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Development of diagnostic immunoassays for tularemia and leptospirosis

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Abstract

The zoonotic bacteria *Francisella tularensis* and *Leptospira* species are the causative agents for tularemia and leptospirosis respectively. Both of these diseases have significant deficits in the diagnostic tools available for efficient early diagnosis, which can result in delayed treatment of these potentially life-threatening infections. Both bacteria are extremely challenging to isolate from patients for definitive diagnosis and instead serological techniques for detection are relied on. Antibody titers can take up to two weeks to reach diagnostic significance, and the assays used to measure this often require extensive laboratory equipment and trained staff to perform. To address the lack of diagnostic options available for these bacteria, libraries of monoclonal antibodies to potential diagnostic targets were produced for inclusion in diagnostic immunoassays. Mice were immunized with purified *F. tularensis* lipopolysaccharide and ten monoclonal antibodies were isolated and confirmed to be reactive to pathogenic strains of the bacteria. The antibodies were screened, and reactive pairs identified to develop a sensitive, quantitative enzyme-linked immunosorbent assay (ELISA) and prototype lateral flow immunoassay (LFI) for rapid point of care detection. The ELISA was optimized in human serum and urine and used to quantify LPS in filtered tularemia patient serum samples. Eight samples contained quantifiable levels of *F. tularensis* LPS, showing that LPS is a viable diagnostic antigen for tularemia. An LFI prototype was developed and determined to be specific for pathogenic *F. tularensis*. A limit of detection of 5 ng/mL purified LPS in normal human serum and urine was determined.

Leptospiral lipoprotein LipL32 was selected as the diagnostic target for production of antibodies specific for pathogenic *Leptospira*. This antigen is conserved in pathogenic *Leptospira* and is not present in saprophytic strains. Mice were immunized with purified

recombinant LipL32 and fifteen monoclonal antibodies were isolated. Reactivity of these antibodies was confirmed with heat inactivated pathogenic *Leptospira* species. These antibodies were used to develop a Western blot for direct detection of LipL32 in patient urine samples. A limit of detection of between $2.72x10^3$ and $1.36x10^3$ colony forming units/mL heat inactivated *L. interrogans* spiked into pooled normal human urine. Previously described clinical ranges indicate that this sensitivity would likely be sufficient for detection of leptospires in symptomatic leptospirosis patients.

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CHAPTER 1

Introduction

1.1 Overview

This research focuses on isolation of monoclonal antibodies and development of immunoassays for detection of zoonotic bacteria *Francisella tularensis* and *Leptospira* species. In this chapter, background information on these bacteria and the selection of relevant diagnostic antigens for development of immunoassays is provided, as well as a brief overview of antibodies and the types of immunoassays that can be developed using them. Chapters 2 and 3 cover development of immunoassays to two different antigens and the associated challenges are discussed. An overall conclusion is provided in Chapter 4.

Chapter 2 covers production of monoclonal antibodies and development of immunoassays for detection of *F. tularensis* lipopolysaccharide (LPS). An antigen-capture enzyme-linked immunosorbent assay (ELISA) was developed for quantification of LPS in tularemia patient samples and a lateral flow immunoassay (LFI) prototype explored as a potential rapid, point of care diagnostic tool. Chapter 3 explores production of monoclonal antibodies to the pathogenic *Leptospira* spp. outer membrane lipoprotein LipL32. The isolated antibodies proved unsuitable for use in antigen-capture immunoassay formats so the focus in this instance was development of a Western blot for direct detection of LipL32 in human urine.

1.2 Tularemia

Tularemia covers a range of syndromes caused by the intracellular Gram-negative bacterium *Francisella tularensis.* First isolated in Tulare County, California after an outbreak in rodents in 1911[1], *F. tularensis* was assigned to its own genus *Francisella,* after

initial classifications as *Bacterium*, *Pasteurella* and *Brucella*[2]. *F. tularensis* can infect a broad range of hosts, including mammals, arthropods, birds and fish and can easily pass to humans through vector transmission or environmental exposure[3].

Much of the research interest around *F. tularensis* stems from its potential for use as a bioterror agent. This organism is considered a Tier 1 select agent through the Federal Select Agent Program, due to its low infectious dose, high mortality rates, and potential for aerosolization[4]. Outbreaks of Tularemia in the 1930s and 1940s triggered early investigations into the pathogenicity of infection[5]. The onset of World War II led to increased interest in *F. tularensis* as a biological weapon, with the US and Soviet Union stockpiling the bacteria and designing weapons to distribute aerosolized *F.tularensis*[6]. Some reports suggest further manipulation of certain strains to exhibit antibiotic resistance[7]. Although the US biological weapons development program has since been dissolved, the impact of *F. tularensis* if deployed as a bioterror agent would be devastating. It has been estimated by the World Health Organization (WHO) that if aerosolized over a population of 5 million people, 50kg of virulent *F. tularensis* could result in approximately 19,000 deaths and even more incapacitated by sickness[8]. Furthermore, the economic impact of exposure of just 100,000 people to an aerosolized attack was estimated to be $5.4 billion (US)[9]. As such there is a need for development of vaccines, diagnostics, and therapeutics for use in the event of widespread exposure.

Geographic distribution and incidence

Tularemia primarily occurs in the Northern hemisphere, with cases reported in North America, Europe and parts of Asia. Cases in North America are almost exclusively caused by *F. tularensis* subspecies *tularensis* and in Europe cases are generally caused by *F.*

tularensis subspecies *holarctica*[10]. The incidence of tularemia cases is low and sporadic, with an estimated 0.5-5 cases per million people in the US per year and approximately 100 cases in 2010[3]. Incidence in Europe is also extremely variable depending on location, with reported annual incidences ranging from 0.15 – 5.2 cases per 100,000[3,11]. Surveillance of wildlife for *F. tularensis* is undertaken in many endemic countries in order to better understand the how these cases are occurring and help predict any future outbreaks based on changes in potential reservoir populations. Currently, environmental surveillance relies on polymerase chain reaction (PCR) assays of soil and water samples[12], and isolation of bacteria from wildlife in affected areas, particularly small mammals[13,14].

F. tularensis has a broad range of zoonotic hosts, but a complex ecological cycle means that no singular reservoir for transmission has been identified. Lagomorphs, such as rabbits and hares, and small rodents are thought to be the primary sources of infection among humans, whilst transstadial transmission in ticks suggests another potential reservoir species[15,16]. These organisms, amongst others, form the basis for terrestrial persistence of tularemia alongside a suggested aquatic reservoir. Outbreaks and isolated cases of oropharyngeal tularemia have been linked to consumption of contaminated water, with one of the largest associated outbreaks in Turkey[17], as well as cases of pneumonic tularemia after inhalation of water in near-drowning accidents in France and Finland[18–20]. Mosquitoes are also vectors for tularemia, and one of the primary ways in which the bacteria is transmitted in Sweden and Finland. Outbreaks in these countries have been aligned with mosquito population dynamics and incidence is positively correlated with proximity to water sources such as lakes and rivers that are breeding grounds for mosquitoes[21–23]. *F. tularensis* has also been detected in aquatic animals such

as crayfish and freshwater fish, and has been isolated from several brackish water samples[20,24–26]. Studies of tularemia incidence in humans across Europe indicate a clear seasonality of cases, with the majority occurring in the period between June and November, consistent with increased outdoor activity and vector dynamics[27].

Both sporadic and clustered reported cases of tularemia have steadily increased in Germany since 2002[28], and re-emerged in the Netherlands in 2013 after a 60 year period without reported isolation[29]. Geographical modeling has suggested that the increase in both tularemia cases and the range of geographical endemicity may be due to expansion of arthropod vector habitats as a result of climate change across Europe[30]. Climate variations also affect the behavior and populations of host organisms such as rodents and lagomorphs[31]. Increased temperatures in endemic areas of Sweden have been linked to outbreaks of tularemia and increased duration of these outbreaks[30].

Subspecies

There are four recognized subspecies of *Francisella* that vary in virulence and geographic distribution: *tularensis* (type A strains), *holarctica* (type B strains), *novicida* and *mediasiatica*. Subspecies *tularensis* has only been found in North America and is estimated to cause 70% of North American cases[32]. Subspecies *holarctica* is responsible for the majority of cases in Europe and generally causes less severe illness than subspecies *tularensis*[2]. *F. tularensis* subspecies *novicida*, also referred to as *F. novicida*, very rarely causes human disease, with nine of the eleven reported cases occurring in immuno-compromised individuals or those with underlying health conditions. *F. novicida* has been widely used as an experimental surrogate for virulent *F. tularensis* as it can be used at Biosafety Level 2 and is exempt from US select agent regulations[33]. Very little is

known about the *mediasiatica* subspecies, which was first identified in Central Asia and later in the Altai region of southern Russia[10,34]. Despite showing similar virulence in rabbits to type B strains, no cases of human tularemia caused by the *mediasiatica* subspecies have been reported[35].

Transmission and virulence

The primary routes of human infection with *F. tularensis* stem from environmental exposures, including contact with contaminated water, infected animal vectors and arthropod bites[36]. As such, there are a number of risk factors that make transmission to humans more likely. Many reported cases stem from occupational or recreational exposures that put people in contact with environmental reservoirs, with examples including hunting, veterinary work, and landscaping, as well as considerable risk to laboratory workers who come into contact with *F. tularensis*[27,37]. There have not been any described instances of person to person transmission; however, there have been reports of cases in organ transplant recipients where *F. tularensis* was isolated from donor tissue samples after recipients became seriously ill[38,39].

The infectious dose and mortality rate for tularemia infection is variable depending on the route of infection. Animal models have indicated that the infectious dose for *F. tularensis* subspecies *tularensis* when administered as an aerosol could be as low as one bacterium[40]. Mortality rates for type A strains are approximately 60% if not treated appropriately. The infectious dose for *F. tularensis* subspecies *holarctica* is reported as less than 10^3 colony forming units (CFU) dependent on the route of infection[41]. Cutaneous infection with *F. tularensis* subspecies *holarctica*, the most common form of natural

infection, has significantly lower mortality rates of less than 0.5%, whilst *F. tularensis* subsp. *tularensis* has a mortality rate of 5-6% in untreated cutaneous infection[2].

Clinical features

Clinical presentations of tularemia can be separated into six broad syndromes that are dependent on the route of infection. All presentations share some key features, primarily fever, lymphadenopathy and non-specific febrile symptoms which generally arise after a 3-6 day incubation period, however there are some more specific symptoms that can complicate diagnosis[16]. Most naturally occurring infections are classed as ulceroglandular tularemia as a result of dermal exposures such as arthropod bites or contamination of cuts in the skin. Although generally not clinically severe, this form can lead to the development of sepsis and death if in an individual who is immunocompromised or untreated[2]. In the event of lymphadenopathy and fever in the absence of a skin ulcer, infection is classed as glandular tularemia. Exposure via the conjunctiva results in oculoglandular tularemia, where a combination of conjunctivitis and swollen lymph nodes on the same side can lead to Parinaud's oculoglandular syndrome[42,43]. Ingestion of contaminated game or water can lead to infection via the oropharyngeal mucosa, resulting in oropharyngeal tularemia which presents with severe pharyngitis[44,45]. Typhoidal tularemia can also occur due to exposure via the oropharyngeal route and presents with non-specific febrile symptoms and sepsis[2]. The most severe syndrome is pneumonic tularemia, which occurs as a result of infection via the respiratory tract and leads to pneumonia[46]. This form is found in patients who have been exposed to aerosolized *F. tularensis* and has been seen in some rare naturally occurring infections, for example inhalation of aerosols from hay contaminated by infected animals, however this form is primarily a concern from a bioterror threat perspective[47,48].

Diagnosis and treatment

Diagnosis of tularemia can be made definitively by direct culture from blood or ulcers, lymph node biopsies and sputum; however isolation from the blood in the early stages of infection is rare and challenging due to low circulating numbers of bacteria and the fastidiousness of the organism with regard to growth conditions[49]. Culture of *F. tularensis* can also take up to 10 days, an unacceptably long time for such a potentially serious illness, especially in the event of widespread exposure. Confirmation of tularemia diagnosis can be made by measuring the fold change in serological response to infection via detection of antibodies to *F. tularensis* lipopolysaccharide (LPS) in patient serum. This approach is limited in that often antibodies do not reach diagnostically significant levels until approximately two weeks post-infection[50], and can persist for decades particularly in endemic areas, thus potentially complicating later diagnosis and meaning that changes in titers over time need to be monitored[51]. Development of PCR-based assays for detection and diagnosis of tularemia have shown promise in terms of increased sensitivity when compared to culture techniques[52]. Unfortunately, these assays cannot be easily integrated at point-of-care and require complex lab equipment and skilled personnel to perform.

Streptomycin and gentamicin antibiotics are thought to be the most effective first-line treatment options for tularemia[2]. Ciprofloxacin and doxycycline have also shown some effectiveness, with ciprofloxacin showing the lowest percentage therapeutic failure in a study of streptomycin, ciprofloxacin and doxycycline treatment during a Spanish tularemia outbreak[53]. In the event of unsuccessful treatment with gentamicin, ciprofloxacin has previously been successfully utilized as a second-line treatment to resolve infection[54]. It is recommended that antibiotic treatment should be continued for at least 10 days depending on clinical presentation[48]. Although susceptible to antibiotics, there are concerns that

release of *F. tularensis* in a bioterror context could involve antibiotic-resistant strains, highlighting a need for development of novel prophylaxis and therapeutics for tularemia[55].

1.3 Leptospirosis

Leptospirosis is a zoonotic bacterial infection that is widespread in tropical regions, especially where sanitation and infrastructure is poor[56]. The disease is caused by spirochetal bacteria of the genus *Leptospira* which are shed in the urine of human and animal hosts as a result of extensive kidney colonization[57]. The full level of global disease burden of leptospirosis is unknown due to a lack of sufficient surveillance and difficulties in diagnosis, but the WHO has estimated that there are more than 500,000 severe human cases every year[58]. Key reservoirs of infection include small mammals and domestic livestock, both of which also play a role in contamination of soil and water sources[57]. Typical presentation of leptospirosis in patients is often non-specific febrile illness, which can result in misdiagnosis as other febrile diseases such as dengue fever, chikungunya fever or malaria[57,59]. Infections can range from mild and self-resolving to life-threatening in severity, often presenting as sudden fever and headache with muscle aches. Incubation periods for symptom development following exposure can vary from three days to a month[60,57]. Leptospirosis is primarily diagnosed via serology; wherein patient sera is assessed for antibodies reactive to a panel of cultured *Leptospira* serovars in a microscopic agglutination test (MAT). This is problematic as there are over 250 identified *Leptospira* serovars and whilst there may be some serological cross-reactivity there is a risk that a patient with an infection from a serovar not included on the MAT panel may not be diagnosed[61].

Geographic distribution and incidence

Geographic distribution of leptospirosis is broad and not well characterized. This is primarily due to the diverse range of animal hosts that can transmit the disease, and high incidence in resource-poor areas where public health surveillance is lacking[62,63]. Leptospirosis is considered a neglected tropical disease as incidence is highest in tropical regions, with 73% of cases and deaths due to leptospirosis occurring in the tropics[64]. Leptospirosis is also an emerging pathogen as outbreaks are often preceded by disruptions such as natural disasters or extreme weather events such as flooding, particularly as it can be transmitted via contaminated water[65]. In 2018 severe flooding in Kerala, India led to over 2500 suspected cases of leptospirosis and 95 suspected leptospirosis deaths in the months following the flood[66]. Other examples of outbreaks following flooding events include a 2014 outbreak in Kelantan, Malaysia which saw incidence of leptospirosis double in the three months following the flood[67], and an outbreak in Fiji in 2012 following two extreme floods that resulted in the largest reported outbreak of *Leptospirosis* in the South Pacific[68].

Although incidence and impact are highest in tropical regions, leptospirosis is a disease of global concern. Cases have been identified as result of exposure to infected rat urine in Baltimore, MD, USA[69], and canine leptospirosis is not uncommon across the USA and Europe[70–72]. Domestic animals can act as vectors for disease in humans due to the shedding of leptospires in urine, as well as becoming sick themselves. Human cases in non-tropical regions not directly linked to exposure to infected animals are often linked to activities that result in close contact with contaminated water or soil such as triathlons, rafting or canoeing and caving[73–75].

Transmission and virulence

Livestock animals and small mammals such as rodents are thought to be the primary reservoirs for human leptospirosis[56]. Infectious spirochetes colonize the proximal tubules of the kidney, are shed into the urine of infected animals and survive in the environment for weeks to months, contaminating soil and water sources[57,76]. Transmission to humans primarily occurs through contact of cuts and abrasions or mucous membranes with contaminated soil or water[57], making leptospirosis a prominent occupational health concern in farming and agriculture, as well as military and sanitation work[57]. Increasing incidence of exposure to those undertaking outdoor recreational activities is also a cause for concern[77,78]. The ease of leptospirosis transmission via contaminated water sources makes it a crucial public health issue in the wake of natural disasters where flooding and damage to sanitation systems can lead to outbreaks[65]. Flooding in countries where the disease is already endemic has led to elevated case numbers, for example a study of hospital admissions for leptospirosis in the Philippines showed increased admissions two weeks after heavy rainfall, a positive association linked to major flooding[79].

It has been estimated through statistical modelling that there are 1.03 million cases and over 58,000 deaths associated with leptospirosis globally per year [64]. This figure is likely an underestimate due to inconsistent surveillance and inadequate diagnostic provisions in endemic countries. Differences in virulence across species and serovars makes ascertaining an infectious dose challenging, however reviews of dose-response experiments indicate a range of median lethal dose from 1-10^7 CFU in hamster models of infection[80].

Subspecies and serovars

There are three subgroups of the genus *Leptospira*, containing 21 species. Group I is known as pathogenic *Leptospira,* and species in this group have been sub-classified into 250 distinct serovars. This group causes disease ranging from mild infections to severe, life-threatening incidences[81]. Group II are also known as intermediately pathogenic strains and generally cause mild, self-resolving illness if any. The final grouping of *Leptospira* species are non-pathogenic saprophytes that are present in the environment and do not cause disease[82]. Classification of these different serovars is down to differences in the carbohydrate moiety of leptospiral LPS, specifically the composition and orientation[83,84].

Clinical features

Typically, symptomatic leptospirosis presents as generalized febrile illness without widespread identifying characteristics. Infection is generally biphasic in nature, consisting of an initial acute phase where initial febrile symptoms develop and leptospires can be isolated from patient blood for approximately seven days, followed by an immune phase where the host immune system begins to make antibodies and isolation of leptospires from the blood is not possible[85]. Some cases can present with subconjunctival hemorrhages and patients often exhibit gastrointestinal symptoms. Presence of a nonproductive cough in some patients can lead to misdiagnosis of leptospirosis as a respiratory illness[57].

In severe cases, multiple organ dysfunction can lead to jaundice and renal failure, resulting in a clinical presentation known as Weil's disease. Bleeding is also common in severe cases of leptospirosis, ranging in severity from petechiae to gastrointestinal or pulmonary hemorrhage[57]. The most severe complications of leptospirosis include renal failure due to

kidney damage and dehydration and acute respiratory distress syndrome leading to lung injury and pulmonary hemorrhage[84,86,87].

Diagnosis and treatment

As mentioned previously, diagnosis of leptospirosis is generally made by using the MAT to determine reactivity of antibodies in patient serum to a panel of *Leptospira* serovars. Briefly, this process involves incubating patient serum samples with live *Leptospira* bacteria. These suspensions are then examined by dark field microscopy for agglutination of leptospires[88]. There are a number of limitations to the widespread use of MAT for diagnosis of leptospirosis. Primarily, the MAT is a complex assay to maintain and perform. A wide range of locally circulating serovars representative of all serogroups need to be maintained in culture to ensure reactivity of patient sera with the correct infecting serovar. Maintenance of these cultures and ensuring there is no cross-contamination between serovars represents a significant burden to laboratory workers, as well as risk of laboratory-acquired infection[89]. Often the MAT cannot reliably determine the specific serovar causing infection[90], and there is a high degree of cross-reactivity between different serogroups, especially in the acute phase[57]. Probable acute infection can be diagnosed by single elevated MAT titer, however this can be complicated in endemic areas where residual serological titers from a previous infection that may have been caused by a different serogroup can be found[63]. For a more definitive diagnosis paired sera showing a fourfold or greater increase in titer can be used as confirmation. The interval between these samples is variable and often dependent on the symptomatic presentation of the patient and the point during infection that samples are taken. In severe cases, infection may prove fatal before adequate seroconversion[57].

