoff dialysis bag against 1mM HEPES and 1 mM DTT (dithiothreitol) for 24 hours. The dialysis was then repeated twice more against water for 24 hours each and stored at 4°C. The azide-fixed prepared cell-free cell lysates of *F. tularensis* LVS, *Staphylococcus aureus* ATCC 25923, *E. coli* O157:H7, *S. godesburg* and

P. maltophilia 555 was then utilized in experiments requiring disrupted cell lysate.

Cell-free, sterile supernatants of *F. tularensis* LVS were required. Following growth in liquid defined media, as described before, cultures were centrifuged (10,000 x g, 4°C, 30 min). The supernatant was decanted and filter-sterilized through a 0.22 µm filter sterilizing flask (Nalgene). The sterile filtrate, defined *F. tularensis* LVS supernatant, was stored at 4°C until needed.

F. tularensis LVS supernatant preparations were concentrated with an Amicon ultrafiltration system pressurized to 55psi with dry nitrogen utilizing a 30 kDa MW cutoff membrane (MWCO) (Millipore Co. Nepean, ON) . The retentate, containing components larger than 30 kDa, was stored at 4°C. Several supernatants from the Amicon ultrafiltration procedure were further concentrated in Amicon 30 kDa MWCO Centriprep YM-30 centrifugal filter device (Millipore Co. Nepean, ON) centrifuged at 1,000 x g for 60 min. Retentate was collected and stored at 4°C until required.

Several flasks of *F.tularensis* LVS were grown in CSM as described, and subsequently filter sterilized (0.20µm, Nalgene). The resultant *F .tularensis* LVS supernatants were then frozen at -70°C and lyophilized. The dry samples were stored in air-tight containers at 22°C until required.

2.3 Polyacrylamide gel electrophoresis

Resolution of high molecular weight components was completed with a separating gel of 7.5% polyacrylamide utilizing the method of Laemmli (1970). Low molecular weight components were separated with a 12% polyacrylamide gel. A 4% stacking gel was used for all samples (appendix C). Native gels were prepared without SDS in a manner identical to that described above. Samples for reversed polarity native gels were prepared with a triple strength (3x) sample buffer containing 40% glycerol (v/v), 4% Tris-HCl (w/v) and methyl-green set to a pH of 8.9. Such gels were run in buffer excluding SDS. All bench-top gel staining and western blotting techniques took place at room temperature on a rotational shaker set at 60 rpm.

A Coomassie R250 staining solution was prepared with 0.025% Coomassie Brilliant Blue R250 (w/v), 40% methanol (v/v), 7% acetic acid (v/v) in water. Initial staining was performed for 1 hour. Destaining was done with several changes of a 40% methanol (v/v), 7% acetic acid (v/v) mixture until a sufficiently clear background was attained. Destained gels were photographed and stored in 5% glycerol (v/v).

Periodic acid oxidizes carbohydrates, such as those in the O-polysaccharide and core region of LPS, and activates them for reaction with silver. The procedure of Bio-Rad silver staining was used (appendix A) with periodic acid used in place of dichromate as the oxidizer. The polyacrylamide gel was fixed in 50 mL fixed containing 40% methanol (v/v), 10% acetic acid (v/v) in triple distilled water for 30 minutes and then discarded. Periodic-acid based oxidizer [50 mL, 0.7% periodic acid (w/v), 40% ethanol (v/v), 5% acetic acid (v/v)] was added, incubated for 5 minutes and discarded. The contents were rinsed with five changes of

distilled water over ten minutes. Silver reagent was added (50 mL Bio-Rad silver staining solution diluted 1:10 in triple distilled water), incubated for 20 minutes and discarded. The gel was lightly rinsed for 30s with distilled water. Developer (1.6% w/v Bio-Rad developer powder in water) was added and allowed to incubate until bands were visually detected. A 50 mL solution of stop bath (5% (v/v) acetic acid) was added to quench the silver reaction. The gel was subsequently stored in 5% (v/v) glycerol.

Molecular weight standards were obtained and electrophoresed with each gel (Appendix D). A prestained broad range set of molecular weight standards was used for samples run on SDS-PAGE (Bio-Rad Laboratories, Mississauga, Ontario). Prestained Rainbow markers were utilized (AmershamPharmacia Biotech) for Western blotting experiments.

Proteins were electro-blotted onto a nitrocellulose membrane (Hybond, ECL Chemicals, Amersham Pharmacia Life Sciences) in a BioRad mini Protean-2-electrotransfer apparatus. Transfer buffer comprised 3 g/L (w/v) Trisma-base (Sigma Chemicals, St. Louis, Minn., USA), 14.4 g/L (w/v) glycine and 20% (v/v) methanol with water. Binding sites were blocked with 5% (w/v) blocking solution reagent (ECL blocking agent, ECL Chemicals Amersham Pharmacia Life Sciences) in PBS with 0.1% (v/v) Tween-20 (PBS-Tween) incubated for 1 hour at room temperature (22°C). The membrane was washed for 10 minutes in PBS-Tween, followed by 3 subsequent washes of 5 minutes each. Serum (primary detector antibody) was diluted either in 1:50 or 1:1000 v/v in PBS-Tween and incubated for 1 hour in PBS-Tween as before. The membrane was washed for 10 minutes in PBS-Tween, followed by

3 subsequent washes of 5 minutes each. The indicator antibody (Caltag, CA., USA) goat anti-mouse (or anti-rabbit) Heavy + Light chain IgG antibody conjugated to horseradish peroxidase (HRP)) was diluted 1:30,000 or 1:3000 (v/v) in 50 mL of PBS-Tween and incubated for 1 hour. The membrane was then washed in PBS-Tween as noted above and then transferred onto a clean, dust-free sheet of acetate. ECL detection fluid (ECL Chemicals, Amersham Pharmacia Life Sciences) was aliquotted onto the membrane, incubated for 1 minute and then removed. A second strip of acetate was layered on top of the membrane and transferred, under safelight conditions, into an exposure cassette containing sensitized X-ray film (ECL Hyperfilm, Amersham Pharmacia Life Sciences). Film was developed in a Kodak GBX developer by inspection, fixed and stored.

2.4 Protein determination

A BCA assay was utilized for protein determination (Smith *et al.*, 1985). Assays were performed in a 96-well, flat bottom, sterile microtitre plate (Nalgene). Samples were diluted 1:1 with BioRad BCA working solution of 50 parts BCA Reagent A (1% (w/v) bicinchoninic acid, 2% (w/v) NaHCO₃.H₂O, 0.16% (w/v) Na tartrate, 0.4% (w/v) NaOH, 0.95% (w/v) NaHCO₃, pH 11.25) to 1 part Reagent B (4% (w/v) CuSO₄). Samples were incubated at room temperature for 10 minutes and read at 595nm in a Thermomax microtitre plate reader (Molecular Devices, Sunnyvale, CA). Protein concentrations were interpolated from a curve constructed from bovine serum albumin standards and the results were analyzed with the

Softmax software (Molecular Devices, Sunnyvale, CA) using the supplied 4-parameter fit. The linear region of this 4-parameter fit normally provided three useful data points for determining the protein content of the sample.

2.5 Enzyme analysis of *F. tularensis* LVS supernatants

A set amount of enzyme (0.83 μ g) was incubated with a set amount (1.88 μ g) of *F. tularensis* LVS liquid culture supernatant, as interpolated from BSA standards, at 37°C for 12 hours. Enzymes were obtained from various sources (Proteinase-K (3.4.21.14 Sigma, P6556, 12 U/mg), lipase (Mann Research, Crude Porcine Pancreas, 3.2 U/mg), DNAase (3.1.21.1, Sigma, DN25, 600 U/mg), RNAase (Sigma, Bovine Pancreas IA, 0.36 U/mg) and lysozyme (3.2.1.17, Sigma, L6876, 62000 U/mg)). Enzyme was inactivated with addition of SDS loading buffer and subsequently boiled prior to loading onto an SDS gel.

2.6 Partial purification of *F. tularensis* LVS cell lysates

A Pharmacia Mono-Q 10/10 column was equilibrated with 10 mM HEPES buffer, pH 7.4, containing 1 mM DTT and 1 mM EDTA. Cell lysate was applied to the column using a 50 mL loop and washed with equilibration buffer until baseline absorbance was achieved. Material bound to the quaternary amine column was eluted by a KCl gradient (0.3 M to 1.5 M) over 80 minutes with a flow rate of 1.0 mL/min. Proteins eluting from the column were monitored in-line at 214nm with 0.5 absorbance units full scale (10% offset) and a chart speed of 2 mm/min.

Fractions were collected in 1 minute intervals and stored at 4°C until required.

2.7 Tissue culture and cytokine determination

Modified minimum essential medium (mMEM) (Hertz *et al.*, 1982) was prepared for cell culture. The macrophage cell line J774.1 was grown on T75 tissue culture plates in mMEM without phenol red, supplemented with streptomycin (10 µg/mL), penicillin (10 U/mL), fungizone (0.25 µg/mL) and 5% (v/v) horse serum (Flow Laboratories, Mississauga, Ontario, Canada). Cells were passaged every 6 days to prevent growth beyond the monolayer and to maintain the cell line. Prior to treatment, J774.1 cells were plated on 96 well plates at a concentration of 5000 cells/well.

J774.1 murine macrophages were seeded into sterile 96 well plates (Falcon) at a concentration of 5000 cells/well. At a concentration of 10^5 cells/well (approximately 6 days growth), cells were treated for 24 hours with various solutions. The NO produced during the 24 hour period was determined by nitrate analysis (Green, *et al.* 1982). The media was removed to a fresh 96 well plate and added to an equal volume (1:1) of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% v/v H₃PO₄, Sigma Chemicals). After incubation at room temperature for 10 minutes, the plate was read at 540 nm in a Thermomax microtitre plate reader (Molecular Devices, Sunnyvale, CA, USA). Sodium nitrate was used as a standard for nitrate quantitation.

Cell viability was assessed using Alamar Blue (AccuMed International, Inc., Westlake,

OH, USA). The dye was added 24 hours post treatment to a final concentration of 10% (v/v) and allowed to incubate at 37°C for 5h. The absorbencies (570-600 nm) were read on a Thermomax titer plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.8 Animal care and handling

Mice (balb/c or CD1, female, 19-21 g, 35 days old) were acquired from Charles River (St. Constant, Quebec, Canada). Mice were cared for in accordance with the guidelines set by the Canadian Council for Animal Care. All procedures were reviewed and approved by the DRES Animal Care Committee (members consist of a veterinarian, scientists and lay people from the community). Protocol JC-98-02 was used for this study.

2.9 Preparation of anti-Tularemia serum

To raise anti-Tularemia mouse sera, twenty balb/c mice were first vaccinated with the *B. abortus* O-polysaccharide (OPS). Each mouse was given 1 µg OPS in 0.1 mL sterile saline by the intraperitoneal route (IP). Two weeks following vaccination, the mice were infected via the interperitoneal route with 10 LD₅₀ of *F. tularensis* LVS diluted in 0.1 mL sterile PBS. Infected mice were then placed into isolator cages (with HEPA filter tops) within an operating BioSafety Cabinet. The cages were then placed into a HEPA filtered Animal Isolator (Thoren Caging, Toronto, Canada). The mice were monitored for 2 weeks. Twelve mice survived (60% of the OPS vaccinated mice survived 10 LD₅₀ of *F. tularensis* LVS) and these were sacrificed for anti-Tularemia serum.

Infection by the intranasal route was completed after mice were anaesthetized with 1:10 diluted Somnitol™. A volume of 0.010 mL of the bacterial suspension was delivered to a single nostril. Blood was collected into 1.5 mL Eppendorf centrifuge tubes. Small volumes (0.1-0.3 mL) were collected utilizing a 26 gauge needle from the warmed tail veins. Larger volumes (1 mL) were collected by performing a heart-puncture with a 1 mL syringe with a 26 gauge needle attached. The blood was centrifuged (5,000 x g, room temperature, 10 minutes) and the serum removed. Glycerol was added to the serum for a final concentration of 10% (v/v). These were stored frozen at -70°C until required.

3.0 Results

3.1 Development of mouse anti-*F. Tularensis* antibodies

Commercially available rabbit anti-*F. tularensis* antiserum (Difco, Detroit, Mich. USA) was initially used in this study but was abandoned. This antibody was used as a probe on a Western blot, following SDS-PAGE of *F. tularensis* LVS cell lysates. The antibody did not appear to identify any significant components in the bacterial lysates possibly because these animals that produced these antibodies were immunized with killed, rather than living (*i.e.*, toxin secreting) bacteria.

Sera were obtained from mice which were infected with *B. abortus*. Two weeks post infection, sera was collected, filter sterilized, and then sterility checked. This antibody were used on Western Blots of SDS-PAGE separated lysates and sterile supernatants of *F. tularensis*. There were no specific bands detected on Western Blot, deemed to be a negative result.

Many of the components of *F. tularensis* and *Brucella abortus* cross-react (Behan, 1982). Using this information, we sought to protect mice with an OPS based *Brucella* vaccine and then challenge them with *F. tularensis* LVS. We hypothesized that these 'Brucella-primed' mice may possess immunity towards challenge against a second intracellular pathogen and that this immunity would be towards specific components important in the pathogenesis of Tularemia.

Mice were then immunized with the Department of National Defense (DND) experimental *Brucella abortus* 1119-3 O-polysaccharide (OPS) vaccine, not to raise

antibodies against *Brucella*, but to provide them with cross-protection against Tularemia. Mice infected with 10 LD₅₀ of *F. tularensis* typically die before they can raise antibodies to its virulence factors. However, once partially protected with anti-*Brucella* vaccine, it was observed that most animals survive even this high dose of infection. It was hoped that the surviving mice would have raised antibodies to immunologically significant components expressed, or exposed by the live bacterium but not found in killed cells.

Little difference was observed between unvaccinated mice and *Brucella* OPS vaccinated mice given 10 LD₅₀ of *F. tularensis* over the first 3 days. All of the hosts became lethargic and less responsive to handling. After three days, health of the unvaccinated mice deteriorated until all but one of the 20 controls perished (the lone survivor was marginally alive for a month and then recovered in the second month). In contrast, most of the vaccinated mice quickly recovered and of 20 mice, 12 or 60% fully recovered (40% perished). Sera from the 12 vaccinated survivors was obtained, pooled and stored at -70°C. However, sera stored at -70°C appeared to lose specificity both with time and increasing number of freeze/thaw cycles.

