

ABSTRACT

Title of Document: METABOLIC VIRULENCE DETERMINANTS
AND RAPID MOLECULAR DIAGNOSTICS
OF PATHOGENIC SPIROCHETES

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Borrelia burgdorferi and *Leptospira interrogans* are pathogenic spirochetes that elicit serious health threats, termed as Lyme disease and leptospirosis. Key areas of spirochete research involve a better understanding of their intriguing biology and infection, including identification of novel virulence factors and improvements in diagnostic and preventive methods. Notably, certain bacterial metabolic enzymes are surface-exposed, having evolved to acquire additional functions referred to as protein moonlighting that contributes in microbial virulence. Comparative genome analysis revealed that certain components of sugar metabolism pathways are either absent or seemingly inactive in pathogenic spirochetes, which were studied herein for their potential roles as metabolic virulence factors. Of nine borrelial enzymes investigated, only phosphomannose isomerase (PMI) was found to be surface-exposed and remained enzymatically active in the spirochete outer membrane. PMI is critical for mannose metabolism and facilitates the interconversion of fructose 6-phosphate and mannose-6-phosphate, although its occurrence in borrelial surface remains enigmatic.

PMI may provide a critical function for *B. burgdorferi* viability as it is constitutively expressed and all attempts to create genetic mutants remained unsuccessful. Active immunization studies using recombinant PMI did not influence the outcome of infection within tick or murine hosts, although a significant reduction in bacterial levels within the joints of mice was recorded, suggesting its involvement in spirochete persistence in a tissue-specific manner.

Despite substantial advancement, the development of more effective diagnostics for leptospirosis and Lyme disease still remains a critical need since human vaccines are unavailable. Antibiotic treatment can resolve these infections but is most effective when administered early during infection, prior to pathogen dissemination to distant organs. As diagnostic methods for spirochete infection still depends on ineffective and antiquated technologies, we sought to develop novel RNA-based assays for better detection of early spirochete infection. Results indicated that targeting specific regions of 16S and 23S ribosomal RNA targets provided the highest possible sensitivity and specificity of detection, which was far superior to current serological, microbiological or molecular methods used to detect presence of invading pathogens.

METABOLIC VIRULENCE DETERMINANTS AND RAPID MOLECULAR
DIAGNOSTICS OF PATHOGENIC SPIROCHETES

By

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Dedication

I would like to dedicate this work to my wife and to my parents for their constant support, encouragement, and patience.

Acknowledgements

First and foremost, I would like to acknowledge my advisor, Dr. Utpal Pal, who has willingly provided aid and guidance to me as a graduate student. The opportunities he gave me to learn and to contribute in the lab will not be soon forgotten.

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Chapter 1: Introduction to pathogenic spirochetes

The spirochetes, order *Spirochaetales*, includes three families of bacteria: *Spirochaetaceae* (genus *Borrelia*, *Brevinema*, *Criptispira*, *Spirochaeta*, *Spironema*, and *Treponema*), *Brachyspiraceae* (genus *Brachyspira*) and *Leptospiraceae* (genus *Leptonema* and *Leptospira*) (Karami, Sarshar et al. 2014). The spirochetes comprise a group of gram-negative bacteria with unique features allowing morphological distinction based upon their size, shape, and motility. These bacteria have a long (3-500 μm) helical or corkscrew-like shape and an extremely narrow width (0.1- 3 μm) that makes them difficult to visualize using routine microscopy (Margulis, Ashen et al. 1993), instead requiring the use of specialized illumination that capitalizes on oblique illumination for contrast enhancement in specimens, called dark-field microscopy. Spirochetes typically consist of an outer and inner membrane that surround periplasm and endoflagella or axial filaments, and a protoplasmic cylinder (Karami, Sarshar et al. 2014). The protoplasmic cylinder (PC) contains a top peptidoglycan layer that surround protoplasm (Charon, Goldstein et al. 2009). Unlike other bacteria, the peptidoglycan layer of spirochetes associates more closely with the inner cytoplasmic membrane rather than the outer membrane (Smibert and Johnson 1973). The outer membrane surrounds the PC with a gap in between the two layers called the periplasm. Within the periplasm, the flagella or axial filaments are found attached at the ends of the protoplasmic cylinder and coiling around the body of the cell (Motaleb, Corum et al. 2000). The number of axial filaments varies between different families of spirochete bacteria. The periplasmic flagella are responsible for the unique motility of the spirochetes and contribute significantly to the shape of the

bacteria (Motaleb, Corum et al. 2000; Lambert, Picardeau et al. 2012). Although most spirochetes are harmless free-living microbes, primarily soil bacteria, a few have evolved to survive in unique ecological and pathogenic niches. These include members of *Borrelia*, the agents of Lyme disease and relapsing fever, *Leptospira*, the agent of leptospirosis, and *Treponema*, the agents of syphilis and oral periodontitis (Karami, Sarshar et al. 2014). The remainder of this thesis will focus on characterization of potential metabolic virulence factors and diagnostic methods for the diseases caused by pathogenic *Leptospira* species and Lyme disease group *Borrelia* species.

1.1 Leptospirosis

Epidemiology, clinical symptoms, and prevention

Leptospirosis is a common zoonotic disease found worldwide that causes severe infections in humans and animals. It is caused by several distinct pathogenic and intermediate pathogenic species of genus *Leptospira* (Levett 2001). Leptospirosis is endemic in many tropical and subtropical climates, especially within urban slums of developing countries due to high density populations living with inadequate sanitation and housing infrastructure in close proximity to infested rat colonies (Ko, Galvao Reis et al. 1999; Reis, Ribeiro et al. 2008). However, infections also occur in industrialized countries, including the United States (Meites, Jay et al. 2004). Numerous surveys have shown infestation among rat populations living within cities of industrialized nations resulting in human cases (Thiermann and Frank 1980; Vinetz, Glass et al. 1996; Forouhar and Mitsani 2011). Additionally, infections are

regularly identified in domestic and farm animals and periodic human outbreaks from natural disasters and recreational hazards like swimming in infected waters (Jackson, Kaufmann et al. 1993; Stern, Galloway et al. 2010; Leibler, Zakhour et al. 2016). It is estimated that more than 800,000 cases and 50,000 deaths occur annually due to leptospirosis (Picardeau, Bertherat et al. 2014). Leptospirosis was removed from the list of national notifiable infection within the United States in 1995, but was recently re-instated to this list in 2013 amidst increasing outbreaks from recreational activities (i.e. triathletes and adventure race participants) and fears of larger emergence due to climate changes (Morgan, Bornstein et al. 2002; Meites, Jay et al. 2004; Reis, Ribeiro et al. 2008; Stern, Galloway et al. 2010).

Exposure of humans and animals occurs through contact between abraded skin and possibly mucous membranes with contaminated urine or water sources. Upon host entry, bacteria proliferate within the blood and disseminate throughout the body infecting various organs (McBride, Athanzio et al. 2005). Some infected individuals present no symptoms of disease, while others may suffer a wide range of symptoms varying from mild febrile illness with flu-like symptoms (fevers, chills, muscle aches, and vomiting) to extreme manifestations known as Weil's disease, including jaundice, kidney or liver failure, meningitis, pulmonary hemorrhaging and sometimes death (Arean 1962). The incubation period to onset of symptoms is usually about seven days, with a range from 2-29 days (McBride, Athanzio et al. 2005). Infections result in fatality rates of approximately 10%, but in cases of severe icteric infection fatality rates may increase (Ko, Galvao Reis et al. 1999). No known

indicators have been able to predict cases of mild illness from cases that will progress to severe clinical disease (Faine, Adler et al.).

There are two overlapping phases of disease characterized by an acute or septicemic phase lasting approximately one week from onset of symptoms, followed by the convalescent or immune phase, which includes clearance of bacteria from the blood and excretion of leptospire in the urine (Levett 2001). Infection is described as septicemic leptospiremia rather than bacteremic because the pathogens are believed to penetrate blood vessels and actually multiply within the blood stream where they attain access to almost any tissue or organ, including the brain (Turner 1967; Mathew, Satishchandra et al. 2006). Immune defense includes phagocytosis of bacteria by immune cells within tissues, and from the second week after onset of symptoms a robust antibody response practically eliminates the leptospiremia and begins to reduce colonization of bacteria in all tissues except the kidneys, where the bacteria continue to persist and may shed in the urine (Adler and de la Pena Moctezuma 2010).

Pathological manifestations are most severe within the liver, kidneys, heart, and lungs (Arean 1962). These include vasculitis, endothelial damage, and inflammatory cell infiltrations. Features of severe icteric Weil's disease may include liver and spleen enlargement, uveitis, interstitial nephritis in kidneys, necrosis and myositis of muscles, myocarditis of the heart, pulmonary hemorrhage and acute respiratory distress, and jaundice (Arean 1957; Faine 1957; De Brito, Freymuller et al. 1966). Barring occasional organ failure or hemorrhaging in some organs (liver and lungs), clearance of the bacteria from tissues allows restoration of normal tissue

function (Turner 1967). However, in the kidneys, the bacteria manage to infiltrate and colonize the lumen of renal tubules to hide in the interstitial tissues without causing damage (De Brito, Freymuller et al. 1966; Tucunduva de Faria, Athanazio et al. 2007; Athanazio, Silva et al. 2008; Coutinho, Matsunaga et al. 2014). From there, leptospires may be shed in the urine to contaminate the host environment (Athanazio, Silva et al. 2008). In the case of accidental hosts, this leptospiuria may last from about eight days post infection to two to three months and eventually cleared, but in carrier reservoir hosts, leptospiuria may persist for years or even the remaining lifespan (Turner 1967).

Research efforts have focused on identification of novel virulence factors (Adler, Lo et al. 2011) and development of subunit vaccines, specifically to identify surface-associated proteins that are conserved among pathogenic isolates and serve as antigenic targets for humoral and bactericidal immune responses. In recent years, availability of full genome sequences of several pathogenic and non-pathogenic *Leptospira* strains (Ren, Fu et al. 2003; Nascimento, Ko et al. 2004; Bulach, Zuerner et al. 2006; Picardeau, Bulach et al. 2008) have renewed interest in the identification of novel cross-protective leptospiral antigens to create more widely-applicable vaccines and treatments against leptospirosis (Nally, Whitelegge et al. 2007; Faisal, Yan et al. 2009). Reverse genetics techniques involving *Leptospira* species are limited, except for creation and partial characterization of a transposon mutagenesis library with greater than one thousand mutated genes (Murray, Morel et al. 2009). Most studies so far have been focused on description of outer membrane proteins as they are usually the target of host immune responses and mostly immunogenic,

although thus far only a few potential vaccine targets have been described (Koizumi and Watanabe 2005; Cao, Faisal et al. 2011). One promising vaccine target, LigA, which contains multiple repeated immunoglobulin-like domains and is induced by high osmolarity, provides protection from death in the hamster model of acute leptospirosis (Silva, Medeiros et al. 2007; Lourdault, Wang et al. 2014). However, it fails to provide sterile immunity since immunized animals still acquire infection as shown by colonization of kidneys and shedding of bacteria in urine.

Although a recombinant vaccine is currently unavailable, in some countries, vaccines have been deployed using attenuated or killed bacteria of the local infectious strains (Koizumi and Watanabe 2005). However, these vaccines offer only strain and serovar specific protection and induce relatively short-term humoral immune responses requiring frequent immunization, providing overall poor protection (Chassin, Picardeau et al. 2009). An ideal vaccine candidate should protect against a broad swath of the diverse *Leptospira* species and serovars while retaining a memory response without the need for frequent immunization boosts. Due to its widespread prevalence, improved diagnostics and preventive strategies such as vaccination remain essential areas for continued leptospirosis research.

Clinical diagnosis

If diagnosed early during infection, antibiotics are usually effective to cure leptospirosis, but diagnosis is difficult to establish because of currently-available inefficient diagnostics and similarity of symptoms with other undifferentiated febrile illness (McBride, Athanzio et al. 2005). Diagnosis of leptospirosis primarily relies

on antiquated serotyping methods and is particularly challenging due to presentation of non-specific symptoms shared by other febrile illnesses leading to misdiagnosis (Limmathurotsakul, Turner et al. 2012; Budihal and Perwez 2014; Niloofa, Fernando et al. 2015). Initiation of antimicrobial therapy during early infection to prevent more serious complications of disseminated infection is often not performed because of a lack of efficient diagnostic tests. Due to confusion from the non-specific clinical manifestations of leptospirosis, it can be impossible to diagnose barring prior clinical index of suspicion (Karande, Gandhi et al. 2005). In fact, failure to diagnose the infection or misdiagnosis has become a significant problem in many developing countries where influenza, yellow fever, dengue, malaria, typhoid and other causes of acute fever are endemic (Biggs, Galloway et al. 2013; Musso and La Scola 2013; Reller, Wunder et al. 2014). The most common methods for diagnosis of leptospirosis include immunoassays against spirochetes or recombinant proteins, direct cultivation of bacteria grown from body fluids, or a microscopic agglutination test (MAT) using paired serum samples and *Leptospira* cultures (Picardeau, Bertherat et al. 2014). Although some of the immunoassays are highly sensitive, they suffer from inherent delays (days to weeks) and variability of host immune responses as well as sequence divergence in target antigens, potentially limiting their use for early diagnosis of leptospirosis (Wynwood, Burns et al. 2015). Even the gold standard methods of direct culture and the MAT are inadequate to be used to aid treatment of the patient (Limmathurotsakul, Turner et al. 2012). Direct culture from tissue specimens may require up to four weeks to grow spirochetes from body fluids, and MAT testing requires paired sera samples, cultures of many different *Leptospira*

species, and highly trained laboratory personnel to perform subjective visual reading of results (Boonsilp, Thaipadungpanit et al. 2011). Some attempts have tried to use blood tests of liver and kidney function, such as creatine, urea, and enzyme markers, but these do not provide a consistent result for diagnostics and may even show normal results (Musso and La Scola 2013). Therefore, in the absence of vaccination, there is a critical need for rapid and effective diagnostics, especially for detection of early infection.

Biology and transmission of pathogenic Leptospira species

The family *Leptospiraceae* includes genus *Leptonema* and *Leptospira*. Genus *Leptospira* contains more than 20 species including nine pathogenic, five intermediate pathogens, and six non-pathogenic or saprophytic species (Adler and Faine 2006). *Leptospira* species are further classified into serovars based upon their antigenic relationship due largely to differences in lipopolysaccharide structures (Clarridge 2004; Adler and Faine 2006). More than 200 unique serovars exist with some serovars spanning multiple different species (Bharti, Nally et al. 2003). All species of *Leptospira* have similar morphological characteristics including a spiral coiled shape of 6-20 μm in length and approximately 0.1 μm in width (Bharti, Nally et al. 2003). Leptospire possess two periplasmic flagella that extend from the cytoplasmic membrane at each end of the bacteria and are essential for motility (Picardeau, Brenot et al. 2001; Lambert, Picardeau et al. 2012). Frequently, leptospire can be distinguished between other spirochetes by a characteristic hook shape at one or both ends of the cell body almost resembling a question mark

(Lambert, Picardeau et al. 2012). Leptospire are cultivable in the laboratory commonly using Ellinghausen-McCullough-Johnson-Harris (EMJH) media typically at 30°C in an aerobic environment (Ellinghausen and McCullough 1965). Long chain fatty acids serve as the primary carbon and energy source *in vitro* via beta-oxidation metabolism, but must be acquired extracellularly, as leptospire lack the ability for fatty acid synthesis (Johnson and Gary 1963; Henneberry and Cox 1970; Staneck, Henneberry et al. 1973). Expression of outer membrane proteins is highly variable between *in vitro* and *in vivo* growth environments (Haake, Martinich et al. 1998; Palaniappan, Chang et al. 2002; Nally, Whitelegge et al. 2007). The outer membrane of leptospire contains an abundant lipopolysaccharide layer and a variety of lipoproteins and transmembrane proteins including numerous virulence factors (Levett 2001; Cullen, Cordwell et al. 2002). A number of potential virulence factors and adhesins have been shown to be induced under specific *in vitro* conditions such as changes in osmolarity, temperature, or pH, as might be encountered by the bacteria during *in vivo* infection (Barbosa, Abreu et al. 2006; Choy, Kelley et al. 2007). Additionally, differences between leptospiral LPS likely contributes to its adaptability and preference towards certain host species and the large diversity of pathogenic leptospiral serovars (Faine, Adler et al. ; de la Pena-Moctezuma, Bulach et al. 1999; Nascimento, Ko et al. 2004).

The genome of pathogenic *Leptospira interrogans* contains two circular chromosomes. Chromosome I contains 4,691,184 base pairs (bp) and is over ten times larger than chromosome II at 358,943 bp (Ren, Fu et al. 2003). The genome encodes for 4,768 putative genes: 4,360 within chromosome I and 367 within

chromosome II. Within chromosome I, 37 genes encode for transfer RNAs and one gene each encode for 23S and 5S rRNA, while two gene copies are maintained for 16S ribosomal RNA (rRNA) (Ren, Fu et al. 2003). Notably, the rRNA genes are not organized into operons as commonly occurs in many bacteria, but are scattered throughout the genome (Nascimento, Ko et al. 2004). Homology between rRNA gene sequences remains high across all *Leptospira* species with 16S rRNA sequences from pathogenic *L. interrogans* exhibiting 90% identity to that of non-pathogenic *L. biflexa* over 100% coverage using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST).

Rodents commonly serve as an asymptomatic reservoir host, while humans and other animals are accidental hosts that can develop disease after infection (Tucunduva de Faria, Athanazio et al. 2007). Large numbers of leptospires colonize the kidneys of rats (*Rattus rattus* and *rattus norvegicus*), up to 10^7 bacteria per gram of tissue (Tucunduva de Faria, Athanazio et al. 2007). Due to the high density of bacteria in kidneys, bacteria slough off and are shed in the urine of infected animals, frequently in high enough concentrations to be observed in urine samples under dark-field microscopy, which contaminates surrounding aquatic environments (Adler and de la Pena Moctezuma 2010). Infection of farm, wild, and domestic animals, as well as humans, occurs through incidental contact of contaminated soil, water, or urine with mucous membranes or abraded skin (Hellstrom and Marshall 1978; Adler and de la Pena Moctezuma 2010). In exceptionally rare cases, transmission has been reported through the bite of infected animal, or human to human through sexual transmission (Luzzi, Milne et al. 1987; Gollop, Katz et al. 1993). Unlike other

pathogenic spirochetes, *Borrelia* and *Treponema*, leptospire are capable of surviving outside of a host in moist environments such as soil and water for long periods of time, aiding their ability to find suitable hosts and persist in nature.(Trueba, Zapata et al. 2004)

1.2 Lyme disease

Epidemiology, clinical symptoms, and prevention

Lyme disease or Lyme borreliosis is a prevalent vector-borne disease in the United States, Europe, and parts of Asia, which is caused by spirochete pathogen *Borrelia burgdorferi sensu lato* (Aguero-Rosenfeld, Wang et al. 2005). In fact, several species of *Borrelia* cause numerous other distinct diseases such as relapsing fever, a recently described illnesses caused by unique species *Borrelia miyamotoi*, and possibly other species such as *Borrelia lonestari* that have been implicated in southern tick-associated rash illness (STARI) (Clinton, Carabin et al. 2010; Herman-Giddens 2012; Krause, Fish et al. 2015). Despite substantial attention, Lyme disease remains underreported globally, including in the United States. In fact, newly revised estimates from the Centers for Disease Control and Prevention (CDC) suggest that actual Lyme cases are at least 10-fold higher than published reports, and that there are likely to be more than 300,000 new cases per year in the U.S. alone (Hinckley, Connally et al. 2014; Nelson, Saha et al. 2015). In Europe, there are 85,000 estimated cases in a year; however, reporting is inconsistent and as such, many infections go undiagnosed (Sood 2011; Leibler, Zakhour et al. 2016). For example, in a single year, there are 213,912 suspected cases in Germany alone (Cook 2015). In addition to known *B. burgdorferi* strains, there are recent reports of emergence of new and more

virulent *Borrelia* species causing human infection globally (such as *B. bissettii* and *B. mayonii*) (Picken and Picken 2000; Pritt, Mead et al. 2016).

Currently, there are as many as 12 different species of *Borrelia* belong to the *sensu lato* complex, but the most frequently identified species associated with human infection are *B. burgdorferi sensu stricto* in the United States, and *B. garinii* and *B. afzelli* in Europe and Asia (Goldings and Jericho 1986; Dufour, Moutou et al. 2008; Stanek 2013). Historically, Lyme disease was first recognized due to an outbreak of rheumatoid arthritis and skin lesions among children in Old Lyme, Connecticut (Steere, Malawista et al. 1977). The causative agent of Lyme disease, *Borrelia burgdorferi*, was later isolated in 1981 in cultures from ticks by Willy Burgdorfer, for whom the bacteria is named, and later cultured from patients showing symptoms of Lyme disease in 1982 (Steere, Grodzicki et al. 1983; Burgdorfer 1984). However, the bacteria likely existed long before identification and isolation in culture. Evidence has shown detection of *B. burgdorferi* DNA in mice specimens from museums dating to the 1800s, and multilocus sequence typing (MLST) creates a pattern indicating possible persistence of *B. burgdorferi* in North America thousands of years prior to European colonization (Marshall, Telford et al. 1994; Hoen, Margos et al. 2009).

Lyme disease remains as the most common vector-borne infection in the United States. Currently, more than 96% of confirmed Lyme disease cases occur within 14 states in the Northeast and upper Midwest, according to the CDC. The bacteria exist through a complex enzootic cycle involving maintenance of the microbe between *Ixodid* ticks, commonly known as deer ticks, and a rodent mammalian host reservoir (Burgdorfer 1984; Kumar, Yang et al. 2010). Incidental

hosts like humans acquire the disease when they are bitten by an infected tick and the bacteria are spread via tick saliva into the skin of the human hosts (Cook 2015).

After mammalian infection, different phases of disease may be observed in human patients: early localized infection, early disseminated infection, and late infection (De Silva and Fikrig 1995). Approximately seven to nine days after infection from the initial tick bite, early localized infection may appear in the form of an identifiable skin rash, erythema migrans, starting near the site of the tick bite as the bacteria spread laterally through the dermis (Barthold, Beck et al. 1990; Steere, Coburn et al. 2004). The erythema migrans occurs in approximately 70% of patients (Steere 1989). Within a few days to several weeks, erythema migrans rash may appear in secondary locations indicating early disseminated infection, often accompanied by fever, regional lymph swelling, carditis, and muscle and joint pains as *Borrelia* colonizes various tissues in the mammalian hosts, notably the joints, heart, and nervous system (Arean 1957; Duray 1989; Magnarelli and Anderson 1989). Within three to four weeks erythema migrans will disappear, even if untreated (Duray 1989; Cerar, Ruzic-Sabljić et al. 2008). Late Lyme borreliosis sometimes occurs in untreated patients months or years after initial infection, characterized by chronic arthritis, central and peripheral nervous system syndromes, and acrodermatitis chronica atrophicans (ACA) (Goldings and Jericho 1986; Brehmer-Andersson, Hovmark et al. 1998).

These minority of patients will have persistent or relapsing nonobjective symptoms of variable intensity, including fatigue, musculoskeletal pain, and cognitive complaints that are collectively termed as chronic Lyme disease or post-treatment Lyme disease syndrome (PTLDS) (Weitzner, McKenna et al. 2015). In fact, about 40% of patients

with erythema migrans would experience such symptoms within 12 months of completing therapy (Berende, ter Hofstede et al. 2016). Treatment at this stage may be ineffective even with long-term dosage of antibiotics, and the underlying mechanisms or pathogenesis of PTLDS remains unknown (Cameron 2009; Delong, Blossom et al. 2012; Weitzner, McKenna et al. 2015; Berende, ter Hofstede et al. 2016).

Despite substantial progress in the efforts for early vaccine discovery in Lyme borreliosis studies, two vaccines were developed against Lyme disease in the late 1990s and tested through Phase III clinical trials, LYMErix (by GlaxoSmithKline, formerly SmithKline Beecham) and ImuLyme (by Pasteur Mérieux Connaught). Both vaccines were developed to protect via production of antibodies targeting *B. burgdorferi* outer membrane antigen called OspA (Fikrig, Barthold et al. 1990). Ultimately, ImuLyme never sought regulatory approval while LYMErix was approved by FDA in 1999. This vaccine showed a 76% reduction in cases of Lyme disease and no serious side effects in a randomized trial involving almost 11,000 adults (Steere, Sikand et al. 1998). However, due to a lackluster recommendation from the CDC's Advisory Committee on Immunization Practices (ACIP) and numerous lawsuits due to misinformation about safety of the vaccine, demand for the vaccine was weak and not cost-effective (Willyard 2014). Ultimately, the vaccine was voluntarily withdrawn from the market in 2002 (Kaaijk and Luytjes 2016). A vaccine based on the same principle does remain on the market for animal use, but no vaccines are currently available for prevention of human infection (Fikrig, Barthold et al. 1990). In the absence of human vaccines, the best method of Lyme disease

prevention is to prevent a tick bite. This can be done by covering all exposed skin while at risk of contact with tick habitats (long pants, long-sleeve shirt, boots, socks, etc.) and application of chemical pesticides (i.e. containing DEET or Picaridin) to repel the blood-sucking arthropods (Bhate and Schwartz 2011). While this can aid in prevention, it can never provide complete protection from the possibility of infection, especially since people are often unaware that they have been bitten by a tick due to its inconspicuous size.

Clinical diagnosis

While relatively easily treated with antibiotics if diagnosed early, undiagnosed and untreated cases often progress from initial symptoms including rash, fever, headache, and nausea to chronic neurologic symptoms and rheumatoid arthritis that can affect the subject for the remainder of their life and severely diminish quality of life (Duray 1989; Stanek 2013; Mead 2015). The lack of available vaccines and limits of preventative measures create a critical need for effective diagnostic methods to detect early infection and enable treatment as soon as possible. Most cases of Lyme disease are differentially diagnosed based on physical symptoms and observed findings (erythema migrans and arthritis), combined with the individuals risk of possible tick exposure (Wilske 2005). Secondly, recommended diagnostic IgM and IgG immunoassays and immunoblotting provide confirmation of diagnosis (Centers for Disease and Prevention 1995). These confirmatory tests, however, do not provide adequate detection until several weeks after infection when the patient develops a robust antibody response (two to four weeks). Other methods for detection have been

attempted utilizing direct culture, microscopic methods, molecular detection, and direct antigen detection to detect the bacteria within tissue samples or bodily fluids (Wilske 2005). Direct culture has become increasingly common, as it may be the most sensitive method and provides direct evidence of active infection (Liveris, Schwartz et al. 2011). However, the bacteria may take up to 12 weeks to grow and require a complex growth media for survival. As a clinical test, this method is resource and time prohibitive to be useful. Microscopic detection is usually not feasible due to the low and inconsistent number of bacteria that may be in a sample. Direct antigen detection has suffered from wide variations in specificity and sensitivity based on the antigen chosen for testing and the phase clinical infection, often showing high rates of false positives and false negatives (Steere, McHugh et al. 2008).

Serology-based testing is most frequently employed, namely immunoblot and ELISA or immunofluorescent assays (Steere 1997). The CDC recommends a two-tiered protocol for present or past infection utilizing a sensitive enzyme immunoassay (EIA) or immunofluorescent assay (IFA) followed by a specific Western blot (Wilske 2005). ELISA is most commonly employed based upon the C6 peptide of an abundant surface protein, VlsE (Bacon, Biggerstaff et al. 2003; Nyman, Willen et al. 2006). Within the first four weeks of infection, IgM and IgG immunoblot should be used, but after this time IgG is preferred (Centers for Disease and Prevention 1995). If a patient tests negative but is suspected to have Lyme disease, paired serum samples should be acquired to test development of antibody between acute and convalescent sera. IgM immunoblot is considered positive if two or more bands are

present at molecular weights of 24 kilodaltons or kDa (representing OspC), 39 kDa (representing BmpA), and 41 kDa (representing FlaB) (Engstrom, Shoop et al. 1995). IgG immunoblot is considered positive if at least five of 10 specific bands are observed at 18 kDa, 21 kDa (OspC), 28 kDa, 30 kDa, 39 kDa (BmpA), 41 kDa (FlaB), 45 kDa, 58 kDa, 66 kDa, and 93 kDa (Dressler, Whalen et al. 1993; Centers for Disease and Prevention 1995). In one study, testing by the two-tiered method showed 100% sensitivity and 99% specificity in late infection patients with chronic neurologic or arthritic symptoms (Steere, McHugh et al. 2008). However, the same two-tiered testing showed just 64% sensitivity with 99% specificity in convalescent phase of early patients with erythema migrans and a dismal 29% sensitivity with 99% specificity in acute phase with erythema migrans (Steere, McHugh et al. 2008).