It is possible to diagnose leptospirosis from culturing of blood, urine or cerebrospinal fluid which can then be either examined microscopically for up to 13 weeks for the presence of leptospires or subjected to molecular detection, either by PCR or direct antigen detection[63]. Radioimmunoassays and ELISAs have been evaluated for direct detection but no technique has become widespread[63,91]. Detection from blood culture is slow, requiring highly specific media and growth conditions to thrive[92]. PCR assays have shown greater sensitivity for early diagnosis, however are often not accessible in endemic areas[93]. It is clear that direct detection from patients, ideally at the point of care is preferable. Leptospiremia can be seen early in the course of disease but often decreases by the end of a week of acute illness, making the timing of blood samples crucial[94]. Urinary shedding of bacteria is thought to last longer, up to several weeks, however recovery rate in culture can be affected by reduced survival rates of leptospires in voided urine[95,96].

The majority of leptospirosis cases resolve without intervention, however if antibiotic treatment is deemed necessary, first line treatment of for severe disease is generally antibiotics from the penicillin family[97], with doxycycline recommended in some cases for prophylaxis and treatment of mild infections, however there is limited data to indicate that a particular antibiotic regimen is most effective[56].

Veterinary concerns

Leptospirosis is not only a disease of human concern. As a zoonosis that affects livestock animals there are economic consequences beyond just acting as a reservoir for human infection. Often chronic infection in animals is minimally damaging to the host, however in situations where animals become immune-compromised, such as during pregnancy, there

can be loss of livestock[98]. It has been suggested that transplacental infection can occur and localization of leptospires to the uterus can cause abortion, stillbirth and neonatal disease[99]. In areas where livelihoods depend on livestock breeding this can have a significant impact. In addition to potential reproductive disease, other symptomatic manifestations can include uveitis and blindness[100], fever, hemolytic anemia, hemoglobinuria and jaundice[99]. One of the most widely studied species for clinical infections are dogs due to their close contact with humans and potential as a source of transmission from rodents. Many canine *Leptospira* infections are asymptomatic, with symptomatic infections presenting similarly to human disease with broadly febrile symptoms and kidney damage[101].

To mitigate the risk of income loss or transmission from livestock animals or pets there are vaccines available for use in dogs, cattle and pigs, however this approach to infection control has its limitations. The efficacy of vaccinations is questionable due to the antigens included in the vaccine and whether or not relevant serovars for the geographic area are included. Where the appropriate serovars are included, it is thought that bivalent cattle vaccines and five-way dog vaccines can provide up to a year of immunity, however these products are also limited by expense[99].

1.4 Diagnostic antigen selection

F. tularensis LPS

Diagnostic antigen discovery studies have indicated that *F. tularensis* LPS may be a diagnostically relevant marker of infection based on the immune response generated by

mice immunized with filtered samples from a tularemia infection model[102]. LPS, also known as endotoxin, forms the majority of the Gram-negative bacterial cell envelope and is implicated in stimulation of the host immune response during bacterial infection[103]. LPS has three main structural components: Lipid A, core region and O-antigen. *F. tularensis* LPS is atypical, primarily in that the lipid A component is tetraacylated with 16-18 carbon fatty acid chains, vs. the more prototypical lipid A which is hexaacylated with 12-14 carbon fatty acid chains[104]. Modification of this lipid A structure is thought to play a key role in the immune evasion strategy of *F. tularensis*, preventing stimulation of the TLR-4 pro-inflammatory pathway common during other bacterial infections[105]. Instead, the TLR-2-mediated signaling pathway is stimulated for production of proinflammatory cytokines, and endotoxicity of *F. tularensis* LPS is extremely low[106,107].

LPS plays a significant role in *F. tularensis* pathogenesis. Mutants lacking the O-antigen component of LPS showed a loss of virulence, leading to survival of mice when challenged with the mutant at doses that were fatal when the parent strain was administered[108]. The O-antigen of *F. tularensis* LPS has also been explored as a potential vaccine candidate and has shown some promise in protecting against intradermal challenge, but has not shown effective protection against respiratory challenge[109]. *F. tularensis* O-antigen antibodies can prolong survival in mice given a lethal challenge, although the mice did eventually succumb to infection [110].

The O-antigen component of LPS is widely regarded as the immunodominant region and is composed of a polysaccharide chain that can vary in length and the sugars present in the chain[111]. Structural characterization of the *F. tularensis* LPS O-antigen has shown that

pathogenic Type A and Type B isolates have identical O-antigen structures, whereas the *novicida* subspecies has antigenically distinct external carbohydrate residue structures[112], differences that are reflected in the O-antigen gene clusters of these organisms[113,114]. Studies have shown that O-antigen-like polysaccharides can also be found on the surface of *F. tularensis* in the form of a capsule, without the lipid A or core components of the typical LPS[115]. It has been shown that O-antigen mAb FB11 is reactive to both the LPS O-antigen and the capsule, however a mAb raised against crude capsule extract only binds capsular O-antigen, indicating that there may be some differences in immunogenicity between the structures[115].

Leptospira LipL32

LipL32 is a 272 amino acid surface-exposed lipoprotein that is the most abundant outer membrane protein of pathogenic *Leptospira*[116–118]. The potential of LipL32 as a diagnostic target stems not only from its abundance but also its association with pathogenic strains of *Leptospira*. The main pathogenic strains of *Leptospira* have more than 94% amino acid sequence identity for LipL32, with intermediately pathogenic strains showing down to 67% indentity[116]. Analysis of *Leptospira* strain reactivity with LipL32 antiserum showed no reactivity to non-pathogenic strains, indicating that LipL32 may be an effective target antigen for exclusively pathogenic *Leptospira*[117].

Despite the abundance of LipL32 on the surface of *Leptospira* cells, its function remains unclear. The presence of LipL32 on the cell surface of pathogenic strains strongly suggests a role in pathogenesis and virulence. An *L. interrogans* mutant lacking LipL32

showed no difference in virulence in both a hamster model of acute infection and a rat model of chronic infection, suggesting that LipL32 is not required for pathogenesis or renal colonization[119]. It has been suggested that, although LipL32 is not found in saprophytic strains of *Leptospira*, presence of orthologs in environmental organisms such as *Pseudoalteromonas tunicata* and other marine bacteria could imply a role in environmental persistance[116,120].

LipL32 has been established as a viable diagnostic antigen for leptospirosis, primarily for molecular detection methods such as PCR. Targeting of LipL32 in RT-PCR assays for direct detection from patient samples has indicated greater sensitivity and early detection of *Leptospira* infection than traditional serological methods[121]. Presence of LipL32 before host antibodies are detectable indicates potential availability of LipL32 for direct detection by immunoassay in resource-poor settings where PCR facilities are unavailable. The detection of LipL32 in patient urine samples reinforces that it is an appropriate target for assay development[122].

LipL32 has also been explored as a potential vaccine candidate due to its conservation across pathogenic *Leptospira* and its ability to provoke an antibody response[123]. Several studies have explored potential vaccine platforms that incorporate LipL32 and have shown reduced and kidney colonization in hamster models of infection however significant protection has not yet been shown[124–126].

1.5 Antibodies

Antibodies are proteins produced by B cells of the immune system in response to antigens that are considered foreign to the body. Also referred to as immunoglobulins, antibodies are a widespread and valuable resource for use in both diagnostic and therapeutic applications due to their ability to bind specifically to pathogenic targets of interest[127,128]. Basic antibody structure is shown in Figure 1. Each antibody consists of two heavy and two light chains that come together to form a Y-shaped structure linked by disulfide bonds. These chains can be divided into constant and variable regions, denoted as V_L and C_L on the light chain and V_H and C_H1, C_H2 and C_H3 for the one variable and three constant regions of the heavy chain. The variable regions of the heavy and light chains form the antigen binding domain, or paratope, and the constant region has a role in effector functions such as antibody-dependent cellular cytotoxicity, phagocytosis and endocytosis[127,129]. The paratope binds a specific site on the antigen, or epitope, which allows for recognition the antigen by B cells[130].

Human antibodies are classed into five isotypes: IgA, IgD, IgE, IgG and IgM depending on their role in the host immune system. IgG antibodies bind to antigens with high affinity to target them for destruction by natural killer cells or monocytes and are the most predominant antibody isotype in the body. The IgG isotype can be subclassed into four forms: IgG1, IgG2a, IgG2b, IgG3 and IgG4[130]. Specific, high affinity binding to target antigens makes the IgG isotype of antibodies desirable for use in an immunoassay format. Antibodies can be isolated from the serum of patients or animals after infection with a pathogen of interest, but a more targeted approach is to immunize animals with an antigen of interest.

Antibodies are widely utilized across a range of diagnostic platforms and immunoassay development is rapidly growing and changing with the introduction of novel technologies and assay formats. Developing an effective immunoassay can be greatly aided by having a library of antibodies available to select from for different assay formats. Both monoclonal antibodies (mAbs) and polyclonal antibodies can be generated by immunizing animals with an antigen of interest. Immunization provokes an immune response and polyclonal antibodies can be purified from the resultant immune serum. Polyclonal antibodies can be advantageous as they are a mixture of antibodies that can react to different epitopes of the same antigen; however, they can be less reproducible due to the variation in antibody responses to immunization. Production of monoclonal antibodies using hybridoma technology can be more costly to develop, however are easier to purify and can provide a more consistent source of reagent[131]. In this process, B cells from the spleens of the immunized animal are fused with an immortal myeloma cell line to form stable antibody-producing hybridoma cell lines that originated from a single hybridoma cell and therefore produce a single antibody. mAbs can be harvested and purified from the media these cells are grown in and characterized for potential applications[132].

1.6 Immunoassays

Diagnostic immunoassays fall broadly into two categories: serological or antigen detection. Serological assays measure the antibody response generated by the host organism to infection, whereas antigen detection assays detect the presence of a pathogen by utilizing antibodies specific to the organism of interest. Antibodies can be used to capture antigen in a sample and labelled with different detection reagents such as enzymes, fluorescent tags or colloidal gold for detection in immunoassays.

Enzyme-linked immunosorbent assay

The ELISA is a simple but sensitive assay format that utilizes enzyme-linked antibodies to detect immune complexes via colorimetric change once substrate is applied[133]. Despite requiring some laboratory equipment and reagents to perform, ELISAs are generally simple assays that have the capacity to screen large numbers of samples and are widely employed for diagnosis of a variety of infectious diseases[134].There are four main types ELISA: direct, indirect, antigen-capture and competitive. The first three forms are shown in Figure 2 with monoclonal antibodies, but polyclonal antibodies can also be used.

Direct ELISAs are the simplest form of this assay and are used to determine the presence of an antigen in a sample coated on a microtiter plate using a primary antibody usually conjugated to horseradish peroxidase (HRP). A colorimetric change occurs when the primary antibody binds to the antigen and the HRP enzyme reacts with a tetramethylbenzidine substrate[135]. Indirect ELISAs function similarly but use a secondary HRP-conjugated antibody to detect the primary, for example use of a goat-anti mouse IgG to detect murine antibodies bound to the antigen of interest[136]. Also known as a 'sandwich' ELISA, the antigen-capture format involves use of a capture antibody coated onto a microtiter plate which binds the antigen of interest. A detector antibody is added that is HRP labelled. A colorimetric change upon addition of substrate is indicative of bound antigen and can be measured with a spectrophotometer. Use of two antibodies rather than the single detection used in the direct detection format allows for signal amplification through binding of multiple or repeating antigen epitopes[137].

Lateral flow immunoassay

LFIs function similarly to ELISAs in terms of an antigen-capture immunoassay or serological detection format but can be easily applied at the point of care, and are able to give a positive or negative result in minutes without any specialized training[138]. Ease of use and low cost per test to produce has meant that since the first use of “paper chromatography” in 1960 to detect insulin in human plasma, the LFI has become a mainstay of clinical diagnostic medicine[139,140].The most widely accessible form of LFI is the home pregnancy test, which detects human chorionic gonadotrophin in the urine of pregnant women, illustrating the ease of use and non-invasive nature of sampling[141].

The adaptability of the LFI platform means that the format is constantly developing and improving, but the core configuration of the assay is shown in Figure 3. A membrane, generally nitrocellulose, that allows the analyte to flow through the strip by capillary action is sprayed with a control line and detection antibody line where binding to the analyte will take place. The sample to be analyzed is added to the sample pad, which can contain the secondary antibody conjugated to a detection molecule, or this antibody conjugate can be incorporated as a separate conjugate pad. The secondary antibody binds to antigen in the sample and the antigen-antibody complex moves up the nitrocellulose membrane until it is captured by the test line antibody, generating a positive signal that can be detected visually or by a specialized reader depending on the detection molecule used. The most common detection molecules are colloidal gold or latex beads which can be seen at the test line. Unbound gold conjugate will bind at the control line, which is often specific for the constant region of the detection antibody and is used to confirm that the test was run successfully. An absorbent wicking pad at the end of the strip maintains capillary flow as the sample runs and absorbs excess reagents, preventing the liquid from flowing back

down the test[140]. These components are all mounted to a backing card for physical support.

Western immunoblot

Western immunoblotting is an extremely common immunoassay for the detection and identification of antigens such as proteins and polysaccharides. This technique is often used as a diagnostic tool due to the high sensitivity for proteins in a sample making it applicable as an early diagnostic when antigen levels are low[142,143].

To detect an antigen of interest via Western blot, the proteins in a sample are separated by molecular weight via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a membrane by application of a current perpendicular to the surface of the gel that forces the antigen onto the membrane[144]. The membrane can then be blocked and probed with antibodies specific for the antigen of interest, either directly with an HRP-conjugated antibody or indirectly with an antigen-specific antibody followed by a detection mAb. Binding can be detected using a chemiluminescent substrate in which the HRP tag catalyzes oxidation of luminol, resulting in a signal that can be imaged digitally[142]. Western blotting can also be used as a serological assay, wherein patient serum can be used to probe a blot containing known antigen to detect an antibody response[143,145].

1.7 Figures

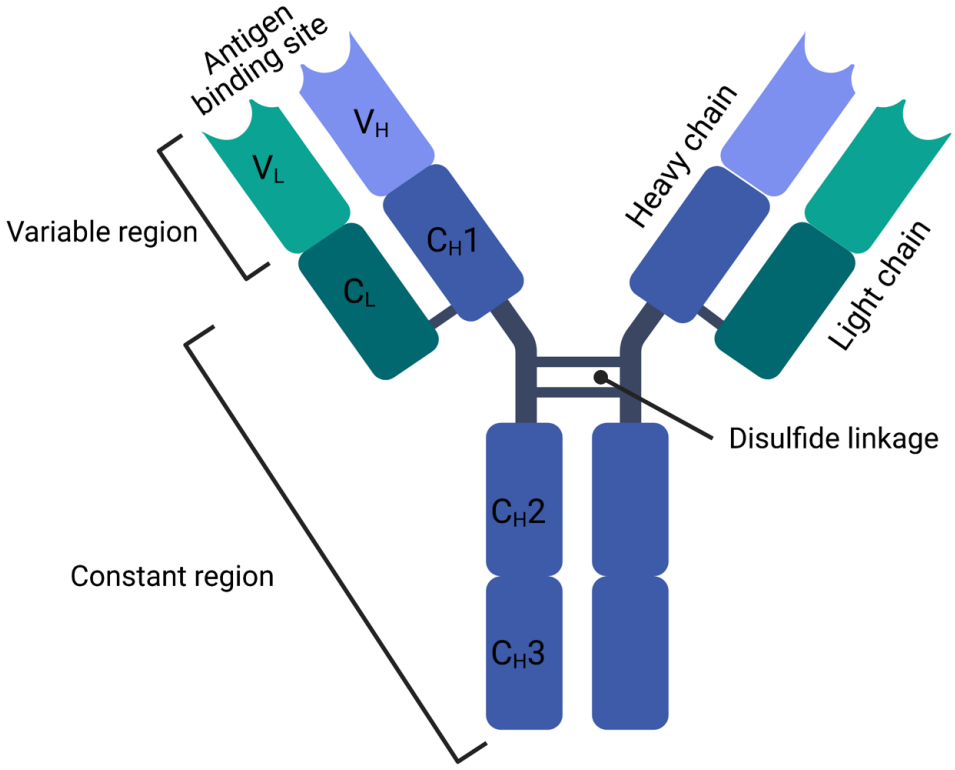

Figure 1. Schematic of an antibody. Antibodies contain two light chains (shaded green) which have a variable region (V_L) and a constant region (C_L), and two heavy chains (shaded blue) which consist of one variable region (V_H) and three constant regions (C_H1, C_H2 and C_H3). The two variable regions at the end of each arm form the antigen binding site and the chains are joined by disulfide bonds. Created with BioRender.com.

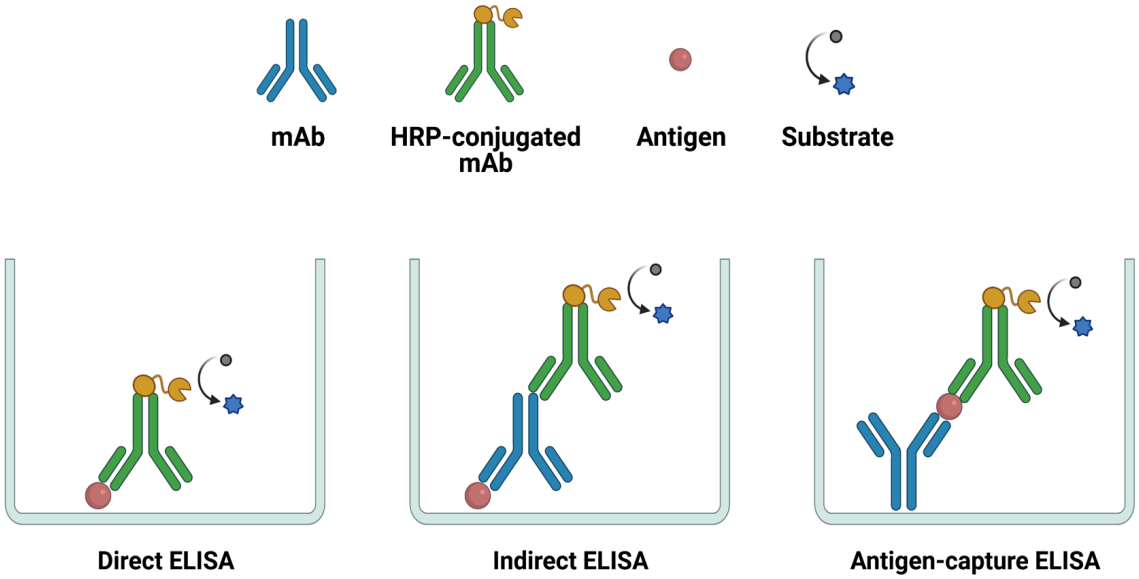

Figure 2. Antibody binding in three types of ELISA. In a direct ELISA, a horseradish peroxidase (HRP)-conjugated antibody is used to detect antigen coated on a microtiter plate well. Indirect ELISAs detect antigen the same way but with a secondary HRP-conjugated antibody detecting the antibody bound to the antigen. Antigen-capture ELISAs use an antibody coated on the microtiter well to capture the antigen, which is then detected by binding of an HRP-conjugated antibody. Created with BioRender.com.

