Delineating host-pathogen interaction of pathogenic *Leptospira* spp.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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Declaration

I hereby declare that this doctoral thesis entitled **"Delineating host-pathogen interaction of pathogenic *Leptospira* spp."** is a genuine work carried out at Department of Infection Biology, Institute of Infection and Global Health, the University of Liverpool under the supervision of Dr Nicholas J. Evans (B.Sc., Ph.D., F.H.E.A). I certify that this thesis has not been submitted in support of an application for another degree or qualification of this or any other institution.

INTAN NOOR AINA KAMARUZAMAN

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Abbreviations

α	Alpha
β	Beta
γ	Gamma
1D	1-Dimensional
2D	2-Dimensional
2-ME	2-beta Mercaptoethanol
APS	Ammonium Persulfate
BeStsel	Beta Structure Selection
BL	Bovine leptospirosis
BSL	Bacterial Subcellular Localisation
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BOMP	The β-barrel Outer Membrane Protein Predictor
CAPITO	CD Analysis and Plotting Tool
CD	Circular Dichroism
CDS	Coding Sequence
CELLO	Subcellular Localization Predictive System
CMI	Cell-mediated immunity
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonuclease Triphosphate
ECM	Extracellular matrix
ELISA	Enzyme-linked Immunoabsorbant Assay
g	Gram
g	Gravitational force
GI	Gastrointestinal
HMM	Hidden Markov Model

IB	Inclusion body
IFN-γ	Interferon gamma
IgG	Immunoglobulin Class G
IgG1	Immunoglobulin Class G Subclass 1
IgG2	Immunoglobulin Class G Subclass 2
IM	Inner Membrane
Interpro	Intergrative protein signature database
I-TASSER	Interative threading Assembly Refinement
kDa	Kilodalton
LB	Luria Broth
LHB	*Leptospira interrogans* Hardjobovis
LHP	*Leptospira interrogans* Hardjopritno
LPS	Lipopolysaccharide
Lsa	Leptospiral adhesin
Lip	Lipoprotein
mA	Milliampere
MAT	Microscopic Agglutination Test
MCC	Matthew's Correlation Coefficient
MCMBB	Markov Chain Model for Beta Barrels
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium chloride
ml	Millilitres
MLST	Multilocus Sequence Typing
mM	Milimolar
NaCl	Sodium Chloride
NCBI	National Centre for Biotechnology Information
NCS	Nitrocellulose Sheet
OM	Outer Membrane
OMP	Outer Membrane Protein
ORF	Open Reading Frame

PBS	Phosphate Buffer Saline
$PBST_{20}$	Phosphate Buffer Saline, Tween 20
PCR	Polymerase Chain Reaction
Pfam	Protein families database
PRED-TMBB	Prediction of TransMembrane Beta-Barrel Proteins
PSORTb	Subcellular Localisation Prediction Tool
RAST	Rapid Annotation using Subsystem Technology
RPM	Revolution per minute
rRNA	Ribosomal Ribonucleic Acid
RV	Reverse Vaccinology
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel
SPHS	Severe Pulmonary Haemorrhagic Syndrome
SVM	Support Vector Machine
svr	Serovar
Th1	Type 1 T helper
TMHMM	Transmembrane Helices Hidden Markov Model
V	Volts
v/v	Volume/volume
w/v	Weight/volume
µl	Microlitre
µM	Micromolar

Abstract

Delineating host-pathogen interaction of pathogenic *Leptospira* spp.- *Intan Noor Aina Kamaruzaman*

Leptospirosis is a highly infectious, global, zoonotic disease affecting the majority of the mammalian species. Leptospirosis is caused by pathogenic bacteria, *Leptospira* spp., with more than >250 serovars identified. Cattle are one of the most susceptible hosts where the infection is commonly caused by serovar Hardjobovis. Bovine leptospirosis (BL) causes severe reproductive disorders and is a significant public health risk.