Many DNA-based and some RNA-based detection methods have been developed that target various genes of *B. burgdorferi* for direct detection (Alby and Capraro 2015; Bil-Lula, Matuszek et al. 2015). DNA-based assays have shown extremely high specificity of detection (up to 100%), but show a wide range of sensitivity depending on sample type and gene target (Ferdin, Cerar et al. 2010; Lee, Vigliotti et al. 2010; de Leeuw, Maraha et al. 2014; Bil-Lula, Matuszek et al. 2015). Previous studies for PCR detection of *Borrelia* DNA in skin biopsy samples of erythema migrans or ACA sites showed sensitivity ranging from 36% to 100% (Brehmer-Andersson, Hovmark et al. 1998). Similar tests from other studies have shown ranges of sensitivities between 0-100% from blood samples, 12-100% from cerebrospinal fluid (CSF), and 42-100% from synovial fluid samples with 93-100% specificities (Aguero-Rosenfeld, Wang et al. 2005; Wilske 2005; Alby and Capraro

2015). In the age of genomics, PCR-based molecular diagnostics are increasingly being utilized for detection of various pathological conditions ranging from bacterial and viral infections to rare form of cancers. PCR assays have not been widely accepted for laboratory diagnosis of Lyme disease to date because of low sensitivity in CSF and blood, even though they are the most promising (Aguero-Rosenfeld, Wang et al. 2005). Improvement in sensitivity of detection may be provided by detection of RNA transcripts rather than genomic DNA.

Biology and transmission of B. burgdorferi

The family *Spirochaetaceae* includes numerous pathogenic and non-pathogenic species belonging to the genera *Borrelia*, *Brevinema*, *Cristispira*, *Spirochaeta*, *Spironema* and *Treponema* (Caimano 2006). The genus *Borrelia* currently includes 36 known species with others potentially undiscovered (Pritt, Mead et al. 2016). These bacteria cause the important hematophagous tick and louse-borne infections, Lyme disease and relapsing fever. Lyme disease in particular is caused by a group of these spirochetes, which are collectively referred to as *Borrelia burgdorferi sensu lato* complex. All *Borrelia* species have similar morphological characteristics including an atypical gram-negative spiral shaped bacteria 5-20 μm long and approximately 0.3 μm wide (Charon, Goldstein et al. 2009; Kariu, Yang et al. 2013). They possess a didermic membrane separated by a spatial region called the periplasm (Barbour and Hayes 1986). The inner membrane is very closely associated with the peptidoglycan layer and contains the cellular cytoplasm and nuclear region to make up the protoplasmic cylinder (Charon, Goldstein et al. 2009). This classifies

Borrelia as a gram-negative bacteria on a structural basis. Near the pole at each end of the protoplasmic cylinder, 7-11 axial filaments or periplasmic flagella attach and wrap around the body of the bacteria (Charon, Goldstein et al. 2009; Guyard, Raffel et al. 2013; Lin, Gao et al. 2015). Most other bacteria have exo-flagella attached to the outside of the cell. These axial filaments are dually responsible for motility of the organism and contribute to the flat spiral cellular shape (Motaleb, Corum et al. 2000). Movement of the flagella causes a wavelike motion along the cell, resulting in forward movement of the bacteria (Goldstein, Charon et al. 1994). The outer membrane surrounds the peptidoglycan-cytoplasmic membrane, periplasmic space, and endoflagella (Motaleb, Corum et al. 2000; Charon, Goldstein et al. 2009). The *Borrelia* outer membrane, uniquely among gram-negative bacteria, lacks lipopolysaccharides and phosphatidylethanolamine and has a low density of transmembrane proteins (Barbour and Hayes 1986; Takayama, Rothenberg et al. 1987) but has numerous abundant outer membrane lipoproteins and glycolipids exposed on the surface of the bacteria (Pozsgay, Kubler-Kielb et al. 2011; Toledo, Crowley et al. 2014).

A large number of the outer membrane proteins have no homology to those of other bacteria and are of unknown function, but may contribute to pathogenesis and persistence within the various phases of the enzootic life cycle (Kraiczy, Skerka et al. 2001; Coburn, Fischer et al. 2005). In fact, many proteins have been shown to be specifically expressed or differentially regulated within the tick or mouse life cycle (Tokarz, Anderton et al. 2004; Brooks, Vuppala et al. 2006; Pal, Wang et al. 2008). OspA, one of the most abundant outer surface proteins is a highly expressed surface

lipoprotein specifically produced during the tick life cycle of *Borrelia* to aid midgut colonization, but down regulated immediately upon mammalian infection (Fuchs, Wallich et al. 1994; Coleman, Gebbia et al. 1997; Pal, Li et al. 2004). Similarly, upon downregulation of OspA, another abundant outer surface protein, OspC, is highly upregulated specifically upon mammalian host entry to aid in early immune evasion (Ramamoorthi, Narasimhan et al. 2005).

Upon transmission between hosts (i.e. from mammal to tick or vice versa) a substantial alteration of genetic expression must occur for the bacteria to survive in the widely different environments (Anguita, Hedrick et al. 2003). One of the important subsets of genes that must be regulated for survival are those encoding metabolic genes and those encoding virulence factors (Tokarz, Anderton et al. 2004). The strict regulation of genetic expression is absolutely essential for the pathogen's ability to survive, infect, and persist within the different host environments and temperatures (Anguita, Hedrick et al. 2003; Bykowski, Woodman et al. 2008; Promnares, Kumar et al. 2009; Bugrysheva, Godfrey et al. 2011). For example, *B. burgdorferi* upregulates expression of an operon necessary for glycerol metabolism during the tick phase of the life cycle (Pappas, Iyer et al. 2011). It was found that hemocoel of the tick contains glycerol which serves as the major carbon source for the bacteria within the tick environment (Pappas, Iyer et al. 2011). Glucose is preferred by *Borrelia* during *in vitro* growth and mammalian infection (von Lackum and Stevenson 2005; Corona and Schwartz 2015). Also of note, numerous novel genes have been found to have increased or decreased expression within the tick versus mouse phase of the life cycle or more specifically within one mammalian

tissue versus another (i.e. heart vs. joint) as a factor of pathogen virulence (Coleman, Yang et al. 2008).

Lyme disease is spread exclusively through feeding of an infected tick on an uninfected host (Burgdorfer 1984). At any stage in the life cycle, *Ixodid* ticks may acquire the bacteria upon feeding on infected mammalian hosts. The bacteria does not spread between ticks by trans-ovarian passage, therefore it may only be acquired via feeding during the nymph, larval, or adult stages of the life cycle (Anguita, Hedrick et al. 2003). The bacteria then persists within the tick gut through molting until the next feeding cycle or end of life cycle (Pal, Li et al. 2004). Upon attachment of an infected tick to a host, *B. burgdorferi* begin to multiply rapidly within the feeding tick gut. The bacteria transmigrates across the gut epithelial cells and escape into the tick hemocoel containing hemolymph (Corona and Schwartz 2015). After a rapid journey through the hemolymph in the open circulatory system of the tick, the bacteria briefly colonize the salivary glands of the tick before being deposited into the dermis of the mammalian hosts (De Silva and Fikrig 1995; Anguita, Hedrick et al. 2003).

1.3 Research objectives

This work focuses on two different bacteria that, while related, exist in separate ecological niches and cause dramatically different diseases. The first part of this work, discussed further in Chapter 2 explores the possibility of metabolic enzymes having functions involved in the virulence of bacteria during infection of an incidental host. The capacity to persist within hosts and ability to continuously

circulate among rodent animal reservoirs are key to *B. burgdorferi* and *L. interrogans* survival in nature. However, after years of research, the intricate mechanisms by which spirochetes survive and infect hosts, and therefore the mechanisms to prevent these infections, are still poorly understood. In recent years, moonlighting proteins have emerged as a new class of virulence determinants in many pathogens, and even provide protective immunity against infection (Jeffery 1999). There is precedent among numerous other organisms from bacteria to parasites that some highly conserved glycolytic enzymes perform dual roles involving both metabolic processes as well as host invasion and colonization (Jeffery 1999; Nogueira, Smith et al. 2012). We specifically investigated expression of glycolytic enzymes during host infection of spirochetes to assess whether their expression is differentially regulated at specific phases of infection. To study potential moonlighting activities of a selected set of candidate enzymes, we utilized animal models and an infectious *B. burgdorferi*, isolate B31, and infectious *L. interrogans* strains. Transcriptional expression and regulation of these genes during *in vitro* and *in vivo* growth as well as studies to observe the localization of these proteins after translation help to indicate the importance of these enzymes and potential for surface localization or secretion. Recombinant proteins and antibodies for moonlight candidates were used to identify cellular localization. Further studies investigated the role of moonlight candidates during infection mimicking the *Borrelia* life cycle in the C3H/HeJ mouse model and *I. scapularis* tick vector for Lyme disease or the acute infection hamster model of leptospirosis (Barthold, Beck et al. 1990; Silva, Medeiros et al. 2007). For promising moonlighting candidates we examined whether they retain their native enzymatic

function, or play an alternative role, such as in host-pathogen interaction and further attempted immunization studies to assess whether they are target of protective host immunity. Antigens that showed immunoreactivity or immunoprotection were further characterized by attempts to develop genetic knockout mutants. As eluded earlier, targeted gene mutation is unavailable for pathogenic *Leptospira* species and was not attempted.

In the second part of this work, as described in Chapters 3, 4, and 5, we attempted and successfully developed novel molecular diagnostic assays utilizing the abundance of RNA transcripts to create sensitive, rapid diagnostic assays for detection of Lyme disease and Leptospirosis infections. We investigated whether highly expressed mRNA or ribosomal RNA targets are viable candidates for a reverse-transcription quantitative PCR (RT-qPCR) assay. We selected a number of highly and constitutively expressed mRNA transcript targets, in addition to the abundant 16S and 23S ribosomal RNA (rRNA), to screen for diagnostic targets in an RT-qPCR format. The specificity of our diagnostic assays are evaluated against various other pathogen and host samples. The sensitivity was explored in simulated infected samples using a series of blood-spiking experiments before finally testing our technique using clinical patient samples, if available. As discussed earlier in Chapter 1, current diagnostic methods for each of these debilitating infections are lacking in sensitivity or specificity to diagnose early infection. These infections are usually easily resolved with antibiotic treatment if treatment can be started in a timely manner. Current tests have low sensitivity during early infection or give high rates of false results. Chapter 3 discusses a method based on detection of 16S rRNA

transcripts that hopes to improve diagnosis of leptospirosis. This study has been previously published in part, but further optimization and validation efforts continue in Chapter 4. Chapter 5 explores a similar assay to diagnose Lyme disease during early infection targeting a specific region of 23S rRNA transcripts. This work provides optimization of the method and proof of concept for the test. Future efforts will be pursued in order to further validate this assay using positive and negative human clinical samples.

Chapter 2: Evaluation of metabolic moonlighting proteins of *L. interrogans* and *B. burgdorferi* as virulence determinants

2.1 Introduction

As detailed in the previous chapter, the spirochetes are a group of bacteria with unique features that amongst other things allow morphological distinction based upon their size, helical corkscrew shape, and internal axial filament based motility (Karami, Sarshar et al. 2014). Although most spirochetes are free-living microbes, a few evolved to be successful pathogens. Two of these, *Leptospira* and *Borrelia*, are responsible for the serious veterinary and clinical diseases leptospirosis and Lyme disease (Bharti, Nally et al. 2003; Auwaerter, Aucott et al. 2004). We set out to examine whether certain metabolic enzymes in these bacteria possess moonlighting virulence activities during infection. Enzymes involved in sugar metabolism pathways appear to moonlight for unrelated functions; in particular, they can support virulence in many pathogens.

The term moonlighting protein refers to a protein with varying function depending on cellular localization, cell type, oligomeric state, or the cellular concentration of a ligand, substrate, cofactor, or product (Jeffery 1999). Moonlighting proteins differ from multifunctional proteins that utilize alternative splicing, post translational modifications, or contain multiple domains (Huberts and van der Klei 2010). The moonlighting functions usually occur in another cellular location than the usual protein function, such as the outer membrane instead of the

cytosol. Most moonlighting proteins identified to date have involved housekeeping genes such as receptors, chaperones, and catalytic enzymes, particularly those used for energy metabolism through glycolysis (Nogueira, Smith et al. 2012).

Moonlighting proteins have been identified in organisms ranging from eukaryotic cells, to yeast, to pathogenic fungi and bacteria (Pancholi and Fischetti 1993). Many moonlighting proteins are highly conserved metabolic enzymes or molecular chaperones possessing housekeeping functions, but also play a role in virulence or host cell invasion as either outer membrane or secreted proteins making them viable target candidates for future drug development (Huberts and van der Klei 2010). The gram-positive pathogens streptococci and staphylococci have been shown to possess most of the glycolytic pathway enzymes on the cell surface (Ling, Feldman et al. 2004). Probably the best characterized bacterial moonlighting protein is the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAP) in *Streptococcus pyogenes*. This glycolytic enzyme was initially reported to be surface exposed and later found to bind fibronectin (Pancholi and Fischetti 1992; Pancholi and Fischetti 1993), plasminogen (Winram and Lottenberg 1996), and possess self ADP-ribosylation activity characteristic of some bacterial toxins (Pancholi and Fischetti 1993). Another glycolytic enzyme, enolase, was shown to moonlight, potentially causing autoimmunity in mammals and involved in virulence of certain bacteria through binding to plasminogen (Nogueira, Smith et al. 2012; Nogueira, Backstedt et al. 2013). Additional moonlight proteins that have been characterized in other organisms include members of the heat-shock protein family, elongation factor

family, and other molecular chaperones (Ling, Feldman et al. 2004; Silveira, Piffer et al. 2013).

Both pathogens, *B. burgdorferi* and *L. interrogans*, are able to survive diverse environments and abrupt environmental changes throughout their life cycles (Anguita, Hedrick et al. 2003; Choy, Kelley et al. 2007; Matsui, Soupe et al. 2012; Corona and Schwartz 2015). This occurs through strict regulation of both metabolic processes and a large number of outer membrane proteins that are indispensable for infectivity (Anguita, Hedrick et al. 2003). Changes in metabolism and virulence have been shown to be strictly regulated and induced under specific conditions such as pH, temperature, osmolality, and etc., making identification and characterization of novel virulence factor challenging (Choy, Kelley et al. 2007). Thus, adequate targets for vaccine prevention and markers of early diagnostic utility have been difficult to define for both Lyme disease and leptospirosis. The unique metabolic needs of these two spirochete pathogens leave many questions unanswered about their metabolism. *B. burgdorferi* maintains a full set of genes necessary for glycolysis, but lacks gene homologs required for oxidative phosphorylation and the TCA cycle (Corona and Schwartz 2015). Glycolysis is the primary method for energy production through the Embden-Meyerhof pathway (Corona and Schwartz 2015). Therefore, *Borrelia*, with a preference for glucose, is capable of ATP production solely through glycolysis resulting in a low energy yield of only two ATP molecules per cycle (von Lackum and Stevenson 2005). On the other hand, leptospire are believed to lack the ability to utilize glucose as a carbon and energy source. *L. interrogans* genome contains a near complete set of glycolytic genes. However, one gene, hexokinase, which

catalyzes the initial step of glycolysis, was absent from the genome (Ren, Fu et al. 2003). Without this key enzyme, leptospire are not believed to utilize glycolysis for energy production. Instead, leptospire are believed to rely upon fatty acid metabolism for energy production through the pentose phosphate pathway (Zhang, Zhang et al. 2011). Therefore the utilization of key sugar metabolism pathways like TCA cycle and glycolysis remains uncertain in *B. burgdorferi* and *L. interrogans*, respectively. Therefore, it remains puzzling as to why these bacteria retain the genes for these metabolic pathways, In many bacteria a number of genes involved in carbon and energy metabolism have been discovered to be exposed on the outer membrane, even though their enzymatic function occurs within the cytoplasm of the cell (Pancholi and Fischetti 1992; Pancholi and Fischetti 1993; Winram and Lottenberg 1996; Ling, Feldman et al. 2004; Gao, Ye et al. 2014). In fact, some of these enzymes have adapted secondary functions acting in host invasion and colonization. For example, the glycolytic enzyme enolase is partially localized to the cell surface in *B. burgdorferi* and secreted in *L. interrogans* (Nogueira, Backstedt et al. 2013). In both of these organisms we previously showed that enolase has the potential to bind to plasminogen (Nogueira, Smith et al. 2012). Here, we investigate the potential for other enzymes involved in energy metabolism of *L. interrogans* and *B. burgdorferi* by investigating possible association with the outer membrane, particularly the bacterial surface, and any role(s) in pathogenesis beyond enzymatic functions.

2.2 Materials and methods

Selection of moonlight candidates

All genes under investigation in the current study have been shown to possess moonlighting function in other pathogens and were previously identified as targets for protective immune responses in other infectious agents (Jeffery 1999). Thus we examined whether spirochete pathogens similarly utilize moonlighting proteins during pathogenesis. The following borrelial proteins are selected for our study: BB0561 (6-phosphogluconate dehydrogenase, 6PGD), BB0055 (triose-phosphate isomerase, TPI), BB0056 (phosphoglycerate kinase, PGK), BB0057 (glyceraldehyde-3-phosphate dehydrogenase, GAP), BB0445 (fructose-bisphosphate aldolase, FBA), BB0407 (phosphomannose isomerase, PMI), BB0368 (glycerol-3-phosphate dehydrogenase, GPS), BB0348 (pyruvate kinase, PYK), BB0683 (hydroxymethylglutaryl-CoA synthase, HMG-CoA), as well as the following leptospiral proteins: LA1532 (FBA), LA1696 (PYK1), LA1703 (PGK), LA1704 (GAP), LA2924 (PYK2), LA3888 (Glucose-6-phosphate isomerase, PGI), LA3939 (GPSa), LA4206 (phosphoglycerate mutase, PGM), and LA4264 (GPSb).

Infectious isolates and hosts

Infectious isolates of *B. burgdorferi*, clone B31-A3 grown in BSK media at 34°C (Elias, Stewart et al. 2002) and *Leptospira interrogans* Fiocruz L1-130 (Ricaldi, Fouts et al. 2012) grown in liquid Elinghausen-McCullough-Johnson-Harris (EMJH) media (Ellinghausen and McCullough 1965; Noubade, Krishnamurthy et al. 2002) at

29°C on a rotating platform at 100 rpm were used throughout this study. Four-week-old female C3H/HeN mice were purchased from the National Institutes of Health and used for *Borrelia* infection experiments. The ticks used in this study were reared in the laboratory as described elsewhere (Kumar, Yang et al. 2010). Four-week-old Golden Syrian hamsters were used for *Leptospira* infection experiments (Coutinho, Matsunaga et al. 2014). All animal experiments were performed in accordance with the guidelines of the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee under approved protocols.

RT-PCR transcript analysis

Cultures of *B. burgdorferi* and *L. interrogans* were respectively grown in BSK and EMJH liquid media to late logarithmic growth phase. One milliliter containing approximately 10^8 cells was pelleted at 10,000 g for two minutes, supernatant discarded, and RNA extracted from cells by homogenization in 1 mL TRIzol reagent (Invitrogen). RNA was purified according to the manufacturer's instructions, treated with DNase I (NEB) to remove DNA contamination, and used as a template for cDNA synthesis kit with or without active reverse-transcriptase enzyme (Invitrogen SuperScript® VILO™). RT-PCR was performed using full-length gene primers, listed in Table 1, and samples containing RNA lacking reverse transcriptase enzyme were included as a control to ensure no amplification due to residual DNA contamination.

Table 1: Primers used for moonlight target RT-PCR and cloning

Name	Sequence (5'-3')
LA1696 F BamH1	CGGGGATCCATGCGTAAGACAATTATTGCAGG
LA1696 R Xho1	GGCCTCGAGTTAGAAAAGTTCAGCGAAGGAACT
LA1704 F BamH1	CGGGGATCCATGACCAGAATAGCCATCAACG
LA1704 R Xho1	GGCCTCGAGTTAACCTTTTTTCGCCATATAGC
LA2924 F BamH1	CGGGGATCCATGAAAATTCTAAACAGGAAAAAAACC
LA2924 R Xho1	GGCCTCGAGTCAATGTATTTTATAAACCTGTAAAAAGTC
LA3939 F BamH1	CGGGGATCCATGAAAATTGGAGTGATCGGAT
LA3939 R Xho1	GGCCTCGAGTCAGACTGAAACGCCCTCTC
LA1703 F BamH1	CGGGGATCCATGGAATTACCTAGACTTGAAAATGT
LA1703 R Xho1	GGCCTCGAGTTACTCGGAAGTTTTTTCTTAAGG
LA4264 F BamH1	CGGGGATCCATGGAAGAAATAAAATCATCCACAA
LA4264 R Xho1	GGCCTCGAGTTATGAGTAAGAGGATTCGTCCG
LA4206 F BamH1	CGGGGATCCATGGAACTCTATTTAATCCGACATACT
LA4206 R Xho1	GGCCTCGAGTTAAAGATTCCAGAAGATTAATTTGGA
LA3888 F BamH1	CGGGGATCCATGATTCGACTAGAAACCAGATTTCG
LA3888 R Xho1	GGCCTCGAGTTAGTTCCTTGAAATCTTTTGCG
LA1532 F BamH1	CGGGGATCCATGATTGATAAAATCAAACCGCT
LA1532 R Xho1	GGCCTCGAGTTAAGCGACCGTTACGTCCT
BB0561 F BamH1	GGGGATCCATGGATGTAGGAATTTATGGACTTG
BB0561 R Xho1	GGCCTCGAGTTATTGCCAAGCGCTATGAA
BB0055 F BamH1	GGGGATCCATGAGAAAAACATTTTTAGCGG
BB0055 R Xho1	GGCCTCGAGCTAAAGAACATTGTTAATTATAGATA
BB0056 F BamH1	GGGGATCCATGTCAATAAAAACAGTAAAAGACT
BB0056 R Xho1	GGCCTCGAG TTAATTCTCCAAAACCTTAATACCC
BB0057 F BamH1	GGGGATCCATGAAATTGGCTATTAATGGCTTT
BB0057 R Xho1	GGCCTCGAGTTATTTTACCAATTTTTGAGCAAGATC
BB0445 F BamH1	GGGGATCCATGGGTGTTTTAGATAAGATTAAACC
BB0445 R Xho1	GCCTCGAGTTAATTTCTATTAATATTATTAAGATTTTTGCATGC
BB0407 F BamH1	GGGGATCCATGAATAATGAAGATAATATTTTTT
BB0407 R Xho1	GGCCTCGAGTTAATTAACCAGCAATAAAAGCT
BB0368 F BamH1	GGGGATCCATGTTTGATTTTAATATTTTTTTAT
BB0368 R Xho1	GGCCTCGAGTTATTGTCTAACATCTCTCATATAC
BB0348 F BamH1	GGGGATCCATGATTTCAAAGTTAACAAAAATTG
BB0348 R Xho1	GGCCTCGAGTTATATATTTTCGTCCTTTGATTGCA
BB0683 F BamH1	GGGGATCCATGAGAATAGGTATTAGTGATATTA
BB0683 R Xho1	GGCCTCGAGTTAGGCTCGATACCCATAAACTC

Production of recombinant proteins and antibodies

The *B. burgdorferi* and *L. interrogans* genes of interest were amplified by PCR using specific primers listed in Table 1. Recombinant proteins were produced in *Escherichia coli* using the bacterial expression vector pGEX6P-1 (GE Healthcare),

and protein expression was induced by the addition of IPTG (isopropyl- β -D-thiogalactopyranoside) to a 1 mM final concentration. The GST-fused proteins were affinity purified by using Glutathione Sepharose 4B (GE Healthcare). Polyclonal antibodies were produced in mice against each recombinant protein and immunoblotting assays were performed as described earlier (Coleman, Yang et al. 2008; Nogueira, Smith et al. 2012).

Triton X-114 phase partitioning

Triton X-114 (TX-114) phase-partitioning assays were performed as previously described (Promnares, Kumar et al. 2009; Nogueira, Backstedt et al. 2013). Briefly, 10^9 spirochetes were centrifuged, washed thrice with PBS and finally re-suspended in PBS (pH 7.4). Samples were incubated overnight at 4°C with agitation in 2% (vol/vol) TX-114 (*Borrelia*), or 1% (vol/vol) TX-114 (*Leptospira*). After incubation, phase-partitioned lysates were centrifuged at 13,000 g for 15 minutes at 4°C to remove insoluble cell debris. The supernatant was removed and placed at 37°C to allow for phase separation. The resulting aqueous and detergent-enriched phases were removed. Samples were again incubated in 2% or 1% TX-114 two more times to wash contaminants. Respective aqueous and detergents fractions were mixed with 10 volumes of ice-cold acetone, followed by centrifugation at 13,000 g for 15 minutes to precipitate proteins. Protein pellets were finally re-suspended in PBS and subjected to SDS-PAGE and immunoblot analysis with antibody against indicated specific antibodies or control antibodies.

Surface proteolysis assay

Proteinase K (PK) accessibility assays were performed as described previously (Brooks, Vuppala et al. 2006; Pinne and Haake 2009). Briefly, *L. interrogans* or *B. burgdorferi* (2×10^8) was washed three times in 1 ml of PBS (pH 7.4) and collected by centrifugation at 4,000 g for four minutes. The cell pellet was re-suspended in 1 ml of PBS and split into two equal 500- μ l volumes. One aliquot received 100 μ g of PK (Sigma), while the other aliquot received an equal volume of PBS without PK. Both aliquots were incubated for 15 minutes at room temperature (*Borrelia*) or 1 hour at 37°C (*Leptospira*) before the addition of 10 μ l of phenylmethylsulfonyl fluoride, PMSF, (Sigma) to stop PK activity. Spirochete suspensions were subsequently pelleted by centrifugation at 10,000 g for 10 minutes and washed three times in PBS before final re-suspension in PBS for immunoblot analysis with specific target and control antibodies.

Assessment of *Leptospira* outer membrane proteins

L. interrogans and *B. burgdorferi* outer membrane (OM) and protoplasmic cylinder (PC) fractions were isolated as described previously (Yang, Promnares et al. 2011; Nogueira, Backstedt et al. 2013) with minor modifications. Five $\times 10^{10} - 10^{11}$ cells were harvested by centrifugation and the pellets were washed twice with phosphate buffered saline pH 7.4 (PBS) supplemented with 0.1% bovine serum albumin (BSA). *L. interrogans* were finally resuspended in 20 mL of ice cold membrane isolation buffer (20 mM Tris-HCl, pH 9.0, 1 M NaCl, 2 mM EDTA) containing 27% sucrose and stirred with a magnetic bar at room temperature for two

hours, after which the sucrose concentration was diluted to 13% by addition of the resuspension buffer and pelleted. *B. burgdorferi* cells were re-suspended in ice-cold 25 mM citrate buffer (pH 3.2) containing 0.1% BSA and subsequently incubated on a rocker at room temperature for two hours and subsequently pelleted. The respective supernatants were collected and further centrifuged at 141,000 g for two hours. The resulting pellets were resuspended in 6 mL buffer, layered onto a discontinuous sucrose gradient (56%, 42%, 26%), and centrifuged at 100,000 g for 16 h at 4°C. The OM and PC fractions were removed by needle aspiration, diluted five to seven fold in cold buffer, and centrifuged at 141,000 g for 4 hours at 4°C. The resulting OM pellet was resuspended in 1 mL buffer, applied to 12 mL of a continuous 10-42% (wt/wt) sucrose gradient, and centrifuged at 100,000 g for 16 hours at 4°C. Finally, the OM pellet was removed by needle aspiration, diluted five to seven fold in cold PBS, centrifuged at 141,000 g for 4 hours at 4°C, and re-suspended in 50-100 µL PBS containing 1 mM PMSF. Equivalent amounts of whole-cell lysate, OM, and PC were separated by SDS-PAGE and immunoblotted with specific antibodies. The known OM protein LipL32, and known inner membrane protein LipL31 were used as controls for *L. interrogans* and the known OM protein OspA, and known inner membrane protein FlaB were used as controls for *B. burgdorferi*.