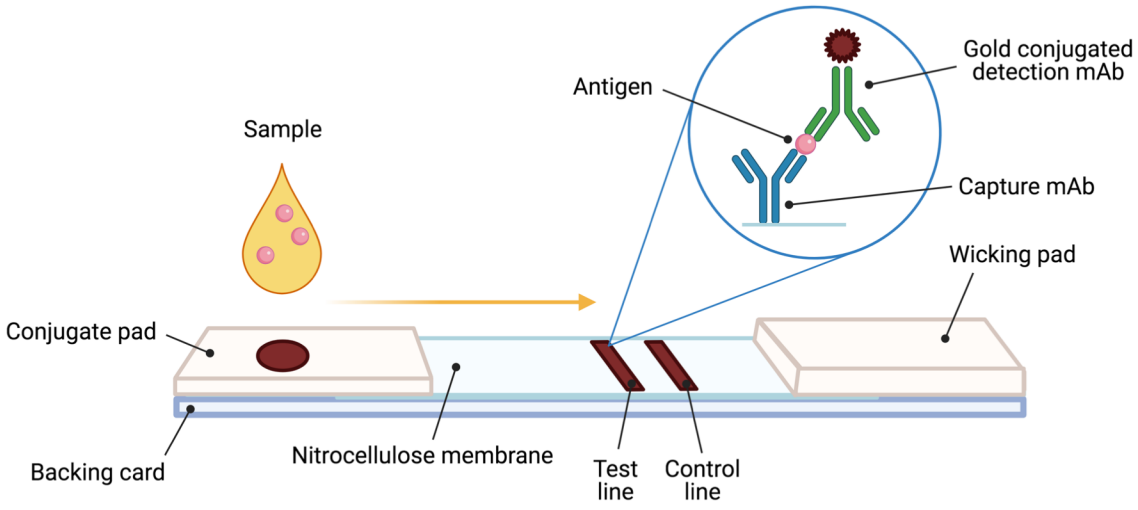

Figure 3. Schematic of a lateral flow immunoassay. In this example, a sample is added to the conjugate pad where a labelled antibody, in this instance gold-conjugated, binds antigen in the sample. Liquid the flows up the nitrocellulose membrane by capillary action and antibody-antigen complex binds to a capture antibody sprayed at the test line. Unbound gold conjugate binds at the control line and the wicking pad absorbs the sample, maintaining capillary flow. Created with BioRender.com.

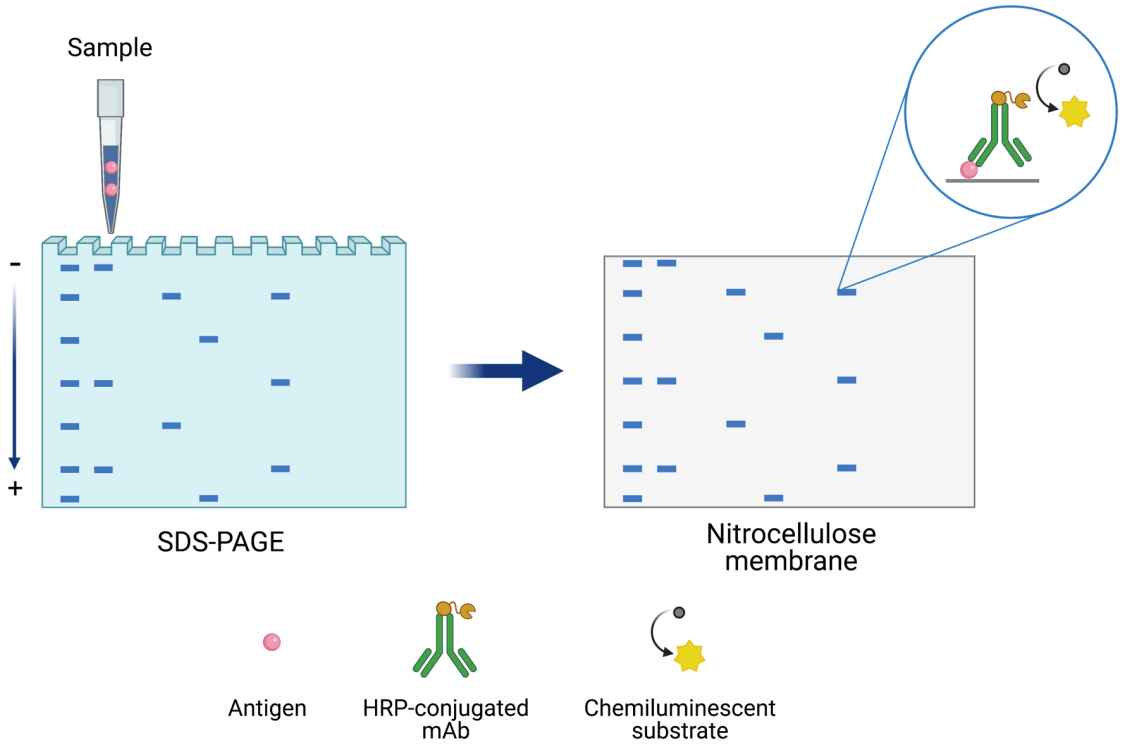

Figure 4. Process of Western immunoblotting. In this example, proteins in a sample are separated by molecular weight and transferred to a nitrocellulose membrane. HRP-conjugated antibodies are used to probe for an antigen of interest and binding is visualized through a chemiluminescent substrate. Created with BioRender.com.

CHAPTER 2

Development of immunoassays for detection of *Francisella tularensis* lipopolysaccharide in tularemia patient samples

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2.1 Abstract

Francisella tularensis is the causative agent of tularemia, a zoonotic bacterial infection that is often fatal if not diagnosed and treated promptly. Natural infection in humans is relatively rare, yet persistence in animal reservoirs, arthropod vectors, and water sources combined with a low level of clinical recognition make tularemia a serious potential threat to public health in endemic areas. *F. tularensis* has also garnered attention as a potential bioterror threat, as widespread dissemination could have devastating consequences on a population. A low infectious dose combined with a wide range of symptoms and a short incubation period makes timely diagnosis of tularemia difficult. Current diagnostic techniques include bacterial culture of patient samples, RT-PCR and serological assays; however these techniques are time consuming and require technical expertise that may not be available at the point of care. In the event of an outbreak or exposure a more efficient diagnostic platform is needed. The lipopolysaccharide (LPS) component of the bacterial outer leaflet has been identified previously by our group as a potential diagnostic target. For this study, a library of ten monoclonal antibodies specific to *F. tularensis* LPS were produced and confirmed to be reactive with LPS from type A and type B strains. Antibody pairs were tested in an antigen-capture enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay format to select the most sensitive pairings. The antigen-capture ELISA was then used to detect and quantify LPS in serum samples from tularemia patients for the first time to determine the viability of this molecule as a diagnostic target. In parallel, prototype lateral flow immunoassays were developed, and reactivity was assessed, demonstrating the potential utility of this assay as a rapid point-of-care test for diagnosis of tularemia

2.2 Introduction

Tularemia is a potentially lethal zoonotic disease caused by the intracellular Gram-negative bacterium *Francisella tularensis*. This organism is considered a Tier 1 select agent through the Federal Select Agent Program due to its low infectious dose, high mortality rates when not treated appropriately, and possibility of aerosolization[4,146]. *F. tularensis* has the potential to be easily disseminated and cause widespread illness and mortality, with estimates suggesting a large scale aerosol dispersal of 50 kg of bacteria over a population of 5 million could result in incapacitating casualties in 5% of the population[48,55]. Natural hosts include insects, mammals, birds and even fish, although the primary reservoir of infection is unknown[147]. Infection of humans can occur through many routes, such as arthropod vectors, direct contact with infected animals, water contamination and aerosol inhalation, thus presenting a rare but significant risk to public health[15,147–149]. Endemic areas primarily fall in the northern hemisphere, including North America, Europe and parts of Asia, with some studies indicating recent increases in the numbers of reported cases, particularly in northern Europe[38].

Severity of human tularemia infection is dependent on several factors, including the strain and route of infection. There are four main subspecies: *tularensis, holarctica, novicida* and *mediasiatica*. *F. tularensis* subspecies *tularensis* is the most virulent subspecies but is responsible for fewer naturally occurring infections worldwide. Type A strains such as *F. tularensis* subsp. *tularensis* are found primarily in North America[10]. Type B strains such as *F. tularensis* subsp. *holarctica* exhibit lower mortality but are responsible for the majority of naturally-acquired infections, predominantly in Europe and Asia[150]. *F. tularensis* subsp. *novicida* has been reported to cause infection in patients who are immunocompromised

or have underlying health conditions and is extremely rare[151]. There are no published accounts of the *mediasiatica* subspecies causing human disease[33].

Symptoms of infection are non-specific and vary greatly in severity. Tularemia presents most commonly as an acute febrile illness with symptoms such as fever, body aches and swollen lymph nodes[149]. Clinical presentation can include more varied symptoms depending on the route of infection, often complicating diagnosis. Respiratory tularemia resulting from inhalation of aerosolized *F. tularensis* is the most severe of the organ-specific infections, particularly involving Type A strains. Without rapid administration of the correct antibiotic therapy, the mortality rate for infection with this form can be as high as 60%[149]. It has been calculated that the infectious dose via the aerosol route could be as low as one bacterium based on animal models, underscoring the serious threat an aerosol release of this pathogen would present to public health[40]. Infection via insect vectors, such as ticks and mosquitoes, has been indicated as the most common route of natural infections, resulting in ulceroglandular tularemia, wherein painless ulcers form at the site of infection[152,153]. This differs from exposure of the eye (oculoglandular tularemia) or infection via contaminated food or water (oropharyngeal tularemia)[154]. While these forms are generally less severe than infection via the respiratory route, they have the potential to lead to systemic infection, sepsis and death if not recognized and treated appropriately[155].

Isolation of *F. tularensis* from biological fluids is challenging as circulating bacterial numbers can be low and *F. tularensis* is notoriously fastidious when grown in culture[49]. Growth in culture can also take up to 10 days, which in the event of widespread exposure

could result in lives lost. Diagnosis of tularemia can also be made by measuring the serological response to infection via detection of fold change in patient antibodies to *F. tularensis*, however diagnostic significance can take up to two weeks to be produced [50]. PCR assays are capable of detecting *F. tularensis* before culture and serological methods are effective, however cannot be implemented at the point of care and requires a laboratory and personnel to perform[52]. There is currently no standardized point-of-care diagnostic for tularemia, and thus recognition of an outbreak or release would likely be entirely dependent on identification by a public health authority after more common infections had been ruled out. The delay of diagnosis and therefore appropriate therapy could result in development of advanced pneumonia or sepsis and death. Thus, a simple, rapid and reliable diagnostic is needed, particularly for use in a mass exposure or outbreak setting [48].

Our laboratory previously identified *F. tularensis* LPS as a potential diagnostic antigen for tularemia in antigen discovery studies due to its reactivity with murine immune sera, abundance on the bacterial outer surface and potential to be shed at detectable levels [102,156]. Host antibodies against *F. tularensis* LPS have been detected in patient serum via Western blot [157] and enzyme-linked immunosorbent assay (ELISA) [158,159]. LPS itself has not been detected and quantified directly and no defined clinical range of LPS concentrations in patient samples has been suggested for diagnostic purposes. Direct detection of LPS from patient samples may serve as an earlier, more accurate diagnostic than currently available assays, especially if implemented at the point of care.

The goal of this study was to isolate a library of high-affinity monoclonal antibodies (mAbs) reactive with *F. tularensis* LPS for use in antibody-based diagnostics capable of detecting LPS in patient samples. Ten mAbs were isolated and reactivity with type A and type B strains of *F. tularensis* was determined. mAbs were evaluated in all pairwise combinations and top performing mAb pairs were integrated into i) a highly sensitive antigen-capture ELISA for laboratory-based detection and quantification of *F. tularensis* LPS and ii) a prototype lateral flow immunoassay (LFI) for rapid point-of-care diagnosis of tularemia. The antigen-capture ELISA was optimized for use with human matrices and used for quantification of LPS in tularemia patient samples. Prototype LFIs were constructed and reactivity with clinically relevant strains was determined

2.3 Results

mAb production and reactivity

Ten mAb-producing hybridoma cell lines were created from female CD1 mice immunized with LPS purified from the CDC Live Vaccine Strain of *F. tularensis* subsp. *holarctica* (BEI Resources, Manassas, VA). mAbs were purified and subclass was determined by indirect ELISA. A combination of IgG1 and IgG2b mAbs were isolated and characterized (Table 1). Western blots were performed using proteinase K treated killed cells to determine reactivity with *F. tularensis* subsp. *tularensis* strain Schu S4, *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *novicida* strain U112 and *Francisella philomiragia*, a near neighbor [16], summarized in Table 1. Representative blots showing reactivity with purified LPS, *tularensis* Schu S4 (Type A) and *holarctica* (Type B) are shown in Figure 1.

Antigen-capture ELISA optimization

To develop a quantitative immunoassay for *F. tularensis* LPS, mAbs were HRP-conjugated and tested in antigen-capture ELISA format. Each mAb was tested in both the capture and detection position at a standard concentration of 1μg/mL diluted in PBS for the capture or blocking buffer for the detection. The cut-off OD value used to determine a positive test for a given antibody pair, or limit of detection (LOD), for LPS in PBS was calculated at 3x background OD 450nm value (no antigen) in technical triplicate and an average taken from experiments on two days to rank the pairs to proceed with optimization (Table 2). The importance of developing a panel of mAbs to test different pairs can be seen in the variation in LOD shown in Table 2. The best performing pairs (Supplemental Figure 1) were selected and antibody coating and HRP conjugate concentrations were

then optimized in pooled normal human serum and urine spiked with *F. tularensis* LPS to determine the LOD in relevant matrices and the most sensitive pairing selected. Optimization in clinically relevant samples is important as these matrices can affect assay performance differently compared to buffer alone. Antibodies 1Ft5 (capture): 1Ft7-HRP (detection) were selected as the optimal pairing at a concentration of 2.5 μg/mL coating and 0.625 μg/mL HRP. These conditions gave an LOD of 0.18 ng/mL in normal human serum and 0.13 ng/mL in normal human urine, the standard curves for which are shown in Figure 2. LOD was calculated at 2x background in triplicate, as replicates provided more consistency when calculating the final LOD.

Quantification of LPS in patient samples

Archived tularemia patient serum samples were obtained from Hacettepe University, Turkey and 0.2 μm filtered to remove any viable bacteria in a BSL-3 laboratory. Samples were verified for sterility using a validated procedure, which allowed for analysis under BSL-2 conditions. Nineteen samples were of sufficient volume for analysis by antigen-capture ELISA for the presence of shed LPS. LPS was detected in eight of the samples and the concentration calculated by comparison to a standard curve of purified LPS. As shown in Table 3, the concentration ranged from 0.22 ng/mL to 109.95 ng/mL. The proximity of the calculated concentrations to the LOD of the ELISA in many of these samples may indicate that additional negative samples may contain LPS below quantifiable levels with this assay.

LFI development

To evaluate the potential of the isolated antibodies in an LFI format for development into a rapid diagnostic test, all mAbs were tested in both the capture and detection position for reactivity with purified *F. tularensis* LPS. Initial evaluation of LFIs involved testing with a standard concentration of LPS in buffer compared to a control of buffer alone. Visual assessment of test line signal intensity was performed for each mAb pairing. In addition, analysis with a Qiagen ESE-Quant lateral flow reader was performed in order to quantify test line intensity and non-specific binding at the test line when LPS was not present in the sample. Details of this testing are in Supplemental Table 1, demonstrating how criteria such as signal minus background with and without a blocking agent at a standard concentration of 500 ng/mL LPS and limit of detection for these prototypes resulted in a ranking system to isolate the top 20 pairs out of a possible 121 combinations.

A prototype LFI was developed using mAb 1Ft6 immobilized on the test line and 1Ft5 as the gold conjugate following further testing and optimization of the top 20 LFIs, including testing for non-specific binding (false positives) in normal human serum. This prototype was selected for its sensitivity, and low levels of non-specific binding in buffer and normal human serum. LFI buffer conditions and components were optimized to increase sensitivity and reduce non-specific binding at the test line. This LFI prototype was used to assess reactivity with purified *F. tularensis* LPS, heat inactivated *F. tularensis* live vaccine strain (LVS), heat inactivated *F. tularensis* strain NIH-B38, formalin inactivated *F. tularensis* subsp. *tularensis* SchuS4, gamma-irradiated *F. tularensis* subsp. *holarctica,* gamma-irradiated *F. tularensis* subsp. *novicida* U112 and gamma-irradiated *F. philomiragia.* The purpose of this testing was to determine reactivity of the prototype assay with both type A and B strains, including BSL-2 (LVS and NIH-B38) and BSL-3 (SchuS4 and *holarctica)* strains as well as *F. novicida,* which has been shown to have a different LPS structure[114] and near neighbor *F. philomiragia.* Reactivity was observed with purified

LPS and both variants of the type A and B strains tested. No reactivity was observed with *F. tularensis* subsp. *novicida* or *F. philomiragia,* as expected due to differences in LPS structure and associated virulence. Representative LFIs are shown in Figure 3. Patient samples were not run on the LFI prototype due to limited sample volume, however whilst the full range of circulating LPS concentrations is unknown, the ELISA data provides a promising target for rapid detection. To provide an indication of the specificity of the LFI, the prototype was tested using purified LPS from Gram negative bacteria and no false positive results were observed (Figure 4).

Purified LPS was serially diluted in pooled normal human serum and urine to assess the limit of detection for the assay in patient matrices. LFIs were assessed visually by three blinded readers and the limit of detection taken as the lowest concentration detectable by all three. In addition to visual assessment, LFIs were read using the Qiagen ESE lateral flow reader to provide a quantitative representation of binding. Visual examples of the dilution series in both serum and urine as well as intensity of the signal is shown in Figure 5. Based on this testing, the LOD of this assay in pooled normal human serum and urine was determined to be ~5 ng/mL. Further optimization of the assay for detection in these matrices can be done to increase the sensitivity to the levels indicated in the ELISA analysis of the patient samples outlined above.

Sample pre-concentration for LFI detection

The potential need for increased sensitivity to detect *F. tularensis* LPS in patient samples using this LFI format led to an exploration of how to increase the amount of antigen available for detection in a sample. A proof-of-concept experiment was designed by adapting a protocol developed for pre-concentration of *Burkholderia pseudomallei*

capsular polysaccharide in patient samples (Unpublished data). The conventional LFI prototype is limited by a maximum sample volume of ~100 μL and is therefore only capable of detecting LPS available in that volume. By employing a pre-concentration step, the amount of LPS present in larger sample volumes can be assayed in a smaller volume that is suitable for the LFI format. Briefly, mAb 1Ft7 was coupled to magnetic particles, which were then added to a 5 mL sample spiked with purified *F. tularensis* LPS. The beads were then removed from the original sample and the LPS eluted with acid. Base was added to neutralize the sample, resulting in a smaller volume that can be run on an LFI, effectively containing the amount of LPS present in a much larger sample, increasing the concentration and thus diagnostic sensitivity. When run on the LFI prototype, a positive signal was visible at 0.3 ng/mL, a significant improvement on the original assay sensitivity. To assess whether LPS was being lost at any stage of the process and highlight where improvements in the protocol could be made, the antigen-capture ELISA described above was used to quantify the amount of LPS in the elution run on the LFI and the amount remaining in the supernatant still available for capture. As indicated in Figure 6, the LPS eluted from the magnetic beads comprised 39% of the total LPS available for capture. Analysis of the supernatant after the beads with the bound LPS were removed from the sample showed that 54% of the available LPS remained unbound. This indicates that there is potential to further improve the sensitivity boost provided by this technique through optimization of the protocol to improve the capacity of the magnetic particles to bind LPS. Implementation of an enrichment protocol could also be useful for removing the antigen of interest from patient matrices before analysis, thus minimizing matrix effects and standardizing the run protocol of an LFI regardless of the sample being analyzed.

