2007

Localization and characterization of C-type lectin-like family of proteins in Leptospira interrogans

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Localization and characterization of C-type lectin-like family of proteins in
Leptospira interrogans

by

Amanda Lee Toot

A thesis submitted to the graduate faculty
in partial fulfillment of the requirement for the degree of
MASTER OF SCIENCE

Major: Immunobiology

Program of Study Committee:
Joan Cunnick, Co-major Professor
Richard Zuerner, Co-major Professor
W. Ray Waters

Iowa State University
Ames, Iowa

2007

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Dedication

I would like to dedicate this thesis to my children, Alyssa and Tayte DePorter. Their unconditional love and understanding helped me through the tough times. Also, I would like to dedicate this thesis in loving memory of my father Stephen C. Toot, who inspired me to be a Microbiologist.
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ACKNOWLEDGEMENTS

I would like to first and foremost thank God for my wonderful life and all the gifts He has blessed me with. I would like to thank my family; my wonderful children Alyssa and Tayte, my mom Janet and her husband Don, my sister Stephanie and her family, my brother Shane and his family and my grandma Marge Toot. They all deserve awards for putting up with me the last few years. They have been very supportive and understanding.

I would like to thank my co-workers, without them this would not have been possible, Ami Frank, Renae DeVries, Rick Hornsby, David Alt and Margaret Elliott. They have all been very supportive and helped me troubleshoot, solve problems and provide me with their expertise.

I would like to thank Dr. Mitch Palmer, Bart Olthoff and Judy Stasko for their help with the histology slides.

I would like to thank my graduate committee, Dr. Richard Zuerner, Dr. Joan Cunnick, and Dr. Ray Waters. Thank you all for your help and support.
ABSTRACT

Genome analysis of *Leptospira interrogans* serovar Copenhageni reveals the presence of proteins that share a unique conserved domain of unknown function (DUF1554). DUF1554 containing proteins are found exclusively among pathogenic *Leptospira* spp., and resemble protein motifs within the C-type lectin protein superfamily. To characterize these proteins further seven fusion proteins from the *Leptospira interrogans* serovar Copenhageni genome were expressed and purified in *Escherichia coli*. These fusion proteins were used to produce polyclonal antiserum in New Zealand white rabbits. Titers were determined by Western blot and antibodies were used to localize the protein in whole cell sonicate and outer membrane fractions. Polyclonal antiserum was also used to show *in vivo* and *in vitro* expression of the proteins. Localization of these proteins is mostly in the outer membrane. The function of these domains has not been determined. C-type lectin-like proteins may play a role in adhesion to extracellular matrix and may be a potential virulence factor in the pathology of leptospirosis.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Leptospirosis is a worldwide zoonotic infection caused by several pathogenic species of *Leptospira* that has been isolated from hundreds of mammalian species including humans. Leptospirosis ranges in severity from a mild disease with few clinical signs to a severe infection resulting in death through organ failure (Faine, 1999). Development of a chronic asymptomatic disease can lead to shedding of leptospires into the environment. This shedding into the environment infects accidental and maintenance hosts thus sustaining the infection (Levett, 2001). Infection occurs when the bacteria are exposed to mucous membranes or abrasions of the host. A single leptospire has the ability to cause infection (Faine, 1994). The host can be either a maintenance host or an accidental host. The maintenance host is chronically infected in the renal tubules, and maintains infection of other animals by shedding leptospires in the urine. Maintenance hosts can transfer infection by direct or indirect routes to accidental hosts. The same host can serve as both the maintenance host and accidental host depending on serovar (Levett, 2001). The disease may progress to a fatal disease causing jaundice and hemorrhage. Leptospires are susceptible to antibiotics such as doxycycline and the disease is treatable if diagnosed early.

Virulence factors for *Leptospira* are still unclear. Studies have shown lipopolysaccharide (Adler, 2004; Nahori, 2005), cytotoxins (Vihn, 1986), hemolysins (Bernheimer, 1986) and outer membrane proteins (Cullen, 2005) are important in the pathology of disease. Of particular interest is the ability of outer membrane proteins
to bind extracellular matrix and other host proteins to cause disease or evade the immune response. According to recent genomic analysis *L. interrogans* serovar Copenhageni (strain 820-K) has a family of proteins belonging to the superfamily of C-type lectin-like proteins. C-type lectin-like domains (CTLDs) occur in many different species of animals, bacteria and viruses. Animal CTLDs have a structure that has a characteristic double loop or “loop in a loop” that is stabilized by two disulfide bridges at the base of the loop and conserved hydrophobic and polar interactions. The second loop is involved in Ca\(^{2+}\)-dependent carbohydrate binding and ligand interaction (Zelensky, 2005). The Ca\(^{2+}\)-dependent loop is absent in bacterial CTLDs. The CTLDs have a region of carbohydrate binding called the carbohydrate recognition domain (CRD) which is a globular structure not similar to any known protein fold (Zelensky, 2005). Bacterial and viral CTLD containing proteins are involved in pathogen-host interaction and are either host derived or imitations of host proteins such as intimin from *Escherichia coli* and invasin from *Yersinia pseudotuberculosis* (Zelensky, 2005).

The same proteins containing CTLDs also contain domains of unknown function (DUF1554) which are in close proximity to the CTLDs. The DUF1554 is a domain of unknown function that is exclusively found in pathogenic leptospires. There are 21 genes with one or two DUF1554 domains in three species of *Leptospira*, 4 in *Leptospira borgpetersenii* serovar Hardjo, 8 in *Leptospira interrogans* serovar Copenhageni, and 9 in *Leptospira interrogans* serovar Lai. In *L. interrogans* serovars Copenhageni and Lai there are 13 C-type lectin-like domains in 9 proteins. These proteins are also weakly related to the endostatin family. The
endostatins are endogenous inhibitors of angiogenesis and tumor growth; they do not share the same fold structure as the CTLDs (O’Reilly, 1997; Zelensky, 2005).

A member of this DUF1554 family is LfhA, a multifunctional protein that binds to factor H, and laminin (Lsa24) (Barbosa, 2006; Verma, 2006). This protein may play a key role in pathogenesis by evading the alternative complement pathway by inactivating C3 while aiding bacterial adherence to tissue by binding to laminin. A second member of this family, LIC12315, is differentially regulated by changes in osmolarity increasing in expression under salt concentration that mimic conditions Leptospira encounter within the host (Matsunaga, 2007).

**Thesis Organization**

This thesis is prepared in the journal format and includes a manuscript prepared to be submitted to a scientific journal. A review of the literature precedes the first manuscript and a general discussion follows the last manuscript. Literature cited in each chapter appears at the end of the last chapter.

**Literature Review**

**Leptospires**

**Taxonomy**

Several species of pathogenic *Leptospira* species are responsible for causing leptospirosis. *Leptospira* was first isolated in 1914 from Japanese rats by Dr. Ryokichi Inada (Inada, 1916). *Leptospira interrogans* was first named *Spirochaeta interrogans* by Stimson in 1907 due to the hooked ends resembling a question mark (Stimson, 1907). Leptospira taxonomic classification is broken down as follows: Bacteria (kingdom), Spirochaetes (phylum), Spirochaetes (class), Spirochaetales
(order), Leptospiraceae (family), Leptospira (genus), interrogans (species) (Brenner, 1999; Turner, 1974).

This classification has changed and expanded to several genera. Leptospires have been classified by two methods; serological and genotypic. Serological classification is based on agglutination after cross absorption with antigen. Agglutination of more than 10% of the bacteria remaining after cross absorption is used to differentiate serovars (Brenner, 1999). There are over 260 different serovars within the genus *Leptospira*.

Genotypic classification uses DNA hybridization to differentiate species based on genetic similarities and differences. The genus *Leptospira* was formerly divided into pathogenic species belonging to *L. interrogans* sensu lato and non-pathogenic belonging to *L. biflexa* sensu lato (Brenner, 1999; Haapala, 1969). There are currently 16 genomospecies of Leptospira that include both pathogenic and non-pathogenic serovars occurring in the same genus (*L. alexanderi, L. biflexa, L. borgpetersenii, L. fainei, L. inadai, L. interrogans, L. kirshneri, L. meyeri, L. nouguchii, L. santarosai, L. weilii, L. wolbachii, and 5 unnamed genomospecies*). Strains within the same species can occur in more than one serogroup based on genetic heterogeneity (Brenner, 1999; Haapala, 1969). Likewise, members of the same serovar may also occur within different species.

**Morphology and Physiology**

Leptospires are motile, helically shaped bacteria with two axial filaments located subterminally within the periplasmic space that wrap around the protoplasmic cylinder. Leptospires are long and thin, 10-20\(\mu\)m in length and 0.1 \(\mu\)m
wide; the use of dark-field or electron microscopy is required to visualize the complexity of the bacteria. The composition of the outer membrane consists of lipids, lipopolysaccharide, lipoproteins and proteins. Outer membrane protein concentration is 100-fold less than gram-negative bacteria. Virulent strains contain more lipopolysaccharide like substance than attenuated strains of *Leptospira* (Haake, 1991). Motility has two forms; either translational or rotational. Translational motility is described as rapid back and forth movements and rotational spinning back and forth along the axis of the bacteria (Goldstein, 1990). Leptospires are both catalase and oxidase positive, optimal growth temperature is 28 to 30°C at pH 7.2 to 7.6 (Levett, 2003). Nutritionally, leptospires have simple requirements for carbon. They consume long chain fatty acids and alcohols (12-18 carbons) as a source of energy through β-oxidation (Adler, 2004). Sugars are not fermented as a source of carbon. Leptospires cannot synthesize the long chain fatty acids themselves. During growth *in vitro*, these long chain fatty acids are provided in culture by addition of Tween (i.e. Tween 80) and detoxified by using bovine serum albumin. Nitrogen sources usable by leptospires are obtained by deamination of amino acids or ammonium salts. Other essential nutrients are vitamin B1 (thiamin), biotin, phosphate, calcium, magnesium, and iron (Fe$^{3+}$ or heme) (Adler, 2004).

Isolation of leptospires requires both liquid and semi-solid phase media. In general the generation time is between 7 to 12 hours. The addition of 5-fluorouracil is useful for selection of the leptospires (Johnson, 1981).

Outer membrane proteins
Several outer membrane proteins (OMP) have been isolated and characterized from pathogenic *Leptospira*. The OMP are classified into three types; transmembrane, peripheral and lipoproteins (Cullen, 2004). Most of the described proteins are lipoproteins, for example LipL32 (Haake, 2000; Zuerner, 1991), LipL21 (Cullen, 2003) LipL41 (Shang, 1996), LipL46 (Haake, 2006) are surface exposed. LipL36 has been localized to the outer membrane but not shown to be surface exposed (Haake, 1998). The functions of these lipoproteins are unknown but may play a role in pathogen-host interaction. OmpL1 is a transmembrane porin with 10 beta-sheet transmembrane domains with short periplasmic epitopes and 5 surface exposed loops forming the porin structure (Cullen, 2004). OmpL1 porin function has yet to be determined but does elicit partial protective immunity in Syrian golden hamsters when given with LipL41 (Haake, 1999). P31LipL45 has been described as a peripheral membrane protein of unknown function (Matsunaga, 2002). Some proteins with known function play a role in extracellular matrix adhesion such as LigA, LigB and Lsa24 (LfhA). LigA and LigB are immunoglobulin like proteins that are structurally similar to bacterial adhesins and are potential members of the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) family of proteins. Other spirochete MSCRAMMs includes decorin binding proteins (DbpA and DbpB) and fibronectin binding protein BBK32 in *Borrelia burgdorferi* and *Borrelia afzelii* (Choy, 2007; Grab, 1998; Haake, 2002). MSCRAMMs are not exclusive to spirochetes; they have also been identified in other bacteria including *Staphylococcus aureus* (fibronectin binding protein A and B), *Enterococcus faecalis* (collagen binding ACE), and *Arcanobacterium pyogenes* (collagen binding protein A).
LigA and LigB bind to extracellular matrix (ECM) proteins including fibronectin, collagen, laminin and fibrinogen (Choy, 2007). Lsa24 was recently shown to bind to laminin (Barbosa, 2006); this protein was also previously described as a factor H binding protein named LfhA (Verma, 2006).

Recent studies using microarrays to study gene expression revealed several leptospiral genes that are differentially transcribed when cells are exposed to different levels of osmolarity or temperatures (Matsunaga, 2007). LigA, LigB and Lsa24 (LIC12906) are up regulated with osmolarity; LipL36 is down regulated with temperature whereas P31LipL45 is up regulated. These data show that the leptospires alter their gene transcription expression profiles when transitioning from the environment to the host (Matsunaga, 2007). LIC12315 is up regulated slightly (1.8 fold) with increased osmolarity (Matsunaga, 2007). This protein is related to Lsa24 (LIC12906), according to the NCBI protein Blast (http://www.ncbi.nlm.nih.gov/). These proteins both contain C-type lectin-like domains indicating they may share the same function of binding to extracellular matrix molecules such as laminin and fibronectin.

Leptospirosis

Leptospirosis is well known for being a worldwide zoonotic infection. Leptospirosis has also been called Canicola fever, hemorrhagic jaundice, infectious jaundice, mud fever, spirochetal jaundice, swamp fever, swineherd’s disease, caver’s flu, sewerman’s flu, Weil’s disease or icterohemorrhagic fever (Chen, 2004). Leptospirosis was first identified by Adolf Weil of Heidelberg, Germany in 1886 when
he reported the clinical signs of fever, jaundice, hemorrhage and renal failure. Acute leptospirosis is generally characterized by loss of appetite, fever, diarrhea, listlessness, frank hemorrhages, jaundice, acute nephritis, and abortion followed by chronic persistence in several organs (Adler, 2004). Leptospirosis has been isolated from several species of animals including humans. Specific serovars are more prevalent in certain regions of the world than others. The host can be either a maintenance host or an accidental host. The maintenance host is chronically infected in the renal tubules, and maintains infection by shedding leptospires in the urine. Maintenance hosts can transfer infection by direct or indirect routes to accidental hosts. The same host can serve as both the maintenance host and accidental host depending on serovar (Levett, 2001).