3.2 Identification of a 52 kDa component in *F. tularensis* supernatant

Supernatant fractions from liquid broth cultures of *F. tularensis* LVS were lyophilized and then, separated by SDS-PAGE. These gels were transblotted onto nitrocellulose and probed with the *B. suis* primed, *F. tularensis* LVS challenged murine serum with a 1:50 dilution (Figure 1 and Figure 2). Figure 1 denotes a prominent band corresponding to a molecular weight of 52 kDa in lanes 1 through six corresponding to the six subcultures

completed. This band is quite prominent in Lanes 1 and 5. Protein loaded onto these two gels was not standardized and differing levels of identification are likely due to differing amounts of antigenic material expressed within the various subcultured samples. In broth subculture 3 (Lane 3 of Figure 1), a doublet band was observed possessing molecular weights of 52 and 51 kDa, relative to protein standards. There were no other immunologically active bands detected. The same 52 kDa band is visible in the separated components visible on the Western blot in Figure 2. The component appeared in all of the CSM broth subcultures electrophoresed on the SDS-PAGE (subcultures 1, 2, 4 and 5 corresponding to lanes 1, 2, 3 and 4). Here, the doublet band was not visible.

Non-lyophilized, filter sterilized *F. tularensis* LVS supernatants, concentrated by pressure ultrafiltration with a MWCO membrane of 30 kDa, were separated by SDS-PAGE, transferred onto nitrocellulose and probed with a 1:1000 dilution of the *Brucella*-primed anti *F. tularensis* antiserum (Figure 3). A band was present at 52 kDa in all of the lanes corresponding with ultrafiltrated concentrates (lanes 2, 4, 6, 8, 10, 11 and 12 in Figure 3). This 52 kDa component was not detected in the lanes corresponding to ultrafiltrated filtrates (lanes 3, 5, 7 and 9 in Figure 3) indicating the 52 kDa component was successfully concentrated. There are several other bands visible corresponding with approximate molecular weights of 90, 48 and 34 kDa. It was thought that these minor bands were likely due to non-specific binding from the higher dilution of primary antibody used. Lane 4, in Figure 3, corresponding to the ultrafiltration concentrated supernatant is darkly stained, likely from increased lysis that occurred in this sample.

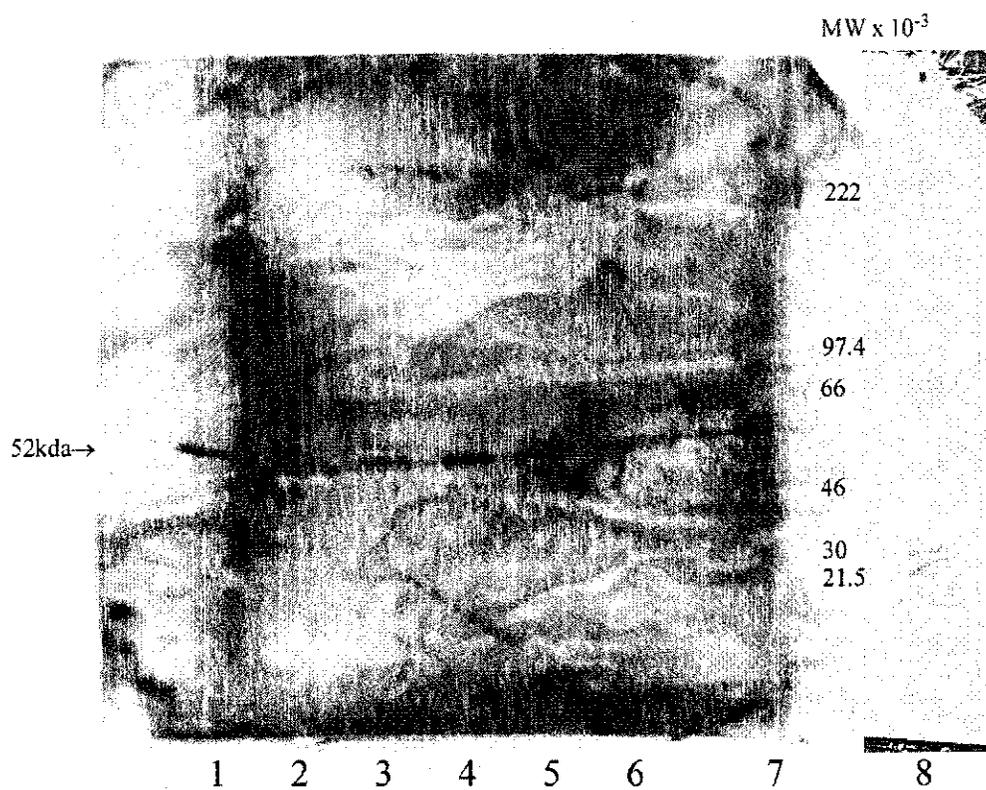


Figure 1

Immunoblot (Western) of *F. tularensis* cellular components from lyophilized samples. Molecular weight determination of antigenic components present in *F. tularensis* LVS supernatant (July 23, 1998). Samples electrophoresed on 8% SDS PAGE and transblotted as described. Primary antibody: 1:50 *Brucella*-primed anti *F. tularensis* murine antisera. Secondary antibody: 1:3000 Caltag anti-mouse-HRP. Lane 1: broth subculture 1, lane 2: broth subculture 2, lane 3: broth subculture 3, lane 4: broth subculture 4, lane 5: broth subculture 5, lane 6: broth subculture 6, lane 7: 5 μ L Rainbow MW marker (weights indicated), lane 8: membrane with Rainbow MW markers indicated.

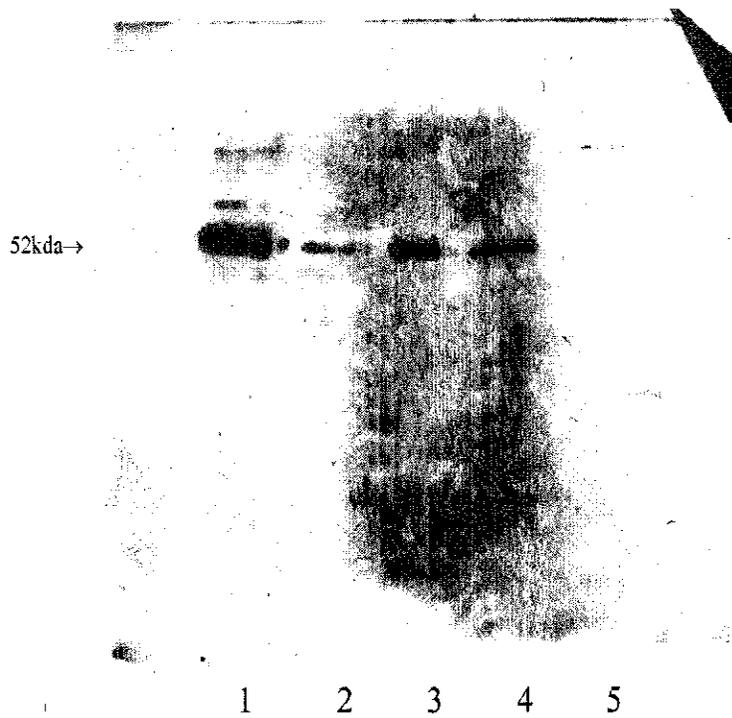


Figure 2

Immunoblot (Western) of *F. tularensis* cellular components from lyophilized samples. Samples electrophoresed on 12% SDS PAGE and transblotted as described. Primary antibody: 1:50 *Brucella*-primed anti *F. tularensis* murine antisera. Secondary antibody: 1:3000 Caltag anti-mouse-HRP. Lane 1: broth subculture 1, lane 2: broth subculture 2, lane 3: broth subculture 4, lane 4: broth subculture 5, lane 5: 5µL Rainbow MW marker.

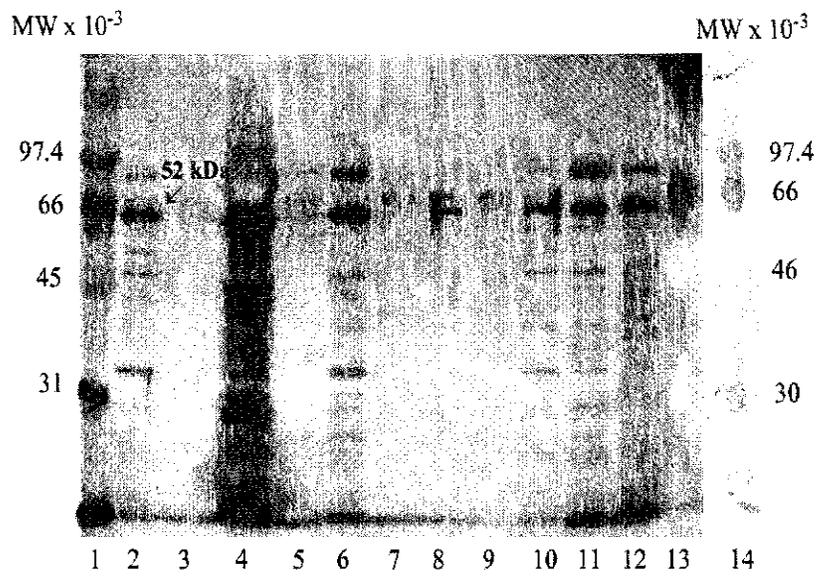


Figure 3

Immunoblot (Western) of *F. tularensis* LVS liquid culture supernatant filtrates and concentrates from pressure ultrafiltration preparation. Samples electrophoresed on a 10% SDS PAGE. Primary antibody: 1:1000 *Brucella*-primed anti *F. tularensis* murine antisera. Secondary antibody: 1:3000 Caltag anti-mouse-HRP. Lane 1: BioRad H/L MW marker (5 μ L), lane 2: 1 $^{\circ}$ Amicon concentrate, lane 3: 1 $^{\circ}$ Amicon filtrate, lane 4: 2 $^{\circ}$ Amicon concentrate, lane 5: 2 $^{\circ}$ Amicon filtrate, lane 6: 3 $^{\circ}$ Amicon concentrate, lane 7: 3 $^{\circ}$ Amicon filtrate, lane 8: 4 $^{\circ}$ Amicon concentrate, lane 9: 4 $^{\circ}$ Amicon filtrate, lane 10: 5 $^{\circ}$ concentrate, lane 11: 6 $^{\circ}$ concentrate, lane 12: 7 $^{\circ}$ concentrate, lane 13: Rainbow MW marker (5 μ L), lane 14: Rainbow MW marker (5 μ L) nitrocellulose membrane:

3.3 Native PAGE of *F. tularensis* supernatant

F. tularensis LVS cultures were grown for one to eight days, supernatant fractions concentrated by pressure ultrafiltration and centriprep filtration, and then separated by PAGE under non-reducing (no SDS) conditions prior to being transblotted onto nitrocellulose and probed with anti *F. tularensis* antiserum (Figure 4). Samples in lanes 2 through 5 were prepared using pressure ultrafiltration only, and samples in lanes 6 through 9 were prepared using ultrafiltration and centriprep filtration. The gel was loaded with 292 ng protein per well, as determined by a BCA assay. A band with an apparent molecular weight of 52 kDa was identified on the immunoblot (Figure 4) in all sample lanes (Lanes 2 through 9). This 52 kDa band was present in increasing intensity up to a maximum after 5 days of growth (lane 6) and then remained in equivocal intensity. In lanes 6 and 7 (5 and 6 days growth), the 52 kDa band appeared as a doublet. There were other bands present on the gel, with apparent molecular weights of approximately 66 kDa and 100 kDa which appeared above the 52 kDa band in all sample lanes. There was also a band which appeared after 96 hours of growth; i.e., the band appeared only in lanes 6 through 9, but was absent from lanes 2 through 5. This second band, appearing with an approximate molecular weight of 38 kDa corresponded with the samples that were secondarily processed with the added step of the centriprep filtration.

The same samples were electrophoresed by PAGE, with reversed polarity, under non-reducing conditions (no SDS), transblotted onto nitrocellulose and probed as above. No antigenic material was identified (Figure not shown).

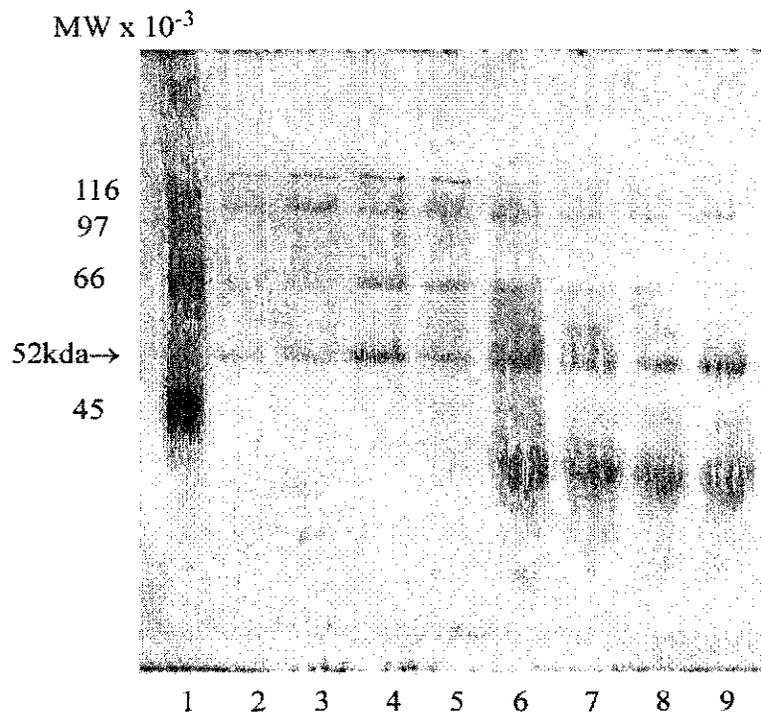


Figure 4

Immunoblot (Western) Characterization of *F. tularensis* LVS cellular components. Samples prepared and electrophoresed under normal polarity on a 7.5% native (no SDS) gel. Protein concentration of samples standardized at 292 ng/well. Primary antibody: 1:50 *Brucella*-primed anti *F. tularensis* murine antisera. Secondary antibody: 1:3000 Caltag anti-mouse-HRP Lane 1: BioRad H/L MW marker (5 μ L), Lane 2: 24 hours growth, Lane 3: 48 hours growth, Lane 4: 72 hours growth, Lane 5: 96 hours growth, Lane 6: 5day growth, Lane 7: 6day growth, Lane 8: 7day growth, Lane 9: 8day growth.

3.4 Identification of antigenic components in *F. tularensis* cell lysate

Sonicated *F. tularensis* LVS cell lysates were prepared as described. A dilution set of cell lysates was prepared and electrophoresed by SDS-PAGE, transblotted onto nitrocellulose and probed (Figure 5). Shown in Figure 5 is a sample result of 1.55 ng probed cell-lysate material (Lane 2) beside a set of pre-stained molecular weight markers (Lane 1). Two major antigenic bands were identified: one with an apparent molecular weight (MW) of 17 kDa and the other with a MW of 52 kDa (Figure 5). Both bands were present in all dilutions and decreased with intensity with decreasing *F. tularensis* LVS cell lysate concentration. There were other immunogenic bands visible, but in lesser intensity, corresponding with molecular weights of approximately 34 kDa, 85 kDa and 100 kDa. The 52 kDa and 17 kDa antigenic bands did not correspond with any component visible in the Coomassie stained gel (Figure not shown).