2.4 Discussion

When attempting to diagnose an infection with the range of clinical symptoms and potential fatality rates associated with tularemia, it is essential to make a quick and definitive diagnosis. This need is compounded by the status of *F. tularensis* as a potential biothreat. In the event of widespread exposure, or exposure in a combat situation a rapid and accurate diagnostic will result in lives saved. Accessible assays for direct detection of *F. tularensis* LPS may also be useful for both field and laboratory analysis of environmental and wildlife samples for monitoring reservoirs of disease. Production of a library of novel antibodies specific to *F. tularensis* LPS allows for development of a sensitive and specific lateral flow immunoassay that could potentially be developed into a rapid point of care diagnostic for tularemia, a necessity for diagnosis and efficient resource allocation for treatment. We have shown that the prototype LFI is reactive with both *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* in both attenuated and fully virulent strains, whilst not reacting with near neighbors. This is an important step towards ensuring that a future diagnostic assay will be specific for tularemia.

Production of a monoclonal antibody library will likely lead to the development of new assays for diagnosis and potentially treatment of tularemia. All mAbs produced in this study were shown to be reactive by Western blot with both *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*, the strains most responsible for causing human disease. In order to produce the most analytically sensitive (lowest LOD) ELISA and LFI it was crucial to test all mAbs in the capture and detection position to determine the best pair of mAbs for each specific assay. This all-by-all testing procedure is labor intensive, however facilitates the development of the most analytically sensitive assay possible,

which can be seen in the variation in sensitivity in the all-by-all testing. Interestingly, although many mAb pairs were able to detect LPS with relative sensitivity, three mAbs were consistently amongst the most sensitive: 1Ft5 and 1Ft6 in the LFI, 1Ft5 and 1Ft7 in the ELISA. mAb pairs were finalized in their respective assays and conditions optimized in patient matrices of interest.

Our previous study that utilized a technique called *In vivo* Microbial Antigen Discovery (InMAD), supports the finding that LPS is shed/secreted into the blood during infection, as LPS reactivity was seen in immune serum from mice immunized with 0.2 μm filtered serum from a tularemia infection model[102]. Very little is known regarding the presence and concentration of soluble *F. tularensis* LPS within clinical samples, primarily due to a focus on detection of anti-LPS antibodies for diagnosis. It was therefore critical to utilize the antigen-capture ELISA to quantify LPS levels in tularemia patient serum samples as a starting point. LPS was quantifiable in 8/19 samples, a promising result given that these samples were filtered to remove viable bacteria, therefore any cell associated LPS was lost. The abundance of LPS on the bacterial surface suggests that it may be a valuable diagnostic antigen. The quantifiable presence in filtered patient samples indicates a portion of LPS was shed/secreted into the blood during infection. Although LPS was detected in patient samples for this study, many of the concentrations are near the calculated limit of detection for the assay. It is possible that this low concentration is due to the loss of cell associated LPS, however it could also be true that serum is not the optimal matrix for detection of this antigen. Previous work from our group studying the shedding of *Burkholderia pseudomallei* capsular polysaccharide (CPS) has determined that the highest concentrations of CPS can be found in the urine, making it the optimal

diagnostic matrix for CPS detection[160]. It has been shown that *F. tularensis* can colonize the kidney in animal models and infected wild animals, therefore it is possible that LPS may also be shed into the urine[161–164]. Further studies are needed in order to determine if LPS is detectable in additional matrices such as urine, lymph node biopsies and abscesses, the latter of which are commonly reported to have high bacterial burden. Analysis of lymph node biopsies or aspirates is a common technique for indicating *F. tularensis* infection[165,166]. In addition to exploring presence of LPS in clinical samples, we would like to examine how LPS is shed/secreted over the course of *F. tularensis* infection in order to characterize how this biomarker can be most efficiently detected. The ELISA developed here is a useful tool that can be employed in future studies for establishment of a clinically relevant range in patient matrices and also in samples collected from animal models of tularemia.

We have shown that the prototype LFI is reactive with both *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* in both attenuated and fully virulent strains, whilst not reacting with near neighbors. The resulting LFI prototype is also non-reactive with purified LPS from several other bacterial species. The next step in assay development for commercialization is a more exhaustive cross-reactivity panel with other microbes that have similar clinical presentations or are commonplace amongst the population. Testing cross-reactivity is important to ensure that the test is specific for tularemia and reduce the likelihood of a false positive test.

When testing the sensitivity of the selected LFI prototype in human serum and urine as two potential matrices of interest, the LOD for both was found to be ~5 ng/mL by three

blinded readers. This LOD is higher than the LPS concentrations quantified in 6/8 samples by ELISA, and therefore raises a question regarding the clinical utility of the assay. It is again important to note that these samples were filtered and therefore do not contain any LPS associated with the bacterial cell and are likely not representative of LPS levels in an unfiltered sample. Furthermore, reduction in sensitivity of an assay prototype when moving from buffer to patient samples is not uncommon, as these samples differ in areas such as protein concentration and host antibodies[167]. As the assay tested is an early prototype, there are many areas of the assay that can be optimized to better accommodate different sample types. Examples include addition of sample pads treated to buffer samples before they reach the nitrocellulose, or additives to the sample or running buffers to neutralize the effects of excess proteins in the sample to be tested [168]. Lateral flow assays have the potential to be able to accommodate many different sample types through optimization of components and minor changes to sample preparation protocols [169,170]. One example of how the assay could potentially be modified to improve sensitivity can be seen in the application of sample enrichment using magnetic particles to boost signal in low antigen concentration samples with sufficient volume and potentially reduce influence on the assay performance from patient matrices.

There are currently no approved vaccines for tularemia. The live vaccine strain was developed for this purpose as a derivative of a virulent Type B isolate, however the mechanism by which this strain is attenuated is unclear[171]. Studies of the immune response to *F. tularensis* have shown that antibodies are produced to LPS components after immunization with LVS[172]. Antibodies generated against *F. tularensis* LPS have been shown to be protective in mouse models of lethal intradermal and intraperitoneal

challenge, with administration of immune serum resulting in survival of mice when challenged[173]. Infection-derived murine antibodies to *F. tularensis* LPS showed 100% protection from infection with *F. tularensis* LVS when administered prophylactically. Administration of anti-LPS mAbs therapeutically significantly increased survival of mice post-challenge[174]. These studies are indicative of a potential role for mAbs to *F. tularensis* LPS as prophylactic or therapeutic options for tularemia and provides an avenue of interest for further research using the mAbs isolated in this study.

2.5 Materials and methods

mAb production

8-week-old female CD1 mice (Charles River Laboratories, Inc., Wilmington, MA) were immunized intraperitoneally with LPS purified from *F. tularensis* subspecies *holarctica* (NR-2627) (BEI Resources, Manassas, VA) either alone or coupled to BSA using the Imject™ EDC BSA Spin Kit (Thermo Scientific, Waltham, MA) to improve immunogenicity, in both instances with Alhydrogel adjuvant 2% (Invivogen, San Diego, CA). An indirect ELISA was used as outlined below to determine antibody titers to LPS in mouse immune serum at 6- and 8-weeks post-immunization. Mice were immunized with a final dose of purified LPS alone three days prior to spleen harvest. Fusions were performed and hybridoma cells produced using standard techniques[132]. Supernatant was collected from hybridoma cells and mAbs purified using recombinant protein A affinity chromatography.

Ethics statement

Laboratory work with animals was approved by the University of Nevada, Reno Institutional Animal Care and Use Committee (Protocol # 00024). All work with animals is supervised by the Office of Laboratory Animal Medicine, which follows the National Institutes of Health Office of Laboratory Animal Welfare policies (Assurance # A3500-01).

Indirect ELISA

96-well medium-binding microtiter plates (Grenier Bio-One, Austria) were coated with 1.25 μg/mL *F. tularensis* LPS overnight. The plate was then washed 3x with PBS containing

0.05% Tween 20 (PBS-T) and blocked for 90 minutes at 37 °C in PBS containing 0.5% non-fat milk and 0.1% Tween 20 (blocking buffer), followed by a second wash in PBS-T. Primary antibody in the form of mouse immune serum, hybridoma supernatant or purified antibody (1 μg/mL) was added to the first well and serial two-fold dilutions performed across the plate. The plate was then washed with PBS-T and incubated with horseradish peroxidase (HRP) labeled goat anti-mouse IgG antibody (SouthernBiotech, Birmingham, AL), either whole IgG or isotype specific, at a 1:1000 dilution in blocking buffer for 1 hour. The plate was washed a final time in PBS-T and incubated with tetramethylbenzidine (TMB) substrate (SeraCare, Milford, MA) for 30 minutes. The reaction was stopped with 1M H_3PO_4 and the absorbance read at OD_{450}.

Western immunoblot

Standard semidry Western blot procedure was performed using Proteinase K-treated $5x10^8$ colony forming units (CFU)/mL formalin inactivated *F. tularensis* subsp. *tularensis* strain SchuS4 cells (NR-15753) (BEI Resources) or using $5x10^8$ CFU/mL gamma-irradiated *F. tularensis* subsp. *holarctica* cells (FRAN-012), *F. tularensis* subsp. *novicida* (FRAN-003) and *F. philomiragia* (FRAN-017) (Department of Defense Critical Reagents Program, Frederick, MD). Samples were boiled with 6x sample buffer, separated on 10% SDS gel (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membrane (Bio-Rad). HRP-conjugated mAbs 1Ft1-10 were used to probe the membrane at a concentration of 1 μg/mL using a Miniblotter system (Interchim, Montluçon, France), which enables probing of one antigen preparation with multiple antibodies. Signal was detected with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA). Images were taken using a ChemiDoc XRS system (Bio-Rad).

Antigen-capture ELISA

96-well microtiter plates were coated with 100 µL/well capture mAb (1 µg/mL) in PBS overnight. Plates were washed in phosphate buffered saline with 0.5% Tween-20 (PBS-T) and blocked at 37 °C with 200 µL/well phosphate buffered saline containing 0.5% Tween-20 and 5% skim milk (blocking buffer). Plates were washed and Purified LPS was added to the first well at a concentration of 100 ng/mL and serial diluted two-fold across each plate in blocking buffer for a final volume of 100 µL/well. Plates were incubated for 60 minutes at room temperature, then washed with PBS-T and incubated with HRP-labelled mAb at 1 µg/mL in blocking buffer for a total of 100 µL/well. HRP labelling of mAbs was done using EZ-link Plus Activated Peroxidase (ThermoFisher). Plates were washed with PBS-T and incubated with 100 µL/well TMB substrate (SeraCare). The reaction was stopped after 30 minutes with 1M H_3PO_4 (100 µL/well). Plates were read at an optical density of 450nm (OD_{450}).

Optimization of antigen-capture ELISA in serum and urine

Checkerboard ELISAs were performed to optimize the concentrations of coating and detection mAbs in pooled normal human serum and urine (Innovative Research, Novi, MI). Capture and detection mAb concentrations were both tested at a range of concentrations from 0.16-20 µg/mL to assess which concentration was the most sensitive without exhibiting non-specific binding. The remainder of the ELISA was performed as described above, with purified *F. tularensis* LPS spiked into pooled normal human serum or urine at a concentration of 50 ng/mL, serially diluted in blocking buffer and incubated for 90

minutes at room temperature. Final optimized conditions were chosen for 1Ft5 capture (2.5 μg/mL) and1Ft7 detection (0.625 μg/mL) as the pair that gave the lowest LOD in both serum and urine for potential future diagnostic applications.

Patient samples

Archived samples from patients with confirmed diagnosis of tularemia either by serological or PCR techniques were obtained from Hacettepe University, Turkey. Experiments using human samples were approved by the University of Nevada, Reno Institutional Review Board. Samples were 0.2 μm filtered to remove viable bacteria in a biosafety level 3 laboratory. Each sample was verified for sterility using a validated protocol and removed to biosafety level 2 for analysis.

Quantitative antigen-capture ELISA

An optimized antigen capture ELISA was performed using the tularemia patient serum samples according to the optimized conditions described above with mAb 1Ft5 coated in PBS at 2.5 μg/mL overnight. Plates were washed and blocked, then purified *F. tularensis* LPS (BEI Resources) was two-fold serially diluted across the plate starting at 50 ng/mL as a standard curve, totaling 100 μL/well. 200 μL patient serum samples were added to the plate and 2-fold serial diluted across prior to incubation for 2 hours at room temperature. Plates were washed again and 100 μL/well HRP-conjugated 1Ft7 was added at 0.625 μg/mL diluted in blocking solution for 1 hour. Plates were washed and incubated with 100μL/well TMB substrate for 30 minutes (SeraCare). Reaction was stopped with 100

μL/well 1M H_3PO_4 and read at OD_{450}. Samples were analyzed in triplicate where possible, however due to limitations in sample volume this was not feasible for all samples.

LFI screening

Initial screening was performed with each mAb in the capture position on the test line and as the detection gold conjugate to test every combination and rank the most sensitive pairings. Testing was done using a default LFI prototype to test reactivity to purified LPS in PBS and non-specific binding in buffer alone. Briefly, 5 μL of gold conjugate at OD 10 was added to the conjugate pad, followed by 40 μL of 500 ng/mL LPS in PBS. The test was then placed vertically in the well of a microtiter plate containing 150 μL chase buffer and allowed to run for 15-20 minutes. LFIs were evaluated visually and read using a Qiagen ESE lateral flow reader then ranked based on the intensity of the test line minus non-specific binding in buffer alone. Top candidates were tested similarly in pooled normal human serum to select the pair with best signal and lowest non-specific binding in human matrices for downstream application.

LFI prototype

Upon selection of 1Ft6 as the optimal capture mAb and 1Ft5 as the gold conjugated mAb according to the above selection criteria, optimization of LFI components and reagents was undertaken. 1Ft6 was applied to CN95 nitrocellulose membrane (Sartorius, Gottingen, Germany) at a concentration of 1 mg/mL in PBS as the test line via contact dispense using a BioDot XYZ platform (BioDot, Irvine, CA). Goat anti-mouse Ig (SouthernBiotech, Birmingham, AL) was dispensed as the control line at 0.5 mg/mL also

in PBS. Nitrocellulose was dried for 30 minutes at 37 °C. LFIs were assembled onto an adhesive backing card with the sprayed nitrocellulose overlapped by CF6 wicking pad (GE Healthcare, Chicago, IL) to allow for capillary flow. Test strips were cut to 4mm width and stored in sealed foil pouches with desiccants. 1Ft5 was passively adsorbed to 40nm colloidal gold particles (DCN Diagnostics, Carlsbad, CA), and diluted to OD_{540} = 10 in 0.05M sodium phosphate, 0.2% 10G, 0.25% BSA, 20% sucrose, 5% trehalose.

LFI testing

LFI prototypes were tested with inactivated cells from various strains of *F. tularensis* and near neighbors to confirm reactivity with clinically relevant *F. tularensis* strains and their derivatives and to ensure no cross-reactivity with near neighbors known to have structurally distinct LPS. Glycerol stocks of *F. tularensis* subspecies *holarctica* LVS and *F. tularensis* subspecies *tularensis* NIH-B38 (BEI Resources) were grown in BHI broth supplemented with cysteine and inactivated by heating to 80 °C for two hours. OD_{600} was taken and the preparations diluted to approximately $1x10^7$ CFU/mL. Formalin inactivated *F. tularensis* subsp. *tularensis* strain SchuS4 (BEI Resources), gamma-irradiated *F. tularensis* subsp. *holarctica* cells, *F. tularensis* subsp. *novicida* and *F. philomiragia* (Critical Reagents Program) and were diluted to $1x10^7$ CFU/mL based on the product information provided. BHI broth supplemented with casein and running buffer alone were used as the negative controls. Samples were tested by placing the strip in a 96 well plate containing 18 μL sample buffer (50 mM borate, 0.5% BSA, 1 μg/mL Mouse IgG, 1% surfactant 10G (Fitzgerald Industries International, Acton, MA)) and 2 μL antigen. Once all liquid in the well was absorbed, the strip was moved to a well containing 15 μL running buffer and left until all buffer was absorbed. The strip was then moved to a well containing 15 μL running

buffer and 5 μL 1Ft5 gold conjugate at OD_{540} = 10. Once all gold was absorbed, the strip was moved to a final well containing 40 μL running buffer. Once all liquid was absorbed the LFI was assessed visually for reactivity as it would be by a clinician. To test reactivity to purified LPS, the above procedure was followed but with 100 ng/mL purified LPS from *B. pseudomallei, Pseudomonas aeruginosa*, *Salmonella typhimurium* and *F. tularensis* instead of inactivated cells. To determine a preliminary LOD in pooled normal human serum and urine, the same procedure outlined above was followed but with 18 μL total volume serum or urine containing 100 ng/mL mouse IgG and 1% surfactant 10G (Fitzgerald Industries International, Acton, MA) and 2 μL purified *F. tularensis* LPS. Signal was assessed as positive or negative by three blinded readers and the LFIs read on an ESE-Quant lateral flow reader (Qiagen, Hilden, Germany).

Sample pre-concentration

To investigate potential ways to improve the sensitivity of the LFI for detection of *F. tularensis* LPS, a magnetic immunoprecipitation protocol was adapted for concentration of antigen from larger sample volumes. Briefly, 1Ft7 was coupled to Dynabeads™ M-270 Epoxy (Thermo Fisher Scientific) according to manufacturer's instructions at a ratio of 20 μg 1Ft7 /1 mg Dynabeads. The conjugate was added to 10 mL samples of PBS spiked with purified *F. tularensis* LPS in two-fold serial dilutions from 5 ng/mL to 0.3125 ng/mL and incubated at room temperature to allow binding to take place. The mAb-Dynabead complex bound to LPS was removed from the original sample with a magnet and the LPS eluted with 1M Glycine-HCl, pH 1.3. Dynabeads were again captured with a magnet and the supernatant containing eluted LPS removed and neutralized with 1M Tris, pH 13.0.

This sample was run on the LFI prototype as described above and read with the ESE-Quant lateral flow reader (Qiagen).

The antigen-capture ELISA described above was used to quantify the presence of LPS in the elution fraction that was run on the LFI prototype and the amount remaining unbound in the supernatant. The protocol remained the same with the exception of substituting the elution and supernatant for the patient samples.

2.6 Acknowledgments

We would like to thank Dr Paul Brett at the University of Nevada, Reno for providing purified LPS from *Burkholderia pseudomallei, Pseudomonas aeruginosa* and *Salmonella typhimurium*.