Clinical Manifestation

Leptospirosis is biphasic, with both acute and immune phases. The acute phase (septic) lasts approximately a week with fever, spirochetemia, spirochetes in the cerebral spinal fluid and urine. The second phase is called the immune phase (convalescent) when antibodies are produced and spirochetes are shed in the urine. Leptospirosis can manifest as anicteric, icteric, ocular, and chronic.

Anicteric leptospirosis is a mild, subacute disease that may or may not need treatment. The symptoms are flu like with fever, chills, headache, myalgia, anorexia, abdominal pain, nausea, vomiting, conjunctival suffusion (redness on the eye surface), skin rash, splenomegaly, hepatomegaly, and lymphadenopathy. These symptoms last for approximately a week followed by the immune phase with some of the same symptoms but milder in addition to uveitis, aseptic meningitis and
pulmonary involvement. The immune phase of anicteric leptospirosis can last 4-30 days (Edwards, 1960; Levett, 2001).

Icteric leptospirosis is much more severe and is commonly called “Weil’s syndrome”. The septic phase is similar to anicteric leptospirosis and the immune phase is much more severe and sometimes fatal. This syndrome is associated with jaundice, renal failure, hemorrhage, azotemia (abnormal levels of urea, creatine, and other body waste materials in the blood from kidney failure), and oliguria (decreased urination) (Heath, 1965; Levett, 2001). Death can occur from septic shock, respiratory failure, myocarditis and multiple organ failure.

Ocular involvement with leptospirosis is a severe manifestation of the disease. The ocular involvement can be conjunctival suffusion, anterior uveitis (inflammation of the middle layer of the eye), and chronic visual obstruction (Martins, 1998; Levett, 2001). The late onset of uveitis can be attributed to autoimmunity. Severe ocular involvement is found in most icteric cases.

Chronic or latent infections are found primarily in chronic ocular infection and meningitis. Autoimmunity in horses with recurrent uveitis has been described (Kalsow, 1998). Chronic infection by maintenance hosts results in shedding of leptospires into the environment.

**Epidemiology**

Leptospires have been linked to outbreaks associated with contaminated water sources especially during times of flooding. Leptospires are able to survive in the environment for extended amounts of time. Leptospires have a worldwide distribution but are common in temperate and tropical areas. According the Centers
for Disease control website (www.cdc.gov) there are 100-200 cases per year reported in the United States with 50% of them in Hawaii. Leptospirosis outbreaks have occurred during triathlons (1998; 110 cases out of 775 exposures) and during floods in Peru, Ecuador and Thailand (www.cdc.gov). Worldwide estimates are nearly 0.5 million cases per year (WHO, 1999). Leptospirosis tends to follow three patterns as described by Faine; the first is in temperate regions involving direct contact between humans and livestock. The second pattern occurs in tropical areas, where rodents and small mammals serve as maintenance hosts and spread the disease through indirect transmission through the environment. The third pattern occurs in urban areas through infected rodents infiltrating the urban areas and causing epidemics, often during seasonal flooding. Leptospirosis is also a direct occupational hazard for veterinarians, miners, sewer workers, farmers (sugar cane plantations and rice workers), slaughterhouse workers and anyone in contact with infected urine or contaminated water (Faine, 1994; Levett, 2001). Recreational sports enthusiasts have some risks for contracting leptospirosis especially after hard rains and flooding that may contaminate water sources.

Treatment

Treatment of leptospirosis is dependent on the stage at which the disease is diagnosed. Treatment can range from antibiotic treatment to hospitalization. Doxycycline is given to treat the infection; hospitalization is required for renal failure and hemorrhaging. Immunizations are not available in Western countries for humans. A polyvalent vaccine is used in the Far East for humans as well as a monovalent vaccine in France (Levett, 2001). However, immunizations are available
in Western countries for dogs, pigs and cattle with mixed success. Veterinary vaccines can induce protective immunity by eliciting a cell-mediated immune response involving CD4+ and γδ T cells (Naiman, 2001), but the exact mechanisms are unclear. Administration of monoclonal anti-leptospiral lipopolysaccharide antibody provided protective immunity in the guinea pig infection model (Jost, 1986). Development of anti-lipopolysaccharide antibodies is the primary outcome of vaccination with a whole cell leptospiral vaccine but these do not appear to protect maintenance hosts from infection, at least in cattle (Naiman, 2001). Another problem with currently used vaccines is development of antibody to only the serovars used to prepare the vaccine; there is no apparent cross protection against other serovars. Development of a recombinant protein vaccine that has is reactive against proteins that are conserved among the pathogenic *Leptospira* serovars could provide the cross protection needed for an effective vaccine. The use of a recombinant vaccine would also make it marketable to the human population.

**Virulence Factors**

The mechanisms by which *Leptospira* spp cause disease are complex and are not well defined. There have been several virulence factors proposed such as hemolytic activity, endotoxicity from leptospiral LPS, and attachment to epithelium. Hemolytic activity is elicited by sphingomyelinases in different serovars (Ballum, Hardjo, Pomona and Tarassovi) but the significance is still undefined (Bernheimer, 1986; del Real, 1989; Segers, 1992). Serovar Canicola has shown to possess phospholipase C activity (Yanagihara, 1982). A hemolysin from serovar Lai was characterized which is neither a sphingomyelinase nor a phospholipase but instead
forms pores in the host cell membrane (Lee, 2002). A protein cytotoxin has been found in serovars Pomona and Copenhageni causing infiltration of macrophages and polymorphonuclear cells; glycolipoprotein was extracted and showed cytotoxic effects such as cell enzyme leakage (Vihn, 1986). Leptospiral lipopolysaccharide (LPS) lacks the endotoxicity of gram-negative LPS making it 10-fold less toxic to animal cells but is active in the limulus lysate assay; although it is structurally, biochemically and immunologically similar to E. coli LPS (Adler, 2004; Haake, 1991). Purified LPS signals through TLR2 and CD14 while the leptospiral lipid A signals through TLR4 (Nahori, 2005).

Leptospires have been shown to attach to kidney epithelial cells, induce antibody-mediated endocytosis by macrophages and associate with neutrophils but are not affected by their cytotoxicity (Ballard, 1986; Cinco, 1983; Cinco, 1981). Attachment of the bacteria to epithelial cells is an important virulence factor that aids in the invasion of host tissue and increases the likelihood of disease. Antibody mediated endocytosis and association of the bacteria with neutrophils are strategies the bacteria has evolved to manipulate the host immune response to avoid being recognized and eliminated from the body.

Serum resistance is also a virulence factor that has yet to be fully described. There is evidence that pathogenic leptospires can survive in non-immune sera (Verma, 2006), and bind to the complement regulator factor H to interfere with the alternative complement cascade (Verma, 2006). The interference of the alternative complement cascade is an important mechanism pathogenic bacterium have employed, this disruption decreases inflammatory mediators and eliminates the
membrane attack complex. Leptospires bind to complement receptor 3 (CR3) on neutrophils and phagocytes in the absence of specific antibody (Cinco, 2002). CR3 binds specifically to the iC3b inactivate subunit of C3b. This inactive subunit is formed by the cleavage of C3b by Factor I which is dependent on the binding of Factor H. iC3b is an opsonin and will bind to the bacterial surface, thus causing phagocytosis of the bacteria without the use of antibody and induce inflammatory cytokines. Fibronectin has also been shown to bind to CR3 and may be acting as a bridge between CR3 and the leptospire (Cinco, 2002).

Outer membrane proteins are potentially the main virulence factor of leptospires; specifically their abilities to bind to extracellular matrix to colonize tissues as previously described in this literature review.

**Immune Response**

The immune response to leptospirosis consists of a robust humoral response know as the immune phase of an acute infection. This immune phase has an increase in antibodies with clearance of leptospires from the blood stream (Levett, 2001). Leptospires stimulate naïve peripheral blood mononuclear cells (PBMC) to proliferate and secrete T-helper 1 cytokines such as IL-12, IFN-γ, and TNF-α. The T cell proliferation consists of both αβ+ and γδ+ T cells (Klimpel, 2003). In vitro stimulation of PBMCs with glycolipoprotein of *L. interrogans* serovar Copenhageni showed increased expression of CD69, HLA DR, IL-10 and TNF-α (Diament, 2002). After vaccination of cattle with *L. borgpetersenii* killed bacterin, there is a CD4+ T cell and γδ+ T cells proliferative response with production of IFN-γ (Naiman, 2001). γδ+ T cells are stimulated to produce IFN-γ without cell proliferation (Klimpel, 2003).
T cells populations are more prevalent in cultures of higher bacterial load, and almost absent in cultures with low bacterial load. It was speculated that the γδ\(^+\) T cells may be playing a regulatory role by regulating αβ\(^+\) T cells, or the high leptospiral load is toxic to αβ\(^+\) T cells (Klimpel, 2003)

**Polyclonal Antibody Production**

To investigate the expression of proteins of *Leptospira* species can be accomplished by producing antigen specific antibody. This antibody can be used to localize the protein to a region within the bacteria. Expression of the proteins *in vivo* can be detected with the polyclonal antibody. Fusion proteins derived from the protein of interest are used to immunize rabbits for polyclonal antibody production.

The use of poly histidine tag provides an excellent tool to visualize the protein of interest. The process begins with the polymerase chain reaction (PCR) amplification of the gene of interest. The PCR primers have 5’ modifications consisting of restriction enzymes to ensure directional ligation after cloning. The PCR product is then transformed into a shuttle vector, in this case pCR8 TOPO vector, with antibiotic resistance to isolate clones containing the shuttle vector. Positive clones are identified by EcoRI digestion. The positive clone is then digested with the restriction enzymes that were added to the 5’ end, *Bam*HI (GGATCC) and *Hind*III (GGAAGCTT) are most commonly used. *Xho*1 (CTCGAG) has also been used if there is an internal *Bam*HI or *Hind*III site.

Once a gene is digested and precipitated from the vector it is ligated with a digested expression vector. In this case pRSETB from Invitrogen is used. pRSETB has a amino-terminal 6x-Histidine tag. pRSETB is regulated by the T7 promoter, T7
RNA polymerase recognizes the T7 promoter. This promoter is induced by the inducer isopropyl-β-D-thiogalactoside (IPTG). Once T7 RNA polymerase is produced it will transcribe the gene inserted into the expression vector. The host cell has a DE3 bacteriophage lambda lysogen that contains the lacI gene that controls the T7 RNA polymerase. The protein will be over-expressed when the inducer is added to the media. Detection of the protein is aided by the poly-histidine tag, which can be detected by immunoblot technique using a monoclonal antibody to histidine.

The histidine tag aids in purification. The histidine tag binds nickel resin on a column. During native purification the histidine will displace the imidazole that blocks non-specific binding. The columns are eluted with a high concentration of imidazole to displace the histidine giving a relatively pure purification. Purification using a denaturant such as 8M urea will rely on pH. The pKa of the histidine residues in the tag is ~6.0. Elution during denature purification is obtained by using a buffer at pH 4.5, this protonates the tag and no longer binds the nickel. Once these proteins are purified they can be positively identified using Western blot analysis with a monoclonal antibody to the histidine tag. The purified protein is then dialyzed against phosphate buffered saline to remove the urea or imidazole. Dialyzed protein was mixed with adjuvant and injected into New Zealand white rabbits. Rabbits were boosted 2-3 times then bled and serum collected.

Summary

Leptospirosis is an important zoonotic infection that has affects worldwide. The disease can affect multiple mammalian hosts as well as maintain itself in the environment and within maintenance hosts. Leptospirosis can be fatal if left
untreated. Virulence factor of leptospirosis are not completely defined. The research in this thesis will characterize and localize a family of proteins, C-type lectin-like proteins, in *Leptospira interrogans* serovar Copenhageni. This family of proteins may play a role in virulence and may be good vaccine candidates in future studies.
References


CHAPTER 2. Factor H binding of *Leptospira spp.*

**Introduction**

Factor H (FH) is a multifunctional protein that is best known for its ability to regulate the alternative complement cascade (Figure 2.1). The alternative complement cascade is an antibody independent process that begins with the spontaneous hydrolysis of C3 into C3b and opsonizes the cell surface. Binding of factor B to C3b(H$_2$O) resulting in C3bB recruits Factor D which cleaves Factor B into Ba and Bb (Pangburn, 1983). Ba is an inflammatory mediator and Bb associates with C3b to for C3bBb (C3 convertase), a second C3b associates with the complex (Pangburn, 1981). Properdin associates and stabilizes the C3 convertase allowing the cleavage of C5 by the C5 convertase (C3b$_2$Bb) into C5a and C5b. This initiates the membrane attack complex. The membrane attack complex is composed of twenty molecules. C5b and C6 bind to C7; this forms the complex C5b67 which binds to the cell membrane. C8 binds to C5b67 and forms a small transmembrane channel, which then acts as a receptor for multiple C9 molecules that form the membrane attack complex (C5b6789) pore lysing the cell (Hu, 1981).
Figure 2.1 Alternative complement pathway.
FH regulates the complement cascade by acting as a cofactor with factor I (FI) to degrade C3b, acting as a decay accelerating factor for C3 convertase, and as a competitive inhibitor for factor B binding to C3b (Zipfel, 1999). FH binding to C3b recruits FI which degrades C3b into iC3b and C3f then degrades iC3b into C3c and C3dg (Figure 2.2) (Ruddy, 1971; Pangburn, 1977). In addition to complement regulation, FH binds to the integrin receptor CD11b/CD18, binds to surface glycoaminoglycans, and pathogenic microorganism surfaces (Zipfel, 1999). FH is a 150kDa protein that has 20 globular domains containing disulfide bonds called short consensus repeats (SCR). The globular domains have been assayed to determine their binding affinities. SCRs 1-4 are responsible for cofactor and decay activity, SCR 4 also has and RGD motif responsible for cell adhesion, SCRs 1-4, 12-14 and 19-20 bind to C3b, SCRs 7, 12-14 and 20 bind to heparin, and SCRs 7-11 bind to C-reactive protein (Zipfel, 2002). Upon purification of FH, factor H-like 1 (FHL-1) is co-purified. FHL-1 is ~42kDa and is made up of the first 7 globular SCR domains of FH. FHL-1 has the same binding affinities as FH except for the lack of SCR8-20. FHL-1 is also known as reconectin because of its ability to bind to cells in a similar fashion as fibronectin (Zipfel, 1999). The ability of bacteria to manipulate this system to their advantage has been utilized by several pathogens such as *Borrelia* spp., *Neisseria* spp., *Treponema* spp., and *Streptococcus* spp (Kraiczy, 2006).
Figure 2.2 Inactivation of C3b by Factor H and Factor I.