Commercially available BL vaccines are typically bacterin and considered limited as they are serovar-specific and confer temporary protection. Bacterial outer membrane proteins (OMPs) are extensively studied as potential vaccine candidates for infectious diseases due to their ability to stimulate robust immune responses and induce cross-protective immunity. Here, seven novels OMPs from *L. borgpetersenii* serovar Hardjobovis L550 were identified using a reverse vaccinology approach. Four OMP genes were successfully cloned, expressed and purified as recombinant proteins. Subsequent functional *in vitro* binding assays showed that these OMPs could adhere to various host components and two OMPs of 37 and 49 kDa with significant binding results were re-assigned as '**L**epto**s**piral **a**dhesin' (Lsa) together with molecular weights as Lsa37 and Lsa49. Immunological evaluation of antibody titres against the OMPs in cattle bulk milk suggests these proteins are expressed by the bacteria and interact with the host immune system with two OMPs, OmpL1 and rLBL0375 exhibiting better discrimination with disease status.

Several leptospiral OMPs exhibit molecular diversity through comparative sequence analysis. Here, two groups of OMP variants, OmpL1 and Lsa49 across five pathogenic genomospecies were selected via phylogenetic analysis to evaluate their functional binding diversities towards various host components. OmpL1 exhibited significant binding variation against various host components, compared to Lsa49. The diversity is strongly correlated with variations on predicted OmpL1 surface-exposed loops contributing to functional loss and gain via molecular evolution, which resulted in binding preferential towards specific host molecules. Immunological evaluation of cattle sera showed that these OMPs are expressed and exposed to the host immune system, and had a strong association against one another. This suggests that these OMPs may have similar structural epitopes that allow antibody binding, and indicates conserved immunogenicity across species. In a final study, we investigated whether the ruminant gastrointestinal (GI) tract was a carriage site for *Leptospira*. Through PCR surveys of ruminant gingival and rectal tissues, the presence of leptospires was identified as extremely low, thus indicating the GI tract does not appear to be an important leptospire carriage site.

In conclusion, here we have identified several novel bovine leptospire OMPs, which may be useful vaccine or diagnostic components for bovine leptospirosis in the future. Additionally, the functional diversity between leptospiral OMP variants identifies OMP genetic evolution resulting in addition or loss of binding function, highlighting the complex host-pathogen interaction of leptospirosis. Lastly, this study does not suggest a role for the ruminant GI tract in leptospire carriage, indicating disease transmission through this route is unlikely.

Chapter 1: Introduction

1.1 The discovery of *Leptospira* and leptospirosis: Historical aspects

The first recorded case of leptospirosis in man can be traced back in 1886 by a German physician, Adolf Weil (Adolf, 1886) who first reported two clinical cases of similar symptoms and clinical signs which include splenomegaly, jaundice, renal dysfunction, nervous symptoms and rapid recovery after a short duration of severe illness. This unknown disease was then referred to as Weil's disease, which often associated with anyone contacting with natural water sources. In 1907, a public report in the United States was published by an assistant surgeon, M. Stimson (1907). He described the organism as black, curved with hook-like extremities at both ends and was restricted to the kidney's tubules. He suggested the organism as *Spirochaeta interrogans* due to the shape of the organism that resembles a question mark.

Seven years later, a group of Japanese scientists made a breakthrough discovery of the causative agent of Weil's disease following inoculation of the blood from an infected human patient to a guinea pig. The guinea pig developed similar symptoms and died shortly after showing signs of jaundice. This was repeated, and in latter investigations, they successfully detected spirochetes in liver tissue of a guinea pig inoculated with infected human blood and described the distribution of leptospires (formerly *Spirochaeta interrogans*) in the liver, adrenal glands, kidneys, spleen, bone marrow and lymph glands. They concluded that this spirochete was the cause of Weil's disease. In the same report from 1916, they obtained a pure *Leptospira* strain designated as *Spirochaeta icterohaemorrhagiae*, by isolation and cultivation *in vitro* for the first time (Inada *et al.*, 1916). Ever since the discovery of *Leptospira* spp., as the causative agent for Weil's disease, further work has been carried out in the early 20th century including to identify the sources of infection, strain isolations and studies on its morphology and the possible pathogenesis in humans (Ido *et al.*, 1917; Kaneko and Okuda, 1917; Noguchi, 1917, 1918a, 1918b). Weil's disease is now referred to as leptospirosis, although this term is still used to describe the severe manifestation of leptospirosis in man.