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2.7 Figures and tables

Table 1. IgG subclass, immunization strategy and Western blot reactivity of mAbs

mAb	Subclass	Immunization	*F. tularensis tularensis*	*F. tularensis holarctica*	*F. tularensis novicida*	*Francisella philomiragia*
1Ft1	IgG1	Ft. LPS-BSA	+	+	-	-
1Ft2	IgG2b	Ft. LPS-BSA	+	+	-	-
1Ft3	IgG2b	Ft. LPS-BSA	+	+	-	-
1Ft4	IgG1	Ft. LPS-BSA + Alum	+	+	-	-
1Ft5	IgG1	Ft. LPS-BSA	+	+	-	-
1Ft6	IgG2b	Ft. LPS-BSA + Alum	+	+	-	-
1Ft7	IgG2b	Ft. LPS-BSA + Alum	+	+	-	-
1Ft8	IgG2b	Ft. LPS-BSA + Alum	+	+	-	-
1Ft9	IgG2b	Ft. LPS-BSA + Alum	+	+	-	-
1Ft10	IgG2b	Ft. LPS-BSA + Alum	+	+	-	-

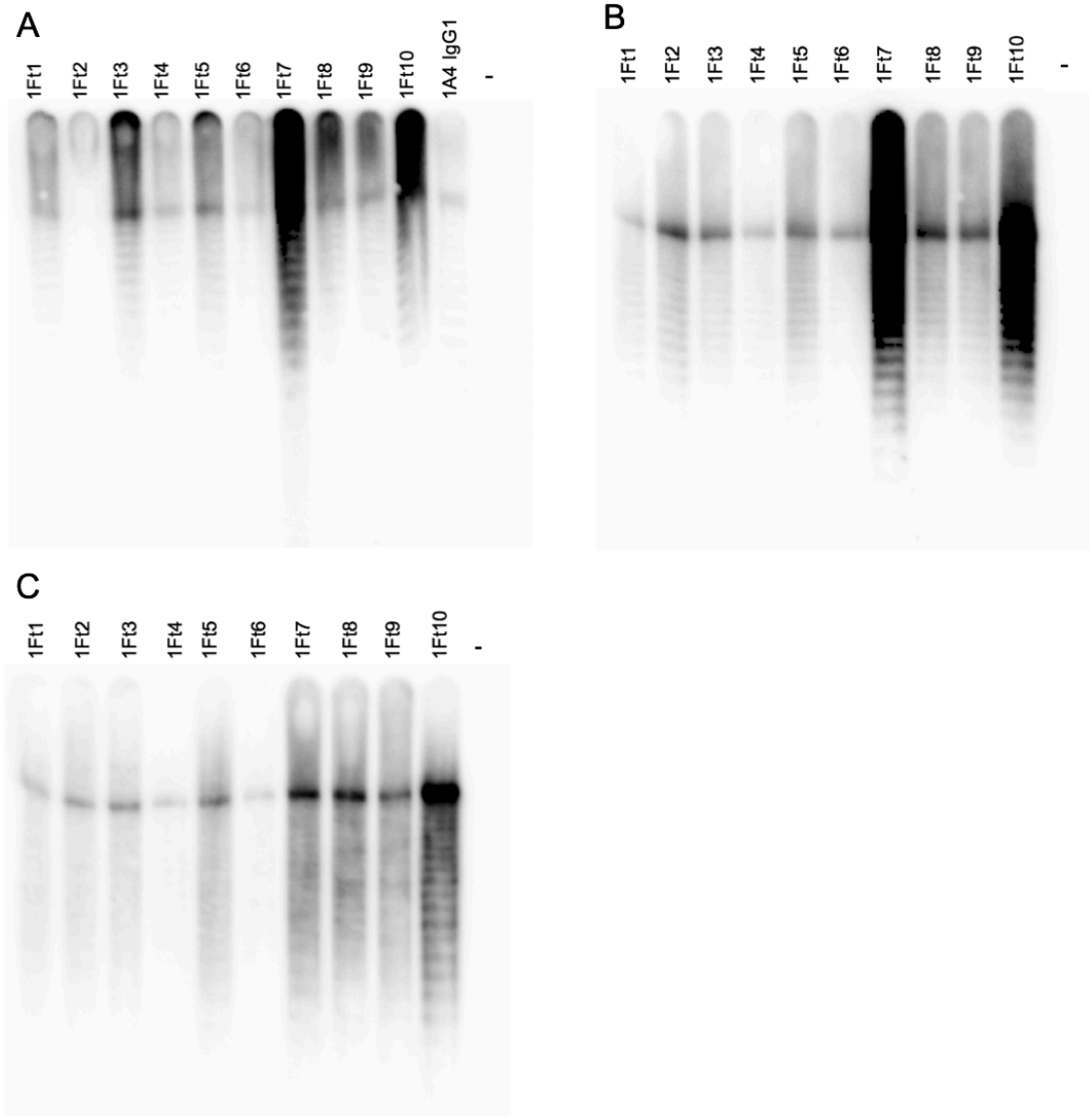


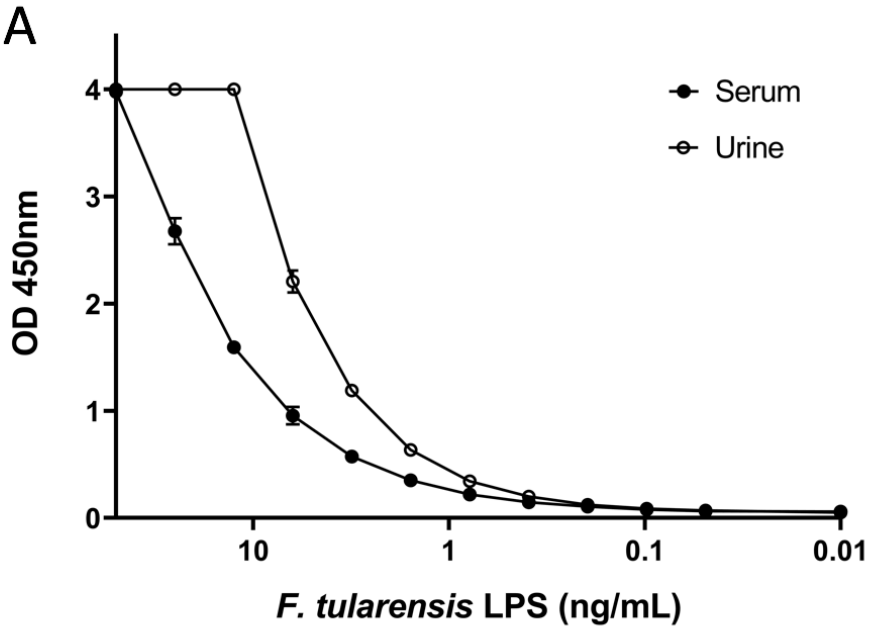
Figure 1. mAb reactivity with purified *F. tularensis LPS and F. tularensis* strains (type A and type B). Purified, HRP-conjugated mAbs were used to probe 1 ug/lane purified LPS (A), $5x10^8$ CFU/mL F. *tularensis* subsp. *tularensis* SchuS4 (type A strain) (B) and *F. tularensis* subsp. *holarctica* (type B strain) (C) by direct Western blot.

Table 2. Preliminary analytical sensitivity of mAb pairs evaluated in an antigen-capture ELISA with *F. tularensis* LPS antigen (ng/mL) spiked into PBS

		Capture mAb[a]									
		1Ft1	**1Ft2**	**1Ft3**	**1Ft4**	**1Ft5**	**1Ft6**	**1Ft7**	**1Ft8**	**1Ft9**	**1Ft10**
Detection mAb	**1Ft1**	2.43	0.56	1.17	17.40	4.81	3.43	1.00	1.54	0.81	12.98
	1Ft2	1.11	1.47	8.35	13.63	1.98	0.54	0.42	1.28	0.80	17.44
	1Ft3	0.61	2.38	0.50	14.83	3.78	1.74	1.18	1.82	1.77	16.72
	1Ft4	7.22	11.53	6.48	52.39	15.36	8.24	5.56	8.61	6.31	91.93
	1Ft5	0.93	2.61	0.89	15.47	3.74	1.49	0.39	0.46	0.68	3.92
	1Ft6	1.69	0.92	16.25	8.92	3.1	0.97	0.98	1.18	0.68	9.39
	1Ft7	0.53	0.99	0.71	13.59	0.47	0.49	0.41	0.45	0.48	2.31
	1Ft8	0.97	0.96	0.79	10.03	2.72	0.95	0.35	0.63	0.40	3.88
	1Ft9	1.97	1.14	0.92	19.96	4.45	0.57	0.45	0.54	0.37	5.11
	1Ft10	4.34	4.91	3.98	56.50	20.37	1.71	0.96	4.15	0.94	13.12

[a] Each mAb was used a standard concentration of 1 µg/mL for both capture and detection

A



B

Matrix	LOD (ng/mL)	SD (± ng/mL)
Serum	0.18	0.067
Urine	0.13	0.028

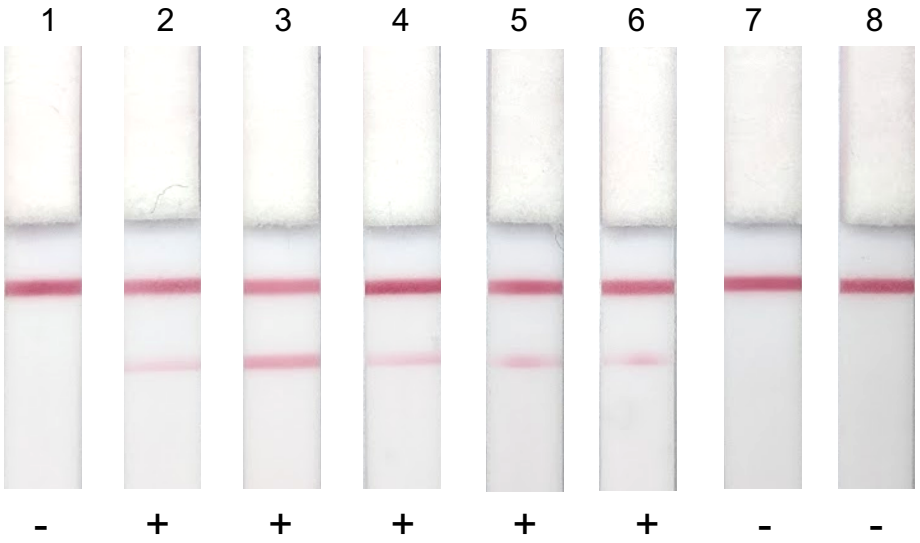
Figure 2. Sensitivity of optimized *F. tularensis* LPS antigen-capture ELISA. (A) Reactivity of the optimized antigen capture ELISA with *F. tularensis* LPS spiked into normal human serum and urine. (B) Limit of detection (LOD) of the assay in each matrix; LOD was calculated using a cutoff value of 2x background.

Table 3. Analysis of LPS concentrations in patient serum samples by antigen-capture ELISA

Sample #	LPS (ng/mL)	Standard deviation	Diagnosis
1	0	-	PCR
2	Insufficient volume	-	PCR
3	0	-	PCR
4	Insufficient volume	-	PCR
5	0	-	PCR
6[#]	0.74	0.0039	PCR
7	0	-	PCR
8	0	-	PCR
9*	0.35	-	PCR
10	0.22	0.044	PCR
11	109.95	12.11	PCR
12	0.41	0.088	PCR
13	0	-	PCR
14	0	-	Serology
15	0	-	Serology
16	0	-	Serology
17	0	-	Serology
18	5.023	0.70	Serology
19	0.36	0.051	Serology
20	0.33	0.16	Serology
21	0	-	Serology

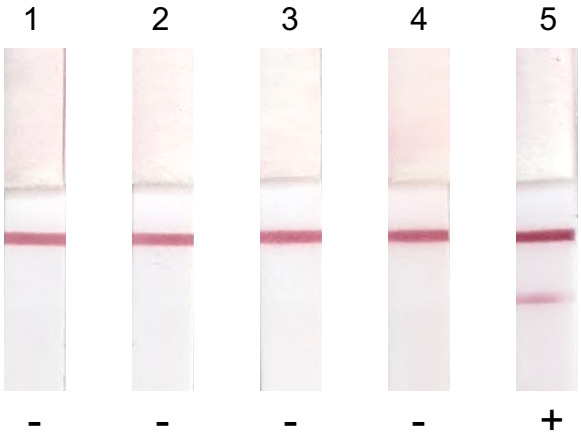
***** Sample analyzed as a single replicate due to sample volume limitations

[#] Sample analyzed in duplicate due to sample volume limitations



LFI	Antigen
1	BHI-c (Negative)
2	100ng/mL purified *F. tularensis* LPS
3	*F. tularensis* LVS
4	*F. tularensis* NIH-B38
5	*F. tularensis* subsp. *holarctica*
6	*F. tularensis* subsp. *tularensis*
7	*F. novicida*
8	*F. philomiragia*

Figure 3. Reactivity of LFIs with clinically relevant *F. tularensis* strains and near neighbors. Prototype LFIs were run with a panel of killed whole cells to determine potential usefulness as a diagnostic of tularemia.



1	Sample buffer
2	*Burkholderia pseudomallei* LPS
3	*Pseudomonas aeruginosa* LPS
4	*Salmonella typhimurium* LPS
5	*Francisella tularensis* LPS

Figure 4. Reactivity of LFIs with purified LPS from different bacteria. Prototype LFIs were tested with 100 ng/mL purified LPS from other species of bacteria to determine potential for cross-reactivity.

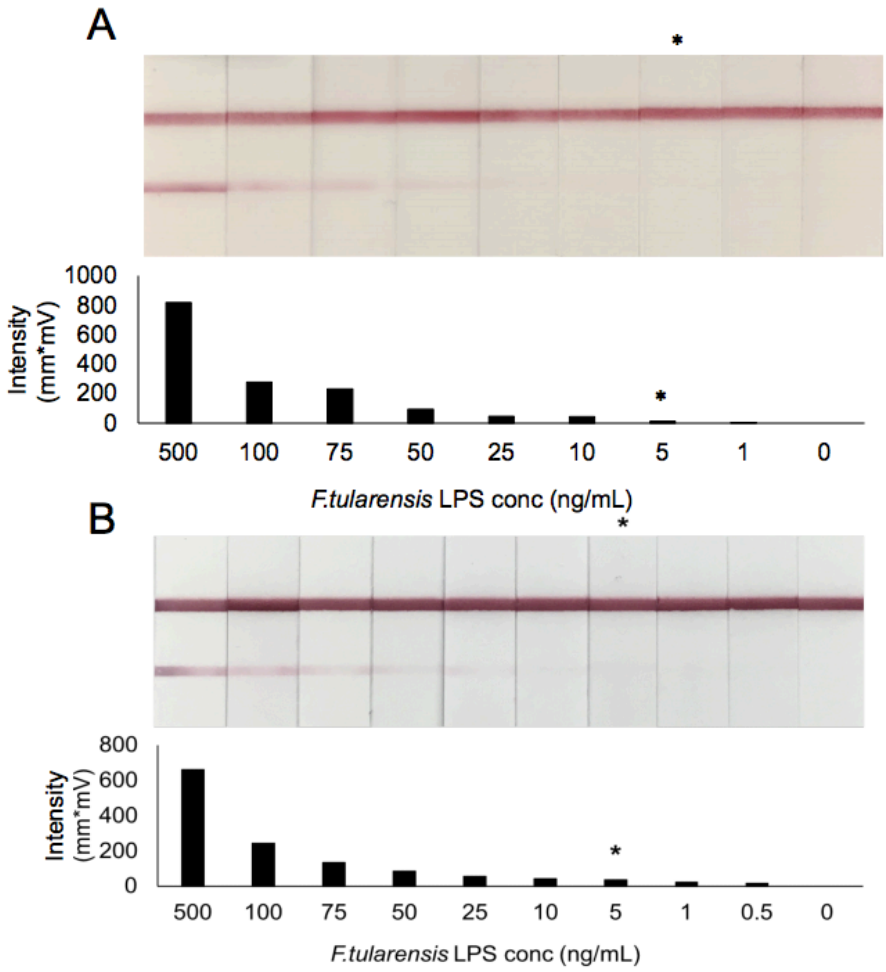
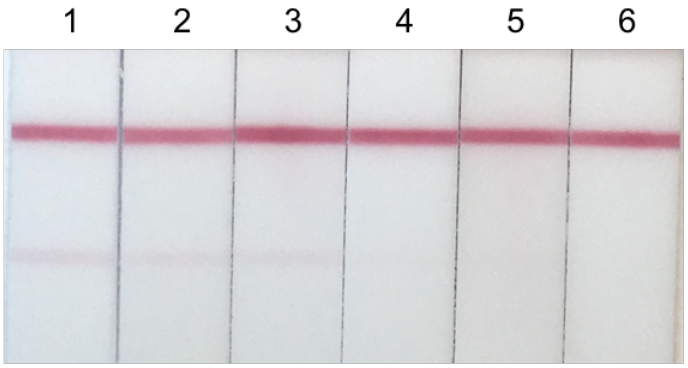


Figure 5. LFI prototype limit of detection in pooled normal human serum (A) and urine (B) spiked with purified *F. tularensis* LPS. Visual LOD is indicated (*) as assessed by three blinded readers. Test line intensity is shown as given by the ESE-Quant lateral flow reader.

A



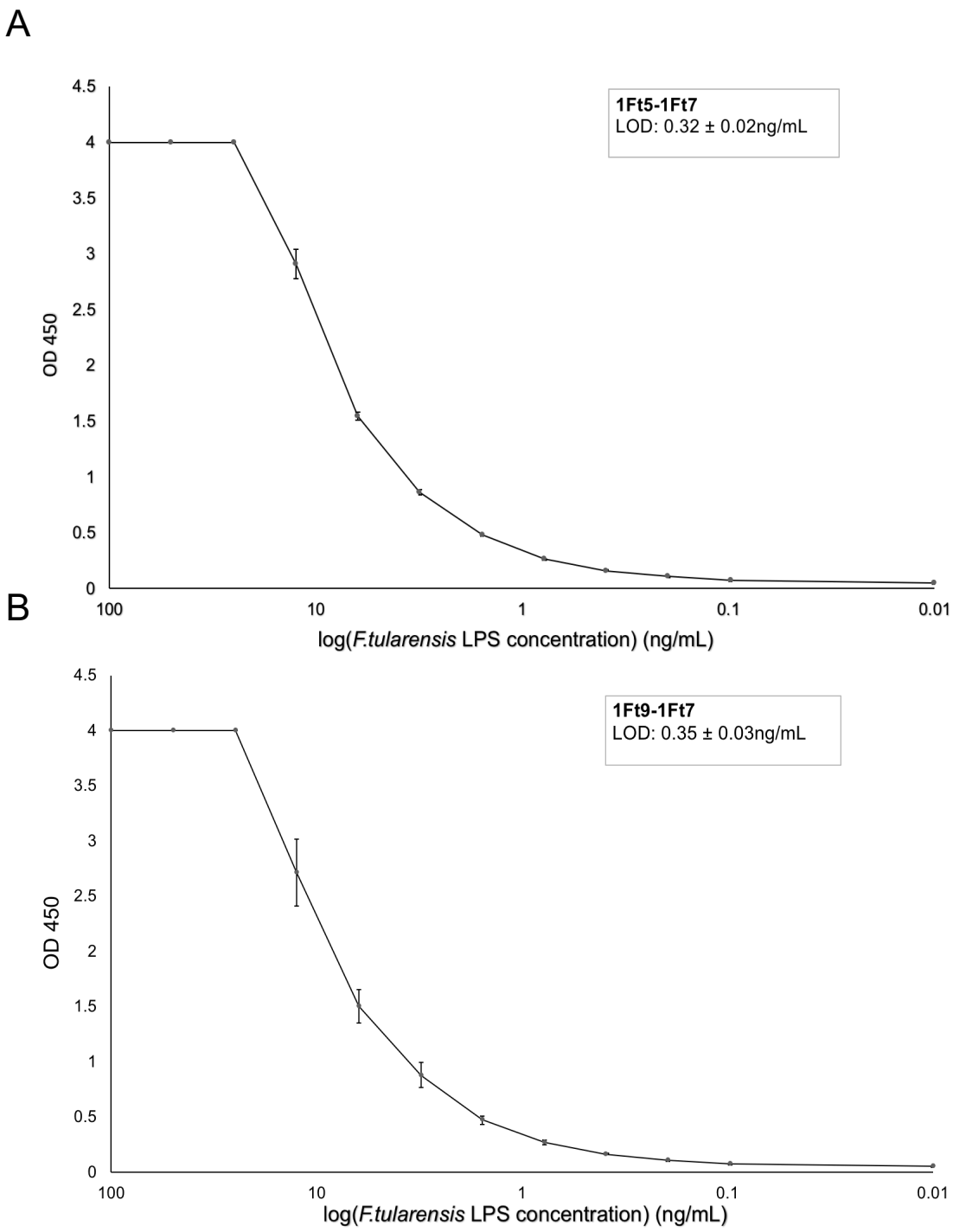
	ng/mL *F. tularensis* LPS	Visual assessment	Intensity mm*mV
1	5.00	+	132
2	2.50	+	83
3	1.25	+	69
4	0.63	+	26
5	0.31	+	10
6	0.00	-	0

B

Fraction	% total LPS
Elution	39
Supernatant	54
Lost	7

Figure 6. Visualization of *F. tularensis* LPS on the LFI prototype following Dynabead pre-concentration protocol samples and quantification of proportion LPS bound. The *F. tularensis* LPS LFI prototype was able to detect 0.31 ng/mL LPS following pre-

concentration of a 10 mL sample (A). Percentage of LPS in the original sample run on the LFI was determined by quantitative antigen-capture ELISA (B).