F. tularensis LVS cell lysate was separated by PAGE under non-reducing (native, containing no SDS) conditions, transblotted onto nitrocellulose and probed (Figure 6). A dilution set of cell lysates was created. Two lanes are shown to indicate the antigenic material detected. Sample loading corresponds with 12.4 ng and 6.2 ng for Lanes 2 and 3, respectively. Two major bands were detected on the immunoblot (see arrows) in the sample lanes (Lane 2 and 3). This indicates that the antigenic component of interest is negatively charged. No molecular weight determination was performed on these samples, as the markers were not visible. There was antigenic material present in the wells which did not migrate into the gel matrix (shown at the top of the gel).

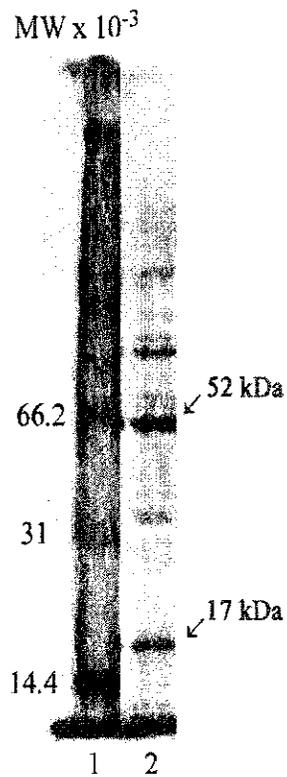


Figure 5

Immunoblot (Western) characterization of *F. tularensis* LVS liquid culture cell lysate. Samples prepared and electrophoresed on 7.5% SDS-PAGE. Gel transblotted onto nitrocellulose membrane and probed under the following conditions: Primary antibody: 1:1000 *Brucella*-primed anti *F. tularensis* murine antisera. Secondary antibody: 1:3000 Caltag anti-mouse-HRP. Lane 1: BioRad H/L MW marker (5 μ L), Lane 2: *F. tularensis* LVS liquid cell culture lysate 1.55ng.

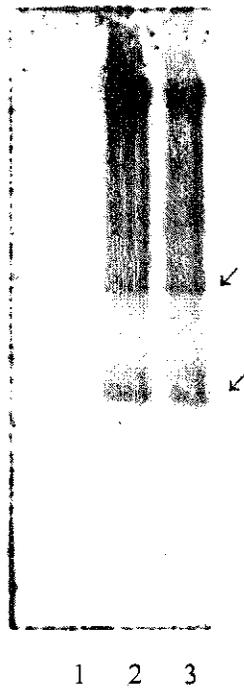


Figure 6

Immunoblot (Western) characterization of *F. tularensis* LVS liquid culture cell lysates using Native PAGE. Samples prepared and electrophoresed under normal polarity on a 7.5% Native (no SDS) PAGE. Gel transblotted onto nitrocellulose membrane and probed under the following conditions: Primary antibody: 1:1000 *Brucella*-primed anti *F. tularensis* murine antisera. Secondary antibody: 1:3000 Caltag anti-mouse-HRP. Lane 1: BioRad H/L MW marker (5 μ L), Lane 2: *F. tularensis* LVS liquid cell culture lysate (12.4ng), Lane 3: *F. tularensis* LVS liquid cell culture lysate (6.2ng).

3.5 LPS stain (silver) of Mono-Q fractionated *F. tularensis* supernatant

F. tularensis LVS cell lysate was fractionated on a Mono-Q anion exchange column (Figure 11). The collected fractions (fractions 11 and 22 through 33) were separated by SDS-PAGE and stained for lipopolysaccharide, using a periodic acid oxidizer and a silver-based staining agent (Figure 7). Numerous components were identified which contained polysaccharide moieties. Fractions 24 and 25, corresponding to Lanes 5 and 6, contained a 17 kDa component (see arrowhead). If this represents the TUL4 17 kDa component of *F. tularensis*, then it would confirm its extracellular nature. A band with an approximate molecular weight of 23 kDa appeared strongly (see arrow) in fraction #23 (Lane 4) and decreased progressively to fraction #30 (Lane 11). A band of 30 kDa was present in fractions #22 through #25 situated just above the 23 kDa component. No component with a molecular weight of 52 kDa was identified within any of the Mono-Q 10/10 fractions, suggesting the intracellular nature of this component.

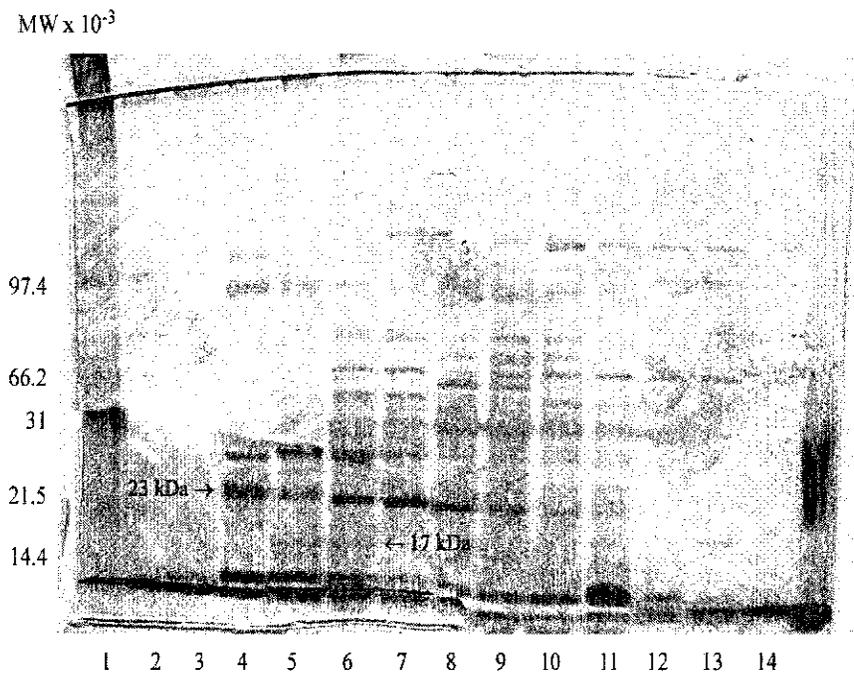


Figure 7

Lipopolysaccharide (LPS) characterization of Mono-Q fractionated *F. tularensis* LVS liquid culture components. Cellular components from January 31 (2°) *F. tularensis* LVS liquid culture was prepared for column chromatography as described. Fractions collected and electrophoresed on a 7.5% SDS PAGE. Gel was stained for LPS components using the technique by Hitchcock and Brown (1983). Lane 1: BioRad H/L MW marker (10 μ L), Lane 2: Fraction #11, Lane 3: Fraction #22, Lane 4: Fraction #23, Lane 5: Fraction #24, Lane 6: Fraction #25, Lane 7: Fraction #26, Lane 8: Fraction #27, Lane 9: Fraction #28, Lane 10: Fraction #29, Lane 11: Fraction #30, Lane 12: Fraction #31, Lane 13: Fraction #32, Lane 14: Fraction #33.

3.6 Enzyme treatment of *F. tularensis* supernatant

Samples of a *F. tularensis* LVS concentrated supernatant were incubated with various enzymes to identify the characteristics of the 52 kDa antigenic component. Following incubation of 1.88 μg *F. tularensis* LVS supernatant with 0.83 μg enzyme for 24 hours and reaction quenching by SDS loading buffer, samples were separated by SDS-PAGE. The gel was transblotted onto nitrocellulose and probed with the 1:1000 Brucella-primed anti *F. tularensis* LVS sera (Figure 8). Samples are displayed in pairs of supernatant/enzyme and enzyme only. Lane 3 consists of the supernatant incubated with Proteinase-K. Lane 5 consists of the supernatant incubated with lipase. Lane 7 consists of the supernatant incubated with DNase. Lane 9 consists of the supernatant incubated with RNase. Lane 11 consists of the supernatant incubated with lysozyme. Molecular weight markers were placed in Lanes 1 and 13. Lanes 4, 6, 8, 10 and 12 consisted of samples containing 0.83 μg Proteinase-K, Lipase, DNase, RNase and lysozyme, respectively.

Six major antigenic components were identified in the stock *F. tularensis* supernatant (Lane 2) with molecular weights of 15 kDa, 17 kDa, 31 kDa, 34 kDa, 44 kDa and 52 kDa respectively (see arrows). Treatment of the *F. tularensis* supernatant with Proteinase-K obliterated the antigenic bands on the immunoblot (Figure 8, Lane 3). The Proteinase-K treated sample had no visible bands on the Coomassie R250 stained gel, whereas prior to digestion, there were bands visible (Figure not shown). There remained a large amount of low molecular weight material that ran with the gel front that was visible on the Coomassie R250 stained gel (Figure not shown).

Lipase treated supernatant (Lane 5) lacked antibody identification of the 44 kDa and 31 kDa components, when compared to the control untreated *F. tularensis* supernatant (Lane 2). Lanes corresponding to RNAse and lysozyme treated supernatant (Lane 9 and 11, respectively) did not differ significantly from their corresponding control lane containing untreated *F. tularensis* supernatant (Lane 2). The same six antigenic components were detected in these lanes. There was no difference between the supernatant treated lanes on either the Coomassie Brilliant Blue R250 stained gel when compared to the control lane of untreated supernatant (Figure not shown).

The lane containing DNAase treated supernatant (Lane 7, Figure 8) exhibited a decreased intensity of the 52 kDa component. There were numerous other changes in the six antigenic components when compared to the control lane (Lane 2). Besides the decreased intensity of the 52 kDa components, there was a second band, corresponding to approximately 50 kDa, identified below the 52 kDa band. While the 44 kDa band was visible, a band with a molecular weight of 47 kDa was present. Interestingly, the 31 kDa band did not appear, rather a new band with a molecular weight of approximately 37 kDa was visible. There was also identified a component with a molecular weight of approximately 13 kDa. DNase appeared to modify the 52 kDa component - possibly cleaving it into two secondary pieces of 37 kDa and 13 kDa respectively.

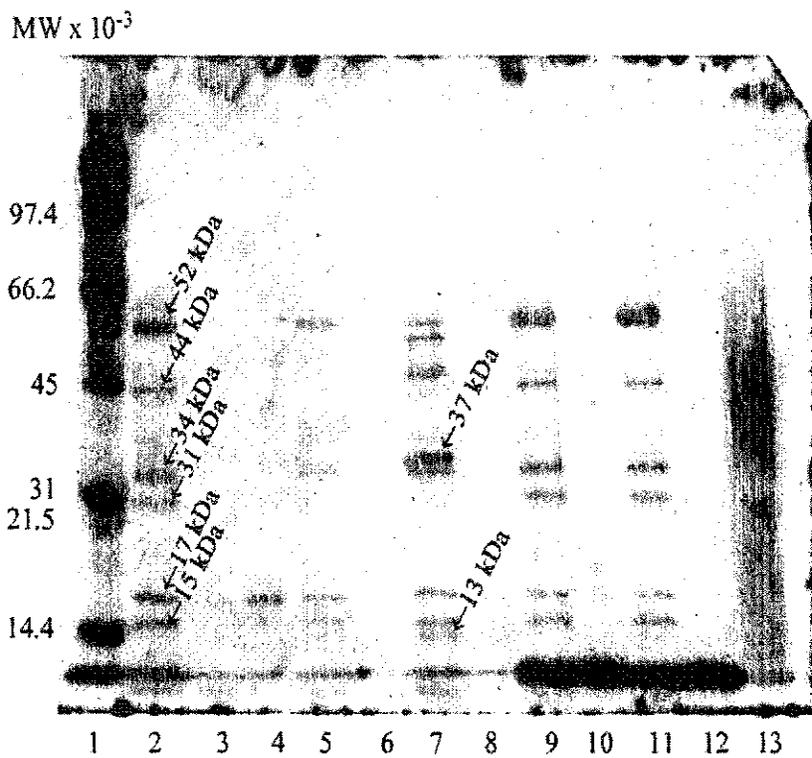


Figure 8

Enzyme characterization and immunoblot (Western) of antigenic *F. tularensis* liquid culture cellular components. Supernatant from Day7 of the multiday growth series used (7D). Supernatant (1.88 μ g) was treated with various enzymes (0.83 μ g). Samples prepared and electrophoresed on a 7.5% SDS-PAGE and transblotted onto nitrocellulose. Membrane probed under the following conditions: Primary antibody: 1:1000 *Brucella*-primed anti *F. tularensis* murine antisera. Secondary antibody: 1:3000 Caltag anti-mouse-HRP Lane 1: BioRad H/L MW marker (5 μ L), Lane 2: 7D supernatant, Lane 3: Proteinase-K digested supernatant, Lane 4: Proteinase-K, Lane 5: Lipase digested supernatant, Lane 6: Lipase, Lane 7: DNase digested supernatant, Lane 8: DNase, Lane 9: RNase digested supernatant, Lane 10: RNase, Lane 11: Lysozyme digested supernatant, Lane 12: Lysozyme, Lane 13: BioRad Kailadoscope MW marker (6 μ L).

3.7 Probing panel of 5 bacteria with *F.tularensis* LVS mouse serum

The antigenic activity present in 4 bacterial cell lysates was compared to the activity present in the cell lysate of *F. tularensis*. These additional bacteria, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* O157:H7, *Salmonella godesburg* and *Pseudomonas maltophilia* 555, were chosen because they all possess sugar components similar to that of the *Brucella* OPS which was utilized to immunize the mice that were subsequently challenged with *F. tularensis* LVS to create sera used in these, and previous experiments. The bacterial product was prepared by sonification as described. Sterile, cell-free bacterial product, at a standard concentration of 12.4 μg , from the five bacteria lysates was separated by SDS-PAGE. The gel was transblotted onto nitrocellulose and probed with the *Brucella* primed anti *F. tularensis* antibody in a dilution of 1:1000 (Figure 9A). Molecular weight markers were run in Lanes 1 and 7. There was a great deal of background bands appearing on the Western blot. *Brucella* primed anti *F. tularensis* antibody identified two major components in each of the six sample lanes. In Lanes 2 and 7 (*F. tularensis* LVS), the major bands corresponded with MW of 52 kDa and 17 kDa, respectively (arrows). Components with these molecular weights were also identified in approximately the same intensity in Lanes 3, 4 and 6 (*E. coli*, *S. godesberg* and *P. maltophilia*, respectively). In Lane 5 (*S. aureus*), a component with a molecular weight of approximately 50 kDa was identified, likely Protein-A. A low molecular weight component of 17 kDa was also identified in Lane 5 (*S. aureus*). The detection of the 52 kDa and 17 kDa components showed these as roughly equal in intensity to each other, but less intense than the

protein-A component. The amount of background banding present was likely indicative of overdevelopment.