There has been significant research with *Borrelia* spp. and FH binding. The proteins responsible for binding of the complement regulator have a coiled coil domain that form a pocket for binding FH (Marconi, 2005). When this coiled coil domain is interrupted the binding capacity is lost. Thus the tertiary protein folding is very important for functionality of the protein (McDowell, 2005).

*Leptospira* spp. has been shown to survive in non-immune serum (Meri, 2005) and bind to FH (Verma, 2006). Serum absorption assays confirm the ability of the leptospires to bind the regulator FH. (see figure 2.11) This assay does not specify which proteins actually bind FH. The protein LfhA is the leptospiral FH binding protein. Recent studies have shown the binding of FH to a ~30kDa protein (LfhA) and a ~50kDa protein. The band intensity at the ~50kDa protein was much more intense than that of the ~30kDa (Verma, 2006).
This finding inspired my thesis research to determine if the 50kDa band truly bound Factor H. Fusion proteins were produced from open reading frames that were predicted to contain coiled coil domains, outer membrane associated, and approximately ~50kDa. Purification of these proteins proved to be troublesome. The proteins required denaturation to allow the histidine tag to bind with the nickel resin columns used in purification. The denaturation disrupted any tertiary structure and rendered the proteins inactive. The theory I propose is the histidine tag on the carboxy terminus is being folded into the coiled coil domains and hindering the tag from being exposed to the column. Due to the lack of success with native purification protocols or denature purification protocols with proper renaturation, this project was abandoned because the functionality of the proteins was lost in purification.

Methods and Materials

Bacteria and cultivation:

*Leptospira interrogans* serovar Kennewicki was isolated from isolated by R. McClellan. *Leptospira borgpetersenii* serovar Copenhageni was isolated from a hamster by Alex Theirman. *Leptospira spp.* were propagated in EMJH (Ellinghausen, McCullough, Johnson, and Harris, 1967) media supplemented with 1% rabbit sera, 10% bovine serum albumin and 100µg/ml 5-fluorouracil until mid log phase at 30°C.

INVαF’ *Escherichia coli* (C2020-03, Invitrogen), and BL21Al *Escherichia coli* (C6070-03, Invitrogen) were grown at 37°C in Luria broth with either 100µg/ml Spectinomycin or Carbenicillin depending on plasmid insert.
Extraction of DNA:

*L. interrogans* serovar Copenhageni and *L. interrogans* serovar Kennewicki genomic DNA was extracted as previously described see Appendix for specific protocol. Briefly, bacteria were grown to mid-log phase (10^9 cells/ml) and centrifuged at 12,000xg for 30 minutes at 4°C, Pellets were resuspended in TE (Tris-EDTA buffer 10mM Tris-HCl, 1mM EDTA, pH 8.0, Invitrogen) and transferred to a pre-spun phase lock gel (LIGHT) tube for overnight incubation. Lysozyme (5 mg/ml in TE, Sigma) was added to suspension and incubated on ice for 15 minutes. 0.1M CaCl_2 and proteinase K (20 mg/mL) were added and incubated at 37°C overnight. Phenol chloroform isoamyl alcohol (PCI) was added and mixed by inversion, then centrifuged at 1500xg for 5 minutes at room temperature. Upper layer was removed and added to a fresh phase lock gel light tube and extract again with PCI. Upper layer was removed again and 2M KCl was added and mix by inversion. 95% ethanol was overlayed and DNA was spooled onto Pasteur pipette until the two phases are completely mixed. Spooled DNA was placed in 70% ethanol then redissolve DNA by incubating overnight at 4°C.

Primer design and PCR:

Primers were designed using Clone Manager 7 ® and Primer Designer 5® software. Restriction enzyme sites were added to the 5’ end of each primer pair. Genes were amplified by PCR with Accutaq ® polymerase, on a MJ research thermocycler. The amplification was 35 cycles; 98°C for 30 seconds, then repeat 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 2 minutes for a total of 35 cycles, then 68°C for 10 minutes. PCR products were separated on a 1% agarose
gel by gel electrophoresis, stained with ethidium bromide and image captured on film.

**PCR product cloning:**

PCR products were then ligated into pCR8® vector (K250020, Invitrogen) as per manufacturer’s instructions. Ligation reaction were used to transform INVαF’ TOP10 chemically competent cells as per manufacturer’s instructions. Transformations were plated on LB agar plates containing 100μg/ml spectinomycin and incubated overnight at 30ºC. Plasmids were isolated as per manufacturer’s instructions using Qiaprep Spin Miniprep kit (27106, Qiagen). Plasmid preps were screened for proper PCR product by digestion with EcoRI. The digestions were separated on 1% agarose by gel electrophoresis, stained with ethidium bromide and image was captured on film. Clones with the proper insert were then digested at a larger scale and enzymes removed using Quick Clean Enzyme Removal Resin® (631770, Clontech) as per manufacturer’s instructions.

**Expression vector ligation:**

Clones from the previously described pCR8 ligation were digested with their respective restriction enzymes and ligated into pRSET vectors (V351-20, Invitrogen) which are the expression vector for high-level expression of recombinant proteins. Ligation reactions were used to transform INVαF’ TOP10 chemically competent cells as per manufacturer’s instructions. Transformations were plated on LB agar plates containing 100μg/ml carbenicillin and incubated overnight at 30ºC. Plasmids were isolated as per manufacturer’s instructions using Qiaprep Spin Miniprep kit (27106, Qiagen). Plasmid preps were screened for proper PCR product by digestion with
the respective restriction enzymes. The digestions were separated on 1% agarose by gel electrophoresis, stained with ethidium bromide and image was captured on film.

**Expression:**

Plasmids containing the proper insert were then chemically transformed into the expression host cell BL21-AI (Invitrogen) as per manufacturer’s instructions. Cultures were grown to OD600 of 0.5. Inducing agent was added to a final concentration of 0.2% L-Arabinose for 4 hours. Expression cultures were separated on 14% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Immobilon-P membrane by western blot. Detection of fusion protein was determined by immunodetection by blocking the membrane with 5% skim milk in phosphate buffered saline-0.1% Tween 20 (PBS-T) for 1 hour at room temperature, then incubated in mouse anti-histidine primary antibody diluted 1:2000 in 5% milk-PBS-T for 1 hour at room temperature. The membrane was then washed three times with PBS-T and incubated with goat anti-rabbit horseradish peroxidase labeled secondary antibody for 30 minutes at room temperature and washed three times with PBS-T. Chemiluminescence was carried out following manufactures instructions Super Signal West Pico (34080, Pierce) detection kits. Kodak MR film for 30 seconds and developed using the X-OMAT®.

**Purification:**

Purification was performed by native and denaturing conditions. Purification of the histidine-tagged fusion proteins under native conditions was carried out using three different methods of purification. Purification using normal conditions from the
manufacturer’s instructions proved to be inefficient in binding these particular histidine tagged proteins. The protocol had to be modified to include zwitterionic detergents (1% NDSB-256, Calbiochem), 0.05% CHAPS (Sigma) and 0.25% Tween 20 (Sigma). Under these conditions the protein was slightly denatured under non-ionic conditions. The first two purification methods used the Ni-NTA columns from Qiagen. The lysates were solubilized and applied to nickel resin columns to bind histidine. The third purification method does not require a column but uses His-Mag® beads from Novagen as per manufactures instructions. After each step, the flow through was collected for analysis on SDS-PAGE for stringency of the assay. After purification the protein elutions were dialyzed using Pierce Slide-a-lyser cassettes against PBS containing 0.2M L-arginine, excess arginine was removed by dialysis against PBS. After dialysis the proteins were concentrated by speed vacuum for 2 hours or using Pierce concentrating buffer as per manufacturer’s instructions.

**Serum Absorption assay:**

*Leptospira spp.* were grown to mid log phase containing approximately $10^9$ cells per mL, harvested by centrifugation and washed three times with PBS. Cells were then resuspended in 200uL diluted monkey serum (1:1 in PBS) and incubated at room temperature for 1 hour. Cells were then washed three times in cold PBS. Resuspended in SDS-PAGE buffer and separated on a 8% SDS-PAGE, Western transferred for 1 hour at 200mA by semi-dry method. Immunodetection of Factor H was performed by blocking overnight with 5% milk in PBS-Tween 20, incubated in primary antibody (1:2000 mouse monoclonal anti-Factor H, Quidel, Cat #A229)
overnight at 4°C, washed three times and incubated in secondary antibody (1:10,000 goat anti-mouse IgG HRP, KPL, Cat# 074-1807) for 30 minutes at room temperature. Washed three times with PBS-T and chemiluminescence was carried out following manufactures instructions Super Signal West Pico (34080, Pierce) detection kits. Kodak MR film for 30 seconds and developed using the X-OMAT®.

**Affinity ligand binding assay:**

Proteins were separated on 14% SDS-PAGE and transferred to PVDF membrane by Western transfer. Membranes were blocked with 5% skim milk in PBS containing 0.01% Tween 20 (PBS-T) for 1 hour at room temperature, then incubated for 1 hour at 4°C with 1:1 diluted monkey serum as a source of Factor H. Membranes were then washed three times with PBS-T and incubated with primary antibody (1:2000 mouse monoclonal anti-Factor H, Quidel, Cat #A229) for 1 hour at room temperature. Membranes were then washed three times with PBS-T and incubated with secondary antibody (1:20,000 goat anti-mouse IgG HRP, KPL, Cat# 074-1807) for 1 hour at room temperature. Membranes were washed three times with PBS-T and chemiluminescence was carried out following manufactures instructions Super Signal West Pico (34080, Pierce) detection kits. Kodak MR film for 30 seconds and developed using the X-OMAT®.

**Reverse Affinity ligand binding assay:**

1:4 dilutions of hamster, guinea pig, rat, dog and monkey serum were separated on a non-reducing 10% SDS-PAGE, and transferred to PVDF membrane by Western transfer. Membranes were blocked with 5% skim milk in PBS-T for 1 hour at room temperature, then incubated with whole cell sonicate of *E. coli*
containing fusion proteins (100µg/mL) overnight at 4°C. Membranes were washed three times with PBS-T and incubated in mouse anti-histidine primary antibody (Qiagen, 34610) diluted 1:2000 in 5% milk-PBS-T for 1 hour at room temperature. The membrane was then washed three times with PBS-T and incubated with goat anti-rabbit horseradish peroxidase labeled secondary antibody for 30 minutes at room temperature and washed three times with PBS-T. Chemiluminescence was carried out following manufactures instructions Super Signal West Pico (34080, Pierce) detection kits. Kodak MR film for 30 seconds and developed using the X-OMAT®.

**Results**

**Sequence analysis, PCR, Cloning, Expression and purification:**

Analysis of the sequence reveals several proteins that contain possible coiled coil domains, are ~50kDa and are predicted to be in the outer membrane (Table 2.1). 19 genes were amplified (Figure 2.3), cloned (Figure 2.4 and 2.5), ligated (Figure 2.6), expressed (Figure 2.7) and purified.

Purification was performed by several methods. Native purification with zwitterionic detergents allowed a mild denaturing of the proteins without complete disruption of the putative coiled coils (Figure 2.8). This method was not successful for all proteins and did not retain the function of the proteins (data not shown). Native purification using His-magnetic beads was performed; this method does not rely on nickel resin columns but rather nickel conjugated to magnetic beads (Figure 2.9). This method was also not successful with all proteins and did not have a very high yield of protein (data not shown). Denature purification was the only method
that was successful with all proteins (Figure 2.10). This method completely denatures the protein and disrupts all putative coiled coil domains leaving the protein functionally inactive (data not shown). Denature purification with nickel resin columns with renaturation steps while the proteins are still attached to the columns was successful with most proteins (Figure 2.11). The histidine tag is still attached to the column and the proteins may not fold in the proper fashion. Function of these proteins was not restored (data not shown).

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Table 2.1 Primers used to make fusion proteins. Table includes gene locus designation, forward and reverse primers, expression vector, restriction enzymes, base pairs and molecular weight.
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Table 2.2 Coils® output for genes that have predicted coiled coil domains. Table includes gene locus designation, number of coiled coils (CC), amino acid sequence, prediction from window 14, 21 and 28.

Figure 2.3 PCR amplification of genes for fusion protein production (lane 1) LIC12906, (lane 2) JB/1/2647057/1646350, (lane 3) LIC11643, (lane 4) LIC12624, (lane 5) LIC13006, (lane 6) 1kb DNA ladder Invitrogen. Figure representative of 5 PCR products.
Figure 2.4 Restriction enzyme digestion of shuttle vector plasmid. Plasmids digested with BamHI and HindIII band at ~2.5kb is vector and lower bands are gene inserts (lane1) 1kb DNA ladder, (lane 2-5) LIC12906, (lane 6-9) JB/1/2647057/1646350, (lane 10-13) LIC11643, (lane 14-17) LIC12624, (lane 18-21) LIC13006. Figure representative of 5 vector digests.