Supplemental Figure 1. Sensitivity of top two antigen-capture ELISA mAb pairings detecting purified *F. tularensis* LPS in PBS. Limit of detection was analyzed for the two best performing pairs 1Ft5-1Ft7 (A) and 1Ft9-1Ft7 (B) in buffer.

Supplemental Table 1. Initial testing and ranking of top 20 mAb pairs in the LFI format to determine the optimal combination to proceed with further optimization

Capture	Gold	PBS signal - background	1% casein in PBS signal – background	Optimized casein %	LOD (ng/mL)
1Ft6	1Ft5	597	714	1.0	1
1Ft6	1Ft1	654	679	0.8	1
1Ft2	1Ft5	107	592	0.4	1
1Ft3	1Ft6	361	581	0.6	1
1Ft5	1Ft2	391	545	0.8	1
1Ft3	1Ft2	381	406	0.2	1
1Ft7	1Ft1	699	768	0.6	5
1Ft2	1Ft1	332	625	0.4	5
1A4	1Ft2	296	569	0.4	5
1Ft3	1Ft3	395	684	0.2	10
1Ft5	1Ft5	394	573	0.4	10
1Ft4	1Ft5	293	479	0.8	10
1Ft5	1Ft3	249	448	0.4	10
1Ft3	1Ft5	342	365	0.2	10
1A4	1Ft5	181	108	1.0	10
1Ft3	1Ft1	526	719	0.2	25
1Ft5	1Ft1	494	484	0.2	25
1A4	1Ft3	419	311	0.4	25
1Ft1	1Ft1	131	415	0.2	50
1A4	1Ft6	330	132	0.6	50

CHAPTER 3

Production of monoclonal antibodies for detection of leptospiral protein LipL32

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3.1 Abstract

Pathogenic spirochetal bacteria of the genus *Leptospira* are the causative agents of leptospirosis, a re-emerging neglected tropical disease that causes extensive morbidity and mortality, particularly in tropical regions. The genus *Leptospira* encompasses over 200 different identified serovars with varying levels of pathogenicity and is often endemic in a population, making efficient diagnosis challenging. Leptospirosis is also a disease of veterinary concern as it is zoonotic, often persisting in domestic and livestock animals where it can be transmitted to humans via infected urine, significantly impacting farming productivity and public health. LipL32 is a surface lipoprotein conserved amongst pathogenic strains of *Leptospira* that is abundant on the cell surface and has been shown to be shed in the urine of infected patients, making it a promising diagnostic biomarker. Female CD1 mice were immunized with His-cleaved recombinant LipL32 cloned from *Leptospira interrogans* strain RGA and their splenocytes used to make antibody-producing hybridoma cell lines. A library of fifteen monoclonal antibodies were purified and shown to be reactive with pathogenic *Leptospira* species and non-reactive with a saprophytic (non-pathogenic) strain by Western blot. These antibodies were used to develop a Western blot for detection of *Leptospira* in pooled normal human urine.

3.2 Introduction

Leptospirosis is an emerging, neglected tropical disease caused by pathogenic species of the genus *Leptospira*[63,175]. Global distribution of leptospirosis is broad, and pathogenic leptospires have been isolated from a range of reservoir animals in both urban and rural environments[64,176,177]. Disease severity in humans varies from asymptomatic to life-threatening febrile illness, with a range of non-specific symptoms including fever, headache and myalgia[63]. Many of these symptoms are shared with other febrile tropical diseases such as dengue fever, chikungunya fever, rickettsial disease and malaria[56,178]. In the most severe cases leptospirosis is associated with pulmonary hemorrhage syndrome[87,179,180] and acute kidney injury (Weil's disease)[181,182], complications that are often fatal, especially if not treated promptly and effectively. Frequent misdiagnosis due to non-specific symptoms combined with a lack of accessible diagnostics have led to under-reporting of cases globally[58]. Statistical modelling generated in 2015 estimated 1,030,000 cases and 58,900 deaths annually due to leptospirosis, with 73% of cases and deaths occurring in tropical areas[64].

The zoonotic nature of leptospirosis makes it a disease of veterinary as well as human health concern. Infection of livestock has been linked to miscarriage and stillbirth in pregnant cattle, leading to economic loss for farmers in endemic areas[98]. In addition, persistent infection in livestock and domestic animals such as dogs can lead to human infection from exposure to infected urine or contamination of soil and water sources[83]. One of the key issues is the prevalence of subclinical infections that can lead to increased spreading within a population and exposure of vulnerable animals or people. Diagnosis in animal species can face many of the same pitfalls as in humans. Immunoassays have

been developed for use in detecting serological responses to infection in livestock, however there can be issues of cross-reactivity in animals who have been vaccinated for a certain serovar[99,183].

The most sensitive and specific way to diagnose leptospirosis is by polymerase chain reaction (PCR), which is able to detect bacterial DNA in patient blood 5-10 days from disease onset, and has been shown to detect *Leptospira* even in culture negative patients[184]. Although extremely sensitive and specific, this testing is largely unavailable in resource-poor endemic areas, meaning that diagnosis relies on bacterial culture and serological testing[185]. The micro agglutination test (MAT) is the standard testing procedure for many laboratories to determine serological reactivity to pathogenic *Leptospira*. Briefly, live leptospires from locally circulating serovars are maintained in the laboratory and agglutination with patient sera is observed via darkfield microscopy. There are over 200 documented serovars of *Leptospira,* including non-pathogenic strains[186], making construction of an exhaustive reference panel to eliminate false negatives extremely challenging[185]. In areas with high levels of *Leptospira* present in the environment, serological positives can occur in the absence of active leptospirosis as titers can take months to years to drop[63,187].

Meta-analysis of studies quantifying shedding of *Leptospira* in host urine has suggested that the quantity of leptospires shed varies across different host species[188]. Due to the variety of locations, species and experimental designs explored, it is difficult to determine a clinical range for the quantity of bacteria shed during infection. Clinically asymptomatic rats experimentally infected with pathogenic *Leptospira interrogans* shed between $1x10^5$

and $1x10^7$ leptospires per mL of urine from day 6 to day 159 of the experiment[189]. A study in dogs indicated an average of $6.22x10^4$ leptospires/mL in urine with a range of 35.5 leptospires – $1.33x10^6$ leptospires/mL[190]. This range of values highlights an important consideration for direct detection assays in terms of the availability of antigen at various stages of infection. A study of febrile patients in Peru indicated a range of 10^2 – 10^6 leptospires per mL of urine at time of admission and one patient continued to have detectable levels of *Leptospira* in the urine for more than 140 days[179]. Investigation of leptospiruria during asymptomatic infection in humans showed a range of $0.32x10^2$ – $4.64x10^4$ leptospira/mL of urine[191], indicating the potential utility of an antigen-capture assay for urine in both symptomatic and asymptomatic infections. Similar ranges of bacterial load in urine have been indicated in cows, deer and mice[192–194], suggesting that a urine-based antigen-capture immunoassay may be of use for multiple species and play a role in environmental and livestock surveillance.

One of the most promising targets for diagnosis of Leptospirosis via direct antigen detection is the outer membrane protein LipL32. This lipoprotein is the most abundant protein on the leptospiral cell surface and is conserved across pathogenic *Leptospira*[116]. PCR assays have been developed targeting LipL32 and studies have shown that it is detectable during the acute phase of infection before host antibodies are generated in the immune phase of infection[121]. Analysis of patient urine for leptospiral antigens showed the presence of LipL32 by Western blot in patients confirmed to have leptospirosis by MAT and was identified in two patients with pyrexia of unknown origin, indicating diagnostic potential when trying to differentially diagnose infections with similar symptoms such as dengue fever[122].

The goal of this study was to create a library of monoclonal antibodies specific to leptospiral LipL32 for use in diagnostics for detection of antigen in patient urine. Fifteen mAbs were isolated and reactivity with killed pathogenic *Leptospira* species established. Antigen-capture immunoassays were explored but showed a lack of reactivity with native protein, leading to a focus on developing an assay for direct detection of samples. mAbs were screened for reactivity in a Western blot format and the optimal conditions used to determine a limit of detection for inactivated pathogenic *Leptospira* in pooled normal human urine.

3.3 Results

mAb production and reactivity

Female CD1 mice were immunized with recombinant LipL32 (rLipL32-1) in an emulsion with Freund's complete adjuvant. Titers to recombinant LipL32 were established by indirect ELISA and three mice with titers over 1:5,000,000 were selected for splenocyte isolation. Prior to splenocyte isolation, immune sera from immunized mice were assessed for reactivity with different heat inactivated *Leptospira* species and strains to indicate the reactivity profile. Immune sera were reactive with pathogenic strains of *Leptospira* by Western blot, less reactive to intermediately pathogenic species *L. inadai* and not reactive with saprophytic strain *L. biflexa* (Figure 1). This pattern of reactivity is reflective of the expected expression of LipL32 across the species of *Leptospira* and suggested that purified mAbs would be specific for pathogenic *Leptospira*. Fusion of splenocytes with a myeloma cell line was undertaken to produce antibody secreting hybridoma cells. Supernatant from these fusions was used to probe heat-inactivated *L. interrogans* L0370 by Western blot to confirm reactivity with native protein (Figure 2).

Fifteen monoclonal antibodies were isolated, purified and their subclass determined, as shown in Table 1. All isolated mAbs were determined to be IgG1, IgG2a or IgG2b subclass by direct ELISA. Reactivity of purified mAbs to heat-inactivated pathogenic *L. interrogans* L0370 serovar Pyogenes by Western blot is shown in Figure 3. All mAbs were reactive to killed cells to varying degrees, however mAbs 1Lp1, 1Lp2, 1Lp10 and 1Lp11 were less strongly reactive than the other mAbs.

Prototype LFI development and optimization in urine

To expedite production of a prototype LFI, screening of isolated mAbs in both the capture and detection position on a prototype LFI was undertaken. All mAbs were optimized and labelled with colloidal gold and sprayed on to nitrocellulose membrane at a standard concentration of 1 mg/mL for preliminary testing of 225 pairings in a basic prototype format. The top pairs were selected for screening in urine based on reactivity to rLipL32-1 in buffer and minimal levels of non-specific binding at the test line (Table 2).

After initial screening for reactivity and non-specific binding, assay development moved directly into optimization in pooled normal human urine. Undertaking the transfer into patient matrices early in assay development was done to minimize the need for re-optimization after preliminary testing in buffer. As anticipated, some optimization was needed to establish a prototype in urine due to the complications of composition involved in biological matrices. Modifications were primarily made to the running buffer to address persistent gold accumulation on the nitrocellulose. The prototype which gave the best reactivity and lowest non-specific binding in urine was 1Lp4 as the test line with 1Lp9 gold conjugate, illustrating how the highest signal-background in buffer does not necessarily translate to the most reactive pairing in human matrices. A comparison of reactivity and non-specific binding in the same prototype in buffer vs. normal human urine spiked with LipL32 is shown in Figure 4. There was a slight reduction in signal and some non-specific binding when run in urine, however this is could likely be resolved with additional downstream optimization.

Recombinant LipL32

As outlined previously, for mAb isolation mice were immunized with His-tagged recombinant LipL32 (rLipL32-1). This protein had a mutation resulting in an additional 40 amino acids at the C-terminus. Screening of immunized mice for reactivity with native protein confirmed that immune sera was reactive via Western blot with killed pathogenic *Leptospira* and therefore that the mutation did not appear to affect the immunogenicity of the protein or the response to the target of interest. Reactivity with native protein was also confirmed throughout the mAb isolation process by Western blot with killed pathogenic *Leptospira*. To confirm reactivity with purified protein that was more similar to native protein and provide antigen for any future applications, LipL32 was re-cloned and expressed in *E. coli* without the extra amino acids (rLipL32-2) for use as an antigen in further screening.

Comparison of rLipL32-1 and rLipL32-2

Concurrent with LFI development, an antigen-capture ELISA for detection and quantification of LipL32 was being developed. Screening of mAbs for use in this format followed a similar pattern to the above LFI screening and involved testing all combinations of mAbs in the capture and detection position to identify the most sensitive pairing. Reactivity was observed when all pairings were tested with the rLipL32-1, however when the newly purified rLipL32-2 was introduced there was little to no reactivity observed (Figure 5). The reactivity observed with 1Lp8 was found to be non-specific binding. A similar lack of binding was observed when rLipL32-2 was tested on the LFI prototype. To determine if the issue only occurred in the antigen-capture format, plates were coated with rLipL32-2 and probed directly with HRP-conjugated detection mAbs. All tested HRP-

conjugated mAbs were strongly reactive with the new protein, indicating that there was not an issue with the detection mAbs. Testing of pairings in the antigen-capture format with heat inactivated pathogenic *Leptospira* yielded very little reactivity not attributable to non-specific binding.

To assess the differences in reactivity, rLipL32-1 and rLipL32-2 were probed with immune sera from the original immunization, an example of which is shown in Figure 6. Immune sera were reactive with both proteins, however there are clear differences in reactivity. rLipL32-1 showed multiple reactive bands rather than a single band at 32kD seen with rLipL32-2, and the band near the same molecular weight was slightly higher, perhaps a result of the extra 40 amino acids.

Western blot

To extract an indicator of diagnostic potential for these antibodies in a direct detection format, Western blot testing was undertaken to determine which antibodies or antibody combinations were the most sensitive when detecting LipL32 from heat-inactivated cells using HRP-conjugated LipL32 mAbs. mAbs 1Lp5, 1Lp6, 1Lp8, 1Lp14 and 1Lp3 were selected for further testing due to their initial reactivity to killed cells shown in Figure 3. mAbs were screened against *L. interrogans* and *L. borgpetersenii* cells alone, and in various combinations to select conditions to move forward with testing in normal human urine (Supplemental Figure 1).

1Lp6, 1Lp8 and 1Lp14 were selected to proceed for analysis in normal human urine to assess diagnostic utility. mAbs were used to probe 1μg/mL rLipL32 spiked into pooled normal human urine by Western blot and the most reactive chosen for a preliminary limit of detection in urine (Figure 7). 1Lp14 was selected for the strength of reactivity in urine but also because it showed strong reactivity with both pathogenic strains *L. interrogans* and *L. borgpetersenii* in Supplemental Figure 1.

Western blot LOD in urine

To indicate the potential sensitivity of 1Lp14 for direct detection of *Leptospira* in patient urine, pooled normal human urine was spiked with serial dilutions of *L. interrogans* and probed with HRP-conjugated 1Lp14. Optimization of HRP concentration was performed and limit of detection was determined by visual appraisal to be between $2.72x10^3$ and $1.36x10^3$ colony forming units (CFU)/mL. Studies of urinary shedding in animals indicate that, although not as sensitive as PCR-based detection, this LOD is promising for detection during the acute phase of infection as it has been shown that asymptomatic individuals can shed up to $4.64x10^4$ leptospira/mL of urine[191]. Although a clear clinical range of shed leptospires in urine has not been established, this limit of detection could potentially be useful for diagnosis of asymptomatic patients and those early in the acute phase of infection where serology is not applicable.

3.4 Discussion

As a neglected tropical disease that can easily be misdiagnosed in endemic areas, Leptospirosis is a key public health concern that can be extremely impactful on community health and wellbeing. A lack of accessible diagnostics, particularly in endemic areas that are often resource-poor highlights a need to develop sensitive and effective diagnostic tools. This need is compounded by a reliance on serology, which can often make diagnosis challenging in the early acute phase of infection and can be complicated by previous infections and the wide variety of serovars that may be circulating in an area. Isolation of a library of monoclonal antibodies reactive to LipL32 provide potential tools for the development of diagnostics specific to pathogenic *Leptospira* that can be used both to diagnose acute infection and in surveillance of asymptomatic individuals. We have produced a library of monoclonal antibodies reactive to killed pathogenic *Leptospira* in a direct immunoassay format and are not reactive to non-pathogenic strains. This is important to prevent cross-reactivity with saprophytic strains in the environment in endemic areas.

Over the course of this study, it became apparent that these mAbs were not effective for antigen detection when paired in an antigen-capture format, despite reactivity to heat inactivated pathogenic *Leptospira* in a direct Western blot and ELISA format. The catalyst for this discovery was purification of recombinant LipL32 without the extra amino acids present in the protein used for the original immunizations. Testing of this new protein (rLipL32-2) on assays developed using rLipL32-1 as a positive control revealed a lack of binding in the antigen capture format, but when tested directly with HRP-conjugated mAbs signal was strong. This contrast in reactivity could suggest a lack of available epitopes for

antigen binding in the native protein, the structure of which could have been altered by the inclusion of extra amino acids allowing for binding of the secondary antibody. A study of immune sera reactivity with LipL32 protein truncations has indicated an IgG immunodominant region containing peptides $LipL32_{148-184}$ associated with the surface exposed area of the protein[195]. An epitope mapping study identified two immunogenic peptides that were considered antigenic regions between $LipL32_{151-204}$[196]. These findings could suggest that the mAbs raised in this study are all reactive to the same epitope of LipL32 and as such the capture and detection antibodies are competing for the same binding sites on the native protein, resulting in the loss of signal. If this is the case, the antibodies could be incorporated into an antigen-capture immunoassay if paired with antibodies raised to a different target epitope or protein, or a polyclonal antibody. Investigation of possible alternative mAbs to pair with the ones isolated in this study may be warranted for development of a quantitative or point of care immunoassay.

Despite the lack of reactivity in an antigen-capture format, the mAbs generated still have diagnostic relevance due to their reactivity with both rLipL32-2 and inactivated pathogenic *Leptospira* in direct ELISA and Western blot formats. Testing and optimization was undertaken to determine the most sensitive antibody and conditions for detection of LipL32 in urine samples via Western blot. Ability to detect LipL32 in urine was key for development of a clinically relevant assay, as shedding of leptospires in urine during infection means that it is a key matrix of interest for diagnosis. Patients with acute illness do have circulating leptospires in the blood, with more than 10^4 leptospires/mL likely to result in a severe outcomes and a range of 10^2 – 10^6 *Leptospira*/ mL suggested[197,198]. However, circulating leptospires in the blood are only present for approximately 7 days during the initial acute

phase of illness before the second immune phase begins[85]. Colonization of the kidney and shedding of leptospires in the urine of chronically infected and asymptomatic individuals suggests that urine may be a more effective matrix for both diagnostic and surveillance purposes, with clinical ranges suggested from 10^2-10^6 leptospires/mL in the urine febrile patients and 10^2-10^4 in asymptomatic individuals[179,191]. The limit of detection for the Western blot presented here was determined to be between $2.72x10^3$ and $1.36x10^3$ CFU/mL *L. interrogans* spiked into pooled normal human urine, meaning that it could potentially be effective for detecting a large proportion of symptomatic and also some asymptomatic infections with sufficient shedding of *Leptospira* in the urine. Further testing of this assay in a range of leptospirosis patient samples is needed to determine clinical relevance.

Several studies have indicated that monoclonal antibodies raised against LipL32 may be of therapeutic value for the treatment of leptospirosis as an alternative to traditional antibiotics. Anti-LipL32 mAbs were able to inhibit *Leptospira* growth in culture as well as reducing the disease severity and preventing up to 61% of deaths depending on the antibody given when administered to hamsters as prophylactic prior to challenge with a lethal dose[199]. A study of two other mAbs found that use in a hamster model of infection protected animals from lethal infections and prevented red blood cell lysis *in vitro*[200]. Analysis of the protective capability of the 15 mAbs isolated here could provide new tools for prophylaxis or treatment of severe leptospirosis.

In conclusion, the mAbs generated in this study have the potential to be utilized for diagnosis or treatment of leptospirosis. We have shown strong mAb reactivity with

pathogenic *Leptospira* spp. and optimized a Western blot for use in detection of purified Lipl32 and killed cells in human urine that could be applied in a diagnostic setting or as an environmental monitoring and wildlife surveillance in endemic countries to identify potential sources of infection and prevent outbreaks.