A similar gel was prepared, using the cell-free sonicated products of *F. tularensiss* LVS, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* O157:H7, *Salmonella godesburg* and *Pseudomonas maltophilia* 555. These products were loaded at a constant protein concentration of 12.4 μ g per well and separated by SDS-PAGE as described. After transblotting onto nitrocellulose, the membrane was probed with a naive (unexposed to either *F. tularensiss* and unvaccinated) balb/c mouse sera as a primary antibody (Figure 9B). No molecular weight markers are shown on this Western blot. The naive mouse sera identified several components within all five of the cell lysates (Figure 9B). In Lane 3 (*S. aureus*), a 50 kDa component was strongly identified. This component likely corresponds to Protein-A (see arrow). Also, in lanes 1, 2, 3 and 5 (*E. coli*, *S. godesberg*, *S. aureus* and *F. tularensis*), the 17 kDa component was again identified. The detection of this 17 kDa component was similar in intensity to that of the protein-A (50 kDa) component. The 52 kDa band was not detected in *E. coli* O157:H7, *S. godesberg* or *P. maltophilia* using naive serum (Lanes 1, 2 and 4). There was a very low level of detection of a 52 kDa component in Lane 5 (*F. tularensis*).

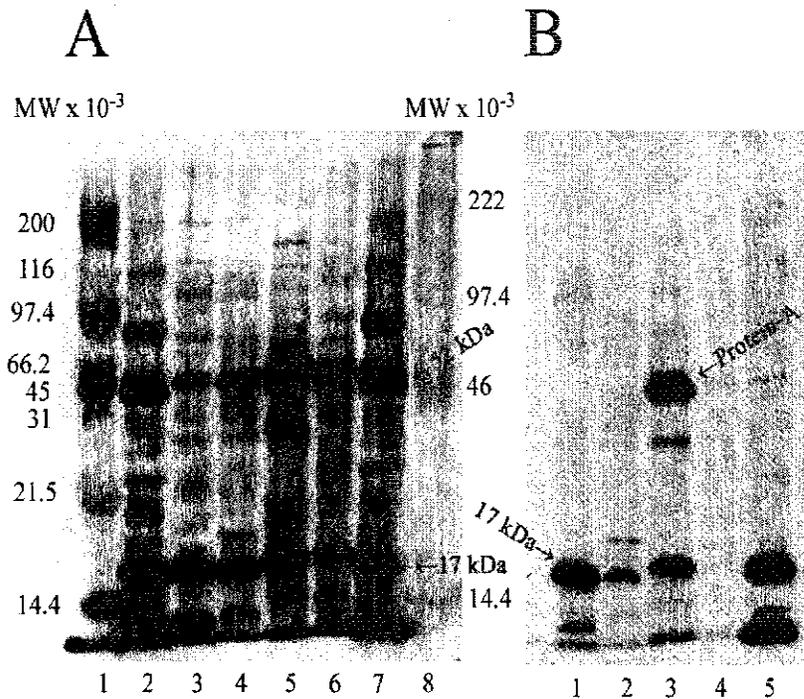


Figure 9
 Immunoblot (Western) comparison of antigenic components in *F. tularensis* LVS to a panel of four other clinically important bacteria. SDS-PAGE of processed bacterial cell lysates. Samples prepared and electrophoresed on a 7.5% SDS-PAGE of 1.0mm thickness as described and transblotted onto a nitrocellulose membrane. Protein concentrations of lysates standardized at 12.4 μg per well.

Immunoblot A: Membrane probed under the following conditions: primary antibody: 1/1000 DND *Brucella/F. tularensis* mouse antisera, secondary antibody: 1/3000 Caltag anti-mouse-HRP. Lane 1: BioRad H/L MW marker (5 μL), Lane 2: *F. tularensis* cell lysate, Lane 3: *E. coli* cell lysate, Lane 4: *S. godesberg* cell lysate, Lane 5: *S. aureus* cell lysate, Lane 6: *P. maltophilia* cell lysate, Lane 7: *F. tularensis* cell lysate, Lane 8: Rainbow MW marker (3 μL)

Immunoblot B: Membrane probed under the following conditions: Primary antibody: 1/1000 naive mouse serum (Nov 95 balb/c), secondary antibody: 1/30000 Caltag anti-mouse-HRP Lane 1: *E. coli* cell lysate, Lane 2: *S. godesburg* cell lysate, Lane 3: *S. aureus* cell lysate, Lane 4: *P. maltophilia* cell lysate, Lane 5: *F. tularensis* cell lysate.

3.8 Probing *F.tularensis* LVS lysate with anti-*F. tularensis* antibodies

Sonicated, cell-free *F. tularensis* LVS cell lysate was prepared as described. A dilution set of cell lysates was prepared and electrophoresed by SDS-PAGE, transblotted onto nitrocellulose and probed with either the Brucella-primed anti-*F. tularensis* LVS serum (Figure 10B) or Canadian Bioconcepts Rabbit anti *F. tularensis* antibody (Figure 10A). Also electrophoresed was a set of pre-stained molecular weight markers (Lane 1 in Figure 10A and 10B). Shown are respective samples of the result of 3.1 μg *F. tularensis* LVS cell lysate sample loaded onto the gel (Lane 2 in Figure 10A and 10B) and probed with the two antibodies. Other lanes appeared similar, with identical bands visible in greater or lesser intensity, depending on the greater or lesser amount of sample loaded onto the gel.

Canadian Bioconcepts anti-*F. tularensis* rabbit serum, prepared using killed whole-cell *F.tularensis* LVS components, identified background components within the *F. tularensis* cell lysate (Figure 10A). While there were bands present, there was no specific band that was of interest. A Western blot of *F. tularensis* LVS components was prepared and probed using Naive (not exposed to either *Brucella* or *Francisella*) mouse sera. A similar background banding pattern was present as was visible in Figure 10A.

Two major antigenic bands were identified by the *Brucella* primed anti-*F. tularensis* LVS sera (Figure 10B): one with a molecular weight of 17 kDa and the other with a MW of 52 kDa (see arrows). Both bands were present in all dilutions and decreased with intensity with decreasing *F. tularensis* LVS cell lysate concentration. This antigenic band did not correspond with any component visible in the Coomassie stained gel (Figure not shown). Also present, but

weaker in intensity, were bands at 34 kDa and approximately 100 kDa. Overall, there was a specific identification of two components: 52 kDa and 17 kDa. We suggest that the different banding patterns present between the DND Brucella primed sera and the Canadian Bioconcepts sera may be due to the live infection process that occurred in the preparation of the DND sera.

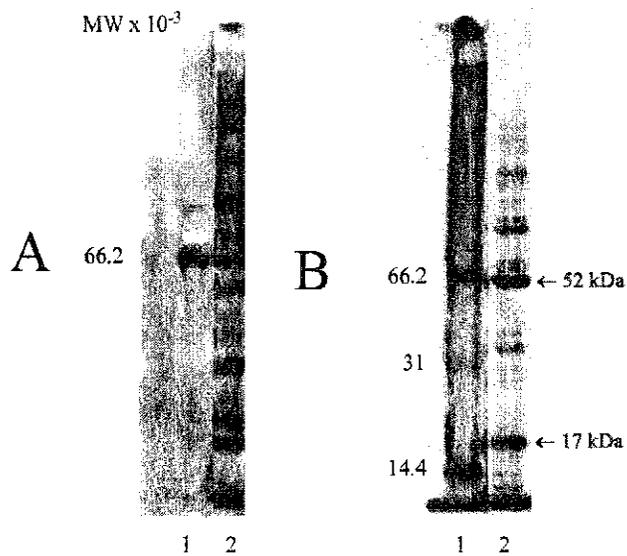


Figure 10

Immunoblot (Western) comparison of DND *Brucella*-primed anti *F. tularensis* murine antisera with rabbit anti-*F. tularensis* antisera. SDS-PAGE of *F. tularensis* LVS liquid culture cell lysates. Samples prepared and electrophoresed on a 7.5% SDS-PAGE and transblotted onto a nitrocellulose membrane Membrane probed under the following conditions:

A: Primary antibody: 1/1000 Canadian bioconcepts Rabbit anti-*F.tularensis* antisera, secondary antibody: 1/3000 Sigma anti-rabbit-HRP. Lane 1: BioRad H/L MW marker (5 μ L), Lane 2: *F. tularensis* LVS liquid cell culture lysate 3.1 ng.

B: Primary antibody: 1:1000 *Brucella*-primed anti *F. tularensis* murine antisera, secondary antibody: 1:3000 Caltag anti-mouse-HRP. Lane 1: BioRad H/L MW marker (5 μ L), Lane 2: *F. tularensis* LVS liquid cell culture lysate 3.1 ng.

3.9 Mono-Q separation of *F. tularensis* LVS cell lysate components

Sonicated, cell-free *F. tularensis* LVS cell lysate was prepared as described, loaded onto a Mono-Q anion exchange column and eluted with a KCl gradient. Material eluted from the Mono-Q 10/10 column was monitored at 214nm and recorded (Figure 11). Fractions were separated by SDS-PAGE, transblotted onto nitrocellulose and probed with a 1:1000 dilution of the Brucella-primed anti-*F. tularensis* LVS sera as described previously (Figure 12).

A molecular weight marker was electrophoresed in Lane 1. A 52 kDa band appeared in Lanes 5 through 11, corresponding to Fractions 25 to 30 (Figure 12), with the strongest band appearing in Fraction 28 (Lane 9). Lane 6 (Fraction 25) had multiple immunogenic components, including the 52 kDa component, the 17 kDa component as well as two with molecular weights of approximately 23 kDa and 20 kDa. Also noted was the presence of an immunogenic band visible in Fraction 24 and 25 (Lane 5 and Lane 6) with an approximate molecular weight of 17 kDa. These bands correspond to the 17 kDa material on visible on the LPS stained gel of the same column fractions (Figure 7). This further supports the extracellular, and immunogenic nature of the 17kDa component. Fraction 29, was chosen for use in tissue culture experiments, as it appeared to have the least amount of extraneous antigenic material with respect to the amount of 52 kDa material present.

A second Mono-Q column was loaded with sonicated, cell-free *F. tularensis* LVS cell lysate. Material eluted from the Mono-Q 10/10 column was monitored at 214nm and recorded (Figure 13). Fractions were separated by SDS-PAGE, transblotted onto nitrocellulose and probed with a 1:1000 dilution of the Brucella-primed anti-*F. tularensis* LVS

sera as described previously (Figure 14). A molecular weight marker was electrophoresed in Lane 1. Fractions 23 through 34 were electrophoresed in Lanes 2 through 13. A 52 kDa band with high intensity appeared in Fractions 26 to 31 (Lanes 5 through 10) and at a lesser intensity in the other sample lanes. The 52 kDa component appeared as an immunogenic doublet in several of the lanes, for example Lanes 4 and 13 (Fraction 24 and 34). There were several other antigenic components present with molecular weights of approximately 80 kDa and 110 kDa, but these were of weaker intensity. Fraction 29 (Lane 8) possessed an acceptable purification of the 52 kDa component, with less non-52 kDa antigenic material than Fraction 28, and was chosen for use in vaccination experiments.

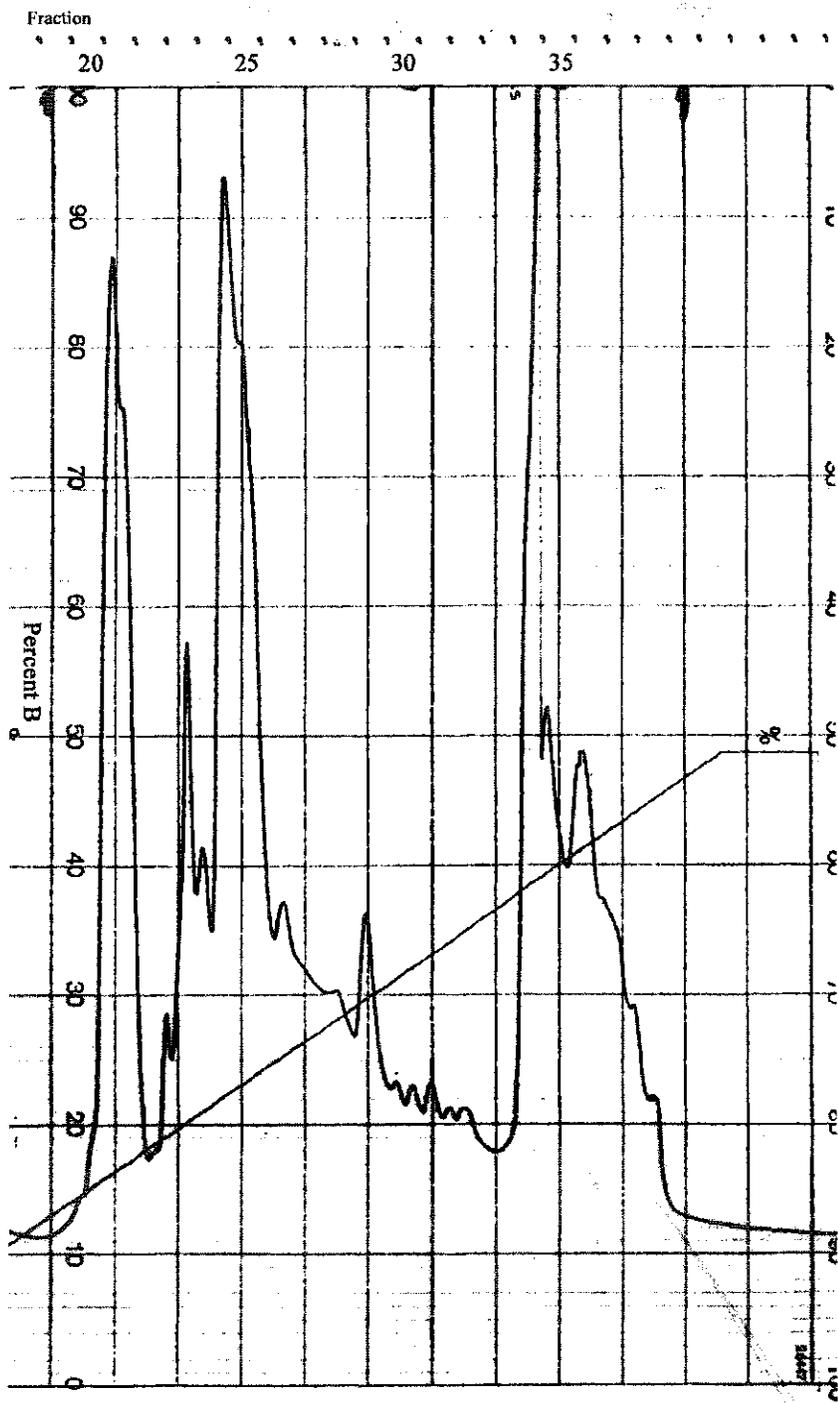


Figure 11
 Protein trace (214 nm) of Mono-Q separation of *F. tularensis* LVS cell lysate components. Fraction number appears between tic marks at top.