Figure 2.5 Large-scale digestion of positive shuttle vector clone for ligation into expression vector, (lane 1) 1kb DNA ladder Invitrogen, (lane 2) LIC12906, (lane 3) JB/1/2647057/1646350, (lane 4) LIC11643, (lane 5) LIC12624, (lane 6) LIC13006. Figure representative of 5 vector digests, more digestions in appendix.
Figure 2.6 Digestion of expression vector ligation, (lane 1) 1kb DNA ladder Invitrogen, (lane 2-8) plasmid digest of LIC12906 insert. Figure representative of one plasmid digestion.

Figure 2.7 Immunoblot of expression of fusion proteins using anti-histidine antibody (lane 1) LIC12906, (lane 2) JB/1/2647057/1646350, (lane 3) LIC11643, (lane 4) LIC12624, (lane 5) LIC12030. Figure representative of 5 fusion protein expressions.
Figure 2.8 Purification of fusion proteins under native conditions with zwitterionic detergents. Figure representative of 5 fusion proteins showing only elution flow through, (lane 1) LIC12030 elution 1, (lane 2) LIC12030 elution 2, (lane 3) Qiagen 6x histidine ladder. LIC12030 is approximately 31kDA.

Fig 2.9 Purification of fusion proteins under native conditions using His-mag purification. Figure representative of one fusion protein, (lane 1) LIC12624 cleared lysate, (lane 2) flow through, (lane 3) wash 1, (lane 4) wash 2, (lane 5) elution 1, (lane 6) elution 2, (lane 7) elution 3, (lane 8) Qiagen 6x histidine ladder. LIC12030 is approximately 31kDA.

Fig 2.10 Purification of fusion proteins using denaturing conditions. Figure representative of one fusion protein, (lane 1) LIC12624 cleared lysate, (lane 2) flow through, (lane 3) wash 1, (lane 4) wash 2, (lane 5) elution 1, (lane 6) elution 2, (lane 7) Qiagen 6x histidine ladder.
Figure 2.11 Purification of fusion proteins under denature-renaturation conditions. Figure representative of 5 fusion proteins showing only elution flow through, (lane 1) LIC12030 elution 1, (lane 2) LIC12030 elution 2, (lane 3) LIC12030 elution 3, (lane 4) Qiagen 6x histidine ladder. LIC12030 is approximately 31kDA

Serum absorption assay:

Serum absorption allows the analysis of the proteins that bind to the bacterial surface. The Leptospira spp. as well as Treponema denticola were allowed to incubate in monkey serum and the proteins analyzed. Figure 2.12 shows the presence of a band at ~150 kDa for L. interrogans serovar Kennewicki and Copenhageni. This band is absent in Treponema denticola, this is consistent with the literature that T. denticola only binds FHL-1 and not FH. FHL-1 is less concentrated in serum than FH; therefore the band does not show up at this dilution. Upon over-exposure of the film the FHL-1 band is visible (data not shown). Therefore FH is bound to the bacterial cell, but the exact protein is not determined by this assay.
Figure 2.1. Serum absorption assay of \textit{L. interrogans} serovar Kennewicki, \textit{L. interrogans} serovar Copenhageni and \textit{T. denticola}. Factor H is detected in both \textit{L. interrogans} serovars but absent in the \textit{T. denticola}.

**Conclusions and discussion**

Purification and functionality of the putative factor H binding proteins proved to be challenging. The purification methods needed to retain the functional epitopes needed to retain factor H binding still have to be optimized. The same purification protocol did not consistently purify proteins. Serum absorption assays did show that factor H is binding to the whole cells of \textit{L. interrogans} serovars Copenhageni and Kennewicki.
Future research to investigate factor H binding would be to make fusion proteins with a cleavable histidine tag or moving the histidine tag to the opposite end of the protein (3’ end) to prevent the tag from hindering binding to the nickel resin columns. Also, the fusion protein should have the entire open reading frame to prevent the interruption of a coiled coil domain. This could be done by using the tag at the carboxy terminus so the tag would not be cleaved during processing as it would be at the amino terminus. Different tags could be used such as glutathione S-transferase, S protein and maltose binding protein. The different tags would prevent the precipitation of the protein during dialysis. Precipitation of the protein was a problem with the histidine tagged proteins.
References


CHAPTER 3. Localization and characterization of Leptospiral proteins belonging to the C-type lectin-like protein family

A paper to be submitted to Infection and Immunity

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Abstract

Genome analysis of \textit{Leptospira interrogans} serovar Copenhageni reveals the presence of proteins that share a unique conserved domain of unknown function (DUF1554). DUF1554 containing proteins are found exclusively among pathogenic \textit{Leptospira} spp., and resemble protein motifs within the C-type lectin protein superfamily. To characterize these proteins further seven fusion proteins from the \textit{Leptospira interrogans} serovar Copenhageni genome were expressed and purified in \textit{Escherichia coli}. These fusion proteins were used to produce polyclonal antiserum in New Zealand white rabbits. Titers were determined by Western blot and antibodies were used to localize the protein in whole cell sonicate and outer membrane fractions. Polyclonal antiserum was also used to show \textit{in vivo} and \textit{in vitro} expression of the proteins. The function of these domains has not been determined. C-type lectin-like proteins may play a role in adhesion to extracellular matrix and may be a potential virulence factor in the pathology of leptospirosis. In this study, we show that most of the proteins are concentrated in the Kupfer cells of the liver and localized to the outer membrane.
Introduction

Leptospirosis is a worldwide zoonotic infection caused by several pathogenic species of *Leptospira* that has been isolated from hundreds of mammalian species including humans. Leptospirosis ranges in severity from a mild disease with few clinical signs to a severe infection resulting in death through organ failure (Faine, 1999). Infection occurs when the bacteria are exposed to mucous membranes or abrasions of the host. The disease can lead to an anicteric disease with flu-like symptoms or it may develop into an icteric disease that begins with flu-like symptoms that progress into multiple organ failure, hemorrhage, and jaundice. The icteric form of leptospirosis can be fatal if left untreated. Human infections are considered accidental hosts. Hosts can be either a maintenance host or an accidental host and become infected by direct or indirect routes. The maintenance host is chronically infected in the renal tubules. Chronic renal infection and passage of viable bacteria in the urine facilitates disease and dissemination by exposing naïve hosts thereby propagating the disease. This shedding into the environment infects accidental and maintenance hosts thus sustaining the infection (Faine, 1994). The same host can serve as both the maintenance host and accidental host depending on serovar (Faine, 1994). Leptospires are susceptible to antibiotics such as doxycycline and the disease is treatable if diagnosed early.

Few virulence factors from *Leptospira* have been characterized. Previous studies suggest that lipopolysaccharide (Adler, 2004; Nahori, 2005), cytotoxins (Vihn, 1986), hemolysins (Bernheimer, 1986) and outer membrane proteins (Cullen, 2005) are important in the pathology of disease. Of particular interest is the ability of
outer membrane proteins to bind extracellular matrix and other host proteins to cause disease or evade the immune response.

According to recent genomic analysis, *L. interrogans* serovar Copenhageni (strain 820-K) has a family of hypothetical proteins belonging to the superfamily of C-type lectin-like proteins. C-type lectin-like domains (CTLDs) occur in many different species of animals, bacteria and viruses. Animal CTLDs have a structure that has a characteristic double loop or “loop in a loop” that is stabilized by two disulfide bridges at the base of the loop and conserved hydrophobic and polar interactions. The second loop is involved in $\text{Ca}^{2+}$-dependent carbohydrate binding and ligand interaction (Zelensky, 2005). The $\text{Ca}^{2+}$-dependent loop is absent in bacterial CTLDs. The CTLDs have a region of carbohydrate binding called the carbohydrate recognition domain (CRD) that forms a unique globular structure (Zelensky, 2005). CTLDs include proteins involved with host interaction, such as intimin from *Escherichia coli* and invasin from *Yersinia pseudotuberculosis* (Zelensky, 2005), and therefore may be involved in pathogenesis.

In *Leptospira*, the same CTLD-containing proteins also have domains of unknown function (DUF1554) that overlap with the CTLD motifs. Genes encoding the DUF1554 motif are found exclusively in pathogenic *Leptospira spp*. There are four DUF1554 genes in *Leptospira borgpetersenii* serovar Hardjo, and 8 to 9 in *Leptospira interrogans*. These DUF1554 containing proteins are also weakly similar to the endostatin family group of proteins. The endostatins are endogenous inhibitors of angiogenesis and tumor growth and that do not share the same fold structure as CTLDs (O'Reilly, 1997; Zelensky, 2005).
One member of the DUF1554 was independently shown to bind Factor H (referred to as LfhA, Verma, 2006) and laminin (referred to as Lsa24, Barbosa, 2006). This multifunctional protein may play a key role in pathogenesis by evading the alternative complement pathway by inactivating C3 while aiding bacterial adherence to tissue by binding to laminin.

In this study we sought to characterize other *Leptospira* proteins that contained DUF1554 and C-type lectin-like domains. Several members of these protein families are expressed during growth in culture and during infection and may function in disease.

**Materials and Methods**

**Bacteria and cultivation:**

*Leptospira interrogans* serovar Copenhageni (820-K) was isolated from a rat kidney (Thiermann, 1977). *Leptospira* were propagated in EMJH (Ellinghausen, McCullough, Johnson, and Harris) (Johnson, 1967) media supplemented with 1% rabbit sera, 10% bovine serum albumin and 100µg/ml 5-fluorouracil until mid log phase at 30°C.

INVαF* Escherichia coli* (C2020-03, Invitrogen), and BL21AI *Escherichia coli* (C6070-03, Invitrogen) were grown at 37°C in Luria broth with either 100µg/ml Spectinomycin or Carbenicillin depending on plasmid insert.

**Extraction of DNA:**

*L. interrogans* serovar Copenhageni and *L. interrogans* serovar Kennewicki genomic DNA was extracted as previously described see Appendix for specific protocol. Briefly, bacteria were grown to mid-log phase (10⁸ cells/ml) and
centrifuged at 12,000xg for 30 minutes at 4°C, Cell pellets were resuspended in TE (Tris-EDTA buffer 10mM Tris-HCl, 1mM EDTA, pH 8.0, Invitrogen) and transferred to a pre-spun phase lock gel (LIGHT) tube for overnight incubation. Lysozyme (5 mg/ml in TE, Sigma) was added to suspension and incubated on ice for 15 minutes. 0.1M CaCl₂ and proteinase K (20 mg/mL) were added and incubated at 37°C overnight. Phenol chloroform isoamyl alcohol (PCI) was added and mixed by inversion, then centrifuged at 1500xg for 5 minutes at room temperature. Upper layer was removed and added to a fresh phase lock gel light tube and extract again with PCI. Upper layer was removed again and 2M KCl was added and mix by inversion. 95% ethanol was overlayed and DNA was spooled onto Pasteur pipette until the two phases are completely mixed. Spooled DNA was placed in 70% ethanol then redissolve DNA by incubating overnight at 4°C.

**Primer design and PCR:**

Primers were designed using Clone Manager 7 ® and Primer Designer 5® software. Restriction enzyme sites were added to the 5’ end of each primer pair. Genes were amplified by PCR with Accutaq ® polymerase, on a MJ research thermocycler. The amplification was 35 cycles; 98°C for 30 seconds, then repeat 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 2 minutes for a total of 35 cycles, then 68°C for 10 minutes. PCR products were separated on a 1% agarose gel by gel electrophoresis, stained with ethidium bromide and image captured on film.

**PCR product cloning:**
PCR products were then ligated into pCR8® vector (K250020, Invitrogen) as per manufacturer’s instructions. Ligation reaction were used to transform INVαF’ TOP10 chemically competent cells as per manufacturer’s instructions. Transformations were plated on LB agar plates containing 100μg/ml spectinomycin and incubated overnight at 30ºC. Plasmids were isolated as per manufacturer’s instructions using Qiaprep Spin Miniprep kit (27106, Qiagen). Plasmid preps were screened for proper PCR product by digestion with EcoRI. The digestions were separated on 1% agarose by gel electrophoresis, stained with ethidium bromide and image was captured on film. Clones with the proper insert were then digested at a larger scale and enzymes removed using Quick Clean Enzyme Removal Resin® (631770, Clontech) as per manufacturer’s instructions.

**Expression vector ligation:**

Clones from the previously described pCR8 ligation were digested with their respective restriction enzymes and ligated into pRSET vectors (V351-20, Invitrogen) which are the expression vector for high level expression of recombinant proteins. Ligations were chemical transformed into was INVαF’ TOP10 chemically competent cells as per manufacturer’s instructions. Transformations were plated on LB agar plates containing 100μg/ml carbenicillin and incubated overnight at 30ºC. Plasmids were isolated as per manufacturer’s instructions using Qiaprep Spin Miniprep kit (27106, Qiagen). Plasmid preps were screened for proper PCR product by digestion with the respective restriction enzymes. The digestions were separated on 1% agarose by gel electrophoresis, stained with ethidium bromide and image was captured on film.
Expression:

Plasmids containing the proper insert were then chemically transformed into the expression host cell BL21-Al (Invitrogen) as per manufacturer’s instructions. Cultures were grown to OD600 of 0.5. Inducing agent was added to a final concentration of 0.2% L-Arabinose for 4 hours. Expression cultures were separated on 14% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Immobilon-P membrane by western blot. Detection of fusion protein was determined by immunodetection by blocking the membrane with 5% skim milk in phosphate buffered saline-0.1% Tween 20 (PBS-T) for 1 hour at room temperature, then incubated in mouse anti-histidine primary antibody diluted 1:2000 in 5% milk-PBS-T for 1 hour at room temperature. The membrane was then washed three times with PBS-T and incubated with goat anti-rabbit horseradish peroxidase labeled secondary antibody for 30 minutes at room temperature and washed three times with PBS-T. Chemiluminescence was carried out following manufactures instructions Super Signal West Pico (34080, Pierce) detection kits. Kodak MR film for 30 seconds and developed using the X-OMAT®.