Host immunoreactivity

Promising *Leptospira* moonlight protein candidates were tested for immunoreactivity against serum from known infected and naïve hosts. Pooled serum samples from 10 clinical patients (provided by our collaborator Dr. Albert Ko at Yale

University) collected during acute phase infection were tested for sero-reactivity against purified recombinant protein ENO, FBA, PGK, and GAP by immunoblotting. Recombinant proteins (0.5 μ g each) were separated by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. After blocking membranes in 5% blocking milk, pooled human serum samples were incubated with membranes at 1:100 dilution for one hour at room temperature. Membranes were washed thrice with PBS-T for 15 minutes each, followed by incubation with HRP-conjugated goat anti-human IgG secondary antibodies for one hour at room temperature. Membranes were again washed thrice with PBS-T for 15 minutes, each wash and developed with SuperSignal West Pico Chemiluminescent Substrate by autoradiography (Kariu, Yang et al. 2013).

RT-qPCR assay

RT-qPCR analysis was performed as previously described (Yang, Coleman et al. 2009). RNA samples were extracted from murine or hamster tissues by grinding under liquid nitrogen and dissolved with TRIzol reagent (Invitrogen), treated with DNase I (Invitrogen), and finally purified by RNA precipitation per manufacturer instructions. RNA was used as a template for cDNA synthesis using SuperScript® VILO™ mastermix (Invitrogen). RT-qPCR analysis was performed using FastStart Universal SYBR Green Master (Roche). For quantitative analysis of gene expression in *Leptospira* the target transcripts were normalized to the number of *flaB* transcripts. For quantitative measurement of *L. interrogans* burden in infected tissues, *16S* transcripts were normalized to hamster β -actin levels. For quantitative analysis of

gene expression in *Borrelia*, the target transcripts were normalized to the number of *flaB* transcripts (Promnares, Kumar et al. 2009). For quantitative measurement of *B. burgdorferi* burden in infected tissues, *flaB* transcripts were normalized to mouse or tick β -actin levels. All RT-qPCR results were checked for specificity by melt curve analysis. Primers used are listed in Table 2.

Table 2: Primers used for detection of bacteria burden by RT-qPCR

Primer	Forward (5' - 3')	Reverse. (5' - 3')
mus β -actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT
tick β -actin	GGTATCGTGCTCGACTC	ATCAGGTAGTCGGTCAGG
<i>Borrelia flaB</i>	TTCAATCAGGTAACGGCACA	GACGCTTGAGACCCTGAAAG
<i>Leptospira 16S</i>	AGCAGCAATGTGATGATGGTAC	ACTTACATGTCCGCCTACGC
hamster β -actin	GCTCTTTTCCAGCCTTCCTT	GCCCTATACCAACCGCATCA

Active immunization and infection studies

For *Leptospira* infection, groups of hamsters (three animals/group) were immunized with Alhydrogel 2% adjuvant containing equimolar mixtures of fructose-bisphosphate aldolase (FBA), phosphoglycerate kinase (PGK), and glyceraldehyde 3-phosphate dehydrogenase (GAP), or PBS containing the same volume of adjuvant and given two boosters 14 days apart. Two weeks after the final boost, animals were infected with 1000 leptospire per animal by intra-peritoneal injection. As *Leptospira* produces a sudden lethal infection in the hamster model, animals were monitored for signs of illness by activity, mood, and weight loss. Animals were euthanized upon the first symptoms of illness or weight loss of greater than 10%. Blood was collected, as well as liver, lung, kidney tissues after perfusion, and samples were frozen in liquid nitrogen. RNA was isolated from infected tissues and *L. interrogans* burden was measured using RT-qPCR.

For *Borrelia* infection, groups of mice (five animals/group) were immunized with Freund's adjuvant containing either recombinant PMI, or PBS containing the same volume of adjuvant and given two boosters 10 days apart. One week after the final boost, infected nymphal *I. scapularis* ticks (five ticks per mouse) were fed on uninfected, immunized mice. The mice were sacrificed 10 days after repletion of feeding ticks. Heart, joint, and skin samples were collected and frozen in liquid nitrogen. RNA was isolated from infected tissues and *B. burgdorferi* burden was measured using RT-qPCR (Nogueira, Backstedt et al. 2013; Kariu, Sharma et al. 2015). To verify whether PMI was involved in tick acquisition of *B. burgdorferi*, separate groups of mice (two animals/group) were immunized with recombinant PMI or PBS (using equal volume of adjuvants), as described above, and 10 days after the last boost, the mice were infected by a single intraperitoneal injection with *B. burgdorferi* (10^5 spirochetes/mouse). After 14 days, sterile *I. scapularis* nymphs (15 ticks/mouse) were allowed to engorge on the mice. Partially fed or fully engorged ticks were collected from mice after 60 hours or post feeding and assessed for pathogen burden using RT-qPCR, as described above. Animal infection studies, including immunization and *B. burgdorferi* challenge studies were independently repeated at least twice.

***In vitro* bactericidal assay**

In vitro bactericidal assay was performed as described previously (Yang, Lenhart et al. 2010). Normal mice serum or serum samples from mice immunized with PMI were used for the bactericidal assays. At 24 hours after the addition of

antiserum (undiluted sera without complement inactivation), 1 μ l of medium containing spirochetes was added to 1 ml of fresh BSK-H medium to assess the spirochetes' ability to regrow in the culture. Spirochetes were incubated at 33°C and enumerated by dark-field microscopy at 24, 48, 60, 72, and 96 h, as described previously (Pal, Montgomery et al. 2001).

Measurement of the phosphomannose isomerase (PMI) activity

PMI activity was determined by measuring the conversion of NADH·H⁺ to NAD⁺, as described previously (Gracy and Noltmann 1968). Briefly, the enzymatic reactions were performed at 25°C in 87 mM triethanolamine HCl buffer pH 7.6 (Sigma) containing 5.5 mM D-mannose 6-phosphate (Sigma), 0.45 mM nicotinamide adenine dinucleotide phosphate (Sigma), 1.33 units phosphoglucose isomerase, PGI (Sigma), 0.0667 units Glucose-6-phosphate dehydrogenase, G6PDH (Sigma), and approximately 1 ng of recombinant *B. burgdorferi* PMI (measured by Biorad Protein Assay) or 0.001 units of PMI control purchased from Sigma. The PMI activity was measured by the increase in absorbance at 430 nm (i.e. increase in the production of NAD⁺ from NADH through the coupled reaction including PGI and G6PDH).

The PMI activity of intact *B. burgdorferi* cells was measured by a direct assay in microtiter format (Gracy and Noltmann 1968). Briefly, 2×10^8 *B. burgdorferi* cells were washed three times with the reaction buffer (100 mM triethanolamine HCl buffer, pH 7.6), centrifuged, and finally suspended in a volume of 400 μ l. Two-fold serial dilution of the cells were mixed with equal volume of the reaction buffer in presence or absence of 5.5 mM mannose-6-phosphate, M6P (Sigma), followed by

incubation at 37°C for 10 min. The bacteria were removed by centrifugation (10,000 rpm for two minutes) after incubation, and the supernatants were measured for the conversion of M6P to fructose-6-phosphate via coupled enzymatic reaction to measure NADP. As a control to verify that enzymatic activity was not due to presence of PMI released into the supernatant from cell lysis, after incubation of live intact cells in the absence of substrate, M6P was added during the coupled enzymatic assay. Additionally, to ensure that the cell permeability is not compromised during the assay, the viability of the *B. burgdorferi* cells was determined before and after the enzymatic assay by using vital fluorescence labeling (Live/Dead BacLight viability kit; Invitrogen), as detailed previously (Yang, Coleman et al. 2009)

Bioinformatics and statistical analysis

The results were presented as means \pm the standard errors of the mean (SEM). Statistical comparisons were performed by using unpaired Student t test. Statistical significance was accepted for $P < 0.05$.

2.3 Results

Moonlighting proteins in *L. interrogans*

Conservation of selected moonlight candidates between related species

We investigated the sequences of each *Leptospira* moonlight candidate, including previously published enolase, ENO, by analyzing the similarity between diverse *Leptospira* species, including numerous pathogenic strains, non-pathogenic strains, and unrelated mammalian host species (Nogueira, Backstedt et al. 2013).

Most of these candidates had nearly 50% similarity in gene sequences (Table 3). One target, FBA, showed no significant similarity to mammalian gene homologs. Three other candidates—PGI, GPS, and PGM—had low levels of similarity (less than 30%) to mammalian and even *E. coli* gene homologs. Also of note, *L. interrogans* genome maintains two gene copies annotated as glycerol-3-phosphate dehydrogenase, LA3939 (GPSa) and LA4264 (GPSb). However, these two genes share no similarity with each other and differ drastically in size: GPSa contains 1008 base pairs while GPSb has 2430 bp. Most candidates indicated a high degree of similarity between *Leptospira* strains. This would be important for any potential vaccine candidate in order to provide cross-protection between different species and serovars of pathogenic leptospires.

Table 3: Similarity of selected moonlight candidate gene homologs between different *Leptospira* species and mammalian hosts

Species	% identity to FBA	% identity to TPI	% identity to PGK	% identity to GAP	% identity to ENO	% identity to PYK	% identity to PGI	% identity to GPS	% identity to PGM
<i>Leptospira interrogans</i> serovar Copenhageni L1-130	100	100	100	100	100	100	100	100	100
<i>Leptospira interrogans</i> serovar Lai	100	99	99	100	100	100	100	99	99
<i>Leptospira interrogans</i> serovar Icterohaemorrhagiae	100	64	99	90	99	N/A	N/A	29	99
<i>Leptospira interrogans</i> serovar Grippityphosa	99	95	43	99	99	N/A	99	98	100
<i>Leptospira interrogans</i> serovar Pomona	99	99	99	99	N/A	N/A	N/A	99	99
<i>Leptospira interrogans</i> serovar Bataviae	98	97	99	N/A	N/A	97	88	98	84
<i>Leptospira borpetersenii</i>	97	82	93	96	96	94	84	96	64
<i>Leptospira kirschneri</i>	97	97	96	99	99	98	95	99	88
<i>Leptospira santarosai</i>	97	82	91	96	95	94	85	95	87
<i>Leptospira licerasiae</i>	88		79	94	89	74	61	77	33
<i>Leptospira biflexa</i>	60	62	72	80	79	34, 74	54	74, 31	38
<i>E. coli</i>	70	43	45	65	62	42	24	43	27
<i>Homo sapiens</i>	*	40	44	59	53	41	25	29	23
<i>Mus musculus</i>	*	39	45	61	52	40	25	29	23
<i>Rattus norvegicus</i>	*	42	44	61	53	40	25	30	23
<i>Mesocricetus auratus</i>	*	40	44	58	53	40	25	31	23

RT-PCR detection of *Leptospira* moonlight candidate gene transcripts

As discussed previously, leptospire are not believed to use glycolysis for energy metabolism, instead relying upon fatty acid metabolism. However, the genome of *L. interrogans* still contains an almost complete set of genes for this pathway (Ren, Fu et al. 2003). To verify that these genes are functionally expressed in leptospire, we performed RT-PCR from *L. interrogans* culture cDNA using gene specific primers. Amplification was observed for all targets showing that all genes are expressed in the respective pathogen (Fig. 1). Appropriate controls confirm that target amplification is not due to genomic DNA contamination of samples.

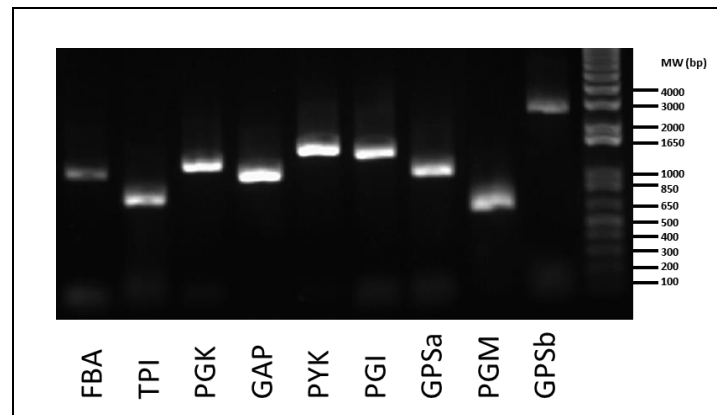


Figure 1: RT-PCR analysis of *Leptospira* moonlight candidates. Transcript expression of all selected gene targets was confirmed by full length gene primers. Controls for DNA amplification showed no amplification.

Protein localization studies

Recombinant protein and polyclonal murine antibodies were generated for each protein as described in the Materials and Methods. The production of antibodies was verified by Western blot using recombinant protein or *L. interrogans* cell lysates and sera from immunized mice to ensure specificity. We were unable to detect the

presence of all proteins in whole cell lysates. However, we were able to detect the presence of PGK and GAP in *L. interrogans* serovar Copenhageni strain Fiocruz F1-130 and FBA in both *L. interrogans* serovars Lai and Copenhageni (Fig. 2A). As moonlighting proteins sometimes associate with the cell membrane or outer surface despite the absences of any recognizable signal peptide, membrane anchors, or transmembrane domains, we analyzed *L. interrogans* for the presence of these enzymes within the membrane. We analyzed aqueous (soluble) and detergent (membrane-enriched) phase proteins separated using TX-114 by Western blotting. Control antibodies detected LipL32 in the detergent phase and OmpL47 in the aqueous phase as previously reported. All three moonlight targets appeared to be present only in aqueous fractions (Fig. 2B). However, this alone is not adequate proof as the control protein OmpL47 is known to be present in the aqueous phase, despite being a surface exposed outer membrane protein. We then analyzed mechanically separated outer membrane vesicles (OM) and protoplasmic cylinder (PC) fractions for the presence of these three enzymes. Antibodies against known inner membrane protein, LipL31, and outer membrane protein, LipL32, served as controls for OM purity. LipL31 was only present in PC fractions, while LipL32 was present in both OM and PC fractions. FBA was observed in PC fraction alone (Fig. 2C). A very faint band might be present for PGK and GAP in OM fractions, although it appeared unclear (Fig. 2C).

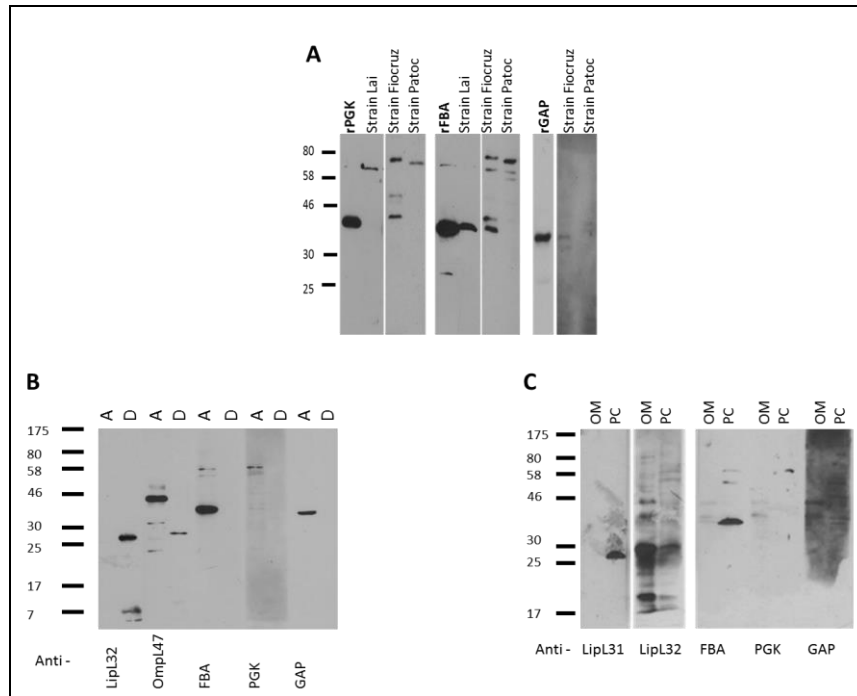


Figure 2: Expression and localization of moonlight candidates in *L. interrogans*. A) Specific antibodies developed against recombinant proteins give robust detection of recombinant protein and detection of indicated target in whole cell lysates. B) Aqueous and detergent phase fraction indicate FBA, PGK, and GAP are not seen in detergent phase versus controls. C) Detection of moonlight targets in highly pure mechanically separated OM and PC fraction.

Moonlight target gene expression during *in vivo* infection

As the exact localization of these proteins on the pathogen surface remained uncertain, we explored whether expression of these genes are regulated during mammalian infection. Two groups of week-old Golden Syrian hamsters were infected with 1000 leptospire. Animals were euthanized at day five and day seven post infection and perfused with PBS before tissue samples were collected. cDNA from infected tissues was analyzed for *Leptospira* gene expression *in vivo*. We were able to detect transcripts of FBA, PGK, GAP, and ENO in infected hamster liver and kidney tissues at day five (Fig. 3A) and day seven post infection (Fig. 3B) with almost identical profiles. Approximately 10-fold higher expression of FBA gene

expression was observed in liver tissues as compared to kidney tissues. Lastly, pooled sera from acute phase human clinical patients was tested for seroreactivity against recombinant protein. We were able to detect the presence of antibodies against FBA, PGK, GAP, and ENO leptospiral proteins in the sera of acutely infected patients, but not normal human sera (Fig. 3C).

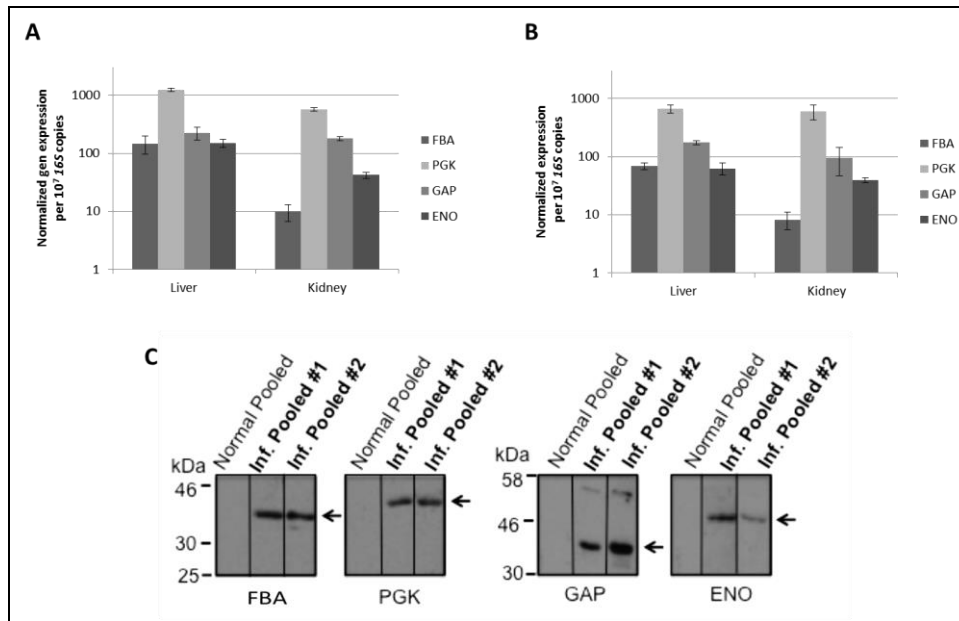


Figure 3: ALD, PGK, GAP, and ENO are expressed during mammalian infection. *In vivo* expression of genes was confirmed during infected hamster tissues. A) Normalized expression of moonlight gene transcripts from infected hamster tissues five days post infection. B) Normalized expression of moonlight gene transcripts from infected hamster tissues seven days post infection. C) Seroreactivity of recombinant protein against pooled human sera from acute phase infected patients.

Immunization does not protect against hamster infection

The development of antibodies in acutely infected patient sera indicates exposure of these proteins to immune mechanisms during infection. This might imply that these proteins play a role during early host infection. We examined whether immunization of hamsters with recombinant FBA, PGK, and GAP, which

has unique divergence in *L. interrogans*, might offer protection against infection in a trivalent vaccination strategy. To accomplish this, three hamsters for each group were immunized with 200 μ L containing either PBS-Alum mixture, or 40 μ g of each of the three recombinant proteins (120 μ g total protein per animal) in PBS-alum. It was necessary to assess whether our proteins were adsorbed to alum particles in order to properly provide immune stimulation for antibody development. Samples were taken from the protein solution mixture to be used for immunization before and after binding to alum for Western blot analysis with specific antibodies. All three antigens are clearly present in the solution before alum binding, but not after, indicating successful adsorption to alum particles (Fig. 4A). After final immunization, we recorded varied antibody responses between immunized animals. All three vaccine-immunized animals developed antibodies to GAP, while only two produced antibodies to FBA (Fig. 4B). Only one animal showed antibody development against PGK. We believe the use of outbred animal populations in this model as well as poor immunogenic stimulus provided by alum adjuvant reduced the production of robust antibody development against all antigens. Animals were infected after verification of antibody responses with 1000 leptospire. As *L. interrogans* causes a lethal illness in hamsters, we euthanized animals on day seven post infection to analyze burden of bacteria in different animal tissues. After euthanasia and blood collection, animals were perfused with sterile PBS so that bacteria within the blood would not affect the results of detection within other tissues. Levels of bacteria in each tissue analyzed are graphed for individual animals because significant variations in bacterial burden were seen between different animals, even within the same group (Fig. 4C-F). This was

somewhat expected, due to the nature of *Leptospira* infection and the use of outbred hamster populations. Variations of up to 100-fold are commonly reported between bacteria levels in different animals (Coutinho, Matsunaga et al. 2014). However, as we were able to detect infection in all tissues of all animals, these antigens do not appear to provide protection from infection and are not considered as vaccine candidates.

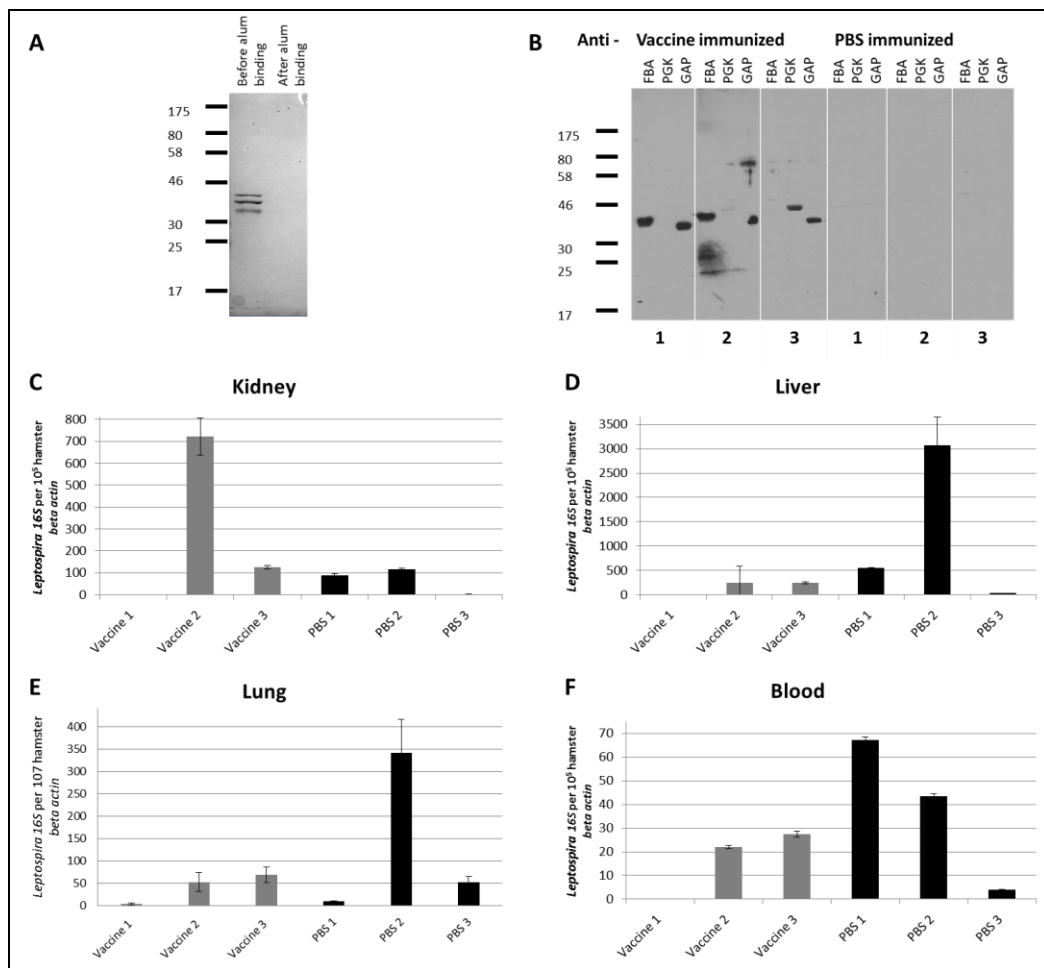


Figure 4: Immunization with FBA, PGK, and GAP fails to protect against *Leptospira* infection. A) Vaccine candidates show complete antigen adsorption to aluminum hydroxide gel. B) Immunized hamsters develop antibodies against selected antigens. C-F) Burden of bacteria in different tissues was analyzed and graphed for individual animals as indicated.

Moonlighting proteins in *B. burgdorferi*

RT-PCR detection of *Borrelia* moonlight candidate gene transcripts

Due to the complex life cycle, *B. burgdorferi* maintains strict control over gene transcript levels under different conditions and hosts. Under different stages of the life cycle, the bacteria has limited resources for energy acquisition and additionally lacks many genes necessary to utilize oxidative phosphorylation or the TCA cycle (Corona and Schwartz 2015). Previous studies indicate that glucose serves as the primary energy source during mammalian infection, while glycerol fulfills that role during the tick phases of the life cycle (Pappas, Iyer et al. 2011). Both of these sugars as well as mannose, N-acetylglucosamine, maltose, and chitobiose are capable of serving the energy requirement of the bacteria (von Lackum and Stevenson 2005). Many enzymes involved in these metabolic reactions serve additional functions for virulence in other pathogens. One glycolytic enzyme, enolase, has already been shown by our laboratory and others as a moonlighting protein with the ability to bind the host factor plasminogen (Nogueira, Smith et al. 2012). We sought to expound on our work with enolase to explore whether other sugar metabolizing enzymes moonlight during *Borrelia* infection. NCBI-BLAST searches revealed *B. burgdorferi* homologues of previously-published moonlighting proteins from other pathogens, specifically those involved in sugar metabolism, and expression of these genes during in vitro growth of *B. burgdorferi*, was verified by RT-PCR (Fig. 5).

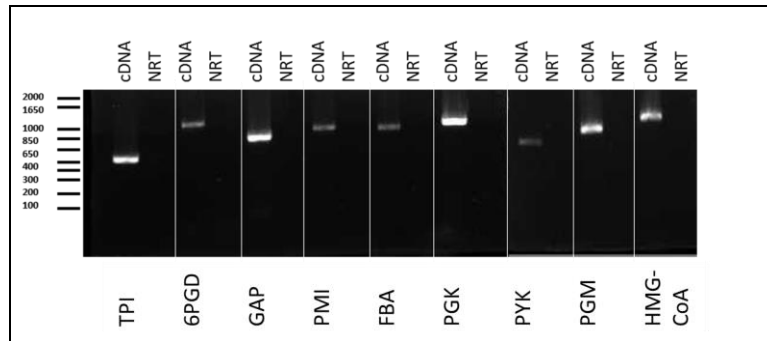


Figure 5: RT-PCR analysis of *Borrelia moonlight* candidates. Transcript expression of all selected gene targets was confirmed by full length gene primers using *B. burgdorferi* cDNA. Controls for DNA amplification (NRT) showed no amplification.

Recombinant protein and antibody production

Moonlighting proteins often associate with cell membranes or outer surface despite lacking any recognizable signal peptide, membrane anchors, or transmembrane domains (Winram and Lottenberg 1996). Therefore, we analyzed *B. burgdorferi* for the presence of these enzymes on the outer surface. Recombinant protein and polyclonal murine antibodies were generated for each protein as described in the Materials and Methods. Recombinant protein was expressed and purified from *E. coli* DH5 α cells. The presence and purity of each protein was confirmed by SDS-PAGE before being used to immunize C3H/HeJ mice 10 micrograms per mouse in Freund's adjuvant. The production of antibodies was verified by Western blot using recombinant protein or *B. burgdorferi* cell lysates and sera from immunized mice to ensure specificity.

Membrane association and surface exposure of PMI

Cellular fractionation techniques were used to detect any association of enzyme targets with the outer membrane or bacterial surface. A previously

established protocol allows for separation of the *B. burgdorferi* outer membranes (OM) while leaving the remaining protoplasmic cylinder (PC) of the cell intact (Yang, Promnares et al. 2011). This allows detection of proteins associated with the OM, whether sub-surface or surface-exposed, without intracellular protein contamination. The *B. burgdorferi* OM and PC cell fraction were separated by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. The results indicated that only one enzyme, phosphomannose isomerase, is present in the OM fractions of *B. burgdorferi* (Fig. 6A). Other targets are either not present, or present at levels below detection in the OM fraction.