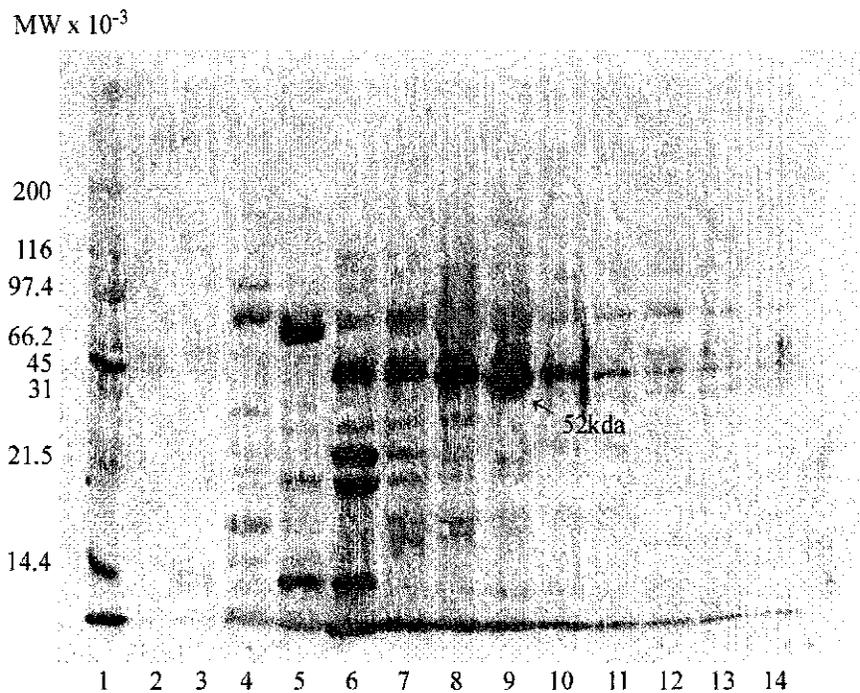


Figure 12

Immunoblot (Western) characterization of Mono-Q fractionated *F. tularensis* LVS liquid culture components. Cellular components from January 31 (2°) *F. tularensis* LVS liquid culture was prepared for column chromatography as described. Fractions collected in one minute intervals and electrophoresed on a 7.5% SDS PAGE of 1.0mm thickness as described and transblotted onto a nitrocellulose membrane. Protein concentrations unknown. Membrane probed under the following conditions: primary antibody: 1/1000 DND *Brucella/F. tularensis* mouse antisera, secondary antibody: 1/3000 Caltag anti-mouse-HRP.

Lane 1: BioRad H/L MW marker (10µL), Lane 2: Fraction #11, Lane 3: Fraction #22, Lane 4: Fraction #23, Lane 5: Fraction #24, Lane 6: Fraction #25, Lane 7: Fraction #26, Lane 8: Fraction #27, Lane 9: Fraction #28, Lane 10: Fraction #29, Lane 11: Fraction #30, Lane 12: Fraction #31, Lane 13: Fraction #32, Lane 14: Fraction #33.

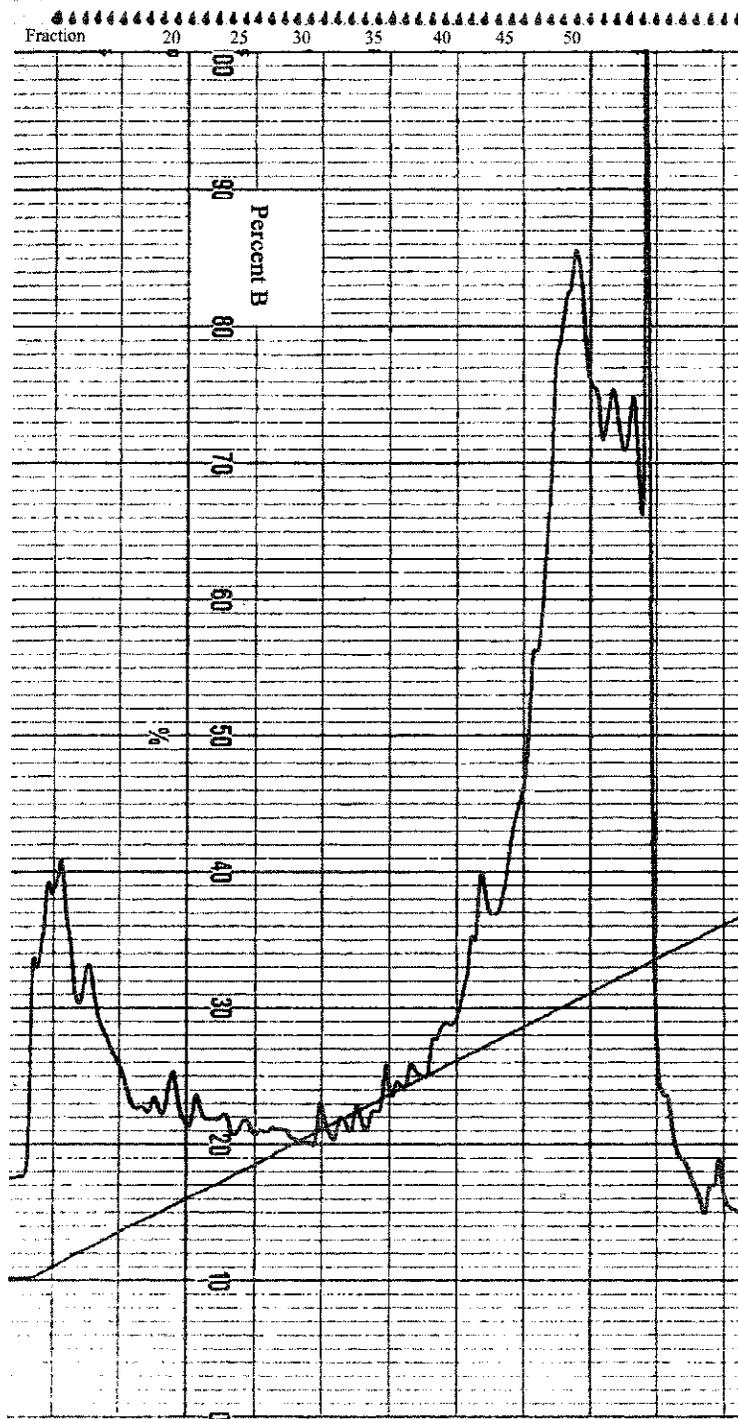


Figure 13
 Protein trace (214 nm) of Mono-Q separation of *F. tulrensis* LVS cell lysate components. Fraction number appears between tic marks at top.

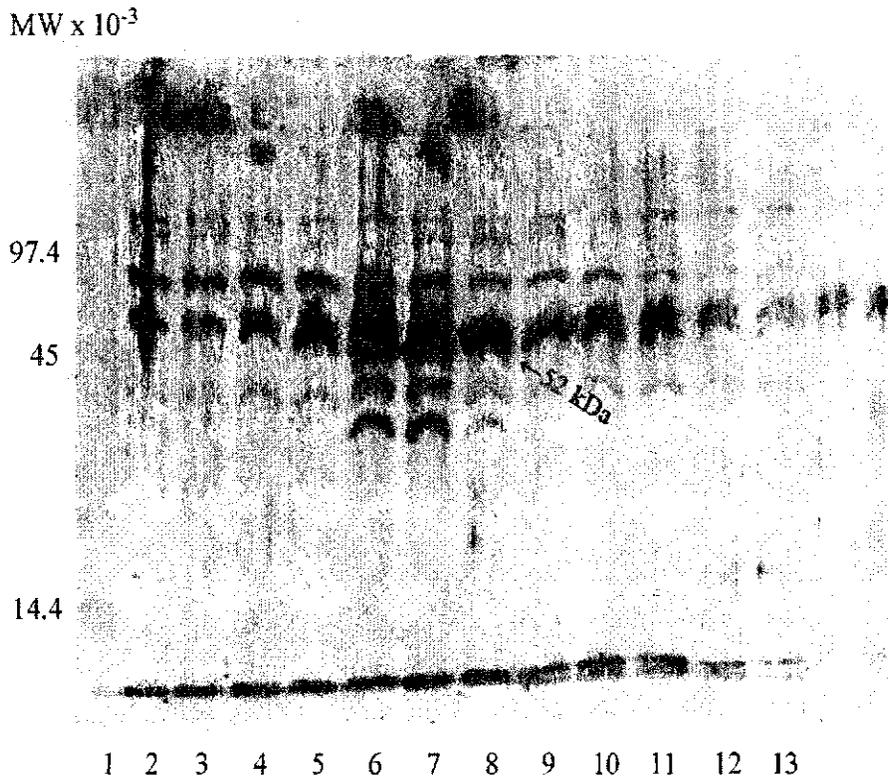


Figure 14

Immunoblot (Western) characterization of Mono-Q fractionated *F. tularensis* LVS liquid culture components. Cellular components from a *F. tularensis* LVS liquid culture was prepared for column chromatography as described. Fractions collected in one minute intervals and electrophoresed on a 7.5% SDS PAGE and transblotted onto a nitrocellulose membrane. Protein concentrations unknown. Membrane probed under the following conditions: primary antibody: 1/1000 DND Brucella primed anti-*F. tularensis* mouse antisera, secondary antibody: 1/30000 Caltag anti-mouse-HRP.

Lane 1: BioRad H/L MW marker (10 μ L), Lane 2: Fraction #23, Lane 3: Fraction #24, Lane 4: Fraction #25, Lane 5: Fraction #26, Lane 6: Fraction #27, Lane 7: Fraction #28, Lane 8: Fraction #29, Lane 9: Fraction #30, Lane 10: Fraction #31, Lane 11: Fraction #32, Lane 12: Fraction #33, Lane 13: Fraction #34.

3.10 Verification of pathogenicity of *F. tularensis* LVS

Pathogenicity of the stored *F. tularensis* live vaccine strain was demonstrated in two strains of mice: balb/c and CD1 (sample sizes of n=6 and n=7, respectively). Mice were given a 250 LD₅₀ dose of *F. tularensis* LVS on day=0 via an intranasal route. Health of the mice was followed for a three week period. Approximately four days post infection, both sets of mice began to exhibit symptoms of infection. These symptoms included: shakiness, ruffled fur, closed eyes and general lethargic behavior when compared to uninfected mice. All mice succumbed to the disease process by the end of the three week period.

All Balb/c mice (n=6) exposed to an IN dose of 250 LD₅₀ *F. tularensis* LVS succumbed by 11 days post infection (Figure 15). These mice exhibited symptoms sooner and than CD1 mice and expired, on average, 96 hours before the CD1 mice. The disease process was fast, with only three days separating the first and last victim of the disease. All Balb/c mice exposed to 250 LD₅₀ *F. tularensis* LVS perished.

All CD1 mice (n=7) exposed to and IN dose of 250 LD₅₀ *F. tularensis* LVS succumbed by 15 days post infection (Figure 15). While CD1 mice showed the same signs of infection as seen in the Balb/c set, the process did not seem as severe; i.e. the mice initially appeared less shaky and their fur was better maintained. CD1 mice survived approximately 96 hours longer than the Balb/c mice. Once established, the disease process was also fast with only four days separating the first and last victims. All mice (100%) within the CD1 group died as a result of the *F. tularensis* infection.

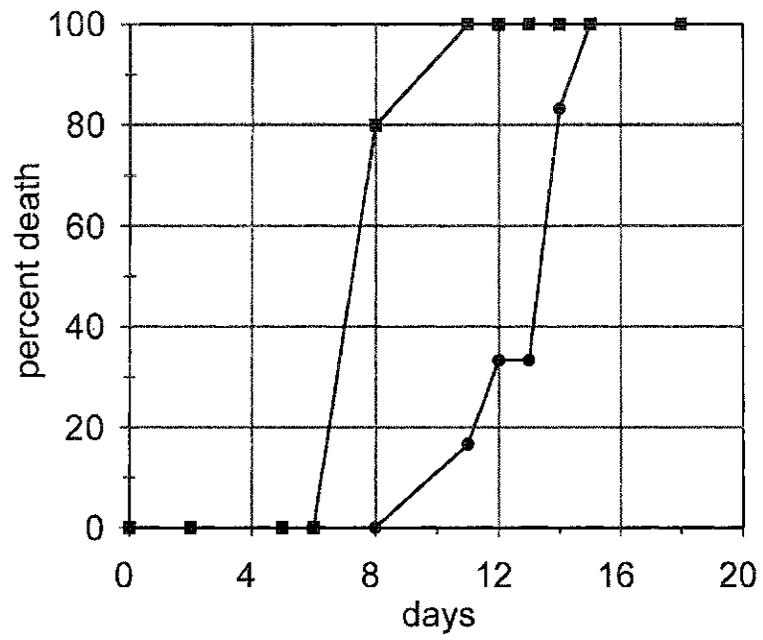


Figure 15
 Progression of Tularemia in control mice. 50 μ L IN injection of 250 LD₅₀ *F. tularensis* LVS in female balb/c and CD1 mice (• - CD1 n = 7, ■ - balb/c n = 6)

3.11 Immunization and challenge of CD1 mice

Forty-nine CD1 mice were used in a vaccination trial against *F. tularensis* LVS using subcellular components. At day=0, mice were given a 0.1 mL intraperitoneal (IP) injection of various components (Table 1). The components used to vaccinate mice were: 1) sterile PBS, 2) 1µg *B. abortus* 1119-3 OPS, 3) 1µg *B. melitensis* 16M OPS/protein, 4) 1µg *B. suis* 145 OPS/protein, 5) 2µg *F. tularensis* LVS cell lysate, 6) 2µg *F. tularensis* LVS cell lysate + 1µg *B. suis* 145 OPS/protein, 7) 2µg >30 kDa *F. tularensis* LVS supernatant (from pressure ultrafiltration), 8) 2µg >30 kDa *F. tularensis* LVS supernatant (from pressure ultrafiltration) + 1µg *B. suis* 145 OPS/protein, 9) Fraction 29 *F. tularensis* LVS cell lysate purification (Section 3.2) and 10) Fraction 29 *F. tularensis* LVS cell lysate purification (Section 2.9) + 1µg *B. suis* 145 OPS/protein. Five mice were in each of the test groups. Four mice were placed in the control group which received sterile PBS as a vaccination product.

The level of protection incurred against *F. tularensis* LVS infection was gauged by superinfection with 250 LD₅₀ of *F. tularensis* LVS delivered intranasally (IN). Test mice in groups one through four were challenged with this dose 33 days post immunization. For groups five through eight, the *F. tularensis* LVS challenge was at 31 days and for groups nine and ten, the challenge was at 26 days. Mice were monitored for two weeks post infection for symptoms of infection.