Purification:

Purification was performed by denaturing conditions. Denature purification of the histidine tagged proteins was performed using the Qiagen Ni-NTA (31014, Qiagen) columns as per manufacturer’s instructions. After purification the protein elutions were dialyzed using Slide-a-lyzer 10k MWCO Dialysis cassettes (66380, Pierce) against PBS with a gradual decrease in urea starting with 6M, 4M, 2M, 1M then ending in PBS containing 0.2M L-arginine, excess arginine was removed by
dialysis against PBS. After dialysis the proteins were concentrated by speed vacuum for 2 hours or using Slide-a-lyzer concentrating buffer (66528, Pierce) as per manufacturer’s instructions.

**Polyclonal antiserum production**

Antisera for LIC12315, 12690, 12906, 13006, 13248, 13296 and 13467 were obtained using female New Zealand white rabbits of approximately 6 months of age. Rabbits were injected with 0.25mg/ml of purified protein using MPL+TDM+CWS adjuvant system (M 6661, Sigma). The rabbits were immunized on day 0, and boosted at 3, 6 and 9 weeks. Test bleeds occurred 10 days after each immunization and at day 0. Sera was tested by immunoblot for reactivity with fusion protein by blocking with 5% skim milk (Sigma), 1:500 dilution of polyclonal antibody and 1:20,000 horseradish peroxidase labeled goat anti-rabbit IgG.

**Octylglucoside outer membrane preparation**

Octylglucoside outer membrane preparations from *L. interrogans* serovar Copenhageni was extracted as previously described by Zuerner 1991. 100 mL of mid log phase cells were harvested by centrifugation at 20,000 x g for 20 minutes at 4ºC, and then resuspended in PBS with 5mM MgCl₂ (PBS+M). Incubate pellets in 1% octylglucoside in PBS+M on ice for 1 hour. Then centrifuge for 20 minutes at 4ºC. Cytoplasmic materials were separated from outer membrane fraction by centrifugation. Supernatant was centrifuged an additional time. Outer membrane fraction was concentrated using Centricon YM-10 (Millipore). Pellet and supernatant were stored at -20ºC.

**Reverse Transcriptase Polymerase Chain Reaction**
Total RNA was isolated from *L. interrogans* serovar Copenhageni as per manufactures instructions using the Qiagen RNeasy Midi protocol for bacteria. RNA was checked for purity using the BioAnalyzer 2100 (Agilent Technologies). RNA was then DNase digested to remove residual DNA using RQ DNase (M6101, Promega) as per manufacturer’s instructions.

Reverse transcriptase PCR was performed using Access RT-PCR kit (A1250, Promega) as per manufacturer’s instructions. 1RT-PCR was performed using the MJ Tetrad thermocycler; 48°C for 45 minutes, 94°C for 2 minutes, then 30 cycles of 94°C for 30 seconds, 60°C for 1 minute, 68°C for 2 minutes, then 68°C for 7 minutes. RT-PCR produces were separated on a 1.5% agarose gel.

**Immunohistochemistry**

Immunohistochemistry procedures were performed on tissue harvested from infected Syrian golden hamsters as previously described by Barnett 1999. Briefly, Syrian golden hamsters were inoculated intraperitoneally with *L. interrogans* serovar Kennewicki isolate RM211. The inoculums contained $10^8$ to $10^9$ viable organisms. Animals were sacrificed when clinical symptoms presented (~5 days) brain, kidney, liver and lungs were collected and either fixed in 10% formalin and paraffin embedded or frozen. Tissue sections were stained with haematoxylin and eosin (H/E) or a modified periodic acid Schiff (PAS)/Steiner silver stain (Steiner and Steiner 1944) to visualize leptospires.

Serial 4µm sections of kidney from infected hamsters were cut. Tissue sections were placed on Probe-on-Plus slides (Fisher). Paraffin was removed from sections with xylene and ethanol following standard procedures. Heat induced
antigen retrieval was performed by boiling slides in a 10mM Citrate buffer pH 6.0 with 0.05% Tween 20 for a total of 20 minutes. Slides were washed with PBS and blocked for 1 hour at room temperature with 10% normal goat serum to block non-specific staining of tissue. Primary antibody of polyclonal antiserum from immunized rabbits was incubated on the slides (1:100) overnight at 4ºC. Control slides included anti-LipL32, pre-bleed rabbit serum, and no primary antibody on both infected and uninfected hamster tissue. Slides were washed three times in PBS and incubated with goat anti-rabbit Alexa Fluor 488 (Molecular Probes) for 60 minutes in the dark at room temperature. Slides were washed three times in PBS and incubated for 5 minutes with 4’, 6-diamidino-2-phenylindole (DAPI; 1.5mg/ml) for nuclear staining. Slides were mounted with Slow Fade Light antifade kit (Molecular Probes). All images were captured on a Spot RT color CCD camera mounted on Nikon Eclipse E800 microscope using an x40 Plan Fluor objective and triple band Excitation (DAPI-FITC-Texas Red) Alexa Fluor 350/488/594 Semrock Brightline Nikon Quadfluor filter (Invitrogen). Exciter center wavelengths were 407, 494, 576 nm; and bandwidth was 14, 20, 20 nm.

Results

Sequence analysis

Genes were chosen based on outer membrane localization and molecular weight (Table 3.1). Primers were designed to amplify the open reading frame excluding the lipidation site (Table 3.2). Most of genes are found in *L. interrogans* serovar Kennewicki by sequence analysis and by PCR using the fusion protein primers (Figure 3.1). One gene (LIC12690) has not yet been identified in *L.*
*interrogans* serovar Kennewicki because the genome has not been fully sequenced. All seven of these genes are present only in pathogenic strains of *Leptospira* and are not present in the saprophytic *Leptospira* (Table 3.4). According to the Wasabi website (http://www.vicbioinformatics.com/research.shtml) (Bulach, 2006) LIC12315 and LIC12690 are predicted to be outer membrane proteins. LIC12906, LIC13006 and LIC13467 locations are unknown. LIC13248 is predicted to be localized in the cytoplasmic membrane. LIC13296 is predicted to be extracellular.

The seven genes are related to each other (Table 3.3) and were identified as containing one or more domain of unknown function (DUF1554) and also domains belonging to the C-type lectin-like Superfamily (Figure 3.2). LIC12315, LIC13006, LIC13248 and LIC13467 contained two DUF1554 and C-type lectin-like domains. LIC 12690, LIC12906, and LIC13296 contained only one DUF1554 and C-type lectin-like domain. LIC12690 has one domain at the amino terminus of the protein while LIC13296 has the domain at the carboxy terminus. LIC12906 is a smaller protein that has the domain covering most of the open reading frame. Therefore there is good evidence these proteins may play an important role in pathogenesis since they are only found in pathogenic strains and mostly localized to the outer membrane of the leptospires.
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Table 3.1. Protein information. Gene designations, pl, base pairs (bp), molecular weight (kDa), number of amino acids (AA) and GC content of genes.

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Table 3.2. Primers used to produce fusion proteins with 6x-Histidine tag. Table includes; LIC numbers are the locus designations in the genome forward and reverse primers used for making PCR products including the 5' restriction enzyme extension, expression vector used, specific restriction enzyme, base pairs of the PCR product and molecular weight of the fusion protein.

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Table 3.3. E-values of the DNA sequences of the seven fusion proteins, and their relatedness to one another, e-values from blast website.
Table 3.4. Homologous genes in other *Leptospira* spp, signal peptide, predicted location and lipoprotein information. Hardjo (*L. borgpetersenii* serovar Hardjo), Biflexa (saprophyte *L. Biflexa*), Lai (*L. interrogans* serovar Lai), Pomona (*L. interrogans* serovar Kennewicki serogroup Pomona).

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![Figure 3.1. Fusion protein primers used to amplify genes in *L. interrogans* serovar Copnehageni and *L. interrogans* serovar Kennewicki serogroup Pomona. (Lane 1) 1 kb DNA ladder, (lane 2) Kennewicki (LIC12315), (lane 3) Copenhageni (LIC12315), (lane 4) Kennewicki (LIC12690), (lane 5) Copenhageni (LIC12690), (lane 6) Kennewicki (LIC12906), (lane 7) Copenhageni (LIC12906), (lane 8) Kennewicki (LIC13006), (lane 9) Copenhageni (LIC13006), (lane 10) Kennewicki (LIC13248), (lane 11) Copenhageni (LIC13248), (lane 12) Kennewicki (LIC13296), (lane 13) Copenhageni (LIC13296), (lane 14) Kennewicki (LIC13467) and (lane 15) Copenhageni (LIC13467).]
Figure 3.2. C-type lectin-like domains and domain of unknown function (DUF1554). This figure shows the full length protein, the location of the fusion protein (blue) and the location of the DUF1554 (pink) and C-type lectin domains (purple). Marker at the bottom indicates number of residues.
Production of fusion proteins and polyclonal antiserum

Fusion proteins were expressed, purified and concentrated for immunization of New Zealand white rabbits. Figure 3.3 shows the fusion proteins after immunoblot with the 6X histidine antibody. LIC12315 is the 46kDa band with cleavage products showing up as the smaller bands. LIC13690 does not show a band due to the possibility of a point mutation in the histidine tag therefore not detected by the histidine antibody, the presence of this protein is visualized in Figure 3.4. LIC12906 is the heavy 19kDa band the larger band at ~50kDa could be a dimer of the 19kDa protein. LIC13006 is the band at 45kDa with cleavage products making up the smaller bands. LIC13248 is visible at ~48kDa with a major cleavage product at ~30kDa. LIC13296 is visible at 61kDa and LIC13467 is visible at ~30 kDa but is predicted to be ~52kDa. The rabbits were bled at day 0; test bled at days 14, 32, 53, and 72. Rabbits were immunized at day 0, and then boosted at days 22 and 43. Serum was checked for reactivity against the fusion protein at a dilution of 1:500. All antibodies reacted with the respective fusion protein (Figure 3.4).

Fig 3.3. Reaction of fusion proteins with 6x-histidine antibody (Qiagen). Lane 1 LIC12315 (46kDa), lane 2 LIC12690 (68kDa), lane 3 LIC12906 (19kDa), lane 4 LIC13006 (45kDa), lane 5 LIC13248 (48kDa), lane 6 LIC13296 (61kDa), lane 7 LIC13467 (52kDa), lane 8 histidine ladder used as positive control. Anti-histidine antibody was used at 1:2000, secondary antibody goat anti-mouse (KPL) 1:5000.
Figure 3.4. Reactivity of fusion protein with polyclonal antibody. Lane 1 LIC12315 (46kDa), lane 2 LIC12690 (68kDa), lane 3 LIC12906 (19kDa), lane 4 LIC13006 (45kDa), lane 5 LIC13248 (48kDa), lane 6 LIC13296 (61kDa), lane 7 LIC13467 (52kDa). Fusion proteins separated on 14% SDS-PAGE gel, western transfer at 200 mA and immunoblotted with rabbit polyclonal antiserum at 1:50 dilution and goat anti-rabbit HRP secondary antibody at 1:20,000.

Localization of proteins

Localization of proteins expressed in culture was determined by using the rabbit polyclonal antiserum and detecting proteins in heat-killed whole cells, whole cell sonicate, cytoplasmic fraction, and octylglucoside outer membrane extraction. Cellular fractions were separated on a 14% SDS-PAGE gel overnight at 20mA, western transferred at 200mA for 2 hours and subjected to immunoblot. Figure 3.5
shows reactivity of the individual rabbit polyclonal antiserum with heat killed *Leptospira interrogans* serovar Copenhageni. All the polyclonal antiserum reacts with proteins ranging between ~35-45 kDa. LipL32 was used as a positive control reacting with a ~32kDa protein and the negative control (pre-bleed) did not react with any proteins. Octylglucoside detergent membrane extraction was performed on *L. interrogans* serovar Copenhageni and *L. interrogans* serovar Kennewicki to help localize the cellular location of these proteins. Whole cell sonicate, cytoplasmic fraction, and octylglucoside outer membrane from both serovars were separated by SDS-PAGE and immunoblotted with the rabbit polyclonal antiserum.

Figure 3.6 shows the reactivity of polyclonal antiserum to LIC12315, 12690, and 12906 to the cellular fractions. LIC12315 reacts with a triple banding pattern in the whole cell sonicate (WCS); strong reaction with a ~55 kDa protein and weakly to a ~40 kDa protein in the cytoplasmic fraction, there is a double banding reaction to the outer membrane preps bands between ~40 – 45 kDa. LIC12690 reacts with a multiple banding pattern in the WCS with a strong reaction around ~40 kDa, double banding pattern with strong reaction to the same ~40 kDa band in the cytoplasmic fraction and a double banding pattern in the outer membrane preps between ~40-45 kDa. LIC12906 has a very weak reaction to the whole cell sonicate, cytoplasmic fraction and outer membrane preparation.