We next used the phase partitioning by a hydrophobic detergent TX-114 to separate *B. burgdorferi* cells into soluble aqueous (A) or membrane associated detergent (D) fractions and assessed the localization of each protein by using immunoblotting with each specific antisera. In agreement with the OM and PC cellular fractionation, the TX-114 showed that phosphomannose isomerase partitioned into both soluble and membrane-associated fractions (Fig. 6B), but all other protein targets were only detected in the soluble fraction. As expected, a control membrane lipoprotein, OspA, was predominantly localized into the detergent fraction.

We further assessed whether each protein is exposed on the microbial surface using a more direct assay. To achieve this, intact spirochetes were subjected to surface proteolysis by a controlled PK digestion and then assessed by immunoblot analysis using antisera against each enzyme target or known surface (OspA) or subsurface (FlaB) antigens. The results indicated that PK digestion slightly reduced

the amount of phosphomannose isomerase (Fig. 6C). Taken together, these data suggest that PMI is in part membrane associated and surface expressed.

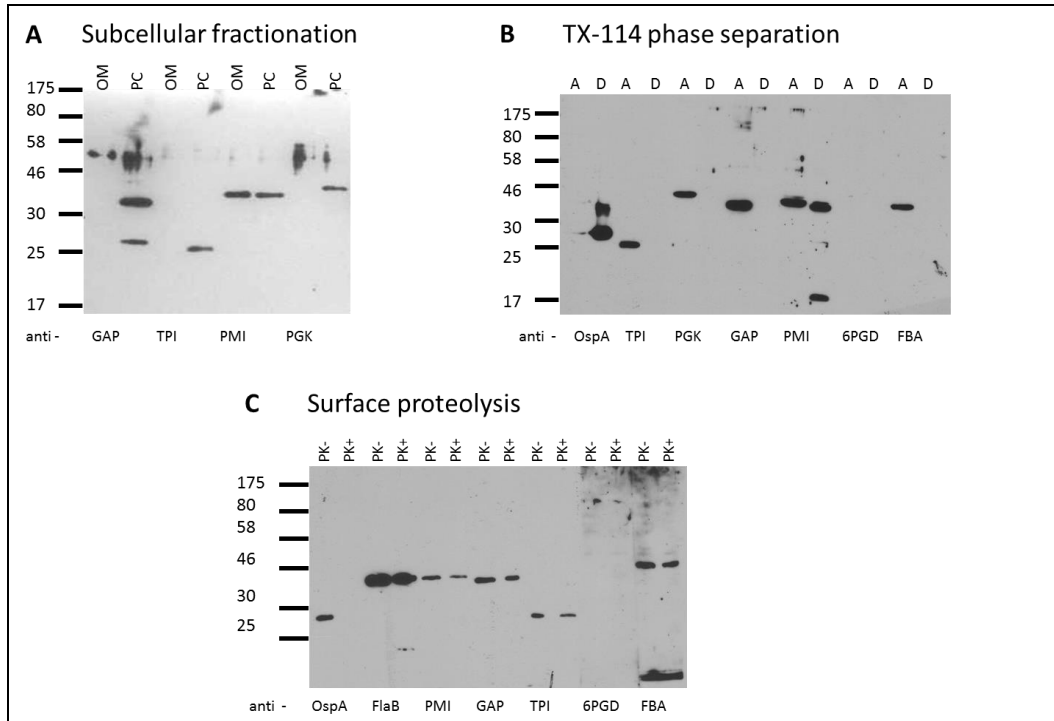


Figure 6: PMI is present on *Borrelia* membrane. A) Outer membrane and protoplasmic cylinder fractions were tested by Western blotting for the presence of moonlighting enzymes. PMI alone was found jointly in OM and PC fractions. B) Aqueous and detergent fractions showed PMI, but other candidates were only detected in aqueous phase. C) Surface proteolysis by partial Proteinase K digestion indicated a reduction in the level of detectable PMI using specific antibodies. OspA and FlaB served as intracellular and extracellular controls.

Immunization of mice with recombinant PMI failed to protect mice from needle injection or prevent acquisition of bacteria by ticks

Since phosphomannose isomerase could be detected on the surface, we examined whether immunization with recombinant PMI had any influence on infection. PMI or PBS immunized mice were intraperitoneal inoculated with *B. burgdorferi* (10^5 spirochetes/mouse). Two weeks after infection, naïve ticks were fed

on the infected mice. Ticks were collected after 60 hours of feeding and after full engorgement for analysis of *Borrelia* levels by RT-qPCR. At 21 days post needle infection, the same mice were sacrificed and tissues collected to analyze the burden in mice tissues after needle injection. No statistically significant change was observed between ticks that fed on PMI or PBS immunized groups (Fig. 7A-B). Additionally, no statistically significant difference was observed in burden of bacteria between PMI and PBS immunized, needle-infected mice (Fig. 7C-F).

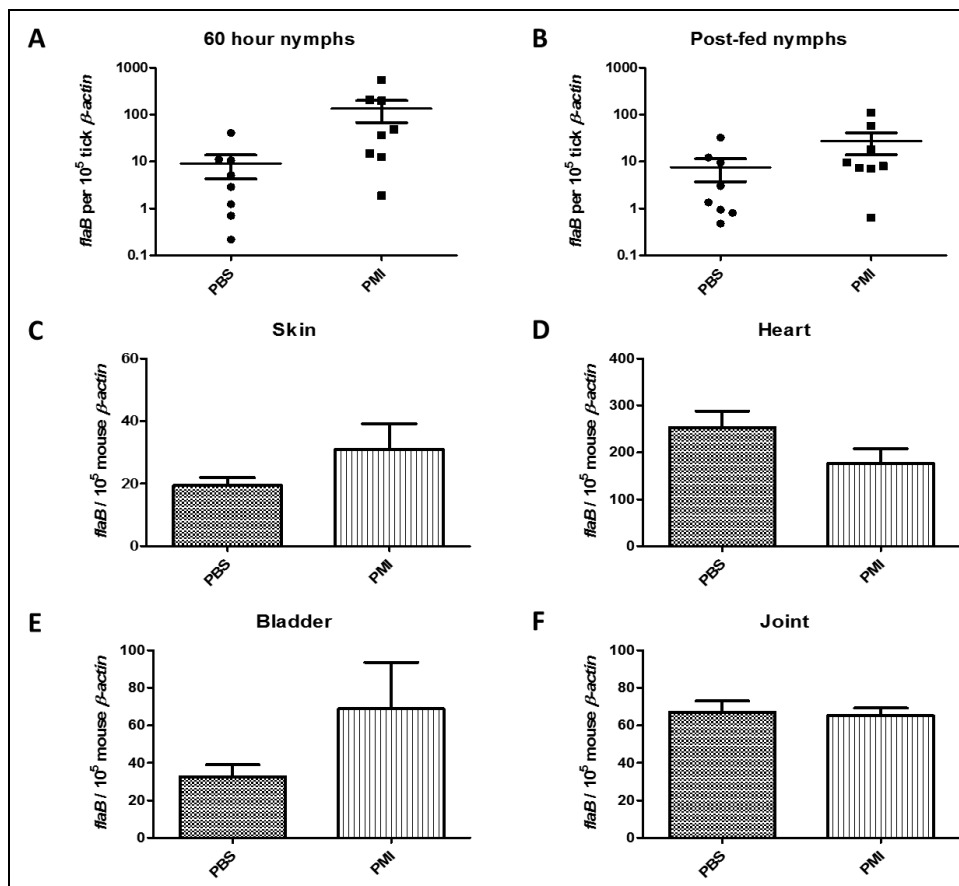


Figure 7: PMI immunization has no effect on *Borrelia* levels in feeding ticks or pathogen burden in needle infected mice. A and B) PBS or PMI immunized mice were infected by needle. Burden of bacteria during tick acquisition was analyzed by RT-qPCR. Antibodies against PMI do not affect the ability of naïve ticks to acquire *Borrelia*. C-F) Mice immunized with PBS or PMI showed no differences in the burden of bacteria from different tissues at day 21 post infection.

Immunization with recombinant PMI caused reduced burden in a selected organ (joints) via tick transmission

To assess whether PMI immunization interferes with spirochete transmission via tick challenge, C3H mice (five animals/group) were immunized with either purified recombinant PMI or equal volume of PBS in Freund's adjuvant with two additional boosts. Ten days after final immunization, sera was collected for ELISA and *Borrelia* infected ticks were fed on both groups of mice. Sera was collected prior to infection by tick feeding and tested for antibody response using Western blotting and ELISA. The ELISA titer of PMI immunized animals was greater than 1:20,000 for all animals (Fig. 8A). All animals developed infection after tick feeding as verified by immunoblotting using serum from 14 day infected mice against *Borrelia* whole cell lysate (Fig. 8B). Additionally, the burden of bacteria in PMI immunized mice was analyzed. Immunization of mice with recombinant PMI reduced *B. burgdorferi* levels within joint tissues of animals by a statistically significant amount, while decreased yet statistically non-significant differences in pathogen burden were recorded in skin, heart, or bladder tissues as compared to PBS immunized animals (Fig. 8C-F). Polyclonal murine antibodies from PMI immunized mice were then tested for borreliacidal activity using OspA antibody as a bactericidal positive control and normal mouse serum as a negative control. Sera from PMI immunized mice were not capable of killing *B. burgdorferi* (data not shown).

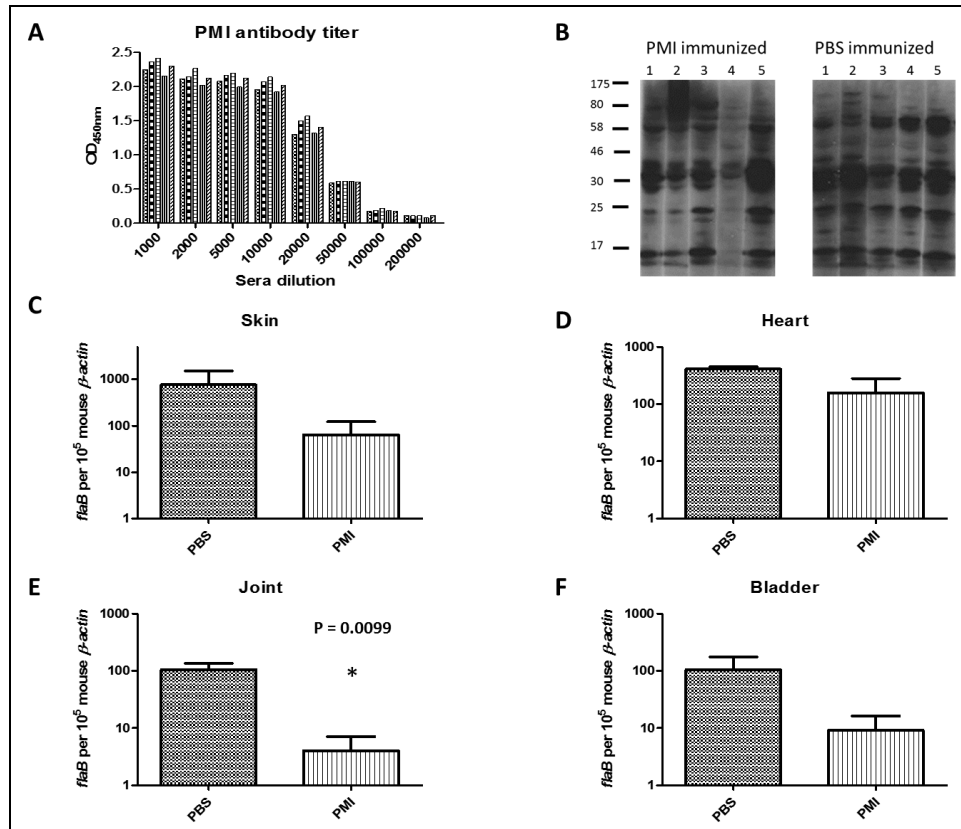


Figure 8: PMI immunization does not protect from infection but reduces burden specifically within joints. A) Mice were immunized against recombinant PMI using Freund's adjuvant and antibody titers were tested by ELISA prior to infection. B) By 14 days post infection, all mice seroconverted against *Borrelia*, indicating all mice developed infection. C-F) Burden of bacteria in mouse tissues 14 days post infection was analyzed by RT-qPCR. Mice immunized with PMI showed reduction of *Borrelia* specifically within the joint.

Enzymatic activity of recombinant and surface-localized native PMI

Finally, we assessed the predicted enzymatic function of recombinant PMI or the native surface localized protein on intact *B. burgdorferi* grown *in vitro*. PMI activity was measured as detailed above to assess the conversion of mannose-6-phosphate using a PGI and GPS coupled enzymatic reaction to assess the production of NADH from β -NAD. Results showed that enzymatic activity of recombinant *B. burgdorferi* PMI became saturated over time, as did a control recombinant PMI enzyme of known activity from *S. cerevisiae* (Fig. 9A). To determine whether the

native PMI is enzymatically active on spirochete surface we incubated live, intact bacteria for 10 minutes in reaction buffer with or without mannose-6-phosphate substrate, but lacking additional enzymes necessary for the coupled reaction to produce a measureable increase in NADH. The results indicated detectable PMI activity in *B. burgdorferi* cells but no activity in samples incubated without substrate, to ensure activity was not due to residual sugars from the growth media (Fig. 9B). An additional set of samples were incubated without substrate, cells subsequently removed by centrifugation, and then substrate added to show that enzymatic activity was not due to cell lysis (lysis control). Lastly, as a secondary control, a Live/Dead bactericidal assay was performed to test the cells were still viable, not lysed, and of similar cell densities (Fig. 9C).

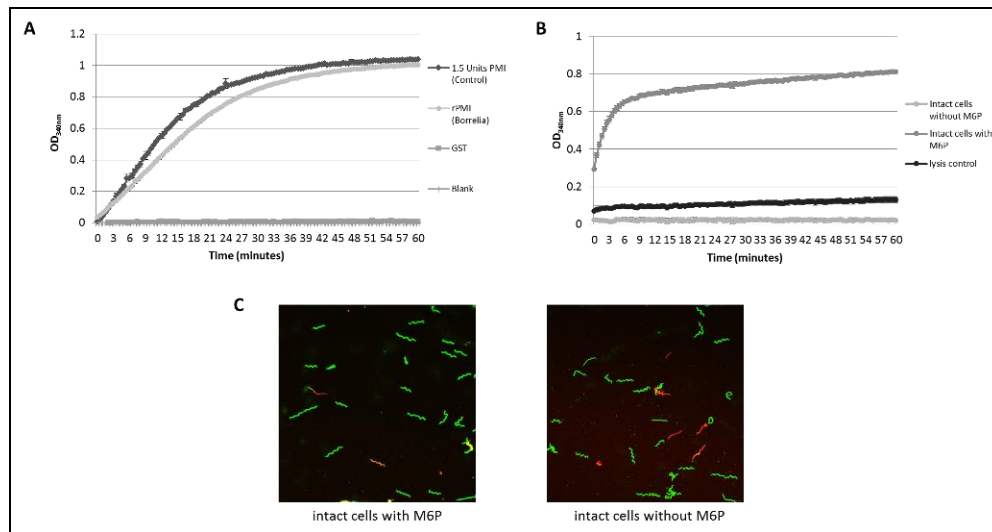


Figure 9: PMI enzymatic activity can be observed on the surface of *Borrelia*. A) Recombinant PMI and controls were tested for enzymatic activity. *Borrelia* PMI retains ability to convert mannose-6-phosphate molecules. B) Viable, intact *Borrelia* cells were tested for PMI activity with and without M6P substrate. Enzymatic activity on the surface is confirmed to be specific to M6P substrate. A control verified enzymatic activity was not due to lysis of cells. C) Viability of spirochetes after the surface enzymatic assay was examined using Live/Dead assay, further indicating that PMI activity did not come from enzyme released by cell lysis.

Transcript profile of PMI during *Borrelia* life cycle

As whether PMI expression is critical for *B. burgdorferi* survival or it has a secondary moonlighting function on the surface remain unknown, we sought to analyze the gene expression profile throughout the *Borrelia* life cycle in tick and mammal hosts. The *pmi* gene transcripts were detected in both tick and mammalian phases but with slightly higher expression seen within the tick (Fig. 10A). A plasmid construct for was cloned for targeted knockout mutagenesis of PMI in *B. burgdorferi* as indicated (Fig. 10B). Numerous attempts were performed to create a genetic mutant clone lacking *pmi* gene from *Borrelia* genome were unsuccessful. Additionally, we found that in a *Borrelia* transposon mutant library no deletion mutant was available for *pmi* while upstream and downstream genes showed viable clones (Lin, Troy et al. 2014). These observations suggest that the attempted deletion of the *pmi* gene causes a lethal mutation for *B. burgdorferi*.

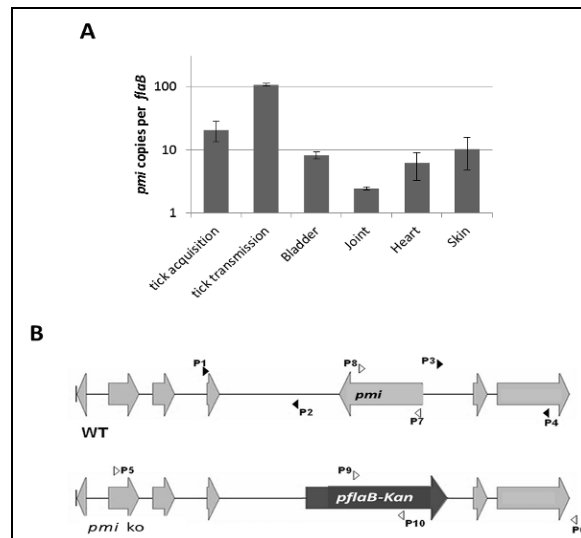


Figure 10: *pmi* gene expression mutagenesis construct. A) *pmi* gene transcripts were amplified throughout the complete enzootic life cycle of *Borrelia* from indicated cDNA samples. *pmi* appears to be constitutively expressed in all phases of *Borrelia* life cycle. B) Diagram illustrating mutagenesis construct created for targeted genetic knockout of *pmi* gene from *Borrelia* genome.

2.4 Discussion

We selected proteins from glycolytic pathway and closely related metabolic branches that had been previously observed to show moonlighting activity in other infectious organisms (Huberts and van der Klei 2010). We compared genetic sequences of these proteins in *L. interrogans* to homologous genes found in *B. burgdorferi*. Glyceraldehyde 3-phosphate dehydrogenase showed the highest gene conservation between these species with 80% identity between pathogenic and non-pathogenic *Leptospira* and 50% identity to *Borrelia* gene homolog. Two gene homologs, fructose bisphosphate aldolase and phosphoglycerate mutase, showed almost no significant similarity between annotated genes. The similarity between all homologs is indicated in Table 4.

Table 4: Similarity of selected moonlight candidate gene homologs between *L. interrogans* and *B. burgdorferi*

Leptospira gene	Borrelia gene	Percent identity	Common Name	Abbreviation
LA1532	BB0445	N/A	Fructose bisphosphate aldolase	FBA
LA1696	BB0055	46.8	Triose-phosphate isomerase	TPI
LA1703	BB0056	48.4	Phosphoglycerate kinase	PGK
LA1704	BB0057	50	Glyceraldehyde 3-phosphate dehydrogenase	GAP
LA2924	BB0348	34	Pyruvate kinase	PYK
LA3888			Glucose-6-phosphate isomerase	PGI
LA3939	BB0368	31	Glycerol-3-phosphate dehydrogenase	GPSa
LA4264		24	Glycerol-3-phosphate dehydrogenase	GPSb
LA4206	BB0648	N/A	Phosphoglycerate mutase	PGM
	BB0683		hydroxymethylglutaryl-CoA synthase	HMG-CoA
	BB0561		6-phosphogluconate dehydrogenase	6PGD

Due to significant differentiation between candidate moonlighting targets, we were unable to fulfill our initial desires of finding an antigen that might provide cross-protection against both pathogens. However, we continued our pursuits with each list of targets in tandem.

B. burgdorferi exhibits an ability to survive incredibly diverse environments through a complex enzootic cycle, and yet it relies solely upon glycolysis as an energy source utilizing glucose in mammalian hosts and glycerol within the tick vector (Pappas, Iyer et al. 2011). We analyzed a set of metabolic enzymes for potential moonlighting function and virulence activity. Our endeavors produced a single candidate from our initial list of proteins, phosphomannose isomerase. While not a direct player in the glycolytic cycle, PMI, catalyzes the reversible interconversion of fructose 6-phosphate and mannose 6-phosphate in the presence of zinc. This is a unique link between mannose metabolism and the glycolytic cycle. Furthermore, mannose is a major component of many glycoproteins and glycans via GDP-mannose biosynthesis or cell wall synthesis in numerous organisms (Smith, Proudfoot et al. 1995).

Our data suggest that at least some fraction of *B. burgdorferi* PMI exists as a cell surface protein although its role as cell surface protein remains puzzling. Also, the mechanism for PMI translocation across the cellular membranes remains unknown as PMI lacks classical membrane localization sequences, a transmembrane domain, or lipidation motifs. PMI might arrive on the surface in *Borrelia* through surface translocation of an interacting partner or other unknown mechanism, or a non-classical marker that was not detected by bioinformatics signal peptide algorithms. It is possible that PMI plays a role in possible exopolysaccharide or oligomannan biosynthesis pathways, but the presence of these substances on the surface of *Borrelia* is poorly characterized and remained unknown at this time (Smith, Proudfoot et al. 1995; Wu, Zhang et al. 2002).

Virulence roles of microbial antigens can be effectively studied by molecular genetics approaches, but our attempts to create PMI mutant spirochetes have been unsuccessful. It is likely that this gene is essential for survival of *B. burgdorferi* due to its enzymatic role in metabolic pathways. PMI mutants have been obtained for some organisms, but often required the addition of free mannose to the growth media. We attempted similar conditions in our attempts. Interestingly, in an *S. cerevisiae* *pmi* deletion mutant, addition of mannose led to regulation of other metabolic enzymes, buildup of mannose-6-phosphate, and resulted in inhibition of growth (Pitkanen, Torma et al. 2004). Through an alternate approach, our antibody-blocking studies provided no evidence for a role in persistence of the bacteria within feeding ticks, as similar levels of *B. burgdorferi* persists in ticks after feeding on PMI or PBS immunized.

While presence of PMI on the surface of *Borrelia* has been shown, antibody against surface PMI did not induce killing or bacteriostatic effect during borreliacidal assays or regrowth assays. Interestingly, a difference in the burden of bacteria was observed specifically in joints of PMI-immunized mice infected via ticks, but not mice infected by needle. Effects of host immunization with recombinant borrelial proteins on the tissue-specific persistence of pathogens were described earlier and possibly linked to the target protein function in bacterial colonization in local microenvironment (Coleman, Yang et al. 2008). This difference is unlikely to be due to an effect on the classical enzymatic function of PMI, but rather some unknown effect since our results during enzymatic assays no reduction of PMI enzymatic

activity from antibody inhibition (data not shown) as epitopes likely did not block the active site of the enzyme.

Three distinct types of PMI enzymes have been identified in different organisms (Wu, Zhang et al. 2002). It has been suggested that type I enzymes participate in mannose metabolism while type II enzymes function in capsular polysaccharide biosynthesis (Roux, Lee et al. 2004). Type I PMI of pathogenic *C. albicans* has become a potential target to combat fungal infections (Smith, Proudfoot et al. 1995). Type I PMI enzymes from bacteria to human beings frequently show high level of sequence identity (>40%), there exist less homology to *Borrelia* PMI giving hope for the possibility of epitopes enabling species-specific inhibition of *Borrelia* PMI. Further work including site-directed mutagenesis will be needed to clarify the catalytic and regulatory mechanism of PMI/GMPs, as well as possible influence of mannose-6-phosphate levels and phosphomannose isomerase activity within joints pertaining to rheumatoid arthritis and collagen production (Bates, Morrow et al. 2006; Xia, Yang et al. 2010).

Chapter 3: Development of novel RNA-based molecular diagnostics of human leptospirosis

3.1 Introduction

Leptospirosis is a prominent zoonotic disease caused by a diverse group of pathogenic leptospires that includes at least nine genospecies and over 200 serovars (Bharti, Nally et al. 2003; McBride, Athanazio et al. 2005; Ko, Goarant et al. 2009). There are an estimated more than 800,000 cases and 50,000 deaths annually due to leptospirosis (Lacerda, Monteiro et al. 2008). The greatest disease burden occurs in subsistence farmers (Sethi, Sharma et al. 2010) and urban slum dwellers (Karande, Gandhi et al. 2005; Reis, Ribeiro et al. 2008), especially in resource-poor settings. In endemic regions, epidemics have frequently been reported following heavy rainfalls (Lau, Smythe et al. 2010). Even in industrialized nations including the United States, outbreaks have been reported following sporting events (Jackson, Kaufmann et al. 1993; Morgan, Bornstein et al. 2002), within military personnel (Corwin, Ryan et al. 1990), and in tourists (van Crevel, Speelman et al. 1994). Additionally, there are increasing cases of the disease globally involving inner-city populations (Thiermann and Frank 1980), climate changes (Lau, Smythe et al. 2010), and expansion of urban slum populations (Ko, Galvao Reis et al. 1999). *L. interrogans* are transmitted from contaminated water, soil, or urine to hosts during contact with abraded skin or mucous membrane. Unlike other pathogenic spirochetes, which cause borreliosis or syphilis in humans and are unable to persist outside of a host body, leptospires can persist in aqueous environments for extended periods of time (Trueba, Zapata et al. 2004). The pathogen can quickly upregulate genes associated with host adaptation

and virulence and can establish serious systemic infection via haematogenous dissemination to multiple internal organs, particularly the kidneys and liver (Gouveia, Metcalfe et al. 2008). While wild rodents serve as major natural reservoir hosts, humans and many other domesticated animals are accidental hosts in the transmission cycle of leptospirosis (Levett 2001).

Pathogenic *Leptospira* spp. cause a spectrum of clinical symptoms ranging from mild febrile disease to severe manifestations such as Weil's disease and pulmonary hemorrhage syndrome, with case fatalities of >10% and >50%, respectively (Bharti, Nally et al. 2003). Although whole cell and recombinant vaccines are shown to interfere with *Leptospira* infection (Cao, Faisal et al. 2011), none of these vaccines offer complete protection, fail to block chronic renal colonization or urinary shedding, can elicit moderate side effects and are mostly effective against local host-adapted serovars (Bolin, Cassells et al. 1991; Zuerner, Alt et al. 2011). Thus, given an absence of effective vaccines, prevention of leptospirosis is primarily reliant on timely diagnosis and antibiotic treatment. Early diagnosis of leptospirosis generally leads to effective antibiotic treatment, thereby preventing the more severe form of disseminated disease; however, there is a lack of rapid diagnostics (Flannery, Costa et al. 2001).

Due to the non-specific clinical manifestations of leptospirosis, failure to diagnose the infection, or misdiagnosis, has become a significant problem in many developing countries where dengue, malaria, typhoid and other causes of acute fever are endemic (Musso and La Scola 2013). Diagnosis of leptospirosis still relies on classical laboratory tests including immunoassays against spirochetes or recombinant

proteins, direct cultivation of bacteria grown from body fluids, or a microscopic agglutination test (MAT) using paired serum samples and *Leptospira* cultures (Boonsilp, Thaipadungpanit et al. 2011). Although some of the immunoassays are highly sensitive, they suffer from inherent delays and variability of host immune responses as well as sequence divergence in target antigens, potentially limiting their use for early diagnosis of leptospirosis. Even the gold standard methods of direct culture and the MAT, require either weeks to grow spirochetes from body fluids or highly trained (Matsui, Soupe et al. 2012) laboratory personnel and paired sera. Therefore, there is a critical need for rapid and effective diagnostics, especially for detection of early infection.

Leptospira disseminate hematogenously and spirochetemia is detectable for many days following initial exposure (Agampodi, Matthias et al. 2012). Although PCR-based diagnostic methods have been developed that can detect leptospiral DNA (Slack, Symonds et al. 2006; Bourhy, Bremont et al. 2011), overall sensitivity of these assays is poor, and in general is less than 60% (Ahmed, Engelberts et al. 2009; Ferreira, Costa et al. 2014), although in some cases, higher sensitivities are reported . Unlike DNA targets, which usually exist as a single copy per cell, each bacterium contains hundreds to thousands of specific RNA molecules. We therefore hypothesized that an assay based on the PCR amplification of cDNA molecules representing highly and consistently transcribed *Leptospira* genes like 16S rRNA (Natarajaseenivasan, Raja et al. 2012), which are also mostly conserved in pathogenic *Leptospira* , could improve the sensitivity of *Leptospira* detection. In addition, detection of *Leptospira* transcripts in the blood would facilitate prompt and

appropriate antibiotic treatment. In the current study, we report a rapid, sensitive, and specific RNA-based PCR diagnostic test for early human leptospirosis. These results could serve as a paradigm for development of novel RNA-based diagnostics of additional bacterial infections in humans, such as Lyme disease, where early diagnostics remains challenging.