The control group of four mice showed signs of disease five days post challenge with 250 LD₅₀ of *F. tularensis* LVS. At nine days post challenge, all control mice succumbed to the disease (Table 2A). This corresponded with results from the initial potency trial which assessed 250 LD₅₀ of *F. tularensis* LVS for infection (Table 1) and confirmed the pathogenicity of the *F. tularensis* LVS stock.

	Injection type	sample size	total dead	percent protection
1	Control (0.1ml PBS)	4	4	0
2	<i>B.abortus</i> 1119-3 OPS (1µg)	5	3	40
3	<i>B.melitensis</i> 16M OPS/protein (1µg)	5	5	0
4	<i>B.suis</i> 145 OPS/protein (1µg)	5	5	0
5	<i>F.tularensis</i> cell lysate (2µg)	5	0	100
6	<i>B.suis</i> OPS/1 day <i>F.tularensis</i> lysate	5	0	100
7	>30 kDa supernatant (2µg)	5	4	20
8	>30 kDa supernatant/ <i>B.suis</i> OPS	5	0	100
9	fraction #29 (52 kDa enriched) (2µg)	5	4	20
10	fraction #29 (2µg)/ <i>B.suis</i> OPS	5	3	40

Table 1:

Protection conferred upon CD1 mice by various vaccine preparations. Mice were immunized with supernatants, lysates and fractions from *F. tularensis* LVS in combination with various *Brucella* OPS isolates. Two weeks post immunization, mice were challenged with a 50µL intranasal injection of 250 LD₅₀ *F.tularensis* LVS and the disease process monitored. Data recorded three weeks post infection.

Table 2A

F. tularensis Potency Trial. The pathogenicity of stock *F. tularensis* LVS was confirmed in both CD1 and Balb/c mice strains. A time=0, mice were exposed by intranasal injection of 2.5 LD₅₀ *F. tularensis* and monitored for 18 days. Number of surviving (s) and dead (d) mice were recorded each day.

Mice Strain	Number Exposed	June 29 (Day 0)	July 4 (Day 5)	July 5 (Day 6)	July 7 (Day 8)	July 10 (Day 11)	July 11 (Day 12)	July 12 (Day 13)	July 13 (Day 14)	July 14 (Day 15)	July 17 (Day 18)
CD1	7	7s	7s	7s	7s	6s/1d	5s/2d	5s/2d	2s/5d	1s/6d	1s/6d
Balb/c	6	6s	6s	6s	2s/4d	1s/5d	1s/5d	1s/5d	1s/5d	1s/5d	1s/5d

Table 2B

Vaccine Protection Trial. The ability of various vaccine preparations to protect against *F. tularensis* LVS was tested in a CD1 murine strain. At time = 0 mice were immunized with various bacterial cell extracts prepared from *B. abortus*, *B. melitensis*, *B. suis*, and *F. tularensis*. Between 26 and 33 days post infection, the mice were challenged with 250 LD₅₀ of *F. tularensis* and monitored for 12 days. Numbers of surviving (s) and dead (d) mice were recorded each day.

Trial #	Treatment	No	Days Pre-Infection				Days Post Infection						
			June02	June04	June09	July05 (0)	July0 6 (1)	July1 0 (5)	July1 1 (6)	July1 2 (7)	July1 3 (8)	July1 4 (9)	July1 7 (12)
1	Control (0.1mL PBS)	4	Immunised			250 LD ₅₀		4s	4s	2d/2s	3d/1s	4d	4d
2	<i>B. abortus</i> 1119-3 OPS (1µg)	5	Immunised			250 LD ₅₀		5s	1d/4s	2d/3s	2d/3s	3d/2s	3d/2s
3	<i>B. melitensis</i> 16M OPS / protein (1µg)	5	Immunised			250 LD ₅₀		5s	1d/4s	3d/2s	4d/1s	4d/1s	5d
4	<i>B. suis</i> 145 OPS/protein (1µg)	5	Immunised			250 LD ₅₀		5s	5s	3d/2s	4d/1s	4d/1s	5d
5	<i>F. tularensis</i> cell lysate (2µg)	5		Immunised		250 LD ₅₀		5s	5s	5s	5s	5s	5s
6	<i>B. suis</i> OPS (1µg) / <i>F. tularensis</i> cell lysate (2µg)	5		Immunised		250 LD ₅₀		5s	5s	5s	5s	5s	5s
7	<30 kDa supernatant (2µg)	5		Immunised		250 LD ₅₀	1d/4s	2d/3s	2d/3s	2d/3s	3d/2s	3d/2s	4d/1s
8	<30 kDa supernatant / <i>B. suis</i> OPS	5		Immunized		250 LD ₅₀		5s	2d/3s	2d/3s	2d/3s	2d/3s	2d/3s
9	fraction #29 (2µg)	5			Immunised	250 LD ₅₀		1d/4s	1d/4s	1d/4s	3d/2s	4d/1s	4d/1s
10	fraction #29 (2µg) / <i>B. suis</i> OPS	5			Immunised	250 LD ₅₀		5s	5s	5s	1d/4s	2d/3s	3d/2s

3.12 Protection conferred with *F. tularensis* LVS components

Three different *F. tularensis* cell preparations were tested for their ability to impart immunity against infection; 1) *F. tularensis* LVS cell lysate prepared by sonication and fixed in azide, 2) >30 kDa *F. tularensis* LVS supernatant fraction obtained from pressure ultrafiltration and 3) Fraction 29 *F. tularensis* LVS cell lysate via Mono-Q anion exchange purification (Section 3.9).

Mice receiving 2µg of the *F. tularensis* cell lysate exhibited no signs of infection (Trial 5, Table 2). At the end of two weeks, no mice (0/5) perished as a result of the disease process. The cell lysate imparted 100% immunity to 250 LD₅₀ *F. tularensis* LVS infection. There appear to be components in the sonicated, azide-fixed *F. tularensis* LVS cell lysate which are able to prime the murine immune system sufficiently to protect the animal against superinfection.

Mice receiving 2µg of the >30 kDa *F. tularensis* LVS supernatant (Trial 7, Table 2) preparation (from pressure ultrafiltration) exhibited a similar pattern to that of the control group which received a placebo of sterile PBS. By 12 days post infection, four out of five (4/5) of the mice succumbed to infection..

Mice which received 2µg of the Mono-Q separated *F. tularensis* LVS (Fraction 29) succumbed to infection (Trial 9, Table 2) in a manner similar to that of the control group, which was immunized with sterile PBS. At 12 days post infection, three out of five (3/5) of the mice succumbed to the disease. Fraction 29, which is a 52 kDa-enriched fraction, appeared to delay mortality, but did not impart immunity.

3.13 Protection conferred with *Brucella* spp. OPS components

Three different *Brucella* spp. OPS preparations were tested for their ability to impart immunity against IN infection of 250 LD₅₀ *F. tularensis* LVS. One microgram of: *B. abortus* 1119-3 OPS, *B. melitensis* 16M OPS/protein and *B. suis* 145 OPS/protein were used to immunize separate groups of mice (Table 1).

Mice receiving the *B. abortus* 1119-3 OPS had a 60% death rate (3/5 mice) 12 days post infection (Trial 2, Table 2). All mice (5/5) receiving the *B. melitensis* 16M OPS/protein (Trial 3, Table 2) and the *B. suis* 145 OPS/protein (Trial 4, Table 2) died by 12 days post infection (100% mortality) (Table 2, Figure 16).

3.14 *F. tularensis* / *B. suis* OPS cross protection

CD1 mice were injected with *F. tularensis* LVS cell lysate (2µg) combined with the *B. suis* 145 OPS/protein (1µg) (Trial 6, Table 2). All mice survived (5/5). They showed no discernable signs of infection after 12 days. This 100% protection to 250 LD₅₀ *F. tularensis* LVS did not differ from the protection imparted by the *F. tularensis* cell lysate alone (Trial 5, Table 2 and Figure 16).

Mice immunized with Fraction 29 (2µg) of the *F. tularensis* cell lysate fractionation combined with the *B. suis* 145 OPS/protein preparation (1µg) exhibited 40% protection (Trial 10, Table 2). An increase in the rate of protection, when compared to Fraction 29 without the *B. suis* OPS, was that of a single mouse and not statistically significant. Mice injected with this combination exhibited a similar pattern of sickness and death as was seen for mice receiving the

52 kDa-enriched fraction (Fraction 29) by itself (Table 2).

CD1 mice receiving the *F. tularensis* LVS supernatant (>30 kDa, from pressure ultrafiltration) combined with *B. suis* 145 OPS/protein (Trial 8, Table 2) exhibited a 100% survival rate, or 5/5 mice. This combination raised the level of protection of the >30 kDa supernatant against 250 LD₅₀ *F. tularensis* LVS from only 20% to 100% (Figure 16).

Although the mice exhibited signs of sickness after five days, they rapidly recovered. At 12 days, three of the five mice were mildly ill and appeared to be recovering. At the end of the study, all mice appeared to be functioning normally and healthy. The *B. suis* OPS, in combination with the material from *F. tularensis* LVS with a MW of >30 kDa, successfully protected the CD1 mice from superinfection with 250 LD₅₀ *F. tularensis* LVS.

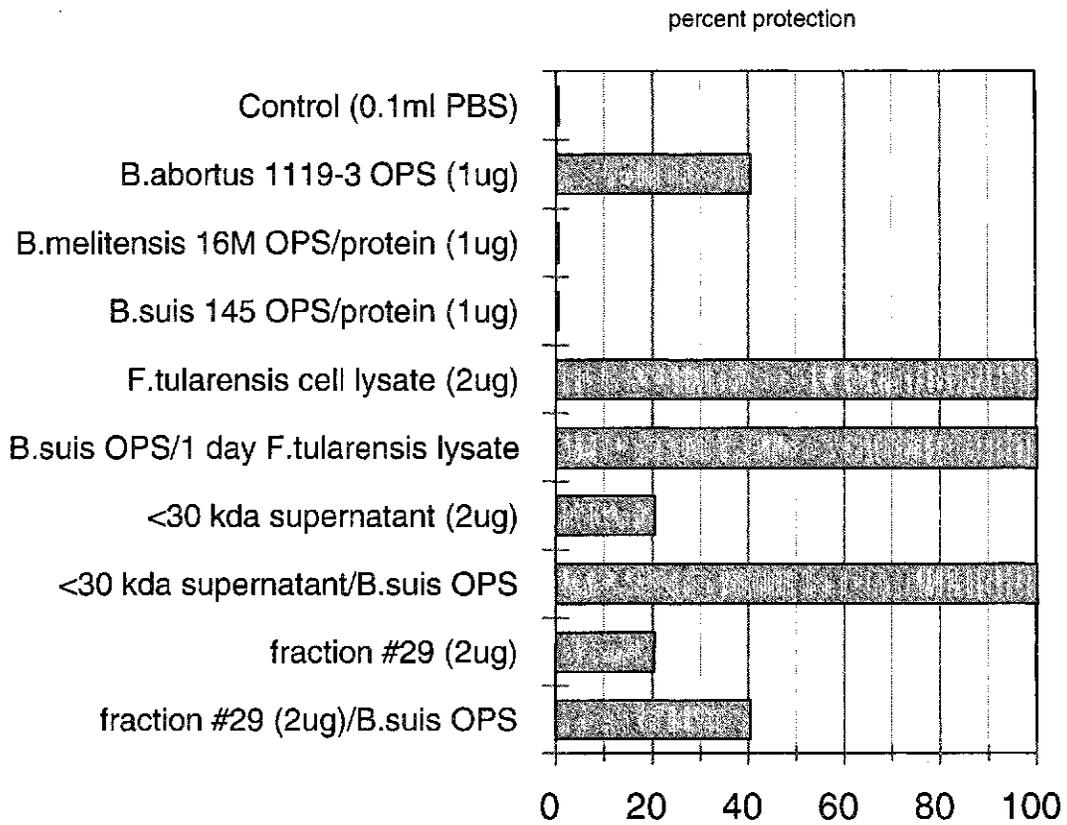


Figure 16

Proportion of protection conferred upon CD1 mice by various vaccine preparation, supernatants, lysates or fractions. 50 μ L intranasal injection of 250 LD₅₀ *F. tularensis* LVS. Data recorded three weeks post infection. Sample size for all non-control groups is 5.

3.15 Viability of J774.1 Cells in Response to *F. tularensis* supernatants

The J774.1 murine macrophage cell line was utilized to investigate the cytotoxic effects of sterile *F. tularensis* LVS supernatants. The cell line was maintained, and prepared as described. The cells were treated with sterile *F. tularensis* LVS supernatant with the protein ranging from 0.002 mg/mL to 0.25 mg/mL (defined as 7D) and 0.002 mg/mL to 2.0 mg/mL (defined as 2°) respectively (Figure 17). A logarithmic scale was utilized to demonstrate the cell viability, due to the large range of protein concentrations used in the experiment. Viability was measured 24 hours post treatment, subjectively by direct observation and quantitatively using the non-toxic indicator dye, Alamar Blue (Accumed International Inc., Westlake, OH). Cell viability controls were created by using a sham inoculation of sterile PBS, which resulted in a determined cell viability of 1. Cell viability of zero was determined when cells were treated with an inoculation of sulfuric acid.

Cells receiving the highest protein concentration of *F. tularensis* supernatant (from 1.005 to 2.010 mg/mL) appeared rounded and detached from the tissue culture support. Untreated control cells maintained their bipolar morphology and remained attached to the tissue culture support. Cells treated with 1.005 to 2.010 mg/mL *F. tularensis* supernatant protein had a reduced cell viability relative to the untreated control cells (Figure 17). Cells receiving protein concentrations of 0.501 mg/mL and less, did not appear to exhibit deleterious effects as assessed by direct observation and viability dye analysis. Cytotoxic effects of the *F. tularensis* supernatant protein on J774.1 murine macrophages was limited to protein concentrations of 1.005 mg/mL and higher.