Figure 3.7 shows the reactivity of polyclonal antiserum to LIC13006, 13248, and 13296 to the cellular fractions. LIC13006 reacts with 5 proteins in the whole cell sonicate with strong reactions to a proteins at 32 and 40kDa, multiple banding pattern is displayed with the cytoplasmic fraction with a strong reaction to the 40kDa
band, serovar Copenhageni outer membrane prep shows a strong reactivity to the ~32 kDa band while the serovar Kennewicki has strong reaction to the 32 and 40 kDa bands. LIC13248 reacts strongly with a ~40kDa band with two weak reacting bands in the WCS, the same bands react with the cytoplasmic fraction and a weak reaction to the outer membrane fractions therefore localizing LIC13248 mostly to the cytoplasmic fraction. LIC13296 reacts strongly with two protein at ~35 and 40kDa in the WCS, with a continued strong reaction with the ~35kDa protein in the cytoplasmic fraction with weak reaction to the outer membrane preparation therefore localizing LIC13296 mostly to the cytoplasm.

Figure 3.8 shows the reactivity of polyclonal antiserum to LIC13467, positive control LipL32, and negative control (pre bleed serum) to the cellular fractions. LIC13467 reacts strongly with a ~40kDa band in the WCS, with a multiple banding pattern in the cytoplasmic fraction with a strong reaction to a ~60kDa protein, moderate reaction to the ~40kDa protein in both outer membrane fractions. LipL32 was the positive control for the experiment since it is localized to the cytoplasm and the outer membrane. The negative control did react mildly with the WCS and cytoplasmic fractions at ~30kDa. This reaction could be to the spirochete flagella proteins since some rabbits can be commensally infected with intestinal spirochetes. These bands are not present in the outer membrane fractions.

In conclusion, this assay demonstrates the localization of the LIC12315, 12690, 13006, 13467 proteins to the outer membrane. There was a weak reaction to the outer membrane fractions for LIC13248 and LIC13296. Therefore, these proteins may be localized mainly in the cytoplasm. This assay does not determine if
the proteins are surface exposed but associated with the outer membrane. They could be exposed to the inner leaflet of the outer membrane or present in the periplasmic space.

Figure 3.5 *Leptospira interrogans* serovar Copenhageni heat-killed whole cell reactivity to rabbit polyclonal antiserum. Lane 1 LIC12315, lane 2 LIC12690, lane 3 LIC12906, lane 4 LIC13006, lane 5 LIC13248, lane 6 LIC13296 (61kDa), lane 7 LIC13467.
Figure 3.6 Localization of proteins in cellular fractions. LIC12315, LIC12690, and LIC12906 (lane 1) Whole cell sonicate (WCS), (lane 2) cytoplasmic fraction (Cyt), (lane 3) octylglucoside outer membrane preparation of \textit{L. interrogans} serovar Copenhageni (OM C), (lane 4) octylglucoside outer membrane preparation of \textit{L. interrogans} serovar Kennewicki (OM K).
Figure 3.7 Localization of proteins in cellular fractions. LIC13006, LIC13248, and LIC13296 (lane 1) Whole cell sonicate (WCS), (lane 2) cytoplasmic fraction (Cyt), (lane 3) octylglucoside outer membrane preparation of *L. interrogans* serovar Copenhageni (OM C), (lane 4) octylglucoside outer membrane preparation of *L. interrogans* serovar Kennewicki (OM K).
Figure 3.8 Localization of proteins in cellular fractions. LIC13467, LipL32, and pre-bleed (lane 1) Whole cell sonicate (WCS), (lane 2) cytoplasmic fraction (Cyt), (lane 3) octylglucoside outer membrane preparation of *L. interrogans* serovar Copenhageni (OM C), (lane 4) octylglucoside outer membrane preparation of *L. interrogans* serovar Kennewicki (OM K).
RNA extraction and Reverse transcriptase PCR

Reverse transcriptase polymerase chain reaction was performed on mRNA from both serovars to determine if the open reading frames of the hypothetical genes are expressed in the bacteria. RNA was extracted, checked for purity, quantitated and DNase digested. The RNA was then subjected to reverse transcriptase PCR to determine if the genes were being expressed in vitro. Two different serovars were studied, *L. interrogans* serovar Copenhageni and *L. interrogans* serovar Kennewicki. The reverse transcriptase PCR primers were designed from the *L. interrogans* serovar Copenhageni sequence, some mismatched mispairing may occur since the sequences are not identical (Table 3.5). PCR was conducted on DNA from both serovars; LIC12690, LIC13006 and LIC13248 did not amplify a product with the RT-PCR primers using serovar Kennewicki DNA as a template (Fig. 3.9). Serovar Copenhageni amplified a product with all the RT-PCR products showing that the primers are adequate to detect the genes. The RT-PCR of the serovar Copenhageni RNA amplifies products for LIC12315, LIC12690, LIC12906, LIC13296 and LIC13467. LIC13296 and LIC13467 have multiple products; the larger product(s) could be false priming and amplification of non-specific binding of the primer. A negative control was performed with each primer set with the RNA; this negative control lacked the reverse transcriptase enzyme. The RT-PCR of the serovar Kennewicki RNA amplified a product for LIC12315, LIC13296 and LIC13467. Negative controls were performed in the same manner as with the serovar Copenhageni RT-PCR. Thus concluding the gene expression profile differs
between serovars when grown in vitro. A conclusion cannot be made as to the expression profiles in vivo.

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Table 3.5. Reverse transcriptase PCR primers. Table includes locus designation, forward and reverse primers and predicted RT-PCR product size in base pairs.

Figure 3.9. PCR of RT-PCR primers used to amplify genes in *L. interrogans* serovar Copenhageni and *L. interrogans* serovar Kennewicki. (Lane 1) 1 kb DNA ladder, (lane 2) Kennewicki (LIC12315), (lane 3) Copenhageni (LIC12315), (lane 4) Kennewicki (LIC12690), (lane 5) Copenhageni (LIC12690), (lane 6) Kennewicki (LIC12906), (lane 7) Copenhageni (LIC12906), (lane 8) Kennewicki (LIC13006), (lane 9) Copenhageni (LIC13006), (lane 10) Kennewicki (LIC13248), (lane 11) Copenhageni (LIC13248), (lane 12) Kennewicki (LIC13296), (lane 13) Copenhageni (LIC13296), (lane 14) Kennewicki (LIC13467) and (lane 15) Copenhageni (LIC13467), (lane 16) empty, (lane 17) 100bp DNA ladder.
Figure 3.10. *L. interrogans* serovar Copenhageni RT-PCR. (lane 1) 100bp DNA ladder, (lane 2) LIC12315, (lane 3) LIC12690, (lane 4) LIC12906, (lane 5) LIC13006, (lane 6) LIC13248, (lane 7) LIC13296, (lane 8) LIC13467, (lane 10) 1kb DNA ladder, (lane 11) empty, (lane 12) 100bp DNA ladder, (lanes 13-19) same as lanes 2-8 without reverse transcriptase, (lane 20) 1kb DNA ladder.

Figure 3.11. *L. interrogans* serovar Kennewicki RT-PCR. (lane 1) 100bp DNA ladder, (lane 2) LIC12315, (lane 3) LIC12690, (lane 4) LIC12906, (lane 5) LIC13006, (lane 6) LIC13248, (lane 7) LIC13296, (lane 8) LIC13467, (lane 10) 1kb DNA ladder, (lane 11) empty, (lane 12) 100bp DNA ladder, (lanes 13-19) same as lanes 2-8 without reverse transcriptase, (lane 20) 1kb DNA ladder.
**Immunohistochemistry with polyclonal antiserum**

Immunohistochemistry of the liver tissue shows the antibodies to LIC12315, 12690, 12906, 13006, 13248, and 13296 (Figure 3.12, 3.13, 3.14) react with leptosiral antigens located in Kupfer cells. It is unclear if the Kupfer cells are activated or resting. The images do not show full-length leptospires inside the cells, but does show fragments of protein. All antibodies reacted the same way with the exception of LIC13467 which did not react with the Kupfer cells (Figure 3.15). Reaction within the Kupfer cells may be easier to detect because the amount of protein within the cell is higher than in the tissue. LipL32 was used as a positive control (Figure 3.16). LipL32 shows full-length leptospires between cells but not inside of Kupfer cells. Pre-bleed serum was used as a negative control and no fluorescence was visible (Figure 3.15).
Figure 3.12. LIC12315 and LIC12690 polyclonal antibody immunohistochemistry with *L. interrogans* infected hamster liver. Polyclonal antibody diluted to 1:50 and secondary Alexa Fluor 488 goat anti-rabbit diluted 1:5000.
Figure 3.13. LIC12906 and LIC13006 polyclonal antibody immunohistochemistry with *L. interrogans* infected hamster liver. Polyclonal antibody diluted to 1:50 and secondary Alexa Fluor 488 goat anti-rabbit diluted 1:5000.
Figure 3.14. LIC13248 and LIC13296 polyclonal antibody immunohistochemistry with *L. interrogans* infected hamster liver. Polyclonal antibody diluted to 1:50 and secondary Alexa Fluor 488 goat anti-rabbit diluted 1:5000.
Figure 3.15. LIC13467 and pre-bleed polyclonal antibody immunohistochemistry with *L. interrogans* infected hamster liver. Polyclonal antibody diluted to 1:50 and secondary Alexa Fluor 488 goat anti-rabbit diluted 1:5000.
Figure 3.16. LipL32 polyclonal antibody immunohistochemistry with *L. interrogans* infected hamster liver. Polyclonal antibody diluted to 1:50 and secondary Alexa Fluor 488 goat anti-rabbit diluted 1:5000.
**Discussion and Conclusion**

In this study, we examined a family of proteins belonging to the C-type lectin-like family of proteins. Some of these proteins were predicted to be outer membrane and share domains of unknown function and C-type lectin like domains. Fusion proteins were made to a large portion of each of these proteins and polyclonal antiserum was produced in New Zealand white rabbits. Using this polyclonal antiserum it was shown that LIC12315, LIC12690, LIC13006, LIC13248, LIC13296 and LIC13467 were expressed by the leptospire as determined by the reaction with heat killed whole cell lysate.

Octylglucoside outer membrane extraction was performed on *L. interrogans* serovar Kennewicki (serogroup Pomona) and serovar Copenhageni. The octylglucoside extraction reveals reactivity with LIC12315, LIC12690, LIC13006, LIC13248, LIC13296 and LIC13467 indicating the presence of these proteins within the outer membrane. Surface exposure has yet to be determined since the protein could potentially be expressed on the inside of the outer leaflet of the bacterial membrane. Polyclonal antiserum to LIC12906 did not react with the cell preparations. This could be because the protein is not expressed at very high levels or is not expressed at a level detectable by the antibody.

Reverse transcriptase PCR reveals the presence of the genes LIC12315, LIC12690, LIC12906, LIC13296 and LIC13467 to be expressed by *L. interrogans* serovar Copenhageni *in vitro* but transcript quantitation was not determined. This analysis proves the genes are no longer hypothetical and they code for a gene that
is expressed by the bacteria. Primers to LIC13006 and LIC13248 did not amplify a product and it is inconclusive if the gene is expressed.

6 of the 7 proteins are present in the liver Kupfer cells of Syrian Golden hamsters infected with *L. interrogans* serovar Kennewicki. Infected hamster tissue from *L. interrogans* serovar Copenhageni (820-K) did not disseminate into the tissue to visualize the leptospires. Serovar Kennewicki infected tissue had a higher concentration of leptospires that could visualize the pathogen for analysis. The concentration of protein within the Kupfer cells was higher than in the tissues that allowed the antibody to detect the protein. It was not determined, however, if the Kupfer cells were activated and degrading the leptospires or if the leptospires were residing there as intact bacteria.

The function of these proteins has yet to be determined. If the function of the protein mimics C-type lectin-like function these proteins could be important virulence factors and vaccine candidates. Further cloning of the genes to obtain full-length proteins and native purification are critical in determining function of these proteins.
References


CHAPTER 4. General Discussion

Discussion

In this study, we examined a family of proteins belonging to the C-type lectin-like family of proteins. Some of these proteins were predicted to be outer membrane and share domains of unknown function and C-type lectin like domains. Fusion proteins were made to a large portion of each of these proteins and polyclonal antiserum was produced in New Zealand white rabbits. Using this polyclonal antiserum it was shown that LIC12315, LIC12690, LIC13006, LIC13248, LIC13296 and LIC13467 were expressed by the leptospire as determined by the reaction with heat killed whole cell lysate.

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Reverse transcriptase PCR reveals the presence of the genes LIC12315, LIC12690, LIC12906, LIC13296 and LIC13467 to be expressed by *L. interrogans* serovar Copenhageni *in vitro* but the number of transcripts was not determined. This analysis proves the genes are no longer hypothetical and they code for a gene
that is expressed by the bacteria. Primers to LIC13006 and LIC13248 did not amplify a product and it is inconclusive if the gene is expressed.

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The function of these proteins has yet to be determined. If the function of the protein mimics C-type lectin-like function these proteins could be important virulence factors and vaccine candidates. Further cloning of the genes to obtain full-length proteins and native purification are critical in determining function of these proteins.

**RECOMMENDATIONS FOR FUTURE RESEARCH**

Future research pertaining to the C-type lectin-like domain family of proteins in *L. interrogans* species should include cloning of the entire open reading frame to produce a full-length protein, *in vitro* analysis of the lectin domain, and *in vivo* analysis of the activation state of the Kupfer cells of the liver.

Cloning of the entire open reading frame to produce a full length protein would enable the protein to fold in the proper fashion for functional activity. This
important in the analysis of the attachment studies to extracellular matrix proteins. The fusion proteins should be cloned to have the histidine tag at the carboxy terminus of the protein. Other tags could be utilized to enhance the purification potential of the protein. Native purification is essential for maintaining the functional epitopes required for the activity of the protein.

*In vitro* analysis of the C-type lectin domain would include attachment studies of extracellular matrix proteins such as laminin, fibronectin, collagen, and fibrinogen as well as attachment to epithelial cells and immune cells (macrophages, dendritic cells, Kupfer cells and neutrophils). This assay could be done by western blot or ELISA.