3.2 Materials and methods

Bacterial strains

Leptospira strains and serovars used in the study are indicated in Table 5. Unless stated otherwise, *Leptospira interrogans* Fiocruz L1-130, a clinical isolate, (Lessa-Aquino, Borges Rodrigues et al. 2013) was used in most parts of the study. In some experiments, additional *Leptospira* serovars were also used, including isolates from 17 pathogenic and five non-pathogenic strains. Spirochetes were grown in liquid Elinghausen-McCullough-Johnson-Harris (EMJH) media (Ellinghausen and McCullough 1965) at 29°C on a rotating platform at 100 rpm. Additional bacterial strains, such as an *Escherichia coli* K-12 derivative, group A streptococcus D471 cells (Raz, Talay et al. 2012) and *Borrelia burgdorferi* clone B31-A3 (Elias, Stewart et al. 2002), were also used in certain experiments and grown by using the standard media and protocols.

Table 5: *Leptospira* species and serovars used in the study

Species	Serogroup	Serovar	Strain
Pathogenic <i>Leptospira</i>			
<i>L. alexanderi</i>	Manhao	Manhoa 3	L 60T
<i>L. alstoni</i>	Ranarum	Pingchang	80-412T
<i>L. interrogans</i>	Canicola	Canicola	Kito
<i>L. interrogans</i>	Icterohaemorrhagiae	Copenhageni	Fiocruz L1-130
<i>L. interrogans</i>	Pyrogenes	Manilae	L495
<i>L. borgpetersenii</i>	Mini	Mini	200901116
<i>L. kirschneri</i>	Grippotyphosa	Grippotyphosa	RM52
<i>L. kmetyi</i>	Tarassovi	Malaysia	Bejo-Iso9
<i>L. noguchii</i>	Autumnalis	ND	Bonito
<i>L. santarosai</i>	Shermani	Shermani	1342 KT
<i>L. santarosai</i>	Tarassovi	ND	AIM
Intermediate <i>Leptospira</i>			
<i>L. broomii</i>	ND	ND	5399T
<i>L. fainei</i>	Hurstbridge	Hurstbridge	BUT 6T
<i>L. inadai</i>	ND	Lyme	10T
<i>L. licerasiae</i>	Iquitos	Varillal	VAR 010
<i>L. wolffii</i>	ND	ND	Khorat-H2T
Non-Pathogenic <i>Leptospira</i>			
<i>L. biflexa</i>	Semaranga	Patoc	Patoc 1
<i>L. meyeri</i>	Sejroe	Hardjo	Went 5
<i>L. terpstrae</i>	ND	Hualin	LT 11-33T
<i>L. vanthielii</i>	ND	Holland	Waz Holland
<i>L. yanagawae</i>	ND	Saopaulo	Sao Paulo

Ethics Statement

Written informed consent was obtained from all participants prior to blood collection. The study protocol was approved prior to study initiation by the Yale Institutional Review Board (HIC#1006006956), the Ethics Committees at the Oswaldo Cruz Foundation (505.490; 16/2013) and Hospital Couto Maia (175), and the Brazilian Ministry of Health National Ethics Committee in Research (15925). All experiments involving human blood, infectious agents and animals were performed according to the guidelines of the Institutional Biosafety Committee, and the Institutional Animal Care and Use Committee.

Samples

Blood samples from uninfected Golden Syrian Hamsters, a species commonly used as an animal model of Leptospirosis (Coutinho, Matsunaga et al. 2014), were collected into BD Vacutainer® whole blood collection tubes containing EDTA (BD Diagnostics). For human samples, twenty five patients were enrolled during active surveillance for leptospirosis at a state-run hospital, Hospital Couto Maia, in Salvador, Brazil, from June 2013 to September 2013, and blood samples were collected during early hospitalization (Ko, Goarant et al. 2009). All patients were confirmed for leptospirosis using previously described methods (hemoculture and/or MAT) (Levett 2001). We determined that 22 out of 25 subjects had laboratory-confirmed leptospirosis as defined by: 1) four-fold increase in MAT titer or seroconversion (0 to $\geq 1:200$) between paired sera, 2) reciprocal MAT titer of greater than 1:800 in one or more samples (Lessa-Aquino, Borges Rodrigues et al. 2013), 3) positive hemoculture, or 4) positive blood DNA PCR results. Three patients had probable leptospirosis based on the presence a single MAT titer value of 1:100-1:400. For samples used in the current study, about 1-2 mL of venous blood was collected directly into BD whole blood EDTA tubes. Within 5 hours of collection, 250 μ L of patient blood was aliquoted from the EDTA tubes into 750 μ L of TRIzol LS, thoroughly homogenized, and immediately frozen at -70°C . All patient samples were bar-coded, monitored during transport for temperature, and all cold chain data including sample receipt, processing time, and freezing time were recorded. Whole blood from 24 healthy individuals residing in non-endemic regions in the U.S. and

Brazil were also collected or purchased (SeraCare Life Sciences) and processed equivalently to patient samples.

Extraction of nucleic acids and cDNA synthesis

Total RNA samples were extracted from samples stored in TRIzol (for bacterial pellets harvested from cultures grown at mid to late log phases), or TRIzol-LS (for blood samples) according to manufacturer's (Life Technologies) instructions (60, 61). After phase separation, RNA samples were either precipitated with isopropanol, dissolved in 20 μ L of RNase-free water and subjected to optional DNase1 treatment (NEB laboratories), or further purified using an RNeasy mini kit (Qiagen). For cDNA synthesis, 0.5 μ g of RNA samples was reversed transcribed using VILO superscript master mix (Life technologies) according to manufacturer's protocols. For extraction of DNA, spiked blood samples were processed using DNeasy mini kit (Qiagen) according to manufacturer's instructions and eluted into 100 μ L nuclease-free sterile water.

Primer design

The primers used for qPCR reaction were designed using NCBI Primer-BLAST primer design program based on the available *L. interrogans* genomic sequences. To identify primers of greatest sensitivity and specificity, we aligned the 16S sequences of 37 *Leptospira* serovars, including all 20 known pathogenic or non-pathogenic leptospiral species, and several other non-target bacterial species using MegAlign program (DNASTAR). We have designed specific sets of 16S forward and

reverse primers expected to amplify a specific region of 16S gene that retains 1) absolute homology between all known highly pathogenic *Leptospira* species and serovars, 2) few base pair mismatches for intermediate pathogenic leptospire and 3) greater number of mismatches to non-pathogenic *Leptospira* species. The specificity of each newly designed primer was initially searched against all reference mRNA sequences using NCBI BLASTn as well as Primer-BLAST programs to rule out possible cross-reactivity with other bacteria species as well as non-targeted species including human, mice, rats, and hamsters. Similarly, primers against additional gene targets, FlaB, LipL31, LipL32, and LipL41 were also designed. All PCR primer pairs had a similar annealing temperature (60°C) and spanned nearly 200 base pairs of each of the target genes. Prior to their use in RNA measurement assays, each primer pair was tested for efficiency and non-specific amplification by melt-curve analysis using *L. interrogans* genomic DNA as a template.

Polymerase chain reaction

The oligonucleotide sequences for each of the primers used in specific PCR reactions are indicated in Table 6. The relative levels of cDNA templates in each sample were assessed by RT-qPCR as detailed (Promnares, Kumar et al. 2009), and whenever necessary, DNA contamination in each sample was measured using an equal volume of purified RNA as a template. All qPCR reactions were performed using the CFX96 real time PCR detection system (Bio-Rad) with the following thermal cycle conditions: 95°C for 10 min, 45 cycles of 95°C for 15 s, 60°C for 60 s, followed by a melt curve from 65°C to 95°C performed at an increment of 0.5°C per

cycle. All qPCR plates included no template control wells to test for non-specific amplification or reagent contamination, and results were further tested for specificity by melt curve analysis. Detection of human or hamster β -actin or GAPDH transcripts by qPCR confirmed the integrity of cDNA samples. For detection of leptospirosis in humans, an optimized DNA qPCR analysis was also performed using nuclease (TaqMan) assay and primers that amplified a sequence of lipL32, a pathogenic *Leptospira*-specific gene, as detailed earlier (Stoddard, Gee et al. 2009).

Table 6: Oligonucleotide primers used for initial *Leptospira* diagnosis target screening

Primer	Amplicon size (bp)	Forward (5'-3')	Reverse 5'-3'
16S-1	211	GCGTAGGCGGACATGTAAGT	AATCCCGTTCACCTACCCACG
16S-2	235	TAAAGGCTCACCAAGGCGAC	TTAGCCGGTGCTTTAGGCAG
LipL32-1	190	GCCGTAATCGCTGAAATGGG	CTTTGGCGATTGGTTCAGGC
LipL32-2	262	TGGCTATCTCCGTTGCACTC	CCCATTTTCAGCGATTACGGC
FlaB-1	192	GCTCGTGCAGGTGGAAGTAT	GCCTTTGAAGTCATCGTGCC
FlaB-2	183	GCTAACGACGTGATCGGTCT	CGAGACAACCTTCTTCCGCCA
LipL41-1	184	GTGCAGACGCAATCAACGAA	GCGAAACCTGCCACTTTCAA
LipL41-2	156	CGTAGGTTTGGCTGTTGAAGC	GCGTCTGCACGTTTACTCAG
LipL-31	180	TCGATGCGATGAGTCGAGTT	AACCGTCTTTTTTCAGCTGCG

Validation of primers

L. interrogans culture was harvested at 2.9×10^8 leptospire per ml by centrifugation at 5000 g at room temperature ($\sim 2.9 \times 10^9$ total cells) and subjected to RNA isolation as detailed above. For relative assessment of each primer set, 2.5 μ g RNA samples were reverse transcribed into cDNA, serially diluted to tenfold, which were used in the qPCR reactions. Standard curves and amplification efficiency of the reactions were calculated by the CFX96 instrument software, as instructed by the manufacturer. cDNA samples were also isolated from a number of pathogenic,

intermediate pathogenic or non-pathogenic species and serovars. For spiking experiments, 250 μ L of untreated whole human blood samples were spiked in triplicate with *L. interrogans* derived from at least three independent cultures at various concentrations ranging from 10^6 - 10^0 cells/mL. Samples were used for DNA or RNA extraction using DNeasy mini kit or TRIzol extraction procedure, followed by cleanup using RNeasy mini kit, respectively, and the RNA samples were further processed for cDNA synthesis. As controls for assessment of specificity, cDNA samples were also isolated from additional bacterial culture grown at late log phases including *B. burgdorferi*, *E. coli*, and group A *Streptococcus* strains as well as from human blood and hamster liver.

RNA stability studies

Triplicate samples of 250 μ l whole human blood were spiked with viable *L. interrogans* (10^0 cells/ml) derived from at least three independent cultures. Aliquots were homogenized with 750 μ L TRIzol LS and stored at room temperature for 0, 4, 8, 24, 72, or 120 h before freezing at -80°C until analysis. In parallel experiments, triplicate aliquots of 250 μ L human blood containing 100 leptospire per ml were stored at room temperature, 4°C , -20°C , or -80°C in the absence of any stabilization reagent, and processed together with other conditions. Samples were collected directly into 750 μ L TRIzol LS after incubating at the aforementioned temperatures for 0 h, 8 h, 24 h, 7 d, and 14 d, and stored at -80°C until completion of all timepoints. RNA was extracted, reverse transcribed to cDNA, and analyzed by qPCR using *Leptospira*-specific 16S primers.

Statistical analysis

Results are expressed as the mean \pm standard error of the mean (SEM). The significance of the difference between the mean values of the groups was evaluated by unpaired Student t test and ANOVA.

3.3 Results

Development of a 16S rRNA-based quantitative PCR assay for detection of pathogenic leptospire

Development of a rapid and sensitive diagnostics test for human leptospirosis, especially for detection of early active infection, is highly warranted. Since pathogenic leptospire are known to disseminate via blood where they are detectable for several days after infection (Lacerda, Monteiro et al. 2008), and since each bacterium may contain hundreds of copies of certain abundant transcripts, we sought to explore whether an RNA-based PCR assay would allow sensitive and specific detection of leptospire in human blood. We adopted a SYBR® Green-based RT-qPCR assay, which is a highly efficient and widely used platform amongst available real-time PCR technologies (Arikawa, Sun et al. 2008), yet relatively simple and cost-effective. For identification of an RNA target that yields most efficient detection, we initially examined a set of characterized, abundant rRNA and mRNA gene targets: 16S rRNA, FlaB, LipL31, LipL32, and LipL41. We selected these genes not only due to their constitutive and abundant expression but also for their sequence conservation in highly pathogenic *Leptospira* spp., and sequence divergence in non-target species.

Although the above-mentioned mRNA genes are unique to pathogenic *Leptospira* species (Noubade, Krishnamurthy et al. 2002), specific regions of their rRNA genes display appreciable species-specific conservation (Clarridge 2004). We therefore used NCBI Primer-Blast software to identify unique regions in 16S gene and created forward and reverse primers 100% identical to pathogenic *L. interrogans* sequences but containing several nucleotide mismatches to non-target bacterial species including non-pathogenic *Leptospira*. These primers also lack significant similarity to mammalian species. All gene-specific primers had a similar annealing temperature and comparable amplicon sizes. The RNA samples from a highly pathogenic species, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 were converted into cDNA and used in a SYBR® Green-based qPCR assay in the absence or presence of 10-fold excess of a control host (hamster) cDNA. While sensitivity of the assay (or the relative abundance of the transcripts) was calculated using the $2^{-\Delta\Delta C_T}$ method (Clarridge 2004), specificity of the target gene amplification was assessed using melt-curve analysis. We found that the 16S rRNA primers offered the most efficient analytical sensitivity, as evidenced by the lowest Ct values, and were nearly 1000-fold more abundant than the next efficient mRNA target, LipL32 (Fig. 11A). The 16S-1 primers (Fig. 11B), or other tested primers, showed PCR efficiencies between 91-99% and without detectable cross-reactivity with spiked control rodent cDNAs. As the 16S rRNA primers offered the highest sensitivity in our assay, we only used these primers in subsequent experiments (16S-1, Fig.11).

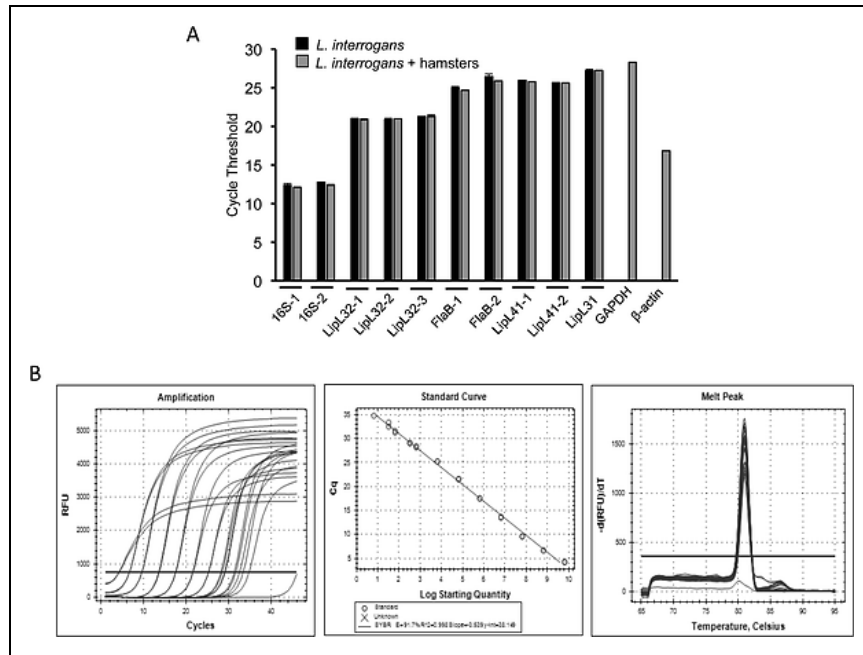


Figure 11: *L. interrogans* 16S rRNA primers offered highest analytical sensitivity. A) Total RNA samples isolated from cultured spirochetes were converted into cDNA and amplification cycles (cycle threshold or Ct value) of various *L. interrogans* target genes are assessed in RT-qPCR assays in the presence (gray bars) or absence (black bars) of hamster cDNA. Data represent results from three independent experiments. B) 16S rRNA primers display a high PCR efficiency. cDNA in RNase-free water (320 ng/μL) was serially diluted to tenfold (10^{-1} to 10^{-9}) and subjected to RT-qPCR assays using 16S-1 primers. Amplification cycles (left panel) were used to calculate standard curve (middle panel), which indicated detection to 10^{-9} dilutions with an amplification efficiency of 91.2%. A melt curve analysis (right panel) showed a melting temperature of 82°C without any non-specific amplification.

Analytical sensitivity

We next assessed whether sensitivity of our 16S RNA-based assay is superior to corresponding DNA-based ones and also tested its specificity with experimentally spiked human blood samples. To accomplish this, we spiked serially diluted *L. interrogans* cells into 250 μl aliquots of human blood and the RNA and DNA samples were isolated using the commercial kits as detailed in the Materials and Methods (Yang, Qin et al. 2013). We then performed subsequent RT-qPCR and qPCR assays using corresponding templates. Using this methodology, we found that

RNA-based assays were at least 100-fold more sensitive than a DNA-based approach, with limits of detection of one bacterium versus one hundred per milliliter of blood, respectively (Fig. 12).

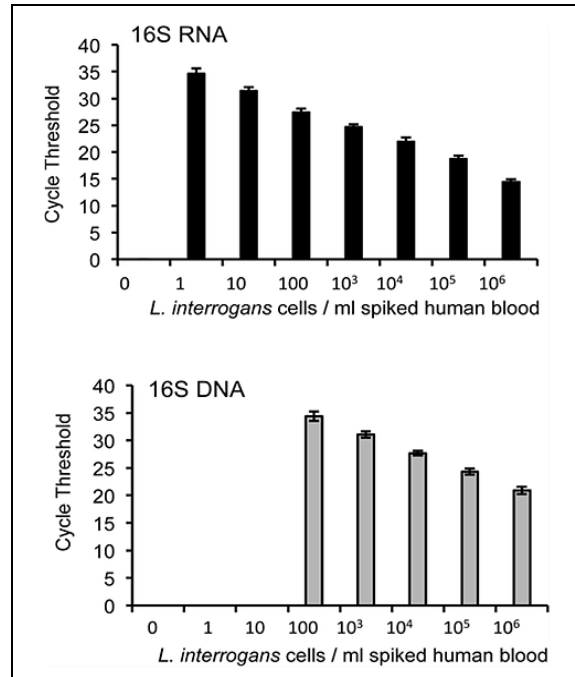


Figure 12: RNA-based detection is more sensitive than DNA. Aliquots of *L. interrogans* bacteria were serially diluted from 10⁶ to 1 bacterium per milliliter of human blood and used for either RNA-based RT-qPCR (upper panel) or DNA-based qPCR (lower panel). Data represent results from three independent experiments.

Analytical specificity

We next determined the sensitivity our 16S RT-qPCR assay in the detection of multiple pathogenic *Leptospira* serovars, and the specificity of the assay using non-pathogenic *Leptospira* species and non-target bacterial species. For this purpose, we prepared RNA samples from 17 highly or intermediate pathogenic *Leptospira* species (Ricaldi, Fouts et al. 2012) and serovars as well as from five non-pathogenic species, *B. burgdorferi*, group A streptococcus, *E. coli* or hamster and human tissues. We found that while the assay was able to detect all pathogenic species with highest

sensitivity, detection of non-pathogenic *Leptospira* species was at least 15 Ct value (or 10,000 fold) higher (Fig. 13). These results indicate a difference of several thousand folds in the concentration of target templates. Further, we detected none of the non-target species suggesting 100% specificity of the assay (Fig. 13).

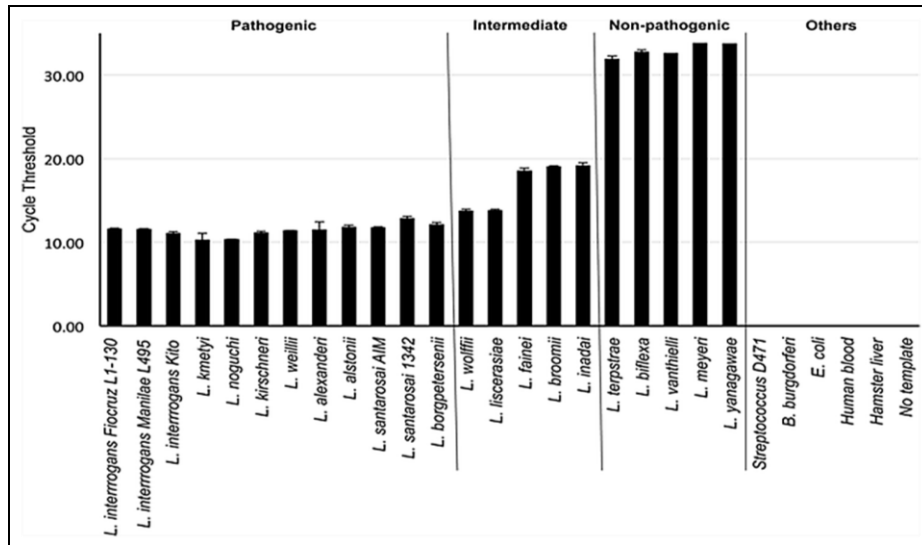


Figure 13: High sensitivity and specificity of 16S rRNA qPCR assay for pathogenic and nonpathogenic *Leptospira* species and serovars. Total RNA was extracted from 17 high or intermediate pathogenic, and five non-pathogenic *Leptospira* species, *Borrelia burgdorferi*, group A streptococcus, and *Escherichia coli*, as well as from uninfected human blood or hamster liver, as described in the materials and methods and converted into cDNA. Equal amount (10 ng) of cDNA templates from each bacterial species were subjected to RT-qPCR assays using 16S-1 primers and amplification cycles (C_t values) were measured. Note that the sensitivity of detection is the best for all tested highly pathogenic species or serovars followed by intermediate species while non-pathogenic strains display the lowest sensitivity (an average of 15 C_t values or 10,000 fold less detectability). All tested non-target bacterial species or mammalian samples remained undetectable. Data represent results from three independent experiments.

Stability of *L. interrogans* 16S transcripts in human blood

An ideal RNA-based diagnostic test for leptospirosis would detect stable targets, allowing for varied blood storage times and temperatures, and not require the use of toxic RNA stabilizing reagents such as TRIzol. Therefore, we compared the limit of detection of 16S transcripts in spiked human blood samples stored at room

temperature in TRIzol to that of untreated spiked blood samples. We also tested how storage temperature of blood samples influenced the window of 16S detection by our assay. To accomplish our objectives, we spiked human blood with *L. interrogans* cells at 100 bacteria per milliliter of blood and stored aliquots at room temperature, 4°C, -20°C, or -80°C for 1, 7 and 14 days either with or without the addition of TRIzol. A sample immediately stored in TRIzol and frozen served as the “0 hour” or baseline control for 16S RNA stability experiments. We measured the transcript levels by RT-qPCR analyses, and observed no appreciable RNA degradation in the samples stored in TRIzol at room temperature (Fig. 14A) or at colder temperatures. Of note, while we recorded a significant loss of 16S RNA in samples stored at room temperature within a day, ~ 5% of the transcripts remained detectable until 7 days (Fig. 14B). In contrast, we were able to detect >20% of 16S RNA transcripts from samples stored at 4°C, and >50% at -20°C and -80°C even after 14 days (Fig. 14B).

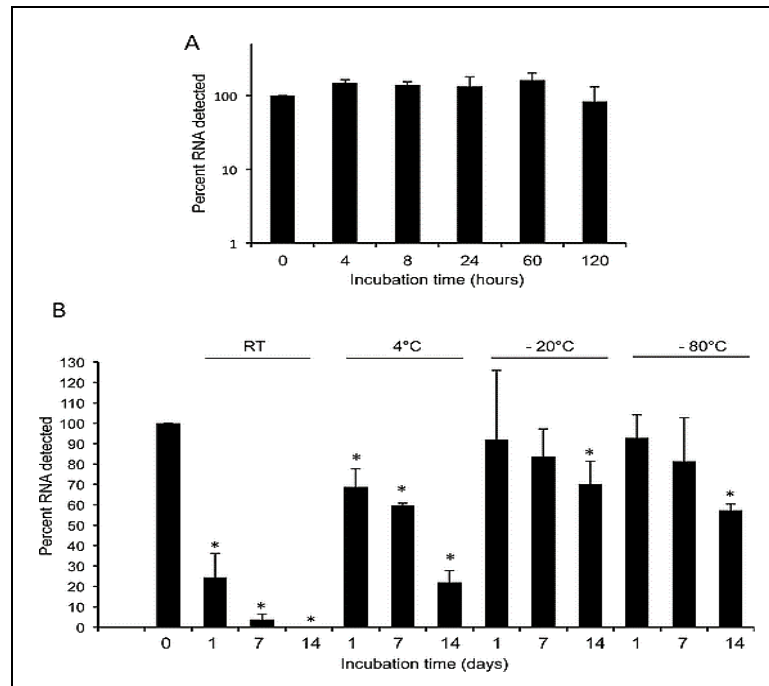


Figure 14: Stability of *Leptospira* 16S transcripts in human blood. A) Transcript stability in the blood treated with an RNA stabilization agent. Aliquots of human blood were spiked with leptospires (100 cells/ml), mixed with an RNase stabilization agent (TRIzol), and each aliquot was stored at room temperature for various times (0–120 hours). Following storage, levels of 16S rRNA transcripts were measured using RT-qPCR assays. Data represent results from three independent experiments. B) Transcript stability in the blood stored at various temperatures in the absence of any RNA stabilization agent. Spiked samples were prepared as described above and stored either at room temperature or at various cold temperatures (4°C, -20°C, and -80°C) up to 14 days, and transcript levels were monitored by RT-qPCR analyses. Transcript levels of “0 hour” were considered as 100%, which served as baseline controls, which displayed significant differences in transcript levels in groups marked by an asterisk (ANOVA, $p < 0.05$). Data represent results from three independent experiments.

Assessment of clinical cases of human leptospirosis

Finally, we tested the sensitivity and specificity of our assay for the detection of *Leptospira* in blood samples obtained from patients, with suspected leptospirosis, as detailed in the Material and Methods section. Total RNA and DNA samples were isolated from these human samples, and detection of *Leptospira* was examined by real-time PCR analyses, as detailed in the Materials and Methods section. Results

indicated that, for 22 confirmed leptospirosis cases, *Leptospira* RNA was detected in 14 samples, yielding a sensitivity of 64% (Table 7). In parallel, we performed qPCR DNA detection assays for the corresponding human samples, which identified 7 positives, thereby reflecting a significantly lower sensitivity (32%, $p=0.035$) for the DNA detection assay compared to the RNA detection assay. Of note, 2 of the 3 probable clinically suspected cases were found to have detectable leptospiral RNA, whereas none of the probable cases had detectable DNA. As expected, all blood samples obtained from the 24 control individuals failed to yield a positive signal, suggesting a 100% specificity of our RNA-based assay.

Table 7: Detection of leptospiral RNA by quantitative PCR in blood samples collected from humans with suspected leptospirosis

Assay results	Confirmed leptospirosis (N=22)	Probable leptospirosis (N=3)	Healthy Subjects (N=24)
Number (%)			
RNA and DNA PCR+	5 (23)	0 (0)	0 (0)
RNA PCR+ alone	9 (41)	2 (67)	0 (0)
DNA PCR+ alone	2 (9)	0 (0)	0 (0)
Total RNA (PP1) PCR+	14 (64)	2 (67)	0 (0)
Total DNA PCR+	7 (32)	0 (0)	0 (0)

3.4 Discussion

As leptospirosis affects nearly a million people annually (Lacerda, Monteiro et al. 2008) and a preventative human vaccine is unavailable, development of a better diagnosis test is critical for effectively treating patients with leptospirosis. However, efficient detection of the infection is difficult to accomplish, primarily due to the fact that pathogenic leptospires not only share features of both gram-positive and gram-negative bacteria including other related spirochetes, clinical manifestation of the infection also share features of many prevalent undifferentiated febrile illnesses, such

as influenza, dengue and malaria (Bruce, Sanders et al. 2005; Biggs, Bui et al. 2011; Boonsilp, Thaipadungpanit et al. 2011; Biggs, Galloway et al. 2013).