Before leptospirosis was recognised, it was thought that the disease had existed for many centuries and was associated with farming activity and migration. In the 17th century, it was thought leptospirosis caused the death of many Native Indians of North America who contracted the disease from the early English pilgrims arriving southeastern coast of the New

World (present-day Massachusetts) (Marr and Cathey, 2010). A similar leptospirosis syndrome was described as 'rice-field jaundice' relating to disease contracted during rice farming in ancient China (Adler, 2015a). Although, there was no scientific evidence to support these claims as the description of the symptoms could be complicated with other febrile illnesses, however, rats were constantly identified as the main source of infection for these outbreaks.

The modern human outbreaks are mainly related to occupations, especially to agricultural and livestock farming activities, which serve as the primary risk factors. As leptospirosis was found worldwide, the disease was formerly known by different local names depending on the types of occupation of those affected, places and apparent symptoms associated with the disease. The list of perceived leptospirosis names across the world is shown in Table 1.1.

Table 1.1: List of former names of leptospirosis associated with countries.

Former name	Country of origin[a]	Disease association[b]	References
Field/ harvest/mild fever	Worldwide	Strawberry farming Rice farming	(Desai *et al.*, 2009) (Izurieta, Galwankar and Clem, 2008)
Cane cutter's disease		Cane farming	
Seven days/Nanukayami fever	Japan	Rice farming	-
Rat catchers yellow	Worldwide	Rat/Dogs	
Canicola fever	Denmark USA		(Rosenberg, 1951)
Fort Bragg or Pretibial fever	USA	Military	(Daniels and Grennan, 1943) (Fraser *et al.*, 1973)
Mud fever	France		(Tatlock, 1982) (Buckland and Stuart, 1945)
Swineherd fever **Dairy farm fever**	New Zealand	Livestock farming	(Kirschner, Miller and Garlick, 1952) (Christman *et al.*, 1974)

[a] *Country of origin that uses a local term to describe the disease*
[b] *Activity with which the disease was observed and where names originated*

Next sections will describe comprehensive details about leptospirosis, *Leptospira* spp., and impact of disease in both human and animals.

1.2 General overview of leptospirosis

Leptospirosis is considered an emerging and potentially fatal zoonotic disease occurring worldwide with a significant number of human and animal cases reported in most continents, particularly in tropical and subtropical regions (Lau *et al.,* 2010a). Recently, Torgerson *et al.* (2015) estimated that more than one million human leptospirosis cases were reported each year with cumulative death index of nearly 60,000 worldwide. These statistics appeared to be higher in temperate countries, where the disease is linked to tropical climates such as flooding after heavy rain and typhoons (Lau *et al.*, 2010b; Mohd Radi *et al.*, 2018). Although the disease poses a global threat, leptospirosis is categorised as one of the neglected infectious diseases of which the term 'neglected' is referring to the disease that has an impact on deprived and marginalised populations in urban settings (WHO, 2019).

The disease is caused by pathogenic *Leptospira* spp*.,* a Gram-negative spirochete which belongs to the family of *Leptospiraceae*. Leptospirosis is zoonotic as it can be transmitted from animals to man through both direct and indirect transmission. Human incidence and outbreaks are often reported following rainy season, lack of sanitation, occupational exposure and recreational activities involving a natural water source (Pappas *et al.*, 2008). Although mortality is not common in human, the disease causes higher morbidity in infected patients (Weeratunga *et al.*, 2015) and fatal outcome is higher when the victims are co-infected with other febrile illnesses (Wongsrichanalai *et al.*, 2003; Sharp *et al.*, 2012; Nhan *et al.*, 2016). Although the disease is treatable, leptospirosis generally presented with undistinguished clinical signs that are similar to other febrile illnesses and is often misdiagnosed (Bruce *et al.*, 2005).