*In vivo* analysis of the activation state of the Kupfer cells containing the reactive proteins would be determined by double staining with the polyclonal antibody and either lysosomal associated membrane protein (LAMP-1 or LAMP-2). LAMP-1 is transported from the Golgi network to the endosome and then to lysosomes. When activated LAMP-1 associates with the plasma membrane, alterations to the carboxy terminal tyrosine based motif allows the protein to associate with the endosome. This assay was performed with optimization needed. The antibody sequence should be changed to determine if the erythrocytes will auto fluoresce green instead of red. The activated lysosome could not be distinguished from auto fluorescing erythrocytes (Figure 4.1 and 4.2). Immunohistochemistry procedures were performed on tissue harvested from infected Syrian golden hamsters as previously described by Barnett 1999. Briefly, Syrian golden hamsters were inoculated intraperitoneally with *L. interrogans* serovar Kennewick isolate.
Inoculums contained $10^8$ to $10^9$ viable organisms. Animals were sacrificed when clinical symptoms presented (~5 days) brain, kidney, liver and lungs were collected and either fixed in 10% formalin and paraffin embedded or frozen. Tissue sections were stained with haematoxylin and eosin (H/E) or a modified periodic acid Schiff (PAS)/Steiner silver stain (Steiner and Steiner 1944) to visualize leptospires.

Serial 4µm sections of kidney from infected hamsters were cut. Tissue sections were placed on Probe-on-Plus slides (Fisher). Paraffin was removed from sections with xylene and ethanol following standard procedures. Heat induced antigen retrieval was performed by boiling slides in a 10mM Citrate buffer pH 6.0 with 0.05% Tween 20 for a total of 20 minutes. 0.1% w/v saponin was incubated on slides for 30 minutes at room temperature (Sander, 1991). Slides were washed with PBS and blocked for 1 hour at room temperature with 10% normal goat serum to block non-specific staining of tissue. Primary antibody of polyclonal antiserum from immunized rabbits was incubated on the slides (1:100) overnight at 4ºC. Slides were washed three times in PBS and incubated with goat anti-rabbit Alexa Fluor 488 (Molecular Probes) for 60 minutes in the dark at room temperature. Slides were washed three times in PBS and incubated primary antibody (LAMP-1, LAMP-2) (Uthayakumar, 1995) at a dilution of 1:500 overnight at 4ºC. Slides were washed three times with PBS and incubated with goat anti-mouse Alexa Fluor 546 for 1 hour at room temperature in the dark. Slides were washed three times in PBS and incubated for 5 minutes with 4′, 6-diamidino-2-phenylindole (DAPI; 1.5mg/ml) for nuclear staining. Slides were mounted with Slow Fade Light antifade kit (Molecular Probes). All images were captured on a Spot RT color CCD camera mounted on
Nikon Eclipse E800 microscope using an x40 Plan Fluor objective and triple band Excitation (DAPI-FITC-Texas Red) Alexa Fluor 350/488/594 Semrock Brightline Nikon Quadfluor filter (Invitrogen). Exciter center wavelengths were 407, 494, 576 nm; and bandwidth was 14, 20, 20 nm.

Figure 4.1. Lamp-1 and Lamp-2 antibody reaction to infected tissues.
Figure 4.2. Double labeling of Lamp-1 or Lamp-2 (Alexa Fluor 546, red) with polyclonal antibody to LIC12690 (Alexa Fluor 488, green)
References


APPENDIX A. RAW DATA

Figure A-1. PCR amplification of inserts for fusion protein production
(lane 1) 1 kb ladder, (lane 2) LIC12315, (lane 3) LIC12690, (lane 4) LIC12906, (lane 5) LIC13006, (lane 6) LIC13248, (lane 7) LIC13296, and (lane 8) LIC13467.

Figure A-2. *Eco*RI digestion of pCR8 (shuttle vector) positive clones (lane 1-4) LIC13467, (lane 5-8) LIC13296, (lane 9-11) LIC13248, (lane 12-15) LIC13006, (lane 16-18) LIC12690, (lane 19-22) LIC12315, (lane 23 and 24) 1 kb ladder, (lane 25-28) LIC12906
Figure A-3. Digestion of fusion proteins from expression vector pRSET (lane 1-6) LIC12315 (BamH1/HindIII), (lane 7-12) LIC13296 (XhoI/HindIII), (lane 13-18) LIC12690 (XhoI/HindIII), (lane 19-24) LIC13248 (BamH1/HindIII), (lane 25) 1 kb ladder, (lane 26-30) LIC13006 (BamH1/HindIII), (lane 31) 1 kb ladder, (lane 32-36) LIC12906 (BamHI/HindIII), (lane 37) 1kb ladder, (lane 38-42) LIC13467 (BamH1/HindIII).

Figure A-4. Purification of histidine tagged protein (lane 1) cleared lysate LIC12906, (lane 2) flow through, (lane 3 and 4) wash, (lane 5 and 6) elution, (lane 7) histidine ladder.
APPENDIX B: Additional Data and Tables

LIC12315 (8830.1)
MKSKFKFVAV LTLTGILFLF SCSETQDGSD NTLPTLLSLL GEKNKPAASC
FHYGKLTDED PSDVEASGEN LLPSSPTYIF LAPAHPHGGL GYIAGDEWCM
RKPVPADLKG PSYSYKALLA DGNRRAVTG NAGGGQINNW VLOASTVVSO
ATTGQPTTGT TNSKFKLFPP LTNKIRPDSI TDFGSITWTG LNPWNTTAPY
NCNNWTDGDAD ITNGTGNY ATSSNAIFDS ALIVPGLMTC SGGKLYCVE
QPAPPIRFVK YIYLPLSLPI GHNNGYGGIA GMDTICNNDT MKPVELLKG
TYKAMAVDQS ITIPSTPATR RATPNNPLNWV LRPNTTYKRV DGATLFTTDDI
NGLFSSTMNR FGETEENNGY ITWLGNLSDFT TATYGNCMNW KSSASYLNGK
IGSANNTVNGS VALSTYNFPC DQTINSEFI RVVCVEQ

LIC12690 (12000.1)
MNYILVCIMT VYLVLGCNTK SQNNPFFFSN FADSILNL
DCAPSYDSSK GYYVILNSYG LSCGRLVSFPS GEQLSLAYPF PANARFADYL
LIWKLDPFQE LSYNGNIQIP FQTHMDSDK TDEISDLRFT GSAQYMSIT
LNWSPGTVTL AMQDFPTQTY PTVETNVTIS SVSDSTFNNI INDMITNNRD
AYIYSGNPV SSSKSKRGYN YTGALNLLK NKTAKNVEV SYILKLLGEFT
LSGMLKHLV PVTNNKKSRS TECSTTSDFP VSI3NYPQGI LVTRFQTPPS
IKFYHADEN IFSDKYHNAP SDCCSSGKNT TFSRNLIDL HAYEPITPVPN
AAGFLDTIKI RIVRGFDQTQ TTNDLSLWG NILTSEFYEA TKKYFKEQI
GQNSSMFIQ DLNQLAVENW YNTRPKTPGK TFLNNLNPDE RLLATMLYVE
FNQKTLNTDL ILYIPQNNG EVTDLYISDF SKIQQGAYAT SNAQLGQSIV
PITNYVLATI SDANIIDTRV FHTINTTGT FHDFPTYQF CLGCPSVAN
ATEYEVNRQ RNLQYTSQNI SIEHLGHTSW HMKMGIRVG DPEPRPFRDF
QHYEGARLGM SVYDDNSIDF FRNMEMYSSK RLRELINOTQ GDILERRULS
RYGNFRSHK YTIVPDPHNN ANFGGITQAD GYCNLDSNKPV SKLLSLGGG
KAMLVGVDTTR ASLSPNAGDG QIDWVLFPLQ EYRRSDDTII G1TNSVGLF
FFQFNSFAFQM YALLWGLDQWT WKNKVISVNQ LNCNLWSTSS SSNLEAFGLA
NWKYGNS1FV QQGICSKTSL PDHTDPYWKN DVPSILCVE Q

LIC12906 (13510.1)
MNLKQGNKIL KTIPLTLILL SGVCTLYSCG DKKEEDNSEL LLFLNNSLG
SNNTSTPVVT SCKDASFCRT FIATNNGAGY NNGLGQIGFA DAKAAAKSS
SLTGAAYKAL VDGPTQRGVI SALDSVDKK DWLYPKNQY RRSDDTTIF
TTNANDIVD NLQNGIDSDT FKFFNWGLGN NVGFLEWEPFLS NCCNWNSTDG
LITQGQGNTTL ELQADITPEG AFTVDNHACN NNLNLLECAEQ

LIC13006 (14270.1)
MRSKLTDNKI KYKIHAVSVSF TLFSVAFACNFK SSQGDNSALS GLISLISDKQ
TLPYCTTNNR QLSQMQSTFP EGYMPYETT SSGVFGHNH FGGISGADAF
CQSHIPSNI SRYIKAMIV DGYNRATLV GPSTVGKQD WVFQPNQYR
RASEDANVMF TNSQGKLEPF QVKEGQWTA LNTNNTWTTWS
NGFPSTCNWS NSGALNDFGI FGSRSTTSD IALALISTNE QVGT8CSLSI
GYGYPELNLG VCEVEFPPP LIFVSTEE WHQDFNGQIA GADAYCQSV
PYNPLLGSIY KAMLVGVDRN VATTIPGNST VQGDRWAVLF NMYFIRYDD
ALIMTNSSG MFIDFTNNREL ENSFQGIAAA QWTLNLSDWT IWTASVGPGR
EPIICNSWIT BDNSFYGYYV MNSRKNDSVL RAAESNGQFT AACSLKFTSY
GNYRGLVCVEQ

LIC13248 (31870.1)
MKIYKYFAE SAPTFLVFAC INKSGGDNDNA VLSGLSILIS NEQQCVGLDC
SKNRTRSRNQ LQSGQSLQAA FPQGYPYI VTSVSGVHN GNFGGIAAGAD
AYCQSNIPGN LSPTGVYKAM LVDGVNRVAT TVGPNSTVGQ KDWFQPHQQ
YRRADDGGIV MTTNASGMD FSGARLQNP FTRDYHAHHW SSINIDWTTW
AVNGPFOACD SWITSEWIPK YAIYGSMDQQ NENIIATKIN FTKPCSDYYA
APLSDGTDWR LGLVCVEQPEF PKYIFVTTSS NGEVYHNANFG GIGADAYCQ
SRIPSNIFPRN NYIKAMVLDVG INRITAIVGPP NSTVQGKDVW FRPNNQQYRA
EDGAIIVMTTN SSGMFDFFDS ARLOQNPFTSN PRAAHWSN IDWTTAVNG
IPTCDSSGW SEWIPKAYI YMDSQVNEHI LTAKINFTKP CSDYHAAPL
DGTWSNLG CVEE

LIC13296 (32320.1)
MSNMCKKVI PLFFFSKETL MLYKIFKTIY SYSFILMVTG FVGCYGGGNS
SFAGISLKN LSLLNIDSHSN NLTATPSGGL YVFTVSYTNS TVSLPLTHNG
NFTGIGADGA YCNHSIPSSL SGTSKAMV DVGVRVATT VGPNNQSSQQK
DWFVKINTSY YRPDKMLFT TNDAGLFFDS TGSLSYADF SYLFWDGIVW
TGLQSNMWTV PSTQCGSSGS PWTQNGNASY QGGITYNYK TGSISAGSI
PCQWKEQIDP SSQGSMEMGI LCYQEKPVTL KAKIGFLEDQ QCYDNNARQG
PFTIPWQKQ CHFKRHFDPAT GVAVSSDVAS PGYRPVPGQ VHLLNPENSTQ
TNPGVCYVSN LQQSLGNINQ VSQINACLKT TPAFPDRVIT VVLYKTNL
YNNSITIRG VWEKTISAPL NLVLYQSKSPT MQTLFEDN TQKQVQYPYE
VVPFLSEWNY ATTVDYDFGT VNLLSIPTS LMSMFLTLA SWQGVYEFHN
RMEAPSESEVE KIHDFMFINN PARNCVTCYT INFNITYGSG AYFGNLSSA
PQADITISSE LSVDIPRLHE FGHTIQSAA FWTIUTTNW AGQFDRSNQ
NYGRAHNLVE YQSMASATE GIVNP1GRYL ISNRGMNIVQ GNYYLGYQTN
GWQLDPFIDVQ TYSDQYFRFY EMWVRGISEN SPEWSRSLQN IQSINSTFTD
RNTNSNNEYR YDGFPYHLLQ VPPYHVDSSY ATGNYAPYVN QFEYRQDMK
LLGIEIDGGVNFKFWYFVD VVHPKNSRVS LKDLLTLNLE YDGSPLAPAN
GSPEFNDKYL ISKSFSPQPS LGWKLIQKGL ISKEVSNLFL RAVGMDQLAN
ICGPWALPD CQ

LIC13467 (33530.1)
MYNQTALKN SIIMLKEQEI SSSLKKSFSY LILIFSYTFL KCNSSENSNL
FVTSDTNKNEI LNLNLPNTHS SSALLEEFGT LFVAPFFHNG GFSYEGISAA
DKYCNANIP GSFAANAGHP KALLANNLGT NLYKRIATVT PNAVGDQQVDW
IFPKNTEYRR IDGVTKVMVT NSVGLFDFTN ENLNSFTST FVNVITGLNP
DWTRGQAVTD DQCGNCIGQW VVGVEDANV YGGTFGANM INSABFDFN
ATCDSTAGVT SQCFAPAPLSL LCVQKQVQRN YKPFVTPTA HNCDGWGVSG
ADAYCQANIP RSIATGTFPYK AMLVAPTRQQ ANTHPNVQDGQ QIDVWFKPDNT
EYRRADDGRVT VMITNSKLF DFGSNLHTN FEGSFAEYI TGLYSDWTAR
ATYREMCHDV PRFGLTHTDS GWEHGNSSGR LGNKSISSR SISHTIDACM
HNVHTFTST VTINFGLCQ EQ

Figure B-1. Amino acid sequence of each of the C-type lectin like proteins. Yellow highlight indicates fusion protein, bold indicates DUF1554 domain and underline indicates C-type lectin like domain.
APPENDIX C. PROTOCOLS

Bacteria and cultivation:

*Leptospira interrogans* serovar Copenhageni (820-K) was isolated from a rat kidney (Thiermann, 1977). *Leptospira* were propagated in EMJH (Ellinghausen, McCullough, Johnson, and Harris) (Johnson, 1967) media supplemented with 1% rabbit sera, 10% bovine serum albumin and 100µg/ml 5-fluorouracil until mid log phase at 30ºC.