Here, we report a use of leptospiral RNA as a diagnostic target for development of a rapid, sensitive and specific quantitative PCR assay for detection of human leptospirosis. While RNA-based diagnostic methods for detection of bacterial pathogens are uncommon, except for a few commercially-available assays for detection of certain sexually transmitted bacterial infections (Chong, Jang et al. 2003; Lowe, O'Loughlin et al. 2006; Cheng and Kirby 2014), to the best of our knowledge, this is the first example of an RNA-based diagnosis of human leptospirosis. Our discovery of the relative stability of 16S transcripts in untreated stored human blood samples indicate that the RNA-based assay could be widely applied for the diagnosis of leptospirosis. Additionally, our results suggest similar approaches can be employed to develop novel diagnostic tests for other bacterial diseases.

Several serological immunoassays are available to date for detection of leptospirosis (Lacerda, Monteiro et al. 2008; Boonsilp, Thaipadungpanit et al. 2011), however, diagnosis of human or animal infection is still inadequate, which is based on classical microbiological methods. The gold standard method for such diagnosis is culture or microscopic agglutination test (MAT), which are extremely slow procedures that also suffer from sensitivity, especially for diagnosis of early infection. Newer diagnostic methods that allow diagnosis of early infection at a relatively fast pace and facilitate initiation of prompt antibiotic treatment could alleviate more severe complications of disseminated infection. As spirochetemia is likely to be associated with early infection, which occurs for at least two weeks following initial

infection, we hypothesized that the detection of nucleic acids with sequence specificity to pathogenic *Leptospira* could surrogate an active infection. Such detection in turn would yield high diagnostic efficiency if the transcripts or corresponding RNA fragments are abundant, and remain stable, in stored blood. Unlike conventional genomic DNA molecules that mostly represent a single copy per bacterial cell, or mRNA molecules, which constitute the minor population of total cellular RNA, ribosomal RNA molecules are likely to be abundant and thus offer more promising diagnostic target. In particular 16S rRNA, due to its high expression, and maintenance of species-specific sequences (Clarridge 2004), has been widely used for taxonomic studies or as a diagnostic target to identify a particular bacterial species. Interestingly, unlike other major pathogenic spirochetes like *Borrelia burgdorferi* (Bugrysheva, Godfrey et al. 2011), *L. interrogans* genome houses at least two copies of 16S rRNA genes also conserved in sequenced genomes of pathogenic leptospires (Tucunduva de Faria, Athanzio et al. 2007). Thus, it is perhaps not surprising that compared to even abundantly and consistently-expressed mRNA gene targets like *lipL32* or *flaB* (Nascimento, Ko et al. 2004), our assay targeting 16S rRNA achieves better sensitivity that allow detection of a single *L. interrogans* cell per milliliter of spiked human blood. This is also a notable improvement in sensitivity, compared to existing DNA-based PCRs where the limit of detection ranges from 10^2 - 10^3 bacteria per milliliter of blood or urine (Smythe, Smith et al. 2002; Slack, Symonds et al. 2006; Natarajaseenivasan, Raja et al. 2012). The fact that leptospiral 16S RNA molecules, at least the regions targeted by the diagnostic primer set used in our study, remain stable in the untreated blood for several hours

that potentially facilitate practical use of an RNA-based diagnosis of *Leptospira* infection at the early stage when the infection is relatively easier to treat. It is difficult to conjecture how 16S RNA maintains notable stability in stored blood and likely to be contributed by the remarkable ability of pathogenic spirochetes to persist in aqueous environment *ex vivo*. Existence of intact or fragmented 16S transcripts in the blood, or within phagocytic cells that enable their detection, also remains as further possibilities.

Although diagnosis of microbial infection based on the detection of a target RNA molecule offers multiple advantages, such as higher sensitivity and specificity or potential indicator of early and active infection, there are inherent limitations that influence successful RNA PCR-based diagnostics. RNA molecules are generally less stable than other biomarkers and their cellular abundance (and thus detection) are likely to be variable, which could also be influenced by a number of additional factors. For example, efficiency of RNA detection depends on successful reverse transcription or absence of intrinsic blood factors that inhibit reverse transcription or subsequent PCR. As each bacterium may contain hundreds of certain RNA molecules transcription of which could vary from cell to population levels, or growth stages, quantitation of microbial cells based on enumeration of RNA molecules might not be possible. Despite these challenges, due to their outstanding abundance and specificity, as also highlighted in our study, 16S RNAs are used in the diagnosis of a number of human infections, primarily the ones caused by viruses (Paulino, de Mello et al. 2002; Jiang, Wu et al. 2012; Jiang, Yu et al. 2013; Zhu, Fan et al. 2013; Maquart, Temmam et al. 2014) and in a limited number of cases, for detection of

bacterial infections, such as *Mycobacteria* species (Beissner, Symank et al. 2012; Jiang, Wu et al. 2012), or *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Chong, Jang et al. 2003; Lowe, O'Loughlin et al. 2006). To the best of our knowledge, the current study represent the first attempt to use RNA detection to develop an improved diagnostic test for leptospirosis. Notably, 16S gene sequences were recently targeted for development of a DNA based real-time PCR assay, denoted as rt-PCR (Matsui, Soupe et al. 2012), which used additional primers tagged with reporter dye and quencher molecules called TaqMan probes to enhance analytical specificity, however the assay achieved overall clinical sensitivity of 34%. While our RNA assay yielded relatively superior sensitivity, we also show that use of a simpler, more widely used, and cheaper in-house platforms like SYBR® Green-based qPCR assay could retain comparable specificities of relatively expensive TaqMan PCR while yielding greater sensitivities. Nevertheless, our results needed to be confirmed with additional studies using larger numbers of patients, including ones from diverse epidemiological settings beyond hospitalized patients from Salvador Brazil involved in our current study.

Although overall sensitivity of our RNA assay is twice as high as parallel TaqMan probe-based DNA assays and yielded statistically significant differences, it detected *Leptospira* RNA in 64% of the laboratory-confirmed cases. The exact reasons why our assay failed to yield positivity in a subset of laboratory-confirmed samples remained puzzling, however, could be linked to the effectiveness of antimicrobial therapy influencing spirochetemia at the time of individual sample collection, or unintended degradation of 16S RNA during less careful handling and

storage conditions, amongst other unknown possibilities. On the other hand, notably, our RNA assay detected positivity in two human samples where routine laboratory diagnosis remained unconfirmed. While this may be a potential issue of specificity, it also suggests enhanced sensitivity of the RNA assay, especially in cases where pathogens are rapidly cleared (such as, via effective anti-microbial treatment) before humoral immune responses could be developed and detected by routine serology-based tests. The latter speculation of enhanced sensitivity of our assay is supported by its high (100%) specificity, where samples from normal humans failed to yield positivity.

Notably, an earlier study also reported positivity in clinical diagnosis of *Leptospira* by PCR and sequencing methods in a subset of clinical samples that are serologically negative using a standard MAT panel (Bourhy, Bremont et al. 2011). Nevertheless, while implementation of additional care towards sample collection and storage would further enhance sensitivity of our RNA assay, use of a real-time PCR platform already has advantage over current serology-based assays in terms of rapidity, allowing quicker diagnosis of early infection and prompt antimicrobial treatment, which could prevent more severe and life-threatening complication of disseminated infection. In addition to currently-adopted SYBR® Green-based qPCR assay, our RNA detection strategy is amenable to additional cheaper PCR formats, such as Loop Mediated Isothermal Amplification (LAMP) reaction for detection of a variety of human pathogens (Parida, Posadas et al. 2004; Jayawardena, Cheung et al. 2007; Lucchi, Demas et al. 2010; Zhang, Brown et al. 2011; Koizumi, Nakajima et al. 2012; Curtis, Niedzwiedz et al. 2014; Iwasaki, Chagan-Yasutan et al. 2016),

including leptospirosis (Sonthayanon, Chierakul et al. 2011). Therefore, our study could have far-reaching implications for development of simple, cost-effective, and rapid RNA-based PCR assays for detection of human leptospirosis as well as other bacteremic human pathogens where efficient diagnosis of early or active infection is warranted yet remains as an unmet need.

A portion of this chapter has been published previously:

Backstedt, B. T., O. Buyuktanir, et al. (2015). "Efficient Detection of Pathogenic Leptospire Using 16S Ribosomal RNA." PLoS One **10**(6): e0128913.

Chapter 4: Improvement of RNA-based diagnostics of human leptospirosis via optimized primer selection

4.1 Introduction

We have previously described a novel approach for molecular diagnosis of leptospirosis using 16S rRNA, performance of which was far superior to current utilized techniques, particularly in cases of early infection and initial onset of symptoms (Backstedt, Buyuktanir et al. 2015). As discussed earlier, the gold standard method for clinical diagnosis of *L. interrogans*, MAT, is reliable only after a patient develops an antibody response against pathogen antigens (Niloofa, Fernando et al. 2015). In fact, by the time a suspected patient can be tested and confirmed by MAT, infection has likely progressed to more serious late stages or been treated without diagnosis (Brownlow, Kavanagh et al. 2014; Waggoner, Balassiano et al. 2014; Wynwood, Burns et al. 2015). Our initial assay showed vast improvements in early diagnosis as compared to DNA-based molecular testing, but still had an overall low sensitivity of detection from infected human clinical samples, as we failed to detect almost one-third of confirmed cases. As our original assay reflected a low level of cross-reactivity with non-pathogenic *Leptospira* species as well as a decrease in the sensitivity of detection for some intermediate pathogenic strains (shown in Fig. 13), we further sought to optimize our primer selection for improvement in the assay efficiency. Here we report the results of an improved rapid, sensitive, and specific RNA-based PCR diagnostic assay for early human leptospirosis.

4.2 Materials and methods

Most of the methods and materials were detailed in Chapter 2 while additional information pertaining to the current studies are described below:

Primer design

The primers used for qPCR reactions were manually chosen. To identify primers of greatest sensitivity and specificity, we further aligned the 16S sequences of 37 *Leptospira* serovars, including all 20 known pathogenic or non-pathogenic leptospiral species, and several other non-target bacterial species using MegAlign program (DNASTAR). We designed primers against 16S rRNA regions that possess at least following traits: 1) absolute homology between all known highly pathogenic *Leptospira* species and serovars, 2) few base pair mismatches for intermediate pathogenic leptospire and 3) greater number of mismatches to non-pathogenic *Leptospira* species. These primers were used in combinations to create primer pairs listed in Table 8. Of note, primer pair PP1 is the original primer set used for this assay in Chapter 3 and our previous publication. The specificity of all primer pairs was initially searched against all reference mRNA sequences using NCBI BLASTn, as well as Primer-BLAST programs to limit the possibility of cross-reactivity with other bacteria species and non-targeted species including human, mice, rats, and hamsters. Each primer pair was then empirically tested for non-specific or cross-reactive amplification. All PCR primer pairs were designed for optimal annealing temperature at 60°C gave an amplicon product of 100-400 base pairs. All observed amplification was verified by melt curve analysis for product specificity.

Table 8: Oligonucleotide primers used for for *Leptospira* 16S primer optimization

Primer pair name	Amplicon size (bp)	Primer name	Sequence (5'-3')
PP1	211	16S lep1 F 550-569	GCGTAGGCGGACATGTAAGT
		16S lep1 R 741-760	AATCCCGTTCACTACCCACG
PP2	283	16S lep1 F 550-569	GCGTAGGCGGACATGTAAGT
		R 16S-810-832	GGTTCGTTACTGAGGGTTAAAAC
PP3	339	F 16S-123-141	GAGTCTGGGATAACTTTCCG
		R 16S-440-461	GTACCATCATCACATTGCTGCT
PP4	447	F 16S-123-141	GAGTCTGGGATAACTTTCCG
		R 16S-550-569	ACTTACATGTCCGCCTACGC
PP5	710	F 16S-123-141	GAGTCTGGGATAACTTTCCG
		R 16S-810-832	GGTTCGTTACTGAGGGTTAAAAC
PP6	130	F 16S-440-461	AGCAGCAATGTGATGATGGTAC
		R 16S-550-569	ACTTACATGTCCGCCTACGC
PP7	393	F 16S-440-461	AGCAGCAATGTGATGATGGTAC
		R 16S-810-832	GGTTCGTTACTGAGGGTTAAAAC

Validation of primers

In addition to validation of primers according to methods in Chapter 3, we performed absolute standard curves from known quantity of DNA gene copies. For standard curves, full length 16S rRNA gene was PCR amplified from *L. interrogans* genomic DNA using primers listed in Table 9 and cloned into *E. coli* DH5 α using the pGEM®-T Easy Vector System. From five milliliters of *E. coli* culture expressing 16S rRNA gene, plasmid was purified using Qiagen Miniprep kit and DNA concentration and purity measured by electrophoresis and Nanodrop quantitation. Utilizing the concentration of purified plasmid DNA and the total size of plasmid containing 16S, the number of gene copies was calculated to develop an absolute standard curve from 10⁹ to 1 gene copies per reaction. Standard curves and

amplification efficiency of the reactions were calculated by the CFX96 instrument software.

Table 9: Primers for full length *L. interrogans* 16S gene amplification

Primer	Sequence (5'-3')
16S full length Forward	AACACGGAGAGTTTGATCCTGG
16S full length Reverse	AAAGGAGGTGATCCAGCCG

4.3 Results

Optimized primer design

We designed new primers targeting leptospiral 16S rRNA by performing multiple sequence alignments of 16S gene sequences from *Leptospira* species and most closely related bacteria. Primers were manually designed from 16S gene regions showing divergence between pathogenic and non-pathogenic leptospires. Each primer set was empirically tested for specificity against cDNA from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 as well as non-target species *B. burgdorferi*, *E. coli*, and group A *Streptococcus* strains, human and hamster samples (Elias, Stewart et al. 2002; Raz, Talay et al. 2012; Ricaldi, Fouts et al. 2012). Primer pairs 1, 2, 3, 4, 6, and 7 all showed sensitive amplification of *L. interrogans* (Fig. 15). Similar to our previous results, PP1 showed low level cross-reactive amplification against non-pathogenic species, but not unintended target species, while PP2 gave non-specific amplification from all species and PP5 showed a much later amplification of *L. interrogans* targets, indicating poor sensitivity. We eliminated PP2 and PP5 from future testing due to low specificity and sensitivity, respectively, but retained PP1 for comparison against our initial assay results.

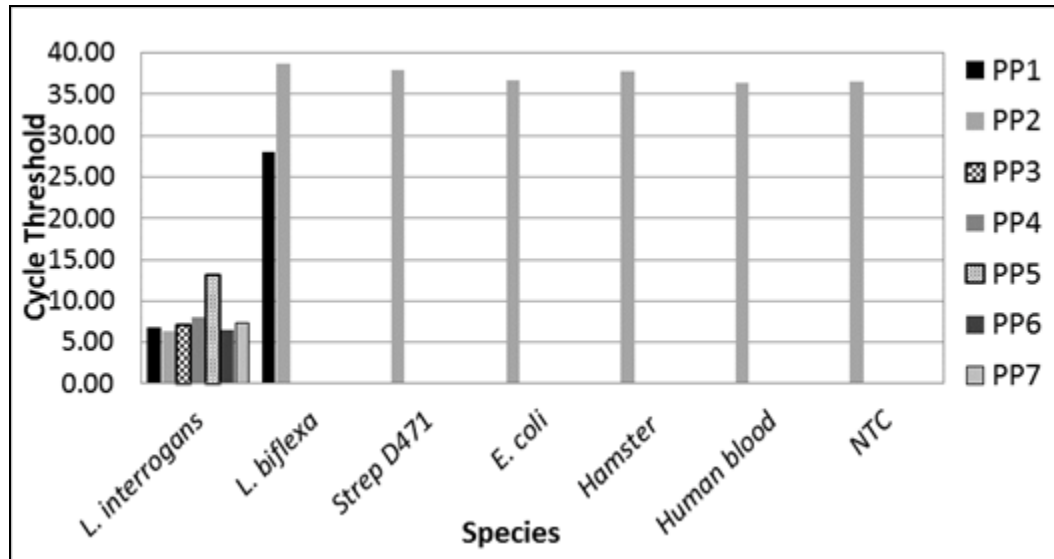


Figure 15: Primer optimization improves specificity against non-target species. cDNA samples were prepared from different species for testing primer specificity. Each primer pair was analyzed for amplification of unintended target species.

As all other primers showed high specificity, we sought to verify whether these new primers maintained the ability to detect all pathogenic and intermediate pathogenic *Leptospira* species without cross-amplification of non-pathogenic strains. Each primer was independently used for RT-qPCR with cDNA from 21 different *Leptospira* species and serovars for 16S detection. Overall, we observed a similar amplification pattern with each primer for all *Leptospira* species. All of the new primers detected pathogenic and intermediate pathogenic strains, but showed no cross-amplification with non-pathogenic species (Fig. 16). PP3 and PP4 showed a significantly lower sensitivity specifically against strains *L. kmetyi* and *L. alstonii*. Cumulatively, a single primer pair, PP6, provided the highest sensitivity and specificity across all tests and was selected for all subsequent studies.

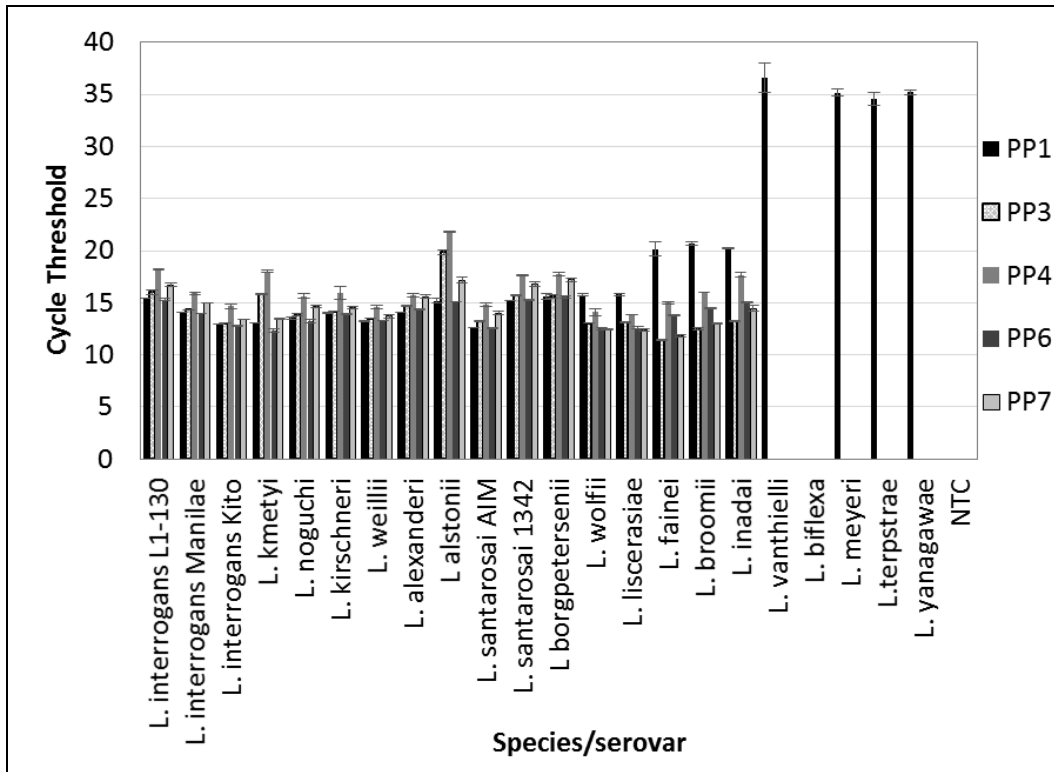


Figure 16: Primer optimization improves specificity against non-pathogenic leptospires. Total RNA was extracted from 17 high or intermediate pathogenic, and five non-pathogenic *Leptospira* species. cDNA templates from each bacterial species were subjected to RT-qPCR assays using indicated primers and amplification cycles (C_t values) were measured. Data represent results from three independent experiments.

Amplification efficiency

To test the sensitivity of the assay using PP6, we initially performed absolute standard curves from known quantity of DNA gene copies. Full length 16S rRNA gene (1509 bp) was PCR amplified from *L. interrogans* genomic DNA and cloned into the pGEM®-T Easy Vector System in *E. coli*. The full length gene was purified and plasmid DNA purity verified by agarose electrophoresis and Nanodrop quantitation. We calculated the exact number of gene copies and made serial dilutions from 10^9 to 1 gene copies to develop an absolute standard curve for the assay. The default molar mass of DNA (650 g/mol/bp) was used with the

pGEM_LIC_16S plasmid size (4,524 bp) and measured DNA concentration to calculate the exact number of DNA molecules using the equation:

$$\# \text{ 16S copies/ng DNA} = (X \text{ ng DNA} * 6.022 \times 10^{23}) / (4524 \text{ bp} * 1 \times 10^9 \text{ ng/g} * 650 \text{ g/mol} * \text{bp})$$

The amplification efficiency of the assay was assessed over serial dilutions containing 6×10^9 to 6 gene copies per reaction well. All replicates amplified down to 6 gene copies per reaction, but not 3 gene copies per well (Fig.17A). The amplification efficiency over the 9-fold serial dilution was 96.9% with a linear regression of 0.997 (Fig. 17B). Additionally, in all wells this amplification gave a single specific product as shown by the specific melt peak at 80.0°C (Fig. 17C). Standard curves and amplification efficiency of the reactions were calculated by the CFX96 instrument software.

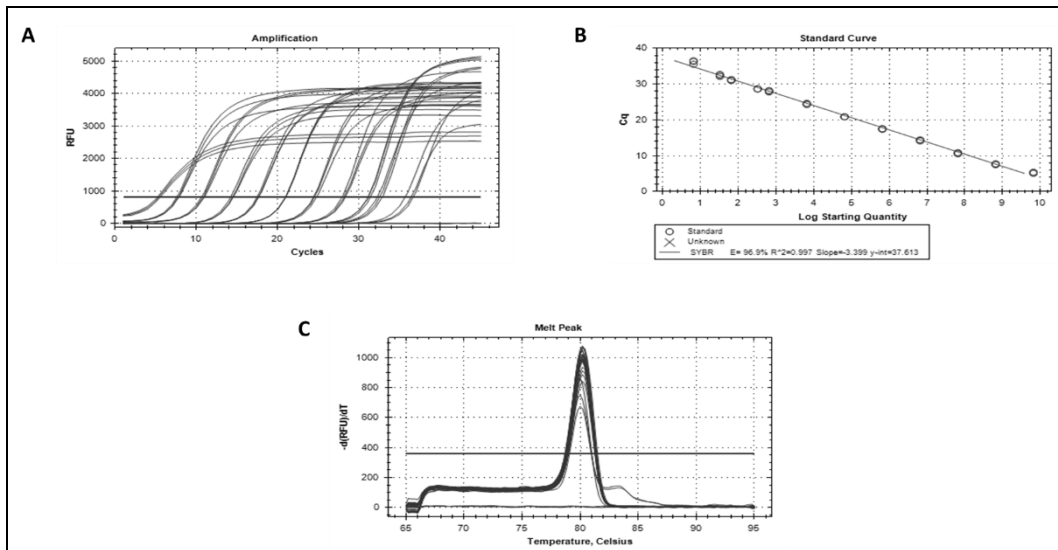


Figure 17: Standard curve for 16S rRNA amplification. The absolute number of 16S gene copies was determined from purified plasmid pGEM_LIC_16S based on size and molecular weight. A standard curve was run on serial dilution of known gene copies from 6×10^9 to 6 gene copies per reaction well showing A) amplification curves, B) amplification efficiency, and C) melt curve analysis.

Analytical sensitivity

We next assessed the sensitivity of our improved 16S RNA-based diagnostic assay as before using spiked human blood samples for comparison of RNA and DNA-based qPCR detection. In simulated infected samples, we found that RNA-based assays were at least 100-fold more sensitive than a DNA-based approach, with limits of detection of one bacterium versus 100 per milliliter of blood, respectively. Amplification was seen in all samples over the range of 10^6 to 1 spiked bacteria per mL of blood (Fig. 18A). Amplification efficiency over this range was 108.0% (Fig. 18B) with a linear regression of 0.988, and all targets gave a specific melt peak (Fig. 18C). qPCR of extracted DNA had a 100-fold lower sensitivity of detection (Fig. 18D).

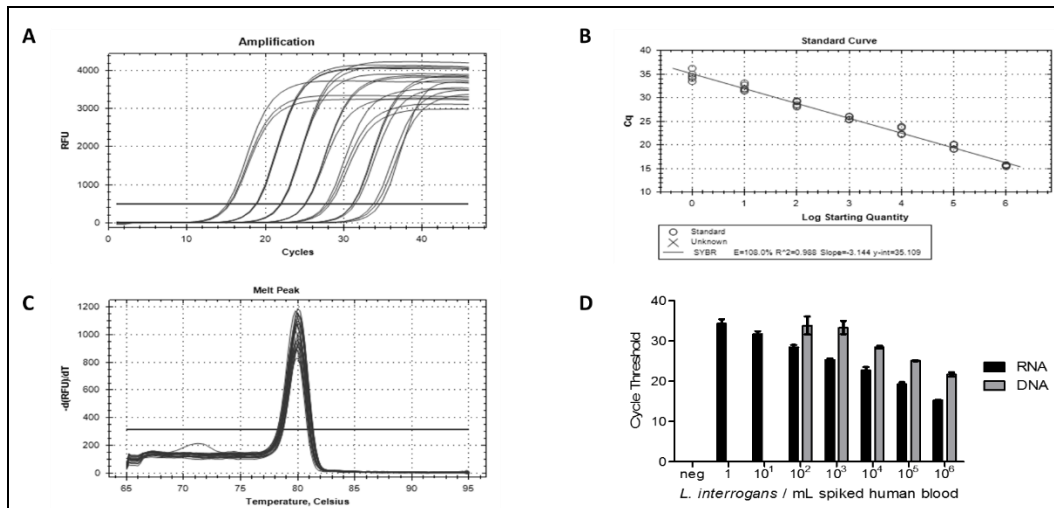


Figure 18: RNA-based detection is more sensitive than DNA. *L. interrogans* were serially diluted from 10^6 to 1 bacterium per milliliter in aliquots of human blood and used for either RNA-based RT-qPCR showing A) amplification curves, B) amplification efficiency, C) melt curve analysis, and D) comparison to DNA-based qPCR performed in tandem. Data represent results from three independent experiments.

Stability of *L. interrogans* 16S transcripts in human blood

We know from previous results and manufacturer's data that TRIzol stabilizes RNA transcripts extremely well. Additionally, we showed previously that leptospiral RNA transcripts were detected with relative stability at varying storage conditions over time (Fig. 14). However, RNA transcripts may not be uniformly stable, as RNA structure will affect the stability of individual transcripts. We compared the window of 16S detection in untreated spiked blood samples with our new assay using primer PP6. Spiked human blood samples containing 100 *L. interrogans* per milliliter of blood were stored aliquots at room temperature, 4°C, -20°C, or -80°C for 1, 4, and 14 days without the addition of TRIzol. Samples immediately homogenized in TRIzol and frozen at -80°C served as the "0 hour" or baseline control for 16S RNA stability experiments. We measured the transcript levels by RT-qPCR analyses for changes in 16S rRNA transcript detection as compared to our baseline control. Similar to our original assay, we recorded a significant loss of 16S RNA in samples stored at room temperature within one day. However, we were still able to detect some level of 16S rRNA after two weeks storage at room temperature (Fig. 19). Additionally, our assay showed no statistically significant decrease in 16S rRNA detection after seven days storage at room temperature and two weeks of storage at -20°C, or -80°C.

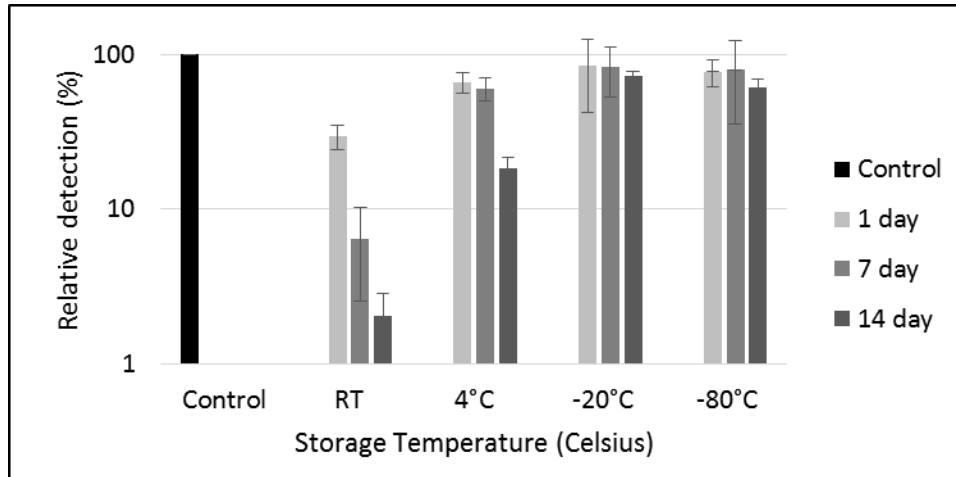


Figure 19: Stability of *Leptospira* 16S transcripts in spiked human blood. Aliquots of human blood were spiked with leptospires (100 cells/ml), and stored at various temperatures in the absence of any RNA stabilization agent for up to 14 days. Detection of 16S was monitored by RT-qPCR analyses, relative to baseline control samples representing 100% detection. Data represent results from three independent experiments.