3.5 Materials and methods

mAb production

Female CD1 mice (Charles River Laboratories, Inc., Wilmington, MA) were immunized with 10 μg recombinant LipL32 (rLipL32-1) in PBS emulsified with Freund's complete adjuvant (MilliporeSigma, St Louis, MO) intraperitoneally. This dosage was repeated at week 4 following initial immunization and a final boost of 5 μg recombinant LipL32 in PBS was given intravenously in the tail in three mice with titers to LipL32 by indirect ELISA at week 10 following immunization. Retro-orbital blood samples were taken from the mice pre-immunization, 4 weeks and 10 weeks post-immunization. Spleens were harvested three days after final intravenous boost from three mice with ELISA titers over 1:5,000,000. Fusions were performed and hybridoma cells produced using standard techniques[132]. Supernatant was collected from hybridoma cells and mAbs purified using recombinant protein A affinity chromatography.

Ethics statement

Laboratory work with animals was approved by the University of Nevada, Reno Institutional Animal Care and Use Committee (Protocol # 00024). All work with animals is supervised by the Office of Laboratory Animal Medicine, which follows the National Institutes of Health Office of Laboratory Animal Welfare policies (Assurance # A3500-01).

Indirect ELISA

96-well medium-binding microtiter plates (Grenier Bio-One, Austria) were coated with 1 μg/mL rLipL32-1 overnight. Plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) 3x and blocked in PBS containing 0.5% non-fat milk and 0.1% Tween 20 (blocking buffer) at 37 °C for 90 minutes. A second wash was done 3x with PBS-T and primary antibody in the form of murine immune sera or hybridoma supernatant added to the first column of the plate and two-fold serial dilutions performed across the plate and incubated for an hour at room temperature. The plate was then washed 3x with PBS-T again and incubated with horseradish peroxidase (HRP) labelled goat anti-mouse IgG antibody, either isotype specific or whole IgG (SouthernBiotech, Birmingham, AL) diluted in blocking buffer for 1 hour at room temperature. A final wash 3x in PBS-T and incubated with tetramethylbenzidine (TMB) substrate (SeraCare, Milford, MA) for 30 minutes. The reaction was stopped with 1M H_3PO_4 and the absorbance read at OD_{450}.

Western immunoblot

For assessing reactivity of murine immune sera to heat inactivated *Leptospira* $1.5x10^8$ CFU/lane was boiled with 6x loading buffer and separated on a 12% gel (Bio-Rad Laboratories, Hercules, CA.) and transferred to a nitrocellulose membrane (Bio-Rad). Immune sera from the mouse with highest titers to rLipL32-1 was diluted 1:1000 in Tris-buffered saline with 0.1% Tween-20 (TBS-T) and 5% milk and used to probe the membrane. When assessing reactivity of murine immune sera to recombinant LipL32, 1μg/mL recombinant protein was separated on a 10% SDS gel and probed with immune sera as described above. For screening reactivity of hybridoma supernatant and purified mAbs to heat-inactivated *L. interrogans*, $1.5x10^8$ CFU/mL cells were boiled with 6x loading buffer and separated on a 10% SDS gel (Bio-Rad) The gel was transferred to nitrocellulose

membrane (Bio-Rad) and either hybridoma supernatant or purified antibody at a concentration of 1 μg/mL was used to probe the membrane using a Miniblotter system (Interchim, Montluçon, France). This system allows for visualization of multiple antibodies probing one preparation of the same antigen on one Western blot.

For all Western blots described in this section a goat anti-mouse IgG HRP-conjugated antibody (SouthernBiotech) was used as a secondary antibody. Signal was detected using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA) and imaged with a ChemiDoc XRS system (Bio-Rad).

LFI screening

To identify which mAbs paired well together to move forward into testing in urine, preliminary screening was performed with each mAb sprayed on CN95 nitrocellulose (Sartorius, Gottingen, Germany) and as the detection gold conjugate. Test strips included a CF6 wicking pad (GE Healthcare, Chicago, IL), the test line was dispensed at 1 mg/mL and the strips cut to 5 mm. Initial testing was performed using 100 ng/mL rLipL32-1 in a running buffer (PBS with 1% 10G (Fitzgerald Industries International, Acton, MA) and 1% BSA). The test strip was placed into a well containing 20 μL 100 ng/mL purified LipL32 in running buffer and left until all buffer was absorbed. The strip was then moved into a well containing 15 μL running buffer, then a new well containing 15 μL running buffer and 5 μL gold conjugate at OD_{540} = 10. Once both of those wells were absorbed, the strip was moved into a final well containing 40 μL running buffer and allowed to absorb that. The LFI was then assessed both visually and with an ESE-Quant lateral flow reader (Qiagen,

Hilden, Germany). Candidates with the best signal relative to the negative control were selected to proceed with optimization in urine.

Urine LFI prototype

1Lp4 sprayed on the test line and 1Lp9 as the gold conjugate was established as the best performing pair through initial testing in pooled normal human urine (Innovative Research, Novi, MI). optimization to improve signal and reduce non-specific binding was undertaken. Optimization primarily involved altering the concentration of protein, surfactant and salt spiked into the urine to address gold aggregation on the nitrocellulose. The final prototype followed the same run protocol as the LFI screening in buffer but with some modifications to the buffers used to accommodate urine as a matrix. Briefly, surfactant 10G (Fitzgerald Industries International, Acton, MA) was added to the spiked urine to a final concentration of 1%. The urine was allowed to absorb then placed into a running buffer of 300mM NaCl containing 1% surfactant 10G (Fitzgerald Industries International) and 1% BSA, which was also used to chase the gold conjugate. The gold conjugate was diluted to OD_{540} = 10 in 50 mM borate, 0.5% BSA, 1% surfactant 10G (Fitzgerald Industries International).

LipL32 expression and purification

Genomic DNA was extracted from *L. interrogans* RGA (BEI resources) using DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany). The *lipL32* gene was amplified by PCR using primers shown in Supplemental Table 1, which excluded the LipL32 signal sequence. The gene was then cloned via Gibson Assembly (New England Biolabs, Ipswich, MA). Sequencing was performed to verify the sequence of the plasmid, which

was then transformed into *E. coli* M15 for expression. The transformed *E. coli* were grown at 37 °C to a log phase OD_{600} of 0.6 and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM for 16 hours. The bacterial pellet was collected via centrifugation and the cells lysed with BugBuster 10X Protein Extraction Reagent (MilliporeSigma), lysozyme to a final concentration of 1 mg/mL and sonication. Protein was purified using Protino Ni-TED resin (Macherey-Nagel, Duren, Germany).

Antigen-capture ELISA

To assess mAb binding to rLipL32-1 and rLipL32-2, all by all testing was undertaken to gain an overview of the differences in reactivity. 96 well microtiter plates were coated with 1 μg/mL each purified antibody per well in 100 μL PBS across each row and incubated overnight at room temperature. Plates were washed 3x with PBS-T and blocked with 200 μL blocking buffer at 37 °C for 90 minutes. 100 μL recombinant LipL32 in PBS was added to each well of the plate and incubated for 60 minutes at room temperature. mAbs were HRP labelled using EZ-link Plus Activated Peroxidase (Thermo Fisher Scientific). Plates were washed 3x with PBS-T and 100 μL each HRP-conjugated mAb at 1 μg/mL in blocking buffer was added to each column of the plates, resulting in an all-by-all matrix. Plates were incubated at room temperature for 60 minutes and then washed a final time before incubation with 100 μL/well TMB substrate (SeraCare). 1M H_3PO_4 (100 μL/well) was added to stop the reaction and plates were read at OD_{450}.

Western immunoblot for direct detection of LipL32

To develop a Western blot for direct detection of LipL32, mAbs were screened for reactivity to rLipL32-2 in pooled normal human urine (Innovative Research). Briefly, 1 ug/mL rLipL32-2 was spiked into urine and separated on a 10% SDS gel (Bio-Rad) and transferred onto a nitrocellulose membrane. HRP-conjugated 1Lp6 and 1Lp14 were used to probe the blot directly, 1Lp8 was used unconjugated, followed by HRP-conjugated goat-anti mouse IgG (SouthernBiotech). SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) was used to image the blot on a Chemidoc XRS system (Bio-Rad).

To determine a preliminary LOD for detection of *Leptospira* using mAb 1Lp14, pooled normal human urine (Innovative Research) was spiked with two-fold serial dilutions of heat inactivated *L. interrogans* beginning at 2.18×10^4 CFU/mL. Samples were boiled with 6x loading buffer, separated on a 10% SDS gel (Bio-Rad) and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked in TBS-T with 5% nonfat milk overnight at 4°C. The membrane was then washed 3x with TBS-T and 5 μg/mL HRP-conjugated 1Lp14 in blocking buffer was used to probe the membrane. Signal was detected with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged with a ChemiDoc XRS system (Bio-Rad).

3.6 Acknowledgements

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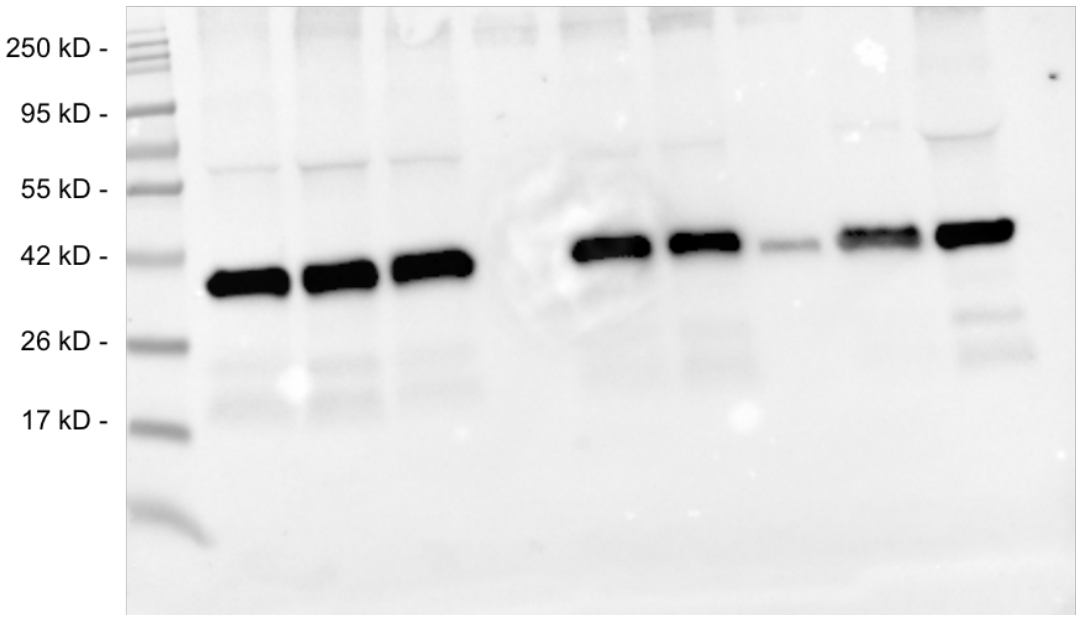
3.7 Figures and tables

A



B

Lane	Species	Serovar	Strain
1	*L. interrogans*	Pyogenes	L0370
2	*L. borgpetersenii*	Javanica	MK146
3	*L. borgpetersenii*	Javanica	L0864
4	*L. biflexa*	n/a	LT17
5	*L. interrogans*	Autumnalis	LP101
6	*L. interrogans*	Bataviae	L1111
7	*L. inadai*	n/a	AT2
8	*L. interrogans*	Undesignated	FPW 2026
9	*L. borgpetersenii*	*Javanica*	L0066

Figure 1. Reactivity of immune sera from mice immunized with recombinant LipL32 to *Leptospira* strains. Pathogenic, intermediate and saprophytic *Leptospira* strains were probed with pooled immune sera from mice immunized with recombinant LipL32 via Western blot (A). A variety of available *Leptospira* species, serovar and strain were used (B).

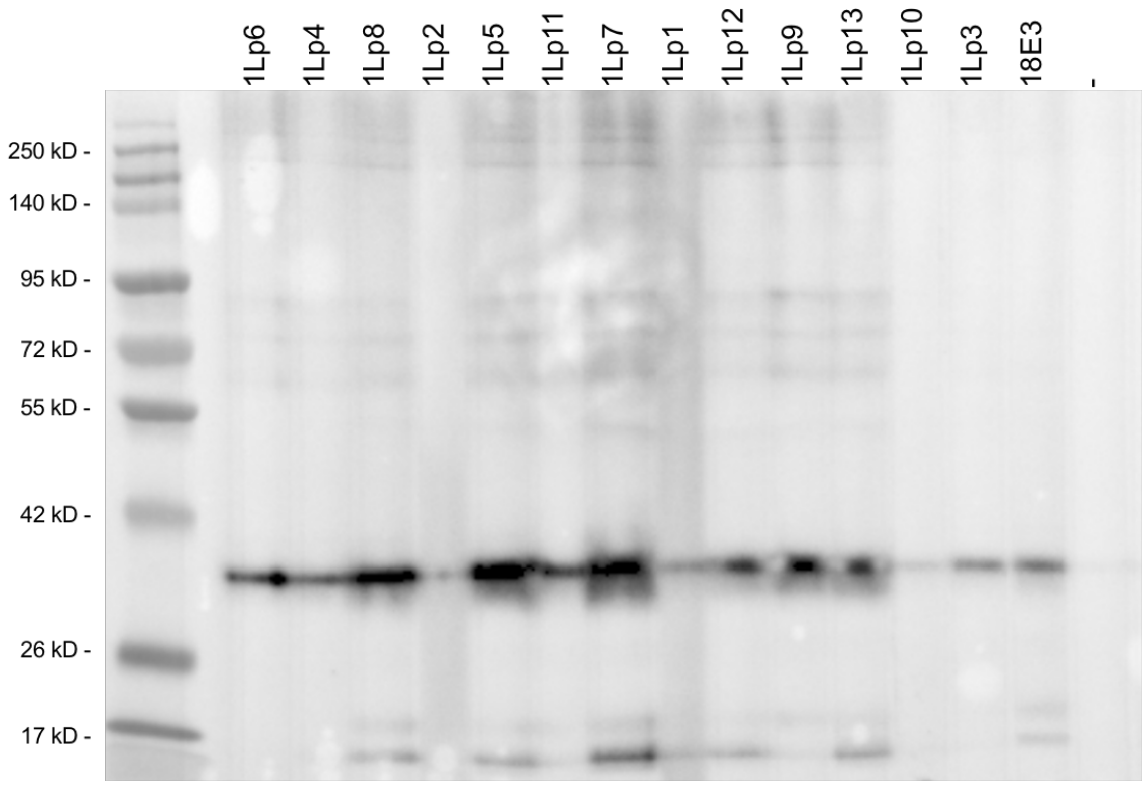


Figure 2. Reactivity of fusion well supernatant with heat-inactivated pathogenic *L. interrogans*. Fusion well supernatant was used to probe $1.5x10^8$ CFU/mL heat inactivated *L. interrogans* L0370 by Western blot.

Table 1. IgG subclass of isolated LipL32 mAbs

Clone name	Subclass
1Lp1	IgG2a
1Lp2	IgG2b
1Lp3	IgG2b
1Lp4	IgG2b
1Lp5	IgG2b
1Lp6	IgG2b
1Lp7	IgG2b
1Lp8	IgG2a
1Lp9	IgG2b
1Lp10	IgG2a
1Lp11	IgG1
1Lp12	IgG2b
1Lp13	IgG2b
1Lp14	IgG2b
1Lp15	IgG2b

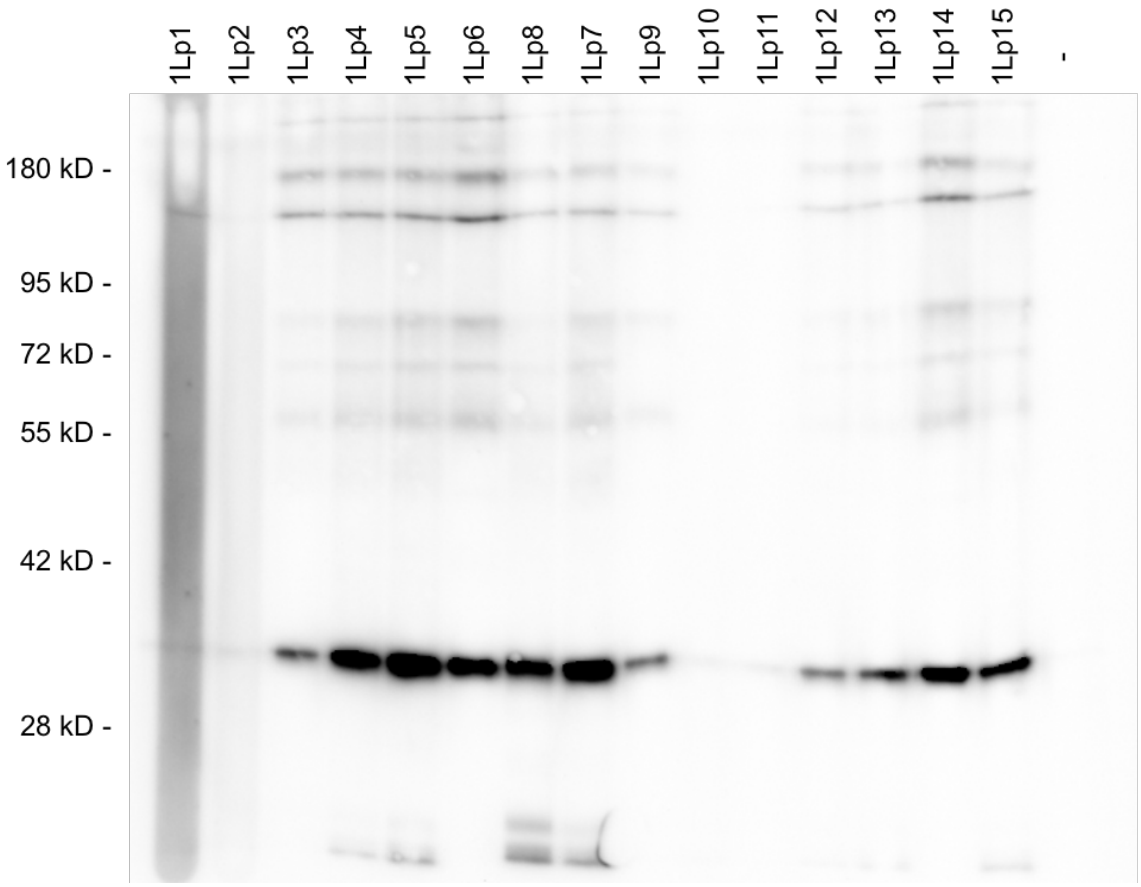


Figure 3. Reactivity of purified mAbs with heat inactivated *L. interrogans.* Purified HRP-conjugated mAbs were used to probe $1.5x10^8$ CFU/mL heat inactivated *L. interrogans* L0370 by Western blot.

Table 2. Top 6 mAb pairs in the LFI format chosen to proceed from initial testing in buffer to urine.