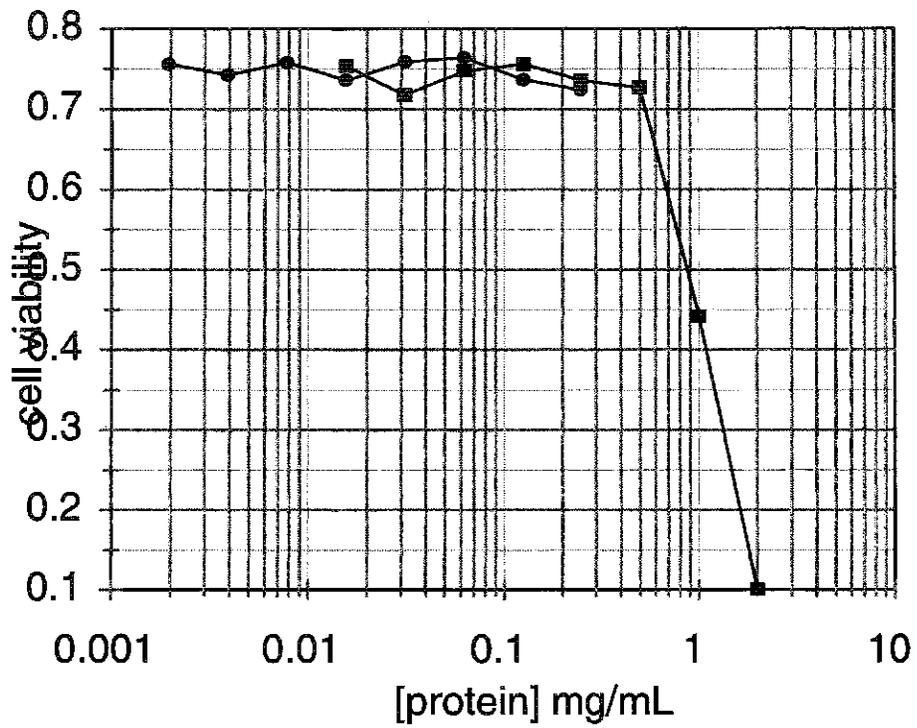


Figure 17
 Alamar Blue cell viability of J774.1 macrophage cells in response to 24 hours treatment of two *F.tularensis* liquid culture supernatants. (● - 7D culture supernatant, ■ - 2° culture supernatant). The range of cell viability was determined as: fully healthy (cell viability = 1), and complete cell death (cell viability = 0).

3.16 NO production in J774.1 Cells in Response to *F. tularensis* supernatants

The J774.1 murine macrophage cell line was utilized to investigate the production of a rudimentary cytokine burst in response to sterile *F. tularensis* LVS supernatants. The cell line was maintained, and prepared as described. The cells were treated with sterile *F. tularensis* LVS supernatant with protein concentrations ranging from 0.002 mg/mL to 0.25 mg/mL (defined as 7D) and 0.002 mg/mL to 2.0 mg/mL (defined as 2°) respectively (Figure 18). A logarithmic scale was utilized to demonstrate the nitric oxide production, due to the large range of protein concentrations used in the experiment. The level of nitric oxide (NO) was measured 24 hours post treatment with sterile *F. tularensis* LVS components and was compared against a standard curve created with stock sodium nitrite.

The total amount of NO produced over the 24 hours followed a linear pattern between *F. tularensis* supernatant protein concentrations 0.031 and 0.503 mg/mL. At a protein concentration of 0.503 mg/mL, the total NO produced over the 24 hours period reached a maximal level of 11 µg/mL. This concentration was eleven times above the baseline level of NO production by the control cells over the same period. Nitric Oxide production decreased sharply in cells treated with two highest protein concentrations of 1.005 and 2.010 mg/mL *F. tularensis* supernatant protein, respectively, likely due to the cytotoxic effects of the treatment solution (see section 3.15).

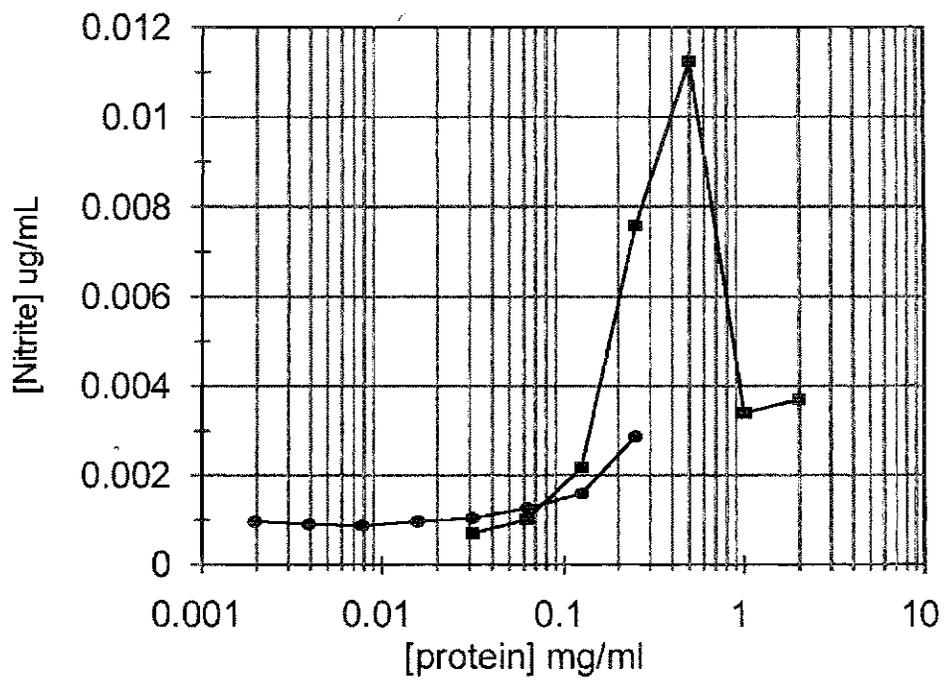


Figure 18
 Nitric oxide (NO) production by J774.1 macrophage cells in response to treatment solution of two *F. tularensis* LVS supernatants for 24 hours (• - 7D liquid culture supernatant, ■ - 2° liquid culture supernatant). Nitrite levels compared against a standard curve created with stock sodium nitrite.

3.17 Viability of J774.1 murine macrophages - 52 kDa treatment

The J774.1 murine macrophage cell line was used to investigate the cytotoxic effects of several *F. tularensis* LVS fractions. The cells were exposed for 24 hours to fractions from a Mono-Q separation of *F. tularensis* LVS liquid culture cell lysate (Section 3.9). Cells were treated with Fractions 19 through 33 from the Mono-Q fractionation of *F. tularensis* LVS cell lysate and were assessed 24 hours post exposure for: 1) viability and 2) amount of nitric oxide (NO) produced.

The *F. tularensis* LVS cell lysate was eluted from the Mono-Q column by a KCl salt gradient and collected in one minute intervals (Section 3.9). The J774.1 murine macrophages were treated for 24 hours with a salt gradient within the range which eluted the 52 kDa protein (0 to 1M KCl). These cells were assessed for viability and NO production. There was no decrease in cell viability indicated by either direct observation or analysis with vital indicator dye (Alamar Blue) noted within this range of salt concentration when compared to control wells receiving no KCl gradient (data not shown). There was also no associated increase in NO production above background levels when compared to control wells receiving no KCl gradient (data not shown).

The viability of J774.1 macrophages was measured 24 hours post treatment by direct observation and using the non-toxic indicator dye, Alamar Blue. Cells receiving aliquots of the Mono-Q fractionated *F. tularensis* LVS cell lysate did not differ morphologically from control cells receiving no treatment. Cells maintained their bipolar morphology and remained attached to the tissue culture support matrix. The viability of treated cells, by analysis with indicator dye,

did not differ from that of the control cells (data not shown). The Mono-Q fractionated *F. tularensis* LVS cell lysate had no cytotoxic effect on the J774.1 macrophages after 24 hours treatment.

The production of nitric oxide in 24 hours by J774.1 murine macrophages in response to fractionated *F. tularensis* LVS cellular components was measured (Section 3.9, Figures 11 and 12). Cells treated with Fractions 25 through 29 produced NO above the baseline level of production by the control cells over the same period (Figure 19). Cells receiving Fractions 25 and 26 produced levels of NO 10 and 8 times above background levels, respectively (Figure 19). This level NO induction corresponds with Lanes 6 and 7 in Figure 12. There are components within the Mono-Q fractionated *F. tularensis* LVS cell lysate which appear capable of inducing nitric oxide synthesis.

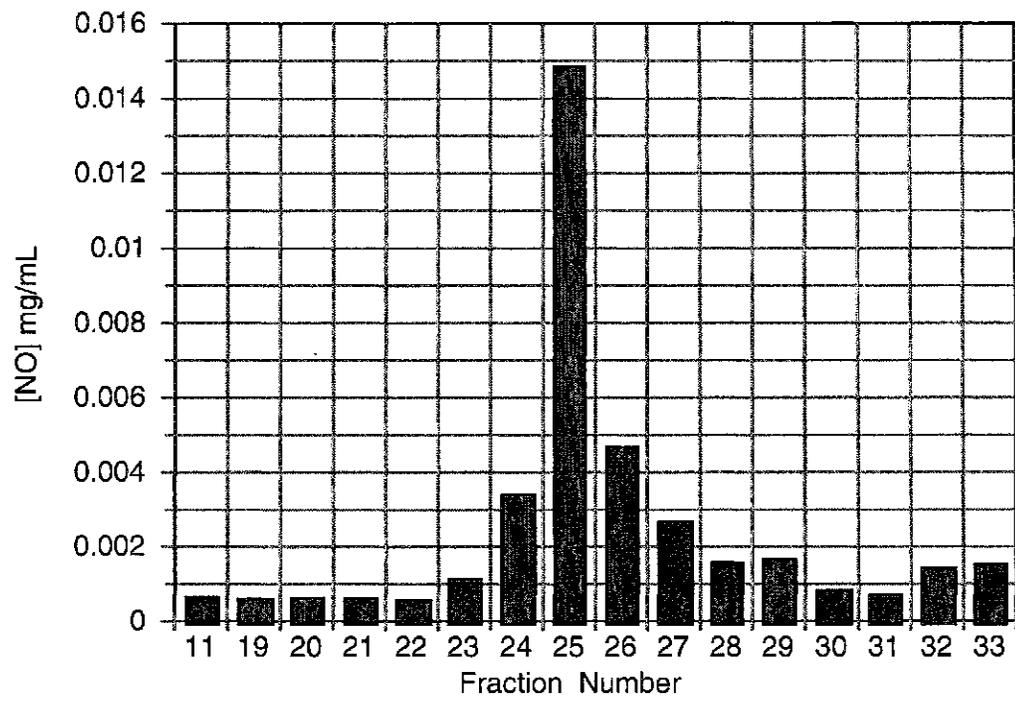


Figure 19
Nitric Oxide (NO) production by J774.1 macrophage cells in response to treatment by Mono-Q separated fractions of *F. tularensis* LVS cell lysate for 24 hours.

4.0 Discussion

The development of long term immunity to *F. tularensis* is a complex and largely unexplored area. The intracellular nature of this organism, coupled with its particularly high virulence, makes it difficult to study. Our research characterizes a cellular component which was identified through a novel method of vaccination. This component is highly immunogenic, is capable of inducing a cytokine response and imparting some degree of protection to infection.

4.1 Antigenic components of *F. tularensis*

A novel approach was used to identify proteins associated with the response of animals to *F. tularensis* infection. The usual method of studying the immune response in animals towards a lethally infectious agent is to immunize a subject with killed whole cell components. Attenuated, or genetically modified strains which are compromised in their ability to elicit infection, can also be used, but the nature of the *F. tularensis* LVS attenuation is not known (Ellis, *et al.*, 2002). With the LD₅₀ of *F. tularensis* LVS for some strains of mice approaching a single organism (Elkins *et al.*, 2003), animals challenged with infection by whole cell pathogens may perish before immune sera can be collected.

Recent work by Kieffer *et al.* (2003) has suggested that the use of *F. tularensis* LPS can protect against challenge by either *F. tularensis* LVS or *F. novicida*. However, this protection was gained only three days post immunization, far before the development of humoral or cellular immunity. Regardless, the protection appeared to be long term. The notion that perhaps polysaccharide could be used to prime the immune response against future

infection was entertained.

It was noted that *F. tularensis* antisera cross reacts with *Brucella* spp. (Francis, 1926; Behan, *et al.*, 1982). Our results indicate that when balb/c mice were immunized with an experimental DND *B. suis* 1193 OPS/protein vaccine, 60% were protected when challenged with 10 LD₅₀ of *F. tularensis* LVS (12/20 survival), suggesting a method of obtaining anti *F. tularensis* serum against the infection process, not just against killed cells. Sera collected from these *Brucella*-primed *F. tularensis* challenged mice were used in all subsequent experiments. The sera appeared to contain antibodies which were useful in protecting animals against live *F. tularensis* LVS infection (Table 1 and Table 2). Identification of a component involved in the live infection may be key to providing protection against disease. Identification of an immunologically significant protein involved in the live disease process may lead to the development of a new vaccine. Such a protein candidate was identified with the aforementioned serum: a 52 kDa component.

Amicon pressure filtration with a 30 kDa molecular weight cutoff membrane (MWCO) was used to concentrate a 52 kDa component from *F. tularensis* LVS liquid culture supernatants. This component was identified using murine anti *F. tularensis* sera, prepared from the *Brucella* primed *F. tularensis* challenged mice (Figure 1 and Figure 3), but not with serum prepared using killed whole cells. While previous researchers have identified immunologically sensitive 17 kDa, 23 kDa and 60 kDa components, this 52 kDa component has not been previously described. This 52 kDa component may represent a potential subcellular vaccine candidate for *F. tularensis*.

The 52 kDa component appeared in all *F. tularensis* cell lysates (Figure 1). It did not appear to be up or down regulated during subculturing (with standardization of protein loading), nor did it appear to be differentially expressed with increasing culture time. The 52 kDa component may be extracellular or intracellular in nature. Although the amount of extracellular material likely increased with increased culture time, the increase was likely due to a greater amount of cell lysis; *i.e.*, total protein released by lysis of the cells increased as the 52 kDa component increased in concentration (Figure 5).

Periodically, the 52 kDa component appeared as a doublet of approximately 51 kDa and 52 kDa, respectively (Figure 1 and Figure 4). We hypothesize that upon subculturing the 51/52 kDa component may be: 1) proteolytically processed in response to external influences thereby decreasing its molecular weight and/or 2) glycosylated with a specific carbohydrate sequence, thereby increasing its molecular weight. This hypothesized modification has unknown significance to the state of the culture or to the activity of the component.

Previous work has been unsuccessful in identifying new immunologically active cellular components. Of note, was a 17 kDa component which has been previously identified as a cell membrane component by Sjostedt *et al.* (1991), a 23 kDa component that is an apparent heat shock protein (Golovliov *et al.* 1997) as well as a 60 kDa chaperonin protein. The 17 kDa component is active in T-cell proliferation assays, suggesting that it may be a candidate for future subcellular vaccines. Our work suggests that there are other components within the *F. tularensis* LVS cell lysate that are capable of eliciting a NO. While the 52 kDa component was detected in all fractions able to elicit NO, it is possible that it may be a key component in

provoking a cytokine burst. With the understanding that a cytokine burst is necessary to control *F. tularensis* infection, this induction shows that this component, or others like it may play an integral part in protecting the host against infection.

Periodate-based stains used to detect carbohydrates attached to the 52 kDa were inconclusive. Staining of a Mono-Q 10/10 fractionated *F. tularensis* LVS cell lysate resulted in the identification of numerous components (Figure 7), including a 17 kDa component which is proven to be extracellular. The 52 kDa component did not stain in these experiments suggesting that there was no carbohydrate associated with it suggesting an intracellular nature to the component.