1.3 *Leptospira* spp.: Taxonomy, classification and features

1.3.1 Taxonomy and classification

The classification of *Leptospira* spp. has been subjected to long-standing discussions among microbiologists due to complicated serotyping classification. Historically, *Leptospira* was classified into two species, *L. icterohaemorrhagiae* (for all pathogenic species) and *L. biflexa* (for all non-pathogenic species) (Johnson and Faine, 1984). In the 1950s, a serological

classification was introduced to further classify *Leptospira* strains into serotypes. Under the new characterisation method, serotype or serovar is defined as "Two strains are considered to belong to different serotypes if, after cross-absorption with heterologous antigen, 10% or more of the homologous titre regularly remains in each of the two antisera in repeated tests" (Wolff and Turner, 1963). A large number of serovars may arise from a single *Leptospira* species, a group of serovars that react to a common antigen are grouped into serogroups (Wolff and Broom 1954). However, serogroups have no taxonomic status but may be useful for epidemiology investigations (Levett, 2001). Distribution of serogroups in different *Leptospira* species is shown in Table 1.2.

Under modern genotypic classification based on DNA-DNA relatedness and by 16S rRNA sequence, the species were further expanded and consist of 15 named species and five new genomospecies namely (Genomospecies 1: *Leptospira alstonii*, Genomospecies 2: *Leptospira alexanderi*, Genomospecies 3: *Leptospira vanthielii*, Genomospecies 4: *Leptospira terpstrae* and Genomospecies 5: *Leptospira yanagawae*) (Brenner *et al.*, 1999; Smythe *et al.*, 2013). Under the phylogenetic classification of *Leptospiraceae* based on 16S rRNA, the species of *Leptospira* are clustered into three groups of phylogenetic classification which comprising pathogenic, intermediate and non-pathogenic group (Figure 1.3) (Levett, 2015). Formerly, there are about 21 recognised species of *Leptospira* and their associated serogroups are known, and recently, a group of 12 novel leptospires of with all pathogenicity classification was discovered in New Caledonia soil by Thibeaux *et al.* (2017, 2018) which adds further to the growing list of *Leptospira* species.

1.3.2 Morphological features

Genus *Leptospira* and two monospecies genera, *Leptonema illini* and *Turneriella parva* also comprise in the *Leptospiraceae* family under the Order of Spirochaetales. Leptospires are normally thin, long, and highly coiled cells with a size of 0.1 µm in diameter and 6-12 µm in length (Carleton *et al.*, 1979) (Figure 1.2). Leptospires are Gram-negative, however, due to its small diameter, the unstained cells are not visible by bright-field microscopy, therefore dark-field or phase-contrast microscopy is needed to visualised unstained cells (Zuerner, 2010). A flagellum is inserted sub terminally at both ends in the periplasm between the peptidoglycan layer and the outer membrane envelope (Goldstein *et al.,* 1996) and enables high motility with movement enhanced by the right-handed helical structure of the cells (Carleton *et al.*, 1979).

1.3.3 Cell envelope

Like typical Gram-negative bacteria, leptospire structure is made up of distinct double membranes; outer membrane (OM) and inner membrane (IM). The OM serves as a selective barrier that regulates the movement of molecules outside the cell in exchange for nutrients and harbours various functions including cell adhesion, cell signalling and transport of waste, whereas the IM is involved in the transport chain during protein synthetisation (Rollauer *et al.*, 2015). Leptospiral outer membrane components however, are somewhat different than its closer relatives such as *Treponema* or *Borrelia* outer membrane, which lack lipopolysaccharides (LPS) and which serves as a serovar identifier (de La Peña-Moctezuma, Bulach and Adler, 2001) and is one of the main virulence factors (Werts *et al.*, 2001; Murray *et al.*, 2010).

The periplasm makes up the second layer of cell envelope, which consists of peptidoglycan, similar to other Gram-negative bacteria. The function of this layer is to provide structural integrity and shape of the bacteria and also supports bacterial motility, which is a unique feature of spirochetes. Their corkscrew motility is generated by endoflagella (Figure 1.1) which adhere within the periplasm (Charon and Goldstein, 2002).