Assessment of clinical cases of human leptospirosis

Finally, we repeated testing of the sensitivity and specificity of our assay for the detection of *Leptospira* in blood samples obtained from patients, with suspected leptospirosis. Results from our RT-qPCR with PP6 were compared to previous RT-qPCR results with PP1 and qPCR from DNA extractions. From 22 confirmed leptospirosis cases, *Leptospira* RNA was previously detected using PP1 in 14 samples, yielding a sensitivity of 64%. **However, in our new assay with PP6, *Leptospira* RNA was detected in 18 samples, yielding a marked improvement in sensitivity to 82% (Table 10).** Two of the three suspected probable clinical cases were again found to have detectable leptospiral RNA. Finally, our new assay retained 100% specificity as all blood samples obtained from 24 healthy control individuals showed no amplification.

Table 10: Detection of leptospiral RNA by optimized quantitative PCR in blood samples collected from humans with suspected leptospirosis

Assay results	Confirmed leptospirosis (N=22)	Probable leptospirosis (N=3)	Healthy Subjects (N=24)
Number (%)			
RNA and DNA PCR+	6 (27)	0 (0)	0 (0)
RNA PCR+ alone	12 (55)	2 (67)	0 (0)
DNA PCR+ alone	1 (5)	0 (0)	0 (0)
Total RNA (PP1) PCR+	14 (64)	2 (67)	0 (0)
Total DNA PCR+	7 (32)	0 (0)	0 (0)
Total RNA (PP6) PCR+	18 (82)	2 (67)	0 (0)

4.4 Discussion

Clinical diagnosis of leptospirosis remains extremely difficult due to inadequate diagnostic methods (Ahmed, van der Linden et al. 2014; Chirathaworn, Inwattana et al. 2014; Panwala, Rajdev et al. 2015; Iwasaki, Chagan-Yasutan et al. 2016). Levels of antigens and antibodies against them remain too low or variable during early infection to be definitively detected, and physical symptoms significantly overlap with numerous other tropical diseases, preventing differential diagnosis (Bruce, Sanders et al. 2005; Musso and La Scola 2013; Reller, Wunder et al. 2014; Waggoner, Abeynayake et al. 2014). Recent efforts have attempted to use molecular diagnosis for detection of highly specific DNA molecules from pathogenic leptospires (Perez and Goarant 2010; Agampodi, Matthias et al. 2012; Natarajaseenivasan, Raja et al. 2012; Iwasaki, Chagan-Yasutan et al. 2016). These assays have shown very high specificity, but often lack the sensitivity to detect the extremely low levels of leptospires (less than 10^2 - 10^3 bacteria per milliliter) in sample biopsies during early infection (Smythe, Smith et al. 2002; Slack, Symonds et al. 2006; Kositanont, Rugsasuk et al. 2007; Stoddard, Gee et al. 2009; Villumsen, Pedersen et al. 2010; Boonsilp, Thaipadungpanit et al. 2011; Koizumi, Nakajima et al. 2012). We

previously reported on a rapid, sensitive, and specific reverse transcription quantitative PCR assay for detection of human leptospirosis utilizing 16S rRNA, which while superior to many other diagnostic methods performed, still resulted in confirmation of infection for only 64% of confirmed cases (Backstedt, Buyuktanir et al. 2015). Here, our new assay shows a remarkable increase in the sensitivity of detection to 82% using the same clinical patient sample set but using a new set of optimized primers. Results from most individual patient samples showed agreement between assays, and both assays showed 100% specificity among human samples.

The dramatic increase in our assay sensitivity, especially while using the same diagnostic target is remarkable, which may be influenced by numerous factors. First, the new assay targets a different region of 16S rRNA. We also found that the amplification efficiency of the assay using PP6 was increased slightly from 93.6% in our initial assay to 96.9% here, using an absolute standard curve. Secondly, certain regions of RNA transcripts may show significant differences in stability due to the binding to transacting-factors, sequence and secondary structures at those locations (Monis and Giglio 2006; Bustin, Benes et al. 2009). Additionally, the secondary structure may affect the ability of a given primer to anneal to template sequences (Gudnason, Dufva et al. 2007). In fact, the predicted structure of leptospiral 16S rDNA sequence at 60°C, the annealing and extension temperature used for our assay, showed that while most of the sequence remains unfolded at this temperature, some regions begin to form small stem-loop structures (data not shown) (Zuker 2003). These structures might affect the ability of primers to anneal to the template sequences in these regions, and thereby decrease amplification of the target.

In addition to SYBR® Green-based qPCR testing as described here, it may be possible to apply this rRNA detection method towards other molecular diagnostic technologies, including digital droplet PCR (ddPCR), which may provide even higher throughput as well as sensitivity to be tested in a centralized facility like hospital, or field tests for resource-limited areas using Loop Mediated Isothermal Amplification (LAMP) (Parida, Posadas et al. 2004; Imai, Ninomiya et al. 2007; Jayawardena, Cheung et al. 2007; Lucchi, Demas et al. 2010; Sonthayanon, Chierakul et al. 2011; Zhang, Brown et al. 2011; Curtis, Niedzwiedz et al. 2014). Applications of these unique technologies could enable vast improvements in epidemiological surveillance and lower the health and economic burden of this prevalent worldwide disease.

Chapter 5: Development of novel RNA-based molecular diagnostics of human Lyme borreliosis

5.1 Introduction

As discussed earlier, Lyme disease is the most common vector-borne infectious disease in the United States and Europe that is caused by spirochetes of the *Borrelia burgdorferi sensu lato* complex, including *B. burgdorferi sensu stricto*, *B. garinii*, and *B. afzelii* (Rudenko, Golovchenko et al. 2011). Despite substantial advances in the field, diagnosis of early stages of the disease or discrimination of active versus resolved infection remains challenging (Nichols and Windemuth 2013; Schriefer 2015). Detection of the pathogen based on identity of microbial components or host response is problematic due to the occurrence of extremely low pathogen levels and highly variable and non-specific immune responses (Auwaerter, Aucott et al. 2004; Liveris, Schwartz et al. 2011). Antibiotic therapy is usually effective if administered early during infection. However, inaccurate or misdiagnosis and inappropriate treatment may result in further complications of disease (Duray 1989; Gerstenblith and Stern 2014; Peacock, Gherezghiher et al. 2015). No vaccine currently exists for the prevention of Lyme disease in humans (Bhate and Schwartz 2011; Kaaijk and Luytjes 2016). Therefore, improvements in the accuracy and sensitivity of diagnostic methods, particularly early diagnostics, are essential for limiting the impact of this global zoonotic disease.

Many distinct diagnostic assays have been evaluated for diagnosis of Lyme disease using both direct and indirect detection methods (Alby and Capraro 2015;

Moore 2015; Leeflang, Ang et al. 2016). The manifestation of erythema migrans is sufficient for clinical diagnosis, but does not develop in up to 30% of cases (Aguero-Rosenfeld, Wang et al. 2005; Stanek 2013). Current recommendations from the CDC and Infectious Disease Society of America (IDSA) advise a two-tiered diagnosis involving an enzyme linked immunoassay followed by immunoblotting to confirm the infection. Direct detection of bacteria from patient samples using culture or specific antigen detection are difficult due to the low bacterial burden in clinical samples (Wilske 2005; Cerar, Ruzic-Sabljić et al. 2008). The utility of direct culture from patient biopsies for diagnosis is negated by the specialized media and length of time required for results (Liveris, Schwartz et al. 2011). PCR detection of samples from total DNA extraction has proven successful (Ferdin, Cerar et al. 2010; Lee, Vigliotti et al. 2010; Lee, Vigliotti et al. 2014; Reiter, Schotta et al. 2015), but is not commonly used due to wide variations in sensitivity in tissues such as cerebrospinal fluid, synovial fluid, and blood.

Previous work in related spirochete pathogens has shown significant increases in the sensitivity of detection when using an RNA-based detection assay, as opposed to DNA-based detection (Backstedt, Buyuktanir et al. 2015). Since, Lyme disease shows a very low level and transient spirochetemia in the blood during early infection, we hypothesized that an assay based on the PCR amplification of cDNA molecules representing highly and consistently transcribed genes like 16S or 23S rRNA (Clarridge 2004; Beissner, Symanek et al. 2012; de Leeuw, Maraha et al. 2014), might be more effective where DNA-based nucleic acid amplification tests have proven inadequate. Notably, we also show that rRNA molecules are remarkably

stable, even in the absence of any chemical preservative. Here we report a reverse transcription quantitative PCR (RT-qPCR) that provides sensitive and specific detection of *B. burgdorferi sensu lato* and can be rapidly performed. These results show the need for further testing using blood samples of human Lyme disease to validate the potential application for clinical diagnostics.

5.2 Materials and methods

Bacterial strains

Borrelia burgdorferi strain B31-A3 (Elias, Stewart et al. 2002), was used in most parts of the study. In some experiments, additional *Borrelia* species were also used. *Borrelia* cultures were grown in Barbour-Stoner-Kelly (BSK) media at 34°C stationary incubator. Additional bacterial strains, such as an *E. coli* K-12 derivative, *Streptococcus pyogenes* Strain D471 (Raz, Talay et al. 2012), and *L. interrogans* (Ricaldi, Fouts et al. 2012) were also used in certain experiments and grown by using the standard media and protocols. *B. hermsii* RNA was provided from Dr. Kishore Allugupalli.

Samples

Naïve or infected tick samples were prepared from a tick colony maintained in the laboratory. Naïve or infected ticks were fed on uninfected mice and collected after repletion for RNA extraction and cDNA synthesis, as detailed (Kumar, Yang et al. 2010). Mice were infected by tick feeding or intraperitoneal injection of 10^5 spirochetes per mouse. Blood samples from naïve and infected C3H/HeJ mice, a

species commonly used as an animal model of Lyme disease (Pal, Wang et al. 2008), were collected into 1.5 mL micro-centrifuge and directly homogenized with Trizol reagent or collected into BD Vacutainer® whole blood collection tubes containing EDTA (BD Diagnostics) at the indicated time points after infection. Whole blood from 24 healthy individuals residing in non-endemic regions in the U.S. were also collected or purchased (SeraCare Life Sciences). Purchased samples were collected fresh and shipped on ice. From the whole blood EDTA tubes, 250 µL of blood was aliquoted into 750 µL of TRIZOL LS, thoroughly homogenized, and immediately frozen at -70°C until RNA purification and cDNA synthesis were performed.

Extraction of nucleic acids and cDNA synthesis

Total RNA samples were extracted from samples stored in TRIzol (for bacterial pellets harvested from cultures grown at mid to late log phases), or TRIzol-LS (for blood samples) according to the manufacturer's (Life Technologies) instructions. After phase separation, RNA samples were either precipitated with isopropanol, dissolved in 20µL of RNase-free water and subjected to optional DNaseI treatment (NEB laboratories), or further purified using an RNeasy mini kit (Qiagen). For cDNA synthesis, 0.5 µg of RNA samples was reverse transcribed using VILO superscript master mix (Life Technologies) according to the manufacturer's protocols. For extraction of DNA, spiked blood samples were processed using a DNeasy mini kit (Qiagen) according to the manufacturer's instructions and eluted into 100 µL nuclease-free sterile water (Backstedt, Buyuktanir et al. 2015).

Primer design

The initial primers used for qPCR reaction were designed using the NCBI Primer-BLAST primer design program based on the available *B. burgdorferi* genomic sequences (Altschul, Gish et al. 1990; Ye, Coulouris et al. 2012). Based on genome data and published information of the transcript abundance, we designed initial primers against gene targets, *16S rRNA*, *23S rRNA bb_0034*, *flaB*, *5S-23S* intergenic spacer region, and *bb_B19 (ospC)* as seen in Table 11.

Table 11: Primers used for initial *Borrelia* diagnosis target screening

Annotation	Sequence (5'-3')
<i>bb_0034</i> F	GCCCTATACCAACCGCATCA
<i>bb_0034</i> R	CGTTTGGGATGAGTGCTGGA
<i>5S-23S</i> intergenic F	AGGGATTCCGGATAAGATTGCCA
<i>5S-23S</i> intergenic R	TGAGTGTCGGGCAAATCCAA
<i>osp_C</i> F	GGTTGAAGCGTTGCTGTCAT
<i>osp_C</i> R	TTGCATAAGCTCCCGCTAACA
B31_23S F	GACAACCATCGCCTAGCAGT
B31_23S R	AGGTCGTCTTCCCAGGGTTA
B31_16S F	ATCCGGACTGAGACCTGCTT
B31_16S R	AAGTCGGAGGAAGGTGAGGA
B31_16S F	TTGCTGATCAAGCTCAATATAACCA
B31_16S R	TTGAGACCCTGAAAGTGATGC

To further identify primers of greatest sensitivity and specificity, we aligned the 16S and 23S rRNA sequences of 15 *Borrelia* species, including Lyme disease and relapsing fever group *Borrelia* species, and numerous other spirochetes with the most closely related rRNA sequences (i.e. *Treponema*) using the MegAlign program (DNASTAR) Clustal V method. We designed several forward and reverse primers to use in paired combination expected to amplify a specific region of 16S or 23S gene.

The primers and pairings used are indicated in Table 12. We identified gene regions that retain near absolute homology between all *B. burgdorferi sensu lato* species that exists globally, but a number of nucleotide mismatches to relapsing fever group *Borrelia* and other species.

Table 12: *Borrelia* specific 16S and 23S primer combinations

Primer pair name	Amplicon length	Primer name	Sequence (5'-3')
Bb1	82 bp	B31_16S_582-603 F	GCGGATATATAAGTCTATGCAT
		B31_16S_643-664 R	CTCTATCAGACTCTAGACATAT
Bb2	260 bp	B31_16S_582-603 F	GCGGATATATAAGTCTATGCAT
		B31_16S_820-842 R	ACTTTTAGTTAACACCAAGTGTG
Bb3	568 bp	B31_16S_582-603 F	GCGGATATATAAGTCTATGCAT
		B31_16S_1131-1150 R	CTTATCTGAGTCCCCACCAT
Bb4	199 bp	B31_16S_643-664 F	ATATGTCTAGAGTCTGATAGAG
		B31_16S_820-842 R	ACTTTTAGTTAACACCAAGTGTG
Bb5	507 bp	B31_16S_643-664 F	ATATGTCTAGAGTCTGATAGAG
		B31_16S_1131-1150 R	CTTATCTGAGTCCCCACCAT
Bb6	330 bp	B31_16S_820-842 F	CACACTTGGTGTAACTAAAAGT
		B31_16S_1131-1150 R	CTTATCTGAGTCCCCACCAT
Bb7	235 bp	B31_23S_157-178 F	TCTAAATAATAGAGGCGATACC
		B31_23S_370-392 R	CTTTGGTTAACTTTCCAGATTA
Bb8	546 bp	B31_23S_157-178 F	TCTAAATAATAGAGGCGATACC
		B31_23S_685-703 R	TCGACTCCAGCACTTCTAT
Bb9	333 bp	B31_23S_370-392 F	TAATCTGGAAAGTTAACCAAAG
		B31_23S_685-703 R	TCGACTCCAGCACTTCTAT
Bb10	249 bp	B31_23S_1554-1578 F	CGATGATCTTAATAGGAAAATCCGT
		B31_23S_1781-1803 R	CTACCTAATTGCTTAGGTCGTAC

The specificity of each newly designed primer was initially searched against all reference mRNA sequences using NCBI BLASTn as well as Primer-BLAST programs to rule out possible cross-reactivity with other bacteria species as well as non-targeted species including human, mice, and ticks prior to empirical testing. Prior to their use in RNA measurement assays, each primer pair was tested for

efficiency and non-specific amplification by melt-curve analysis using *B. burgdorferi* genomic DNA as a template (Gudnason, Dufva et al. 2007).

Polymerase chain reaction

The oligonucleotide sequences for each of the primers used in specific PCR reactions are indicated previously in Table 11 and 12. The relative levels of cDNA templates in each sample were assessed by qPCR and RT-qPCR as detailed, and whenever necessary, DNA contamination in each sample was measured using an equal volume of purified RNA as a template. All qPCR reactions were performed using the CFX96 real time PCR detection system (Bio-Rad) with the following thermal cycle conditions: 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds, followed by a melt curve from 65°C to 95°C performed at an increment of 0.5°C per cycle, unless otherwise indicated. All qPCR plates included no template control wells to test for non-specific amplification or reagent contamination, and results were further checked for specificity by melt curve analysis. Detection of human, mouse, or tick β -actin transcripts by qPCR confirmed the integrity of cDNA samples.

Validation of primers

One milliliter of *B. burgdorferi* culture was harvested at 1.2×10^8 spirochetes per ml by centrifugation at 5000 g at room temperature and subjected to RNA isolation as detailed above. For relative assessment of each primer set, 2.5 μ g RNA samples were reverse transcribed into cDNA, which were used in initial RT-qPCR

reactions for all primers. cDNA samples were also prepared from a number of other *Borrelia* strains and species. For standard curves, full length 16S and 23S rRNA genes were PCR amplified from *B. burgdorferi* genomic DNA using primer in Table 13 and cloned into *E. coli* DH5 α using the pGEM[®]-T Easy Vector System.

Table 13: Primers for full length *Borrelia* 16S and 23S gene amplification

Primer	Sequence (5'-3')
16S full length Forward	AAATAACGAAGAGTTTGATCCTG
16S full length Reverse	AAAGGAGGTGATCCAGCC
23S full length Forward	GGTCAAAGTAATAAGAGTCTATGGT
23S full length Reverse	ATACGGGTAATTAGTATTAGTCAGC

From 5 mL *E. coli* culture, plasmid was purified using a Qiagen Miniprep kit and DNA concentration and purity was measured by electrophoresis and Nanodrop quantitation. Utilizing the concentration of purified plasmid DNA and the total size of each plasmid containing 16S or 23S rRNA, the number of gene copies was calculated to develop an absolute standard curve from 10⁹ to one gene copies per reaction. Standard curves and amplification efficiency of the reactions were calculated by the CFX96 instrument software, as instructed by the manufacturer.

For spiking experiments, 250 μ L of untreated whole human blood samples were spiked in triplicate with *B. burgdorferi* strain B31 derived from three independent cultures at various concentrations ranging from 10⁶ - 10⁰ cells/mL. Samples were used for DNA or RNA extraction using a GenElute Blood Genomic DNA Kit (Sigma) or TRIzol extraction procedure, and the RNA samples were further processed for cDNA synthesis. As controls for assessment of specificity, cDNA samples were also isolated from additional bacterial culture grown at late log phases

including *L. interrogans*, *E. coli*, and group A *Streptococcus* strains, as well as from uninfected human blood, mouse blood, and *Ixodes scapularis* ticks.

RNA stability studies

We tested the stability of the rRNA molecules using various parameters, in the presence or absence of preservatives. Triplicate samples of 250 μ l whole human blood collected in EDTA tubes were spiked with viable *B. burgdorferi* strain B31 (100 cells/ml) derived from at least three independent cultures. Aliquots were homogenized with 750 μ L TRIzol LS and stored at room temperature for 0, 24, 72, or 120 hours before freezing at -80°C until analysis. In parallel experiments, triplicate aliquots of 250 μ L human blood containing 100 bacteria per ml were stored at room temperature, 4°C , -20°C , or -80°C in the absence of any stabilization reagent. Samples were collected directly into 750 μ L TRIzol LS after incubating at the aforementioned temperatures for 0 hours, 24 hours, 7 days, and 14 days, and stored at -80°C until completion of all timepoints. RNA was extracted, reverse transcribed to cDNA, and analyzed by qPCR using *Borrelia*-specific 23S detection primers.

Statistical analysis

Results are expressed as the mean \pm standard error of the mean (SEM). The significance of the difference between the mean values of the groups was evaluated by unpaired Student t test and ANOVA.

5.3 Results

Development of a 23S rRNA-based quantitative PCR assay for detection of *B. burgdorferi sensu lato* complex

We wanted to assess if an RNA-based RT-qPCR assay could give sensitive and specific detection of *Borrelia* in human blood samples. We sought to develop a SYBR® Green-based RT-qPCR assay due to its ease of setup and low cost as compared to TaqMan® assays. For identification of an RNA target that yields the most sensitive detection, we initially examined a set of highly expressed rRNA and mRNA gene targets: *16S rRNA*, *23S rRNA*, *bb_0034*, *flaB*, the *5S-23S* intergenic spacer region, and *bb_B19*. We selected these genes due to their known constitutively high transcript expression levels, sequence homology within the *B. burgdorferi sensu lato* (s.l.) complex, and divergence from other non-target species. We used NCBI Primer-BLAST software to identify unique regions in 16S and 23S gene and created forward and reverse primers specific to *B. burgdorferi* sequences but containing several nucleotide mismatches to non-target bacterial species including relapsing fever group *Borrelia* species (Ye, Coulouris et al. 2012). Primers were also checked against nucleotide similarity to mammalian and tick species. All gene-specific primers had a similar annealing temperature and comparable amplicon size. Total RNA was extracted from *B. burgdorferi* strain B31 culture grown until log phase, converted into cDNA, and used in a SYBR® Green-based qPCR assay. Sensitivity of the assay (or the relative abundance of the transcripts) was assessed based on the cycle threshold for target amplification, while specificity of the target gene amplification was assessed using melt-curve analysis. We found that the 16S

and 23S rRNA primers offered the most sensitive detection, as evidenced by the lowest C_t values, and were nearly 200-fold more abundant than the mRNA target, *bb_0034* or integral membrane porin P13 (Fig. 20).

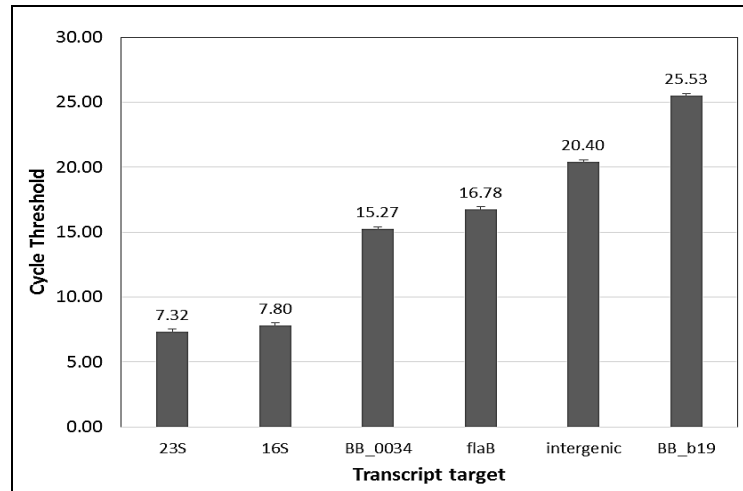


Figure 20: RT-qPCR detection of *B. burgdorferi* cDNA with various gene targets. RT-qPCR was performed using cDNA from *in vitro* grown *B. burgdorferi* with different gene targets. All samples contained equal amount of cDNA. Differences in sensitivity were estimated based upon the detection threshold cycle where lower value determines more sensitive detection.

Since 16S and 23S rRNA sequences are highly conserved among prokaryotes, we performed multiple sequence alignments of 16S and 23S rRNA from various *Borrelia* species and most closely related rRNA sequences of other non-target species. Based on the sequence alignments, we designed and evaluated several different primer sets for each target to select the highest sensitivity and specificity, and an optimized amplification efficiency under our assay conditions. The primer pairs as indicated in Table 12 were tested by RT-qPCR using cDNA from *in vitro* grown *B. burgdorferi*. Our results gave several promising primer pairs targeting both 16S and 23S rRNA at a similar level of detection (Fig. 21). Based on further analysis

of gene sequence alignments and empirical testing with uninfected human, mouse, and tick cDNA, this list of primers was narrowed down to select primer pair Bb10 as the optimal candidate.

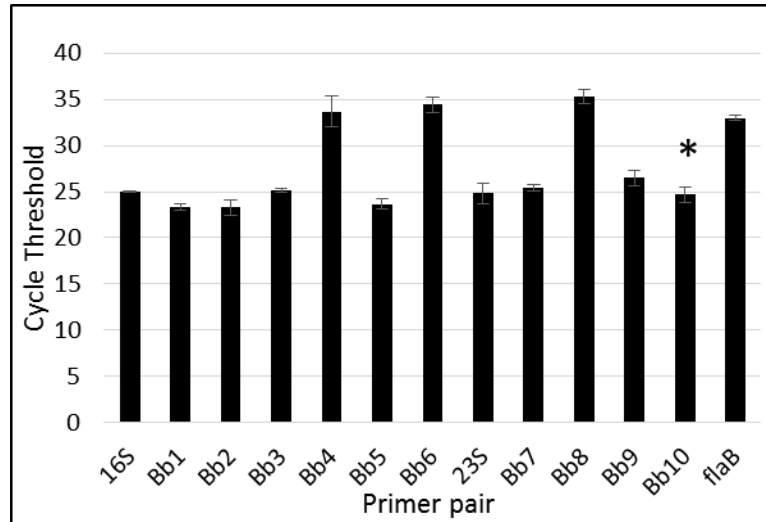


Figure 21: RT-qPCR detection of *B. burgdorferi* cDNA with various primer pairs targeting 16S and 23S rRNA. RT-qPCR was performed using cDNA from *in vitro* grown *B. burgdorferi* with different gene targets. All samples contained equal amount of cDNA. Differences in sensitivity were estimated based upon the detection threshold cycle where lower value determines more sensitive detection.

Amplification efficiency

To test the amplification efficiency, we created a plasmid containing the full-length 23S gene amplified from genomic DNA and cloned into pGEM T-easy plasmid as stated in the Materials and methods section. This plasmid was amplified and purified from *E. coli* and used to develop an absolute standard curve with a known number of gene copies. The default molar mass of DNA (650 g/mol/bp) was used with the pGEM_B31_23S plasmid size (5,949 bp) and DNA concentration to calculate the exact number of DNA molecules using the equation:

$$\# \text{ 23S copies/ng DNA} = (X \text{ ng DNA} * 6.022 \times 10^{23}) / (5949 \text{ bp} * 1 \times 10^9 \text{ ng/g} * 650 \text{ g/mol} * \text{bp})$$

We previously selected a primer set targeting 23S rRNA, primer pair Bb10, as the ideal candidate for all subsequent experiments. In order to further optimize conditions for amplification, we analyzed the amplification efficiency curves of template serial dilutions against temperature gradient annealing and extension conditions from 57.2°C to 60.0°C. The results showed up to 10000 fold decrease in amplification at the lower end of the temperature range as we could only detect down to 10^5 gene copies at 57.2°C annealing/extension. However, from 58.9°C to 60°C, we could similarly detect over the entire 7-log range of dilution at a similar level with strong linearity and amplification efficiencies between 90-100% (Fig. 22).

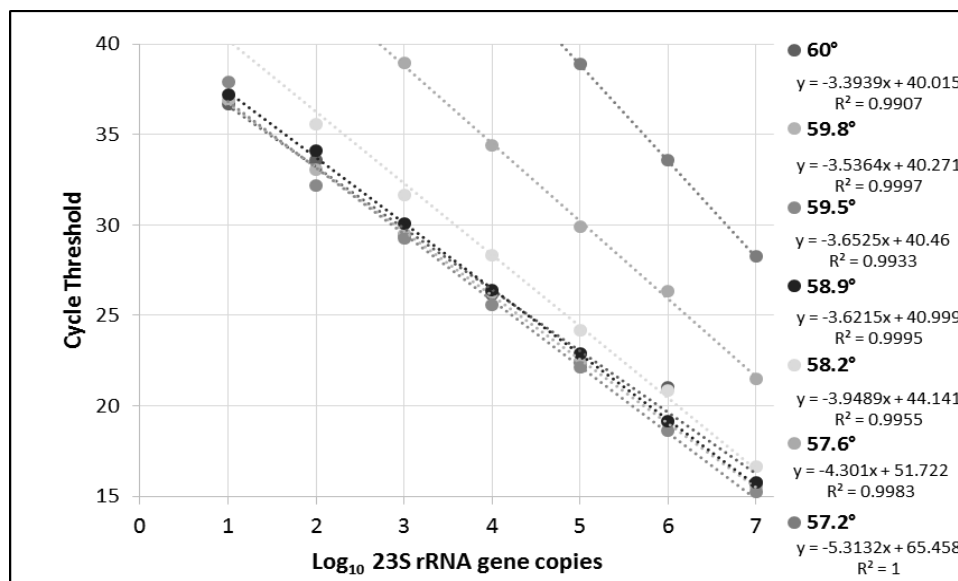


Figure 22: Optimization of assay for 23S detection using primer pair Bb10. Optimal temperature for annealing and extension in 60°C. A serial dilution of 10^7 - 10^1 23S gene copies was amplified by qPCR using primer pair Bb10 over a thermal gradient.