Capture	Detection	-	+	Signal-Background
1Lp6	1Lp12	96	364	269
1Lp6	1Lp4	56	245	189
1Lp14	1Lp9	22	193	171
1Lp4	1Lp9	57	205	148
1Lp4	1Lp6	39	182	143
1Lp11	1Lp9	36	77	41

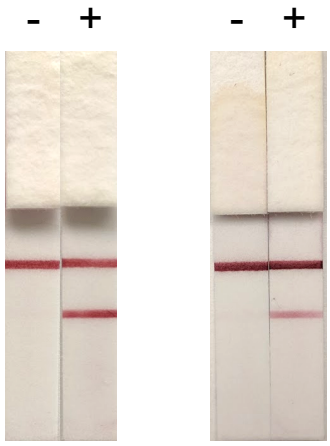


Figure 4. Reactivity of LipL32 LFI prototype in buffer and urine. LFI prototype 1Lp4-1Lp9 was tested with 1 μg/mL rLipL32-2 either spiked into a running buffer or pooled normal human urine.

rLipL32-1

Coating mAb \ HRP-conjugated detection mAb	1Lp1	1Lp2	1Lp3	1Lp4	1Lp5	1Lp6	1Lp7	1Lp8	1Lp9	1Lp10	1Lp11	1Lp12	1Lp13	1Lp14	1Lp15
1Lp1	0.267	0.202	0.183	0.153	0.14	0.092	0.105	2.327	0.089	0.078	0.081	0.093	0.088	0.088	0.089
1Lp2	0.241	0.181	0.145	0.138	0.106	0.087	0.1	1.893	0.086	0.085	0.083	0.102	0.088	0.093	0.105
1Lp3	0.19	0.259	0.485	1.395	1.425	0.935	2.813	1.838	0.764	0.089	0.62	2.171	1.14	1.864	2.191
1Lp4	0.195	0.156	0.251	0.309	0.286	0.231	0.521	2.08	0.204	0.083	0.203	0.409	0.335	0.532	0.565
1Lp5	0.315	0.224	0.296	0.334	0.327	0.214	0.464	4	0.184	0.083	0.172	0.289	0.25	0.498	0.475
1Lp6	0.316	0.331	0.454	0.679	0.683	0.3	1.166	4	0.355	0.087	0.328	0.768	0.689	0.721	1.242
1Lp7	0.329	0.324	0.637	1.739	0.893	0.595	1.493	4	0.594	0.089	0.513	0.958	0.668	1.237	1.539
1Lp8	0.384	0.845	2.765	4	4	2.804	4	4	2.589	0.128	4	4	4	4	4
1Lp9	0.099	0.135	0.291	0.574	0.636	0.373	1.211	4	0.225	0.083	0.337	0.689	0.874	1.008	1.013
1Lp10	0.09	0.087	0.097	0.107	0.11	0.095	0.13	4	0.094	0.084	0.097	0.12	0.098	0.112	0.116
1Lp11	0.1	0.474	1.166	4	4	2.543	4	4	2.373	0.106	1.605	4	4	4	4
1Lp12	0.16	1.717	4	4	4	4	4	4	4	0.158	4	4	4	4	4
1Lp13	0.102	0.623	4	4	4	4	4	4	4	0.115	4	4	4	4	4
1Lp14	0.107	0.61	3.522	4	4	2.707	4	4	4	0.108	4	4	4	4	4
1Lp15	0.104	0.29	1.212	2.014	2.049	1.547	4	4	1.487	0.094	1.397	2.525	1.343	2.719	2.326

rLipL32-2

Coating mAb \ HRP-conjugated detection mAb	1Lp1	1Lp2	1Lp3	1Lp4	1Lp5	1Lp6	1Lp7	1Lp8	1Lp9	1Lp10	1Lp11	1Lp12	1Lp13	1Lp14	1Lp15
1Lp1	0.056	0.045	0.044	0.049	0.049	0.051	0.055	0.206	0.06	0.054	0.064	0.049	0.052	0.057	0.05
1Lp2	0.049	0.056	0.045	0.045	0.044	0.049	0.051	0.406	0.046	0.063	0.047	0.051	0.051	0.054	0.049
1Lp3	0.054	0.051	0.057	0.044	0.079	0.045	0.046	1.815	0.044	0.045	0.044	0.047	0.052	0.055	0.054
1Lp4	0.055	0.045	0.047	0.047	0.049	0.046	0.046	1.75	0.047	0.047	0.042	0.046	0.05	0.058	0.059
1Lp5	0.065	0.044	0.049	0.045	0.046	0.046	0.049	1.573	0.046	0.045	0.047	0.047	0.052	0.056	0.059
1Lp6	0.071	0.051	0.048	0.048	0.05	0.05	0.059	2.321	0.046	0.056	0.053	0.05	0.05	0.051	0.053
1Lp7	0.054	0.07	0.069	0.053	0.051	0.05	0.06	1.665	0.048	0.057	0.048	0.054	0.058	0.048	0.059
1Lp8	0.078	0.105	0.061	0.06	0.081	0.057	0.053	1.709	0.054	0.043	0.047	0.059	0.05	0.052	0.08
1Lp9	0.075	0.058	0.051	0.052	0.054	0.052	0.057	1.927	0.067	0.059	0.092	0.071	0.054	0.05	0.051
1Lp10	0.053	0.055	0.061	0.056	0.056	0.049	0.047	2.053	0.059	0.065	0.064	0.063	0.051	0.055	0.055
1Lp11	0.063	0.058	0.054	0.057	0.06	0.046	0.047	0.066	0.064	0.074	0.055	0.054	0.055	0.053	0.057
1Lp12	0.063	0.065	0.058	0.061	0.062	0.049	0.064	1.919	0.051	0.06	0.053	0.057	0.052	0.061	0.065
1Lp13	0.048	0.052	0.046	0.051	0.051	0.053	0.063	1.649	0.058	0.062	0.057	0.062	0.055	0.054	0.064
1Lp14	0.051	0.114	0.053	0.058	0.049	0.047	0.056	1.331	0.061	0.061	0.061	0.073	0.066	0.047	0.05
1Lp15	0.059	0.069	0.073	0.068	0.063	0.051	0.05	1.243	0.065	0.071	0.066	0.085	0.056	0.049	0.062

Figure 5. All by all antigen-capture ELISA testing of reactivity to rLipL32-1 and rLipL32-2. Reactivity of all LipL32 mAbs in the capture and detection position with rLipL32-1 and rLipL32-2. Little to no reactivity was observed with rLipL32-2 in pairs that were strongly reactive to rLipL32-1.

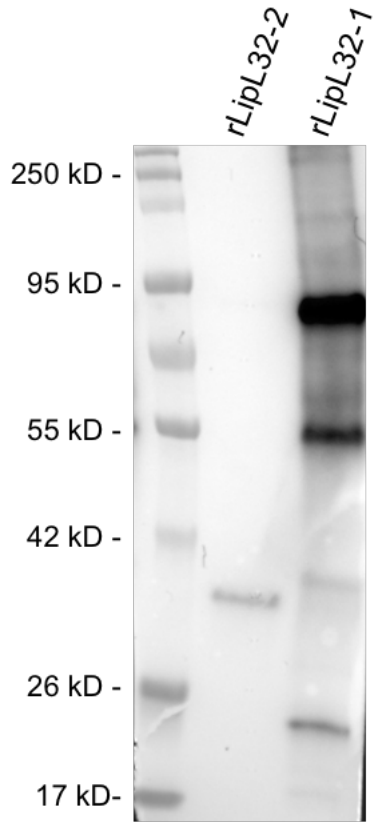


Figure 6. Comparison of immunized mouse immune sera reactivity with newly purified rLipL32-1 and rLipL32-2. Both forms of rLipL32 were reactive with murine immune sera from the original immunizations. rLipL32-2 showed a single band of reactivity at ~32 kD, whereas rLipL32-1 showed multiple reactive bands.

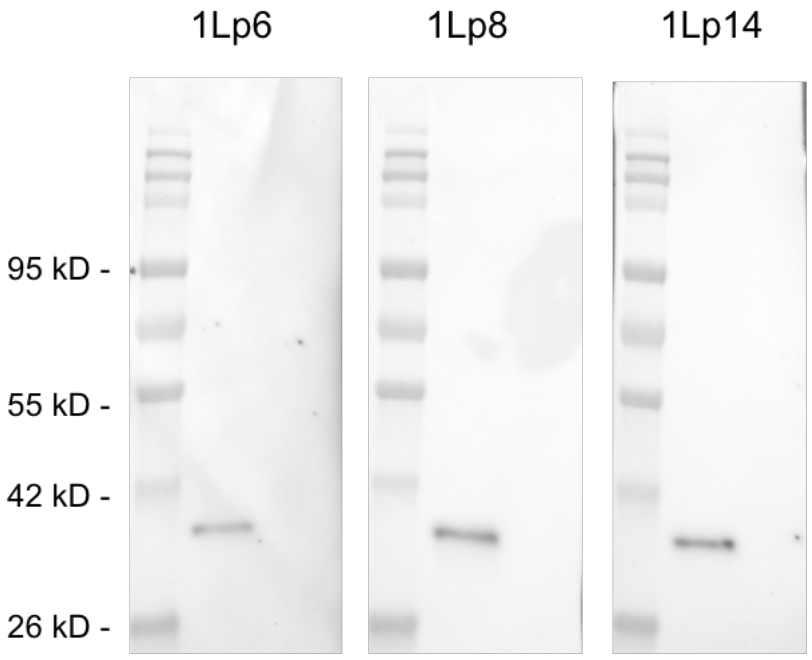


Figure 7. Western blot reactivity of three mAbs to rLipl32-2 in pooled normal human urine. Reactivity of three LipL32 mAbs to rLipL32-2 spiked into pooled normal human urine was assessed to identify a suitable antibody for sensitive detection. 1Lp14 was chosen to proceed.

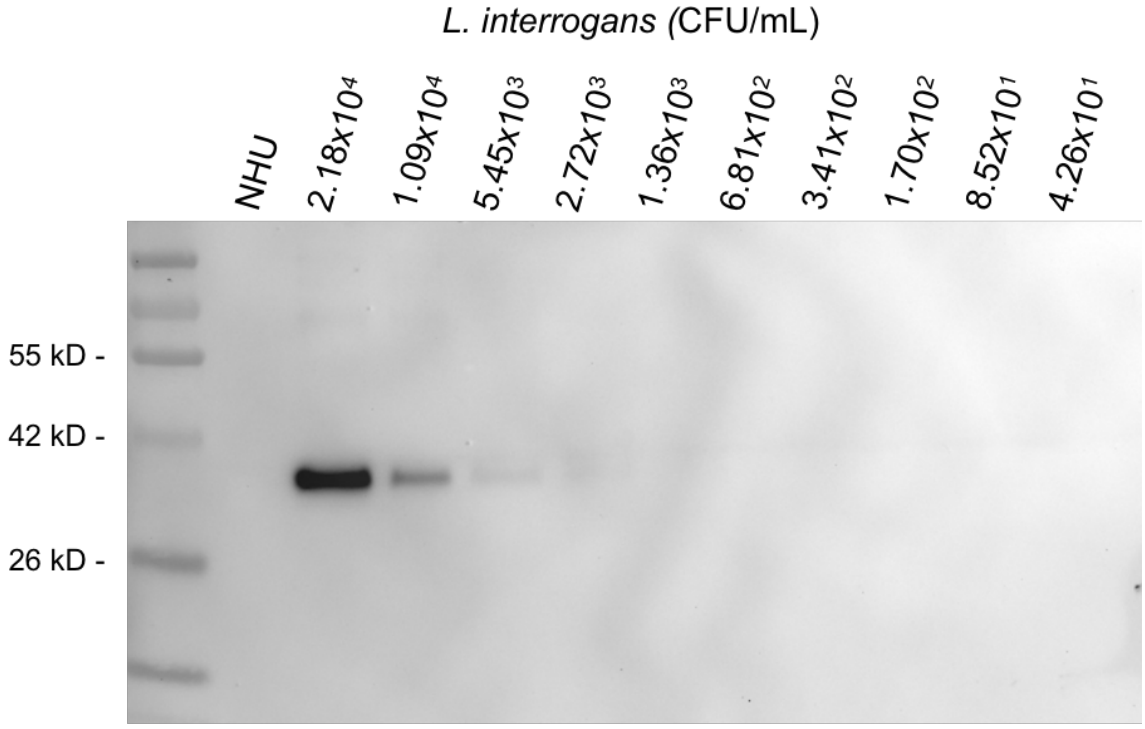
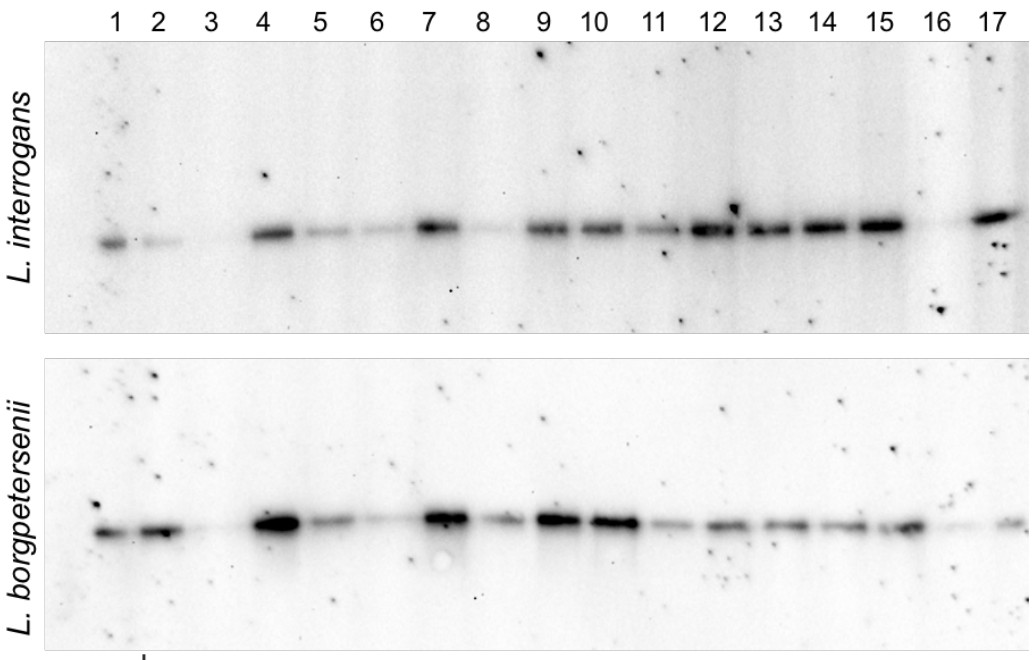


Figure 8. Preliminary limit of detection of killed *L. interrogans* spiked into pooled normal human urine. Serial dilutions of heat-killed *L. interrogans* was probed with HRP-conjugated 1Lp14 to establish the sensitivity of the assay.

Supplemental Table 1. Primers used to clone the LipL32 gene from *L. interrogans* strain RGA via Gibson assembly

Gene		Primer
lipL32	F	5'-GGATCTGGTATCGAGGGAAGGGGTGCTTTCGGTGGTCTG-3'
	R	5'-CAGGAGTCCAAGCTCAGCTATTACTTAGTCGCGTCAGAAG-3'



Lane	Detection mAb
1	1Lp5
2	1Lp6
3	1Lp8
4	1Lp14
5	1Lp5, 1Lp6
6	1Lp5, 1Lp8
7	1Lp5, 1Lp14
8	1Lp6, 1Lp8
9	1Lp6, 1Lp14
10	1Lp8, 1Lp14
11	1Lp5, 1Lp6, 1Lp8
12	1Lp5, 1Lp6, 1Lp14
13	1Lp5, 1Lp8, 1Lp14
14	1Lp6, 1Lp14, 1Lp8
15	1Lp5, 1Lp6, 1Lp8, 1Lp14
16	1Lp3
17	1Lp5, 1Lp6, 1Lp8, 1Lp14, 1Lp3

Supplemental Figure 1. Reactivity of potential detection mAb combinations with heat inactivated pathogenic *Leptospira*. mAbs that had shown good reactivity with *L. interrogans* previously were assessed alone and in combination for reactivity to pathogenic strains *L. interrogans* and *L. borgpetersenii.*

CHAPTER 4

CONCLUSION AND REFERENCES

Conclusion

Both tularemia and leptospirosis are bacterial zoonoses that face diagnostic challenges. Tularemia, caused by *Francisella tularensis,* can present as range of syndromes and a rarity of natural infections mean that diagnosis is complicated and time consuming[16]. A lack of efficient, accessible diagnostics is especially problematic given the classification of *F. tularensis* as a Tier 1 Select Agent, with the potential to cause widespread illness and death if released deliberately[4,8]. Leptospirosis is a neglected tropical disease that severely impacts communities in endemic areas through illness, death and loss of livestock, especially following natural disasters like flooding events[65]. Diagnosis of leptospirosis is challenging due to the large number of pathogenic serovars that cause disease and complicate serological testing, as well as non-specific clinical symptoms that are often mistaken for dengue fever, chikungunya fever or malaria[57,59]. Both infections rely heavily on serological assays for diagnosis, which can present issues with cross-reactivity, residual titers from previous infections and a need for multiple time points that can delay diagnosis unacceptably in instances of acute infection. More effective diagnostic tools are needed for both infections.

Immunoassays for detection of circulating antigens represent an important diagnostic resource that has the potential to diagnose infection before a serological response is raised to clinical relevance. In the studies presented here, libraries of monoclonal antibodies (mAbs) were produced to antigens of diagnostic interest: the lipopolysaccharide (LPS) of *F. tularensis* and outer membrane lipoprotein LipL32 of pathogenic *Leptospira* spp. Production of mAb libraries is allows for selection of candidates that are best suited for the immunoassay of interest and can provide flexibility

when optimizing assays for different patient matrices. The selection process shown in Chapter 2 illustrates that, although a group of mAbs was consistently amongst the most sensitive, different pairings gave optimal results in the enzyme-linked immunosorbent assay (ELISA) format versus the lateral flow immunoassay (LFI) format, and successful pairing on one platform does not necessarily translate to another.

In the first study presented, an antigen-capture ELISA was developed using mAbs specific to *F. tularensis* LPS. This assay was optimized in pooled normal human sera and used to quantify the amount of LPS present in filtered tularemia patient serum samples. Of the 19 samples assayed, LPS was detected by ELISA in 8 samples, with concentrations ranging from 0.22 ng/mL to 109.95 ng/mL. As the samples were filtered, these figures likely underrepresent the true clinical range of LPS available for detection in patient samples as there was no cell associated LPS present. Detection of LPS in the absence of bacterial cells indicates that LPS is a promising antigen for diagnosis of tularemia. In conjunction with ELISA development, an LFI prototype was produced as a rapid, point of care diagnostic. The prototype was determined to be reactive with killed cells from pathogenic type A and type B strains of *F. tularensis* and non-reactive with killed near neighbors and LPS from other bacteria. The results of this testing provide a promising indicator of a lack of cross-reactivity, although a more exhaustive cross-reactivity panel is needed to confirm this. The limit of detection for the LFI was 5 ng/mL, which is a little higher than is desirable for clinical relevance, so further optimization is needed. Overall, this study has confirmed the viability of *F. tularensis* LPS as a diagnostic antigen and developed sensitive and specific tools for improving diagnostic options.

The second study covers isolation of 15 mAbs reactive with LipL32, the most abundant outer membrane protein of pathogenic *Leptospira* spp. Reactivity with pathogenic heat inactivated *Leptospira* was established and mAbs were non-reactive with saprophyte *L. biflexa,* which lacks LipL32. Screening and optimization of mAb pairs in an LFI and ELISA format for detection of LipL32 in urine was undertaken, however expression and purification of recombinant LipL32 that was more similar to native protein than the recombinant antigen used for immunization revealed that antigen-capture assays were not able to detect native protein, as confirmed by testing with killed *Leptospira* cells. As mAbs were strongly reactive in a direct Western blot format, optimization of a Western blot for detection of LipL32 in urine was undertaken. Horseradish peroxidase (HRP) - conjugated mAbs were assessed for reactivity with inactivated pathogenic *Leptospira* and recombinant LipL32 spiked into pooled normal human urine and the most sensitive antibody selected. The resultant assay was able to detect between $2.72x10^3$ and $1.36x10^3$ CFU/mL *L. interrogans* spiked into normal human urine. It is difficult to determine a defined clinical range for shedding of leptospires in the urine, however this limit of detection is promising for clinical relevance based on studies of symptomatic and asymptomatic leptospirosis patients. Overall, this study describes new tools for diagnosis of leptospirosis in the form of a mAb library of antibodies specific for LipL32 and a Western blot for detection of leptospires in urine samples.

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