F. tularensis may harbor a plasmid that encodes toxins. This plasmid might be released into the media, or differentially expressed when the bacterium is stressed. However, when the bacteria were exposed to the stressful condition of growth on synthetic media (CSM) and serially subcultured no plasmids were detected.

Enzymatic digestion of *F. tularensis* LVS liquid culture supernatant was carried out to characterize the 52 kDa component. Lipase, RNAase and lysozyme had no effect on detection of the 52 kDa component by *Brucella*-primed anti *F. tularensis* antiserum suggesting that the 52 kDa component lacks an RNA, lipid or N-acetylmuramic acid moiety. A general protease also was used to determine if the 52 kDa component was a protein. Proteinase-K, which cleaves peptide bonds, effectively eliminated all antibody-based detection of the 52 kDa component, making it apparent that the 52 kDa component is mostly protein in nature.

Treatment of the 52 kDa protein with DNAase lowered its molecular weight from 52

kDa to 37 kDa (Figure 8). This shift in molecular weight following treatment by DNAase suggests that the 52 kDa protein may be a nucleoprotein. Autolysis may have occurred within the bacterial culture, releasing this component into the solution. Likewise, during infection, destruction of the bacteria takes place as the immune system protects the host, thus allowing the component to be displayed to the host's cellular immune system. Such a component may only be detectable as a result of a live challenge. Given the pathogen's intracellular nature and the innate ability of the host to fight infection, protection against such an intracellular component may be useful in conferring long term immunity.

4.2 Cytotoxicity of *F. tularensis*

It has been noted that sterile *F. tularensis* LVS supernatants grown in liquid CSM are able to kill Balb/c mice (Cherwonogrodzky, unpublished data). Because screening in animals is expensive, a tissue culture model was adopted to investigate this property further. Macrophages are the primary defense against cellular pathogens. *F. tularensis* is an intracellular pathogen that is capable of surviving within macrophages and evading the immune response. A murine macrophage cell line, J774.1a, was chosen to mimic macrophage infections in culture and act as a model for *F. tularensis* induced cell cytotoxicity. Cell viability was assayed using AlamarBlue, a vital dye which only is taken up by viable cells.

Macrophages were treated with a KCl salt gradient to mimic the range of concentrations used in Chamberlain's synthetic media and *F. tularensis* cell lysate FPLC fractions used in our experiments. There was no increase in cellular death associated with the

salt concentrations tested. This indicated that the J774.1 murine macrophages remained fully viable within the range of salt concentrations present in the treatment solutions and any cytotoxic effects observed were due to other components in solution. Cytotoxic effects were not observed in the J774.1 cultures treated with cell fractions prepared using FPLC. However, nitric oxide was induced by a few of these fractions without induction of cell death. Cytotoxic effects were observed when cells were treated with high (>1.00 mg/mL) concentrations of *F. tularensis* LVS cell lysate. This suggests that there are components within *F. tularensis* LVS which are capable of inducing cell death. Apoptosis has previously been identified as an important factor in fighting infection (Lai *et al.*, 2001); defense against such intracellular pathogens may include triggering the apoptotic pathway (Kauffman, 1999).

4.3 Cytokine and Immune Response

The immune response against intracellular pathogens is a complex one and the relationship between the cytokine response and cellular action appears to be key to understanding the pathogenesis of disease. Research has pointed to a duplex role for T-cells and B-cells in the pathogenesis of disease. Work has shown that T-cell deficient mice can control infection, but not successfully clear disease. This is almost expected, given the intracellular nature of the bacterium. Interestingly, B-cell deficient mice can also control infection as well as successfully clear the disease, but are impaired in their ability to clear a second infection (Elkins *et al.* 2003). This impairment of long term immunity is consistent with the loss of B-cell function and again points to the requirement of functioning T-cells for defense

against *F. tularensis*. It appears that the role of B-cells in controlling infection is somewhat mixed and that T-cells may be the determining factor in preventing outright disease. To support this notion, Poquet *et al.* (1998) documented the expansion of a particular class of T-cells in response to *F. tularensis* infection. This increase in T-cell number occurred within 1-3 weeks post infection and appeared related to the presence of phospho-antigens. Such an expansion indicates the requirement for intact T-cell function in the long-term defense against *Francisella*.

The cytokine response to infection is complex and is largely not understood. Research has indicated that mice deficient in the ability to produce NO or IFN- γ are exquisitely sensitive to *F. tularensis* infection (Elkins *et al.* 2003). But it has also been noted that the LPS from *F. tularensis* does not induce either NO or IFN- γ , pointing to the importance such cytokines have in bacterial defense (Kieffer, *et al.*, 2003). In our study, we show that NO is produced by J774.1 macrophages after exposure to *F. tularensis* LVS cell lysates for 24h. Production of NO by murine macrophages in response to *F. tularensis* non-LPS components has not been demonstrated previously. With the importance that such cytokines have in the defense against *Francisella*, identifying an inducer of the cytokine burst is key to boosting immune reaction to disease. We showed that NO production increased when concentrations of *F. tularensis* LVS cell lysate was increased (Figure 18). At high concentrations of cell lysate, a cytotoxic effect resulting in the death of exposed macrophages was noticed which caused a marked decrease in NO production (Figures 17, 18). These results support the notion that NO production by macrophages is important to the development of an immune response to *F. tularensis* and that our novel 52 kDa component may be capable of inducing such a cytokine burst.

However, not all fractions from a Mono-Q fractionated *F. tularensis* LVS cell lysate were able to induce NO production in J774.1 cells. The highest concentration of NO was induced with Fraction 24 of the extract (Figure 19), indicating that a limited number of cell constituents are involved in the induction of NO. Fraction 24 contained a high concentration of protein in solution and included immunogenic components with molecular weights of 17 kDa, 34 kDa and 52 kDa. We believe that these components are important to the host response against *F. tularensis*.

4.4 Development of a Tularemia vaccine

In this study, we demonstrated that CD1 mice are susceptible to *F. tularensis* infection. Three vaccination scenarios for CD1 mice were tested: 1) the ability of *F. tularensis* LVS cellular components to evoke cross protection against a *F. tularensis* LVS challenge 2) ability of *Brucella* spp. OPS components to evoke cross protection against a *F. tularensis* LVS infection and 3) the ability of *Brucella* spp. OPS-based vaccine mixed with various *F. tularensis* components to provide immunity to *F. tularensis* infection.

Components in the *F. tularensis* LVS cell lysate appeared to possess components which were able to prime the murine immune system sufficiently to confer 100% protection against 250 LD₅₀ of *F. tularensis* LVS. Lower protection rates were observed with the 52 kDa enriched fraction (#29) (Figure 16, Table 1) and the >30 kDa concentrate, 40% and 20% respectively. This suggests that mixtures of protein and cellular constituents may be required to induce complete protection against bacterial infection by *F. tularensis*.

Polysaccharide (OPS) is a powerful immunomodulator and can be used as a vaccine against various disease bacteria, such as *Meningococcus*, *Streptococcus* and, to some extent, *Francisella* (Kieffer, *et al.*, 2003). It has been noted that the OPS of *F. tularensis* LVS is not an effective immunomodulator and has been described as having only 1/1000 the activity of *E. coli* polysaccharide. It has also been noted that *Brucella* spp. has cross reactivity with *Francisella* spp. Using this knowledge, we hypothesized that it might be useful to 'prime' the murine immune system with *Brucella* OPS prior to *F. tularensis* infection.

In these studies we demonstrate that *Brucella* OPS primed, *F. tularensis* LVS challenged murine sera are cross reactive with proteins from *F. tularensis* LVS extracts. By itself, *Brucella* spp. OPS inferred very little protection against a 250 LD₅₀ *F. tularensis* LVS challenge (table 1). Only *B. abortus* 1119-3 OPS provided protection (40%) while *B. melitensis* 16M and *B. suis* 156 OPS did not provide any protection against a *F. tularensis* LVS challenge. However, when *B. suis* 145 OPS was combined with various *F. tularensis* LVS substituents, some level of protection was observed. *B. suis* OPS combined with *F. tularensis* LVS cell lysate showed no increase in protection as one cannot have higher than 100% protection. When *B. suis* OPS was combined with a 52 kDa enriched fraction (#29) (Figure 16, Table 1), an increase in protection from 20% to 40% was observed. While statistically insignificant, this may suggest that *B. suis* OPS is capable of boosting the immune response to *F. tularensis*. Similarly, when *B. suis* OPS was combined with >30 kDa Amicon concentrated *F. tularensis* components, a 100% protection against a 250 LD₅₀ *F. tularensis* LVS challenge was achieved (Figure 16, Table 1).

While *B. suis* OPS is not an effective vaccine against 250 LD₅₀ of *F. tularensis* LVS, it is able to prime the murine immune system enough to allow it to respond to various *F. tularensis* LVS cellular components in a vaccination trial. These results indicate that there are fractions of *F. tularensis* LVS which would be good candidates for vaccines.

4.5 Conclusion

Our work has contributed to many of these research areas. With the identification of a novel 52 kDa component using a method which requires complete resolution of infection, our component constitutes an immunologically significant protein. The characterization suggests that this 52 kDa component is protein in nature, is non-glycosylated and may be intracellular with a partial DNA component. We have also shown that the protein invokes a basic cytokine response, something key to the defense against disease. Our vaccine work, albeit preliminary, suggests a role for the 52 kDa protein in protecting against *F. tularensis* infection.

If the FDA approves the live-attenuated *F. tularensis* LVS for general use, the 52 kDa component may have potential to be used as a 'quality control' measure in vaccine preparations. Lastly, our detection of this component appeared to be very specific, with no cross reactivity occurring to either *Brucella* antisera, or with sera prepared using killed whole-cell *F. tularensis*. This protein may therefore be useful in early detection systems similar to those that employ the 17 kDa TUL4 lipoprotein.

There remains a great deal of work to be completed to fully characterize the 52 kDa protein. Our work suggests a role for this protein in infection as well as immunity and may

therefore be a powerful tool in further *Francisella* research. With the constant threat of bioterrorism, we believe it prudent that such proteins be characterized and investigated as thoroughly as possible. There is a great deal of potential present in this 52 kDa *F. tularensis* antigen.

4.6 Future Work

Francisella is a difficult organism to work with. There remains many unknowns with respect to the pathogenesis of disease, structure, genome as well as virulence. Some researchers have commented that *Francisella* is an 'enigma' (Titball, *et al.*, 2003). Many groups around the globe are investigating various aspects of the disease process. Major research areas include: epidemiology, early detection, genomic sequencing, 2D gel electrophoresis with protein identification (encompassing the greater class of proteomics), identification of virulence factors, immune response characterization and finally, improvement of the current vaccine.

We hope to further characterize this component. It would be prudent to repeat our experiments using 2D electrophoresis and attain a partial sequence for this 52 kDa protein. Once sequenced, we could clone this protein and gain sufficient, pure protein to perform immunological experiments. We might characterize further cytokine responses to this component, including TNF α , IFN- γ , and IL-12. The interaction of this component in tissue culture, using T-cells and macrophages, could also be explored.

As a product, the 52 kDa protein component has potential in a number of areas. It

may be useful as a sub-cellular vaccine to *F. tularensis*. Further testing with virulent strains is necessary to properly characterize the protein/host interaction.

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Appendix A

LPS Silver Stain

A six step silver staining method was utilized to preferentially stain LPS components present on the polyacrylamide gel. This method was modified from the standard Bio-Rad protocol. A periodic acid oxidizer was utilized in place of the supplied dichromate oxidizer.

Step	Action	Time
1	Fixer	2x15 min
2	Oxidizer	5 min
3	Wash	10 min
4	Silver Reagent	20 min
5	Wash	1-30 sec

Fixer:

25% (v/v) isopropanol

7% (v/v) acetic acid

Oxidizer

0.7% Periodic acid

40% ethanol (or isopropanol)

5% acetic acid

(Hitchcock and Brown (1983) Journal of Bacteriology 154:269-277)

Silver Reagent

1:10 dilution of Bio-Rad silver staining reagent with 3X distilled water

Developer

1.6% (w/v) Bio-Rad powder developer

Stop Bath

5% (v/v) acetic acid

Appendix B

Chamberlains Synthetic Media (CSM)	Component	mg/L	
	L-arginine (free base)	400	
Add all components to 90% volume of triple distilled water except L-Cysteine-HCl. Raise pH to 10 with 1.0N NaOH to bring undissolved substituents into solution. Bring pH to 7.4 with 1.0N HCl. Add remaining amino acid and bring to volume. Filter sterile through a 0.2µm filter sterilization unit (Nalgene 1.0L unit).	L-Aspartic Acid	400	
	L-Cysteine-HCl	200	
	L-Histidine (free base)	200	
	DL-Isoleucine	400	
	L-Leucine	400	
	L-Lysine	400	
	DL-Methionine	400	
	Chamberlain RE. Evaluation of Live Tularemia Vaccine Prepared in a Chemically Defined Media. Applied Microbiology 1965 13:232-235	L-Proline (hydroxy-L-Proline-free)	2000
		DL-Serine	400
		DL-Threonine (allo-free)	2000
L-Tyrosine		400	
DL-Valine		400	
Spermidine Triphosphate		40	
Thiamine-HCl		4	
DL-Calcium Panthothenate		2	
Glucose		4000	
Sodium Chloride		10000	
MgSO ₄ •H ₂ O		135	
FeSO ₄ •7H ₂ O		2	
KH ₂ PO ₄		1000	
K ₂ HPO ₄	1000		

Appendix C

Conditions for polyacrylamide gel electrophoresis

Component	7.5% gel	4% stacking gel
water (triple distilled)	6.08 mL	3.7 mL
3M Tris pH8.9	1.45 mL	0.6 mL
40% Acrylamide (29:1 acrylamide:bisacrylamide)	1.88 mL	0.5 mL
2% SDS (w/v)	0.5 mL	0.25 mL
TEMED	10 μ L	5 μ L
10% w/v Amonium persulfate	100 μ L	50 μ L

2X Sample Buffer

100 mM Tris-HCl, pH 6.8

20% glycerol

2% SDS

200 mM DTT

0.1% bromophenol blue

10X running buffer

30g Tris base

144g glycine

10g SDS

to 1.0L water

Appendix D

Prestained Molecular Weight Standards (Bio-Rad product numbers)

Low Range 161-0306

High Range 161-0311

Broad Range 161-0319

Protein	MW (kDa)	Low Range	High Range	Broad Range
Myosin	200		X	X
β -galactosidase	116.25		X	X
Phosphorylase b	97.4	X	X	X
Bovine Serum Albumin	66.2	X	X	X
Ovalbumin	45	X	X	X
Carbonic Anhydrase	31	X		X
Soybean Trypsin Inhibitor	21.5	X		X
Lysozyme	14.4	X		X
Aprotinin	6.5			X