After optimization of the annealing temperature of the assay for this primer set to 60° C, the amplification efficiency was validated more in depth using a serial dilution containing 10^9 to 1 gene copies per reaction well (Fig. 23A). All replicates

amplified down to five gene copies per reaction, as well as 50% of replicates at a concentration of two and one gene copies per reaction. The amplification efficiency over the 9-fold serial dilution was 93.6% with a linear regression of 0.995 (Fig. 23B). Additionally, all wells gave amplification of a single specific product as shown by the specific melt peak at 81.0°C (Fig. 23C) and analysis of product amplicons by DNA electrophoresis on a 1.5% agarose gel (Fig. 23D).

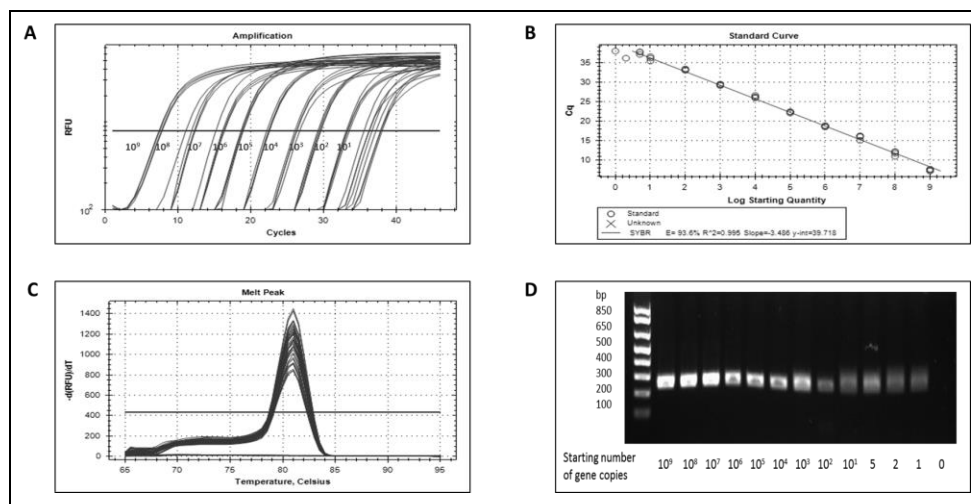


Figure 23: Standard curve for 23S rRNA amplification by primer pair Bb10.

The absolute number of 23S gene copies was determined from purified plasmid pGEM_B31_23S based on size and molecular weight. A standard curve was run on serial dilution of known gene copies from 10^9 to 1 gene copies per reaction well showing A) amplification curve, B) amplification efficiency, C) specific melt temperature, and D) specific amplicon by DNA electrophoresis.

Analytical sensitivity

We next assessed whether sensitivity of our 23S RNA-based assay is superior to a DNA-based assay and tested its specificity with experimentally spiked human blood samples. We spiked serially diluted *B. burgdorferi* cells into 250 μ l aliquots of human blood and isolated RNA and DNA samples as detailed in the Materials and Methods. We subsequently performed RT-qPCR assays using corresponding cDNA

and DNA templates. Amplification was seen in all samples over the range of 10^6 to 1 spiked bacteria per mL of blood (Fig. 24A). Amplification efficiency over this range was 104.1% with a linear regression of 0.981 (Fig. 24B) and all targets gave a specific melt peak (Fig. 24C). qPCR of extracted DNA was also detectable over the range of 10^6 to 10 spiked bacteria per mL of blood, however with higher Ct values, lower amplification efficiency, and greater standard deviation (Fig. 24D). Based on the differences in Ct values between comparative RNA and DNA based sample detection, we found that RNA-based assays were at least 100-fold more sensitive than a DNA-based approach.

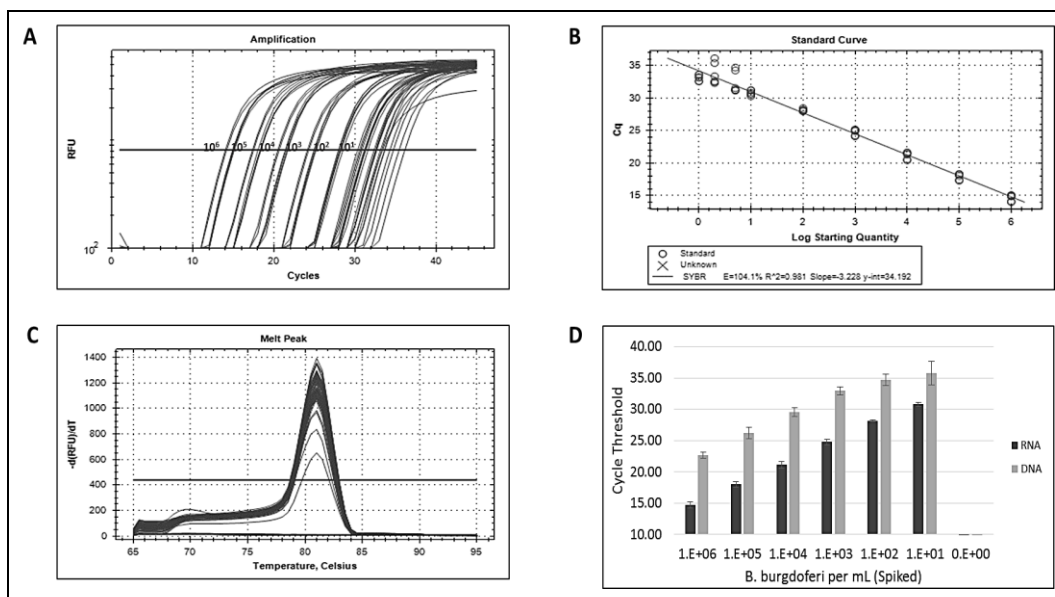


Figure 24: Detection of *B. burgdorferi* in spiked blood by qPCR. RNA-based detection provides higher sensitivity of detection. Samples of 250 μ L of human blood were spiked with *B. burgdorferi* at a concentration from 10^6 to 1 bacteria per mL in triplicate from independent cultures for subsequent RNA or DNA extraction. A) Resulting amplification curves from cDNA of spiked human samples. B) Amplification efficiency from cDNA of simulated infected samples. C) All amplified products gave a singular, specific melt peak. D) Comparison of detection from spiked human blood between cDNA (from total RNA extraction) and total DNA templates.

Analytical specificity

We next determined the specificity of our 23S RT-qPCR assay in the detection of multiple *B. burgdorferi* species and the specificity of the assay against non-target species, including *Borrelia hermsii*, human, mouse, and tick cDNA. For this purpose, we prepared cDNA samples from six different *Borrelia* strains, *L. interrogans*, group A *Streptococcus*, *E. coli*, and uninfected tick, mouse and human samples. We found that the assay was able to detect all five *B. burgdorferi sensu lato* complex species tested, but did not cross react with any other bacterial species tested, including the relapsing fever pathogen, *Borrelia hermsii* (Fig. 25A). Additionally, no amplification was observed from uninfected tick, mouse, or human samples. Furthermore, we screened our 23S rRNA RT-qPCR assay using cDNA prepared from 20 unique human blood samples and saw no cross-amplification from our primers with unintended targets (Fig. 25B). A positive control gene for human cDNA samples, human β -actin, could be detected in all samples at equivalent levels, as well as a primer positive control that amplified *Borrelia* cDNA.

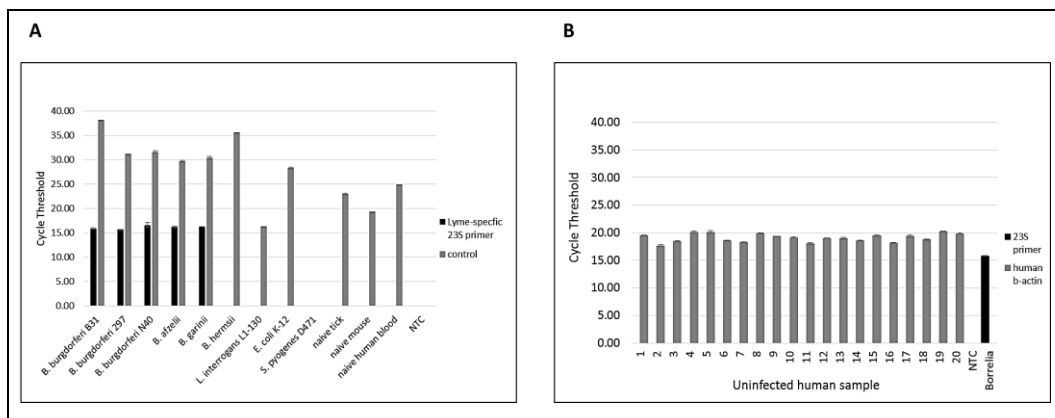


Figure 25: Specificity of assay for *Borrelia burgdorferi sensu lato* (s.l.) complex. RT-qPCR amplification has 100% specificity. A) Primer pair Bb10 specifically amplifies *B. burgdorferi sensu lato* complex species, but does not cross react with relapsing fever group *Borrelia* or other species. B) From 20 independent human blood samples, no cross reaction

was seen from primer pair Bb10. Specific and non-specific controls were used to exemplify cDNA was present in all samples.

Stability of *B. burgdorferi* 23S rRNA transcripts in human blood

The logistics of samples collection and RNA stabilization are potential concerns for our assay due to the relatively unstable nature of RNA molecules. Ideally, the biomarkers or the diagnostic targets should remain stable in the event of variable blood storage times and temperatures after collection. Therefore, we compared the amplification of 23S rRNA transcripts in spiked human blood samples stored at room temperature in TRIzol to that of untreated spiked blood samples under various storage conditions. We also tested how storage temperature of blood samples affected the detection of 23S rRNA targets in our assay. To perform these experiments, we spiked human blood with *B. burgdorferi* using 100 bacteria per milliliter of blood and stored aliquots at room temperature, 4° C, -20° C, or -80°C for up to 14 days either with or without the addition of TRIzol. A sample immediately stored in TRIzol and frozen at -80°C served as the baseline for our stability experiments.

We performed RNA extraction and cDNA synthesis, and measured the transcript levels by RT-qPCR analyses. Interestingly, we could detect similar levels of 23S rRNA targets from samples stored at room temperature up to our 14-day endpoint (Fig. 26A). In contrast, a significant degradation of transcripts for a highly abundant control gene, human β -actin, was observed (Fig. 26C). This indicates possible lysis of blood cells and degradation of human RNA in samples, but perhaps survival of bacteria and persistence of *Borrelia* and its rRNA molecules. As

expected, we observed equivalent levels of 23S rRNA and human β -actin target detection from all samples up to five days stored in TRIzol at room temperature (Fig. 26 B and D), as RNA had been stabilized.

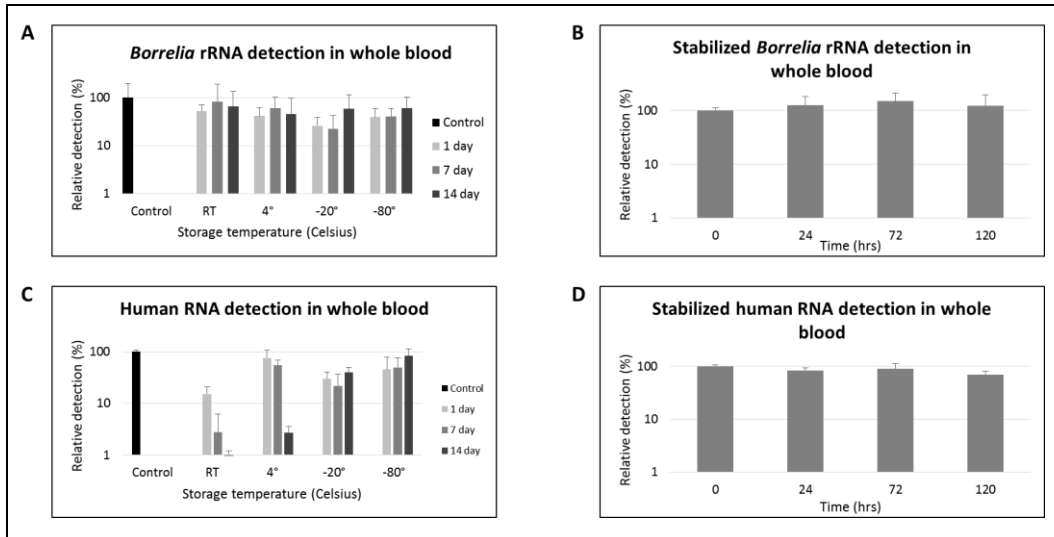


Figure 26: Stability of 23S transcript detection in spiked human blood sample. Detection of *B. burgdorferi* 23S transcripts remains remarkably stable in spiked blood samples. A and C) 23S transcripts were detectable for up to two weeks without the use of any RNA stabilization agents, whereas as control human RNA transcript beta-actin was depleted under various storage conditions. B and D) TRIzol provides protection from *B. burgdorferi* and human RNA degradation in samples at room temperature.

Assessment of early murine infection using 23S rRNA assay

As a reliable test of early Lyme disease for humans is currently unavailable, we used a murine RT model of Lyme disease to test our hypothesis for developing the rRNA-based efficient Lyme diagnostics test. Specifically, we used an *in vivo* mouse model of *B. burgdorferi* infection to explore whether 23S rRNA is an efficient diagnostic target for detection of early murine infection. Groups of three mice were infected by intraperitoneal injection of 10^5 bacteria per animal or by feeding of infected ticks to full engorgement. For needle-infected mice, approximately 20 μ L blood samples were collected on days 1, 4, and 7 post infection (dpi) and analyzed by

qPCR using primer pair Bb10. For tick-infected mice, approximately 20 μ L blood samples were collected on days 1, 4, 7, 10, 14, 21, and 28 post tick-repletion for qPCR analysis using primer pair Bb10. Such a small blood sample size was used for this experiment due to animal welfare guidelines for frequent blood collection in mice. Infection was detected in all infected mice on day four and day seven. We could not detect *Borrelia* in needle-infected mice at 1 dpi, however we were able to detect at day four and day seven post-infection (Fig. 27A). We were able to detect *B. burgdorferi* in one out of three mice one day post-tick repletion (Fig. 27B). Lack of detection in other animals after one day was likely due to the use of such small sample volumes. Since infection via tick feeding gives a more natural model, we extended the study to see the length of time that *B. burgdorferi* remained detectable in the blood. In mice infected via tick feeding, we detected the presence of *Borrelia* in the blood continuously from day 4 through day 28 post-tick repletion (Fig. 27B). We confirmed the results from 28-day infected mice by culture of blood samples. Uninfected mouse blood samples and NRT control showed neither specific nor non-specific amplification of any products using our qPCR assay.

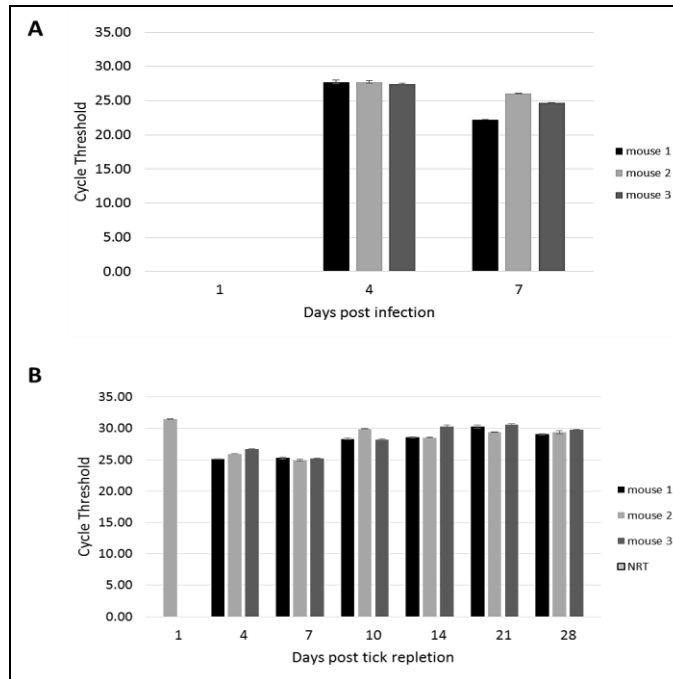


Figure 27: Detection of borrelial RNA by quantitative PCR in blood samples collected from mice during early infection. A) Detection of *B. burgdorferi* in needle infected mice using 23S rRNA. B) Detection of *B. burgdorferi* in tick infected mice using 23S rRNA.

5.4 Discussion

Lyme disease is the most commonly reported vector-borne disease in the United States and many other parts of the world, yet efficient diagnostics and vaccines to prevent the infection in humans are currently unavailable (Willyard 2014; Kaaijk and Luytjes 2016). We have developed a highly sensitive and specific RNA-based qPCR assay targeting the 23S rRNA of *B. burgdorferi* for positive diagnosis of infection. Similar diagnosis methods have been developed for numerous viral and bacterial infections, including human immunodeficiency virus (HIV), hepatitis C virus (HCV), enterovirus, tick-borne encephalitis virus (TBEV), chlamydia, gonorrhea, and leptospirosis (Parida, Posadas et al. 2004; Lowe, O'Loughlin et al. 2006; Jayawardena, Cheung et al. 2007; Curtis, Rudolph et al. 2008; Maquart,

Temmam et al. 2014). Our results indicate that human blood could be suitable for efficient detection and confirmatory diagnosis of Lyme disease infection.

In the absence of universal confirmatory clinical manifestation or other assays, confirmation of Lyme disease currently relies on the detection of antibodies against the bacteria. The CDC recommends a two-tiered serologic system that 1) screens for antibodies using an enzyme immunoassay or an immunofluorescence assay, followed by 2) an isotype-specific Western blot for positive samples (Centers for Disease and Prevention 1995). Alternatively, in certain cases diagnosis can be confirmed by clinical manifestations of disease, particularly the characteristic erythema migrans rash, or positive culture of bacteria from patient samples (Wilske 2005; Gerstenblith and Stern 2014). Unfortunately, up to 30% of cases do not develop the erythema migrans rash, and culture requires careful sample handling and specialized growth media as well as up to 12 weeks of growth to visualize bacteria by microscopy (Alby and Capraro 2015). Furthermore, seropositive detection in immunoassays can require up to a month for development of antibodies against infection to reliably be detected (Bacon, Biggerstaff et al. 2003; Halpern, Jain et al. 2013). Due to the inconsistency of clinical manifestations and the length of time required for serology or culture diagnosis, it is essential to develop new, improved diagnostic methods to allow earlier diagnosis and faster treatment of Lyme disease.

A number of alternative testing methods of Lyme disease have been developed that included direct antigen detection, T-cell proliferation assays, and nucleic acid amplification tests (Leeflang, Ang et al. 2016). Direct antigen detection and T-cell proliferation assays have largely proved inadequate due to lack of

sensitivity and specificity, respectively (Alby and Capraro 2015; Leeflang, Ang et al. 2016). The latter series of assays also requires days to accomplish and may not be cost-effective. Nucleic acid amplification test (NAATs) have similarly shown a lack of sensitivity, even while exhibiting extremely high specificity (Bil-Lula, Matuszek et al. 2015). Previous NAAT have focused on detection of both plasmid and chromosomal *Borrelia* DNA gene targets, including *ospA*, *ospC*, *vlsE*, *flab*, and 16S and 23S rRNA genes (16S and 23S) by PCR methods (Gooskens, Templeton et al. 2006; Ivacic, Reed et al. 2007; de Leeuw, Maraha et al. 2014). We hypothesized that the sensitivity of molecular diagnosis could be greatly improved by using RNA molecules as the template for target detection rather than DNA, as many RNA transcripts can be generated within a cell versus 1-2 gene copies. In particular, 16S and 23S ribosomal RNA, which compose approximately 90% of all RNA molecules, presented attractive targets due their abundance and constitutive expression. Indeed, 16S and 23S rRNA targets gave more than a 200-fold increase in sensitivity of detection in *B. burgdorferi* cDNA, based on the relative cycle of quantitation using our assay, than the next best target.

Since, ribosomal RNA genes are highly conserved among prokaryotic species, we aimed to target sequences that were conserved among *B. burgdorferi sensu lato* complex species but would not cross react with related relapsing fever group *Borrelia* or other unintended prokaryotic and eukaryotic targets. *B. burgdorferi* 16S and 23S rRNA nucleotide sequences show up to 97% and 95% sequence identity, respectively, as compared to relapsing fever group spirochetes such as *B. hermsii*, and up to 83% identity to *Treponema* spirochetes from NCBI Blastn. Due to the highly conserved

nature of these target sequences, we performed multiple gene alignment on the 23S and 16S sequences from representative *Borrelia* species as well as other bacterial species with most closely related sequences using MegAlign program (DNASTAR) ClustalV method. Primers were designed with specific nucleotide mismatches compared to unintended pathogen species. Indeed, our assay was able to specifically detect all tested *B. burgdorferi* strains, as well as *B. garinii* and *B. afzelii* isolates, without cross-reacting with *B. hermsii* or eukaryotic host species RNA molecules.

The kinetics of Lyme disease infection involve deposition of bacteria into the skin from the bite of a tick, followed by dissemination through the skin into blood and finally to other tissues (Auwaerter, Aucott et al. 2004). During early infection, Lyme disease is easily treated by antibiotics, but the difficulty and delays in providing a definitive diagnosis of *Borrelia* infection allow the bacteria to disseminate to various tissues throughout the body, such as the joints where infection may become more difficult to treat. In the early phases of infection, although transient and variable amongst subjects, low levels of bacteria are present in the blood of the host (Liveris, Schwartz et al. 2011). Due to the presence of bacteria in blood during early infection and the relative ease of sample collection for both patients and clinicians, we elected to test blood as the source of nucleic acid for our assay. Previous molecular tests have shown a low level of sensitivity when testing DNA amplification of gene targets in the blood. We hypothesize that the superior abundance of RNA transcripts allows for more efficient detection of bacteria in samples, even in cases of low bacteremia. Notably, the sensitivity of detection from blood will inherently depend upon the sample volume used for nucleic acid extraction and the stage of infection at the time

of sampling. With 100% specificity testing against our uninfected controls, a positive result indicates definitive infection; however, a negative test result does not prove the absence of infection. In the absence of clinical samples to test the sensitivity of our assay, we tested for detection of early infection in low volume samples from mouse blood in animals infected by either needle inoculation or infected tick-feeding. In both groups, we could reliably detect early infection at day four post-infection. In one animal, detection could be detected at day one post tick-repletion. Additionally, a detectable level of spirochetemia persisted from day 4 through day 28 in all tick infected mice. It was previously reported that *B. burgdorferi* could be detected in blood of infected mice by culture up to 30 days post infection (Barthold, Beck et al. 1990). While human infection likely differs in kinetics of infection, as well as the burden of bacteria in particular tissues or organs (Babady, Sloan et al. 2008), these data highlight that our assay may have practical use for detection of *B. burgdorferi* in human blood during early infection. In particular, this assay may be applicable for filling the current gap in diagnosis for patients lacking the erythema migrans rash, but not yet having developed a detectable antibody response. Further testing of clinical samples is warranted to validate our assay, which can fill a critical unmet in diagnosis of Lyme disease.

Chapter 6: Conclusion

The identification of spirochete virulence determinants and improved diagnostic methods are important focus areas of ongoing research. In this thesis work, we explored: 1) the role of glycolytic enzymes as potential moonlighting proteins to support leptospiral or borreliac virulence and their use as broad-spectrum vaccine targets, and 2) the development of novel, highly sensitive molecular diagnostic techniques against *B. burgdorferi* or *L. interrogans* rRNA transcripts for potential applications in early clinical diagnosis of leptospirosis and Lyme disease.

In Chapter 2, we explored the possibility of metabolic enzymes having functions involved in the virulence of bacteria during mammalian infection. The candidate moonlight genes showed significant divergence between pathogenic *Leptospira* and *Borrelia* species, which also reflected remarkable sequence divergence from mammalian homologs. Based on our studies of protein expression and subcellular localization, we narrowed our focus to three enzyme candidates—FBA, PGK, GAP in *L. interrogans*—and one, PMI, in *Borrelia* for further immunization studies. For both groups, immunization failed to provide protection from infection in animal models. In leptospires, glycolysis is not utilized for energy metabolism (which is missing an orthologs for classical hexokinase enzyme, yet genes representing an almost complete glycolytic pathway remain present). It is most likely that these enzymes are utilized for production of intermediates of carbohydrate metabolism synthesized by means of the tricarboxylic acid cycle and the non-oxidative pentose phosphate pathway in anaplerotic reactions (Ren, Fu et al. 2003).

In contrast, in case of *B. burgdorferi*, energy metabolism is solely produced via glycolysis (von Lackum and Stevenson 2005). While PMI is not directly involved in the glycolytic pathway, it is an important intermediary between mannose metabolism and glycolysis. Gene expression of *pmi* in *Borrelia* is at least somewhat regulated by *rrp1*, a global regulator of *Borrelia* genes that is particularly important with tick phase of the life cycle (Rogers, Terekhova et al. 2009). As we recorded higher *pmi* gene expression within the tick vector as compared to mammalian infection, PMI enzymatic activity may be more essential during the resource limited tick life cycle than during mammalian infection where glucose is abundant. The appearance of PMI on the surface of *B. burgdorferi* may be due to the importance of this enzyme in mannose metabolism and contribution towards production of exopolysaccharides as seen in other organisms (i.e. *Pseudomonas aeruginosa*, *Cryptococcus neoformans*), but unstudied in *Borrelia* (Roux, Lee et al. 2004; Zaragoza, Rodrigues et al. 2009; Franklin, Nivens et al. 2011).

Chapter 3 discusses a method for improved molecular diagnosis of leptospirosis. Current laboratory diagnostic tests for leptospirosis rely on antiquated methods and suffer from low sensitivity, especially in the first days of illness. In fact, the gold standard diagnostic method, MAT testing, may give poor diagnostic results creating imprecise standards for development of future techniques (Limmathurotsakul 2012). Existing PCR methods to detect *Leptospira* DNA, at best only have a sensitivity of approximately 60% (Villumsen, Pedersen et al. 2010; Stoddard 2013). We designed and presented proof of concept for a novel testing modality to detect pathogenic *Leptospira* bacteria in human blood samples (Backstedt, Buyuktanir et al.

2015). Our preliminary results demonstrate that a greatly higher sensitivity can be achieved through the PCR amplification of cDNA molecules derived from 16S RNA, due to the abundance of 16S rRNA over that of genomic rDNA. We successfully showed that our assay detects 16S rRNA from all pathogenic species of *Leptospira* with high sensitivity and specificity. Future application of this concept is ongoing with blood and urine samples in different testing formats: (1) real-time PCR amplification, with or without a fluorescent TaqMan probe; (2) droplet digital PCR, with improved sensitivity and specificity over real-time PCR; and (3) loop-mediated isothermal amplification, a low-cost PCR technique that may be beneficial in resource-poor settings where diagnosis of *Leptospira* remains challenging.

Chapter 4 explores a similar RNA-detection assay to diagnose Lyme disease during early infection. After empirical testing of various highly expressed gene transcripts, representing either mRNA and rRNA targets, we concluded that rRNA offers far superior levels of sensitivity as expected. Gene sequence analyses and empirical testing of specific primers against 16S and 23S rRNA indicated 23S as the preferred target in *B. burgdorferi* due to extremely high 16S gene conservation among *Borrelia* spp, more broadly within family *Spirochaetaceae*. We were able to show highly specific and sensitive detection of *B. burgdorferi sensu lato* complex 23S rRNA transcripts. Additionally, results in early mouse infection with extremely low blood sample volumes indicated that spirochetemia is detected early during infection and persists at detectable levels for up to one month. Further testing of this method using human clinical samples is warranted to validate the assay.

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