INVαF’ *Escherichia coli* (C2020-03, Invitrogen), and BL21Al *Escherichia coli* (C6070-03, Invitrogen) were grown at 37ºC in Luria broth with either 100µg/ml Spectinomycin or Carbenicillin depending on plasmid insert.

Extraction of DNA:

*L. interrogans* serovar Copenhageni and *L. interrogans* serovar Kennewicki were grown to mid-log phase (10⁹ cells/ml) and centrifuged at 12,000xg for 30 minutes at 4ºC, then washed with phosphate buffered saline (PBS) and spun again. Pellet was resuspended in a smaller volume and washed a third time. Pellet was resuspended in TE (Tris-EDTA buffer 10mM Tris-HCl, 1mM EDTA, pH 8.0, Invitrogen) and transferred to a pre-spun phase lock gel (LIGHT) tube for overnight incubation. 500 µL lysozyme (5 mg/ml in TE, Sigma) was added to suspension and incubated on ice for 15 minutes. 30 µL 0.1M CaCl₂ and 60 µL proteinase K (20 mg/mL) were added and incubated at 37ºC overnight. 4 mL phenol chloroform isoamyl alcohol (PCI) was added and mixed by inversion, then centrifuged at 1500xg for 5 minutes at room temperature. Upper layer was removed and added to a fresh phase lock gel light tube and extract again with PCI. Remove the upper layer and
add 100 µL 2M KCl and mix by inversion. Overlay 95% ethanol, spool DNA onto Pasteur pipette until the two phases are completely mixed. Place spooled DNA into 1 mL 70% ethanol for 2 minutes. Place pipette tip in 200 µL TE, incubate at room temperature for 20 minutes, redissolve DNA by incubating overnight at 4ºC.

Primer design and PCR:

Hypothetical proteins (LIC12315, 12690, 12906, 13006, 13248, 13296 and 13467) were selected from the *L. interrogans* serovar Copenhageni genome based on the predicted value of their potential coiled coil domains, their molecular weight and association with the C-type lectin-like superfamily. The genetic sequence was uploaded into the Clone Manager 7® and Primer Designer 5® software. The 5′ ends were modified with *Bam*HI (GGATCCC) for the forward primer and *Hind*III (GGAAGCTT) for the reverse primer, extra bases were added to ensure the gene would be in frame with the histidine tag. Genes were amplified by PCR with Accutaq® polymerase, on a MJ research thermocycler. The amplification was 35 cycles; 98ºC for 30 seconds, then repeat 94ºC for 15 seconds, 60ºC for 30 seconds, 68ºC for 2 minutes for a total of 35 cycles, then 68ºC for 10 minutes. PCR products were separated on a 1% agarose gel by gel electrophoresis, stained with ethidium bromide and image captured on film.

Shuttle vector preparation:

Fresh PCR products were then ligated into pCR8® shuttle vector. While shuttle vector was thawing on ice, ligation reaction (2µL PCR product, 1µL salt solution, 2µL dH₂O and 1µL vector) incubated for 5 minutes at room temperature. Chemical transformation was carried out by adding 2µL ligation reaction to TOP10
chemically competent cells and allowed to incubate on ice for 30 minutes. Heat
shock was carried out at 42°C for 30 seconds; 250μL SOC media was added and
recovered, shaking at 37°C for 1 hour. Once recovered, 50μL was plated on LB
agar plates containing 100μg/ml spectinomycin and incubated overnight at 30°C.
Four isolated colonies were picked and inoculated into 5mL of LB liquid media
containing 100μg/mL spectinomycin. Inoculation was incubated overnight shaking at
37°C. Overnight culture was subjected to plasmid isolation as per Qiagen technical
were screened for proper PCR product by digestion with EcoRI. 3μL of plasmid prep
was incubated for 2-3 hours with 17μL digestion master mix. The digestions were
separated on 1% agarose by gel electrophoresis, stained with ethidium bromide and
image was captured on film. Clones with the proper insert were then digested at a
larger scale with the restriction enzymes that were added to the 5’ end of the gene of
interest and subjected to the same electrophoresis conditions. After large scale
digestion the enzymes were removed by ethanol/sodium acetate precipitation or
Quick Clean Enzyme Removal Resin® as per manufacturer’s instructions.

Expression vector ligation:

Expression vector (pRSET) has to have the same compatible ends as the
digested insert. Therefore the expression vector has to be digested with the same
restriction enzymes as the gene of interest. Once the expression vector has been
prepared the insert and vector are ligated using T4 DNA ligase as per
manufacturer’s instructions. Vector was incubated with digested insert at a 1:5 ratio.
Ligations were incubated overnight at 16°C. Chemical transformation was carried
out with TOP10 (Invitrogen) cells as described previously. 50μL of the recovered cells were plated on 100μg/ml carbenicillin or ampicillin LB agar plates. 6-8 colonies were inoculated into 5 mL LB liquid media and incubated at 37°C shaking overnight. Plasmids were purified from overnight cultures by Qiagen mini prep as described previously. Prepared plasmids were digested with appropriate restriction enzymes and screened on 1% agarose by gel electrophoresis, stained with ethidium bromide and image was captured on film.

Expression:

Plasmids containing the proper insert were then chemically transformed into the expression host cell BL21-AI (Invitrogen) or Novablue (DE3) (Novagen) as per manufacturer’s instructions. BL21-AI host cells require L-Arabinose and Novablue (DE3) requires IPTG as the inducing agent. Plasmids were chemically transformed into host cells and streaked to antibiotic containing LB agar plates. A starter culture was started from a single colony into 5mL LB broth containing 100μg/mL carbenicillin shaking, overnight at 37°C. The starter culture was diluted 1:20 and allowed to grow shaking at 37°C until OD600 reached 0.5. Inducing agent was added to a final concentration of 0.2% L-Arabinose or 0.4mM IPTG for 4 hours. Expression cultures were separated on 14% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Immobilon-P membrane by western blot. Detection of fusion protein was determined by immunodetection by blocking the membrane with 5% skim milk in phosphate buffered saline-0.1% Tween 20 (PBS-T) for 1 hour at room temperature, then incubated in mouse anti-histidine primary antibody diluted 1:2000 in 5% milk-PBS-T for 1 hour at room temperature.
The membrane was then washed three times with PBS-T and incubated with goat anti-rabbit horseradish peroxidase labeled secondary antibody for 30 minutes at room temperature and washed three times with PBS-T. Chemiluminescence was carried out following manufactures instructions for either Amersham BioSciences ECL-Plus kit or Pierce West Pico detection kits. Membranes were wrapped in plastic wrap and enclosed in a light protective case and exposed to Kodak MR film for 30 seconds and developed using the X-OMAT®.

**Purification:**

Purification was performed by native and denaturing conditions. Purification of the histidine-tagged fusion proteins under native conditions was carried out using three different methods of purification. Purification using normal conditions from the manufacturer’s instructions proved to be inefficient in binding these particular histidine tagged proteins. The protocol had to be modified to include zwitterionic detergents (1% NDSB-256, Calbiochem), 0.05% CHAPS (Sigma) and 0.25% Tween 20 (Sigma). Under these conditions the protein was slightly denatured under non-ionic conditions. The first two purification methods used the Ni-NTA columns from Qiagen. The lysates were solubilized and applied to nickel resin columns to bind histidine. The third purification method does not require a column but uses His-Mag® beads from Novagen as per manufactures instructions. After each step, the flow through was collected for analysis on SDS-PAGE for stringency of the assay. Denature purification of the histidine tagged proteins was performed using the Qiagen Ni-NTA columns as per manufacturer’s instructions. After purification the protein elutions were dialyzed using Pierce Slide-a-lyser cassettes against PBS.
containing 0.2M L-arginine, excess arginine was removed by dialysis against PBS. After dialysis the proteins were concentrated by speed vacuum for 2 hours or using Pierce concentrating buffer as per manufacturer’s instructions.

**Polyclonal antiserum production**

Antiserum for LIC12315, 12690, 12906, 13006, 13248, 13296 and 13467 were obtained using female New Zealand white rabbits of approximately 6 months of age. Rabbits were injected with 0.25mg/ml of purified protein using MPL+TDM+CWS adjuvant system (Sigma) as previously described (Haake 1998, 2000, Matsunaga 2002, Shang 1996). The rabbits were immunized on day 0, and boosted at 3, 6 and 9 weeks. Test bleeds occurred 10 days after each immunization and at day 0. Sera was tested by immunoblot for reactivity with fusion protein by blocking with 5% skim milk (Sigma), 1:500 dilution of polyclonal antibody and 1:20,000 horseradish peroxidase labeled goat anti-rabbit IgG.

**Octylglucoside outer membrane preparation**

Octylglucoside outer membrane preparations from *L. interrogans* serovar Copenhageni was extracted as previously described by Werts, 2001 and Zuerner 1991). 100 mL of mid log phase cells were harvested by centrifugation at 20,000 x g for 20 minutes at 4°C, and then resuspended in PBS with 5mM MgCl$_2$ (PBS+M). Incubate pellets in 1% octylglucoside in PBS+M on ice for 1 hour. Then centrifuge for 20 minutes at 4°C. Cytoplasmic materials were separated from outer membrane fraction by centrifugation. Supernatant was centrifuged an additional time. Outer membrane fraction was concentrated using Centricon YM-10 (Millipore). Pellet and supernatant were stored at -20°C.
Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated from *L. interrogans* serovar Copenhageni as per manufactures instructions using the Qiagen RNeasy Midi protocol for bacteria. RNA was checked for purity using the BioAnalyzer 2100 (Agilent Technologies). RNA was then DNAse digested to remove residual DNA. To 20µl RNA (5ng/µl), 5µl RQ DNAse buffer (Promega), 2ul RNAse Out (Invitrogen), 2.5ul RQ DNAse (Promega) incubate at 37ºC for 30 minutes. Add another 2.5µl of DNAse and incubate 1 hour at 37ºC. Add 5µl DNAse stop, incubate for 2 minutes at 95ºC, store at -70ºC.

Reverse transcriptase PCR was performed using Access RT-PCR kit from Promega. 1.5µl of each primer, 1µl digested RNA and 1µl AMV RT were added to the master mix containing 5x buffer, dNTPs, MgSO$_4$ and Tfl DNA polymerase. RT-PCR was performed using the MJ Tetrad thermocycler; 48ºC for 45 minutes, 94ºC for 2 minutes, then 30 cycles of 94ºC for 30 seconds, 60ºC for 1 minute, 68ºC for 2 minutes, then 68ºC for 7 minutes. RT-PCR produces were separated on a 1.5% agarose gel.

Immunohistochemistry

Immunohistochemistry procedures were performed on tissue harvested from infected Syrian golden hamsters as previously described by Barnett 1999. Briefly, Syrian golden hamsters were inoculated intraperitoneally with 1 mL of the Dinger zone containing $10^8$ to $10^9$ *L. interrogans* serovar Copenhageni (strain 820-K isolated by Alex Thiermann) per mL. Hamsters were euthanized at 3, 7, and 14 days. Hamsters did not show clinical signs of disease. At 7 days, hamster liver was homogenized and passed to uninfected hamster. At each time point brain, kidney,
liver and lungs were collected and either fixed in 10% formalin and paraffin embedded or frozen. Tissue sections were stained with haematoxylin and eosin (H/E) or a modified periodic acid Schiff (PAS)/Steiner silver stain (Steiner and Steiner 1944) to visualize leptospires.

Serial 4µm sections of kidney from infected hamsters were cut. Tissue sections were placed on Probe-on-Plus slides (Fisher). Paraffin was removed from sections with xylene and ethanol following standard procedures. Heat induced antigen retrieval was performed by boiling slides in a 10mM Citrate buffer pH 6.0 with 0.05% Tween 20 for a total of 20 minutes. Slides were washed with PBS and blocked for 1 hour at room temperature with 10% normal goat serum to block non-specific staining of tissue. Primary antibody of polyclonal antiserum from immunized rabbits was incubated on the slides (1:100) overnight at 4ºC. Control slides included anti-LipL32, pre-bleed rabbit serum, and no primary antibody on both infected and uninfected hamster tissue. Slides were washed three times in PBS and incubated with goat anti-rabbit Alexa Fluor 488 (Molecular Probes) for 60 minutes in the dark at room temperature. Slides were washed three times in PBS and incubated for 5 minutes with 4’, 6-diamidino-2-phenylindole (DAPI; 1.5mg/ml) for nuclear staining. Slides were mounted with Slow Fade Light antifade kit (Molecular Probes). All images were captured on a Spot RT color CCD camera mounted on Nikon Eclipse E800 microscope using a x40 Plan Fluor objective and triple band Excitation (DAPI-FITC-Texas Red) Alexa Fluor 350/488/594 Semrock Brightline Nikon Quadfluor filter (Invitrogen). Exciter center wavelengths were 407, 494, 576 nm; and bandwidth was 14, 20, 20 nm.