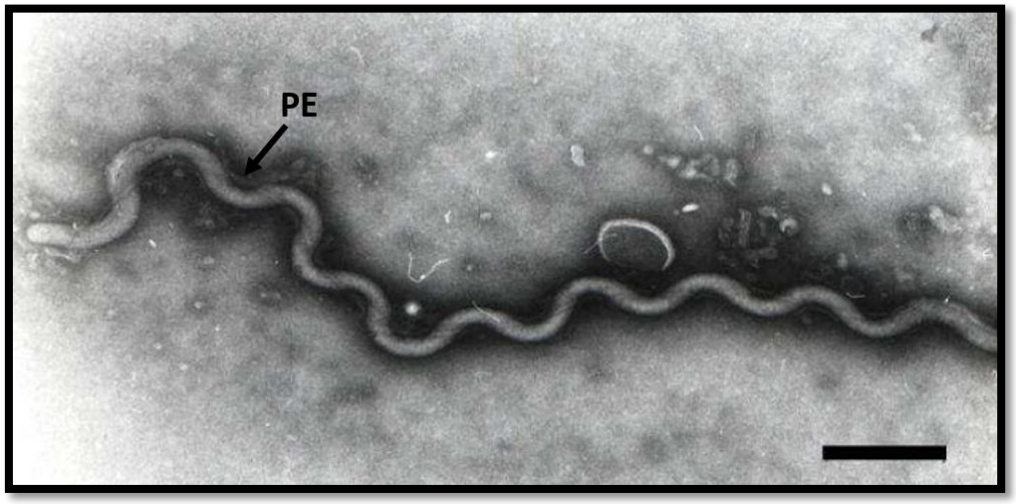


Figure 1.1: Transmission electron microscopy of negatively stained Leptospira interrogans. The periplasmic endoflagella (PE) is highlighted in the image. Image adapted from Catroxo and Martins (2015).

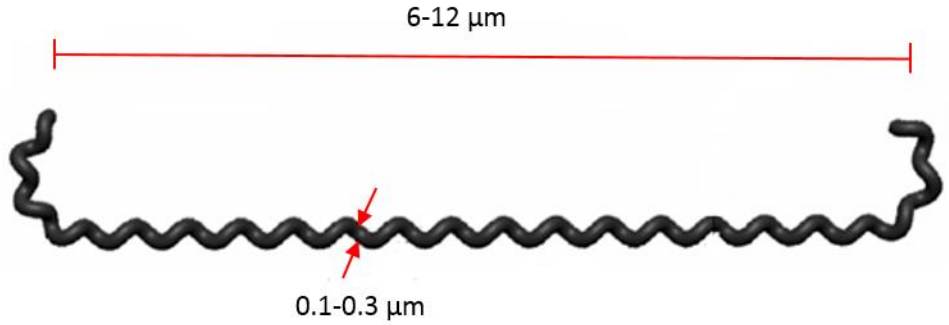


Figure 1.2: The schematic diagram of Leptospira structure. Image reproduced from Wong-ekkabut et al. (2009).

1.3.4 *Leptospira* metabolism

Leptospira acquire their energy and carbon through beta-oxidation of long fatty acids (Nascimento *et al.*, 2004) or from a glucose source. However, *Leptospira* do not utilise glucose in a normal metabolic pathway, instead, they possess a unique glycolysis pathway in which glucose molecules are broken down by a substitute enzyme (pyrophosphate-fructose-6-phosphate 1-phosphotransferase) instead of glucokinase (Nascimento *et al.*, 2004; Picardeau *et al.*, 2008; Zhang *et al.*, 2011). Metabolism of *Leptospira* spp. also differs between pathogenic and non-pathogenic species. For instance, Johnson and Rogers (1964) observed reduced growth of *L. interrogans* when the leptospires cells were cultivated with the addition of 8-azaguanine. In contrast to non-pathogenic species, the growth of this species was unaffected.

Leptospires can grow under aerobic conditions and survive depending on the temperature. Pathogenic leptospires are likely to survive at 28-30°C, whereas the non-pathogenic leptospires can also survive and grow in lower temperatures (11-13°C). Fresh isolated pathogenic *Leptospira* strains take longer to grow *in vitro* compared to non-pathogenic strains (Cameron, 2015). Altogether, most pathogenic leptospires can survive inside the host and extensively in the environment with favourable climates and humidity level. The exception to *Leptospira borgpetersenii* strains of which have undergone a reduction in genome level thus was believed to have host restriction transmission (Bulach *et al.*, 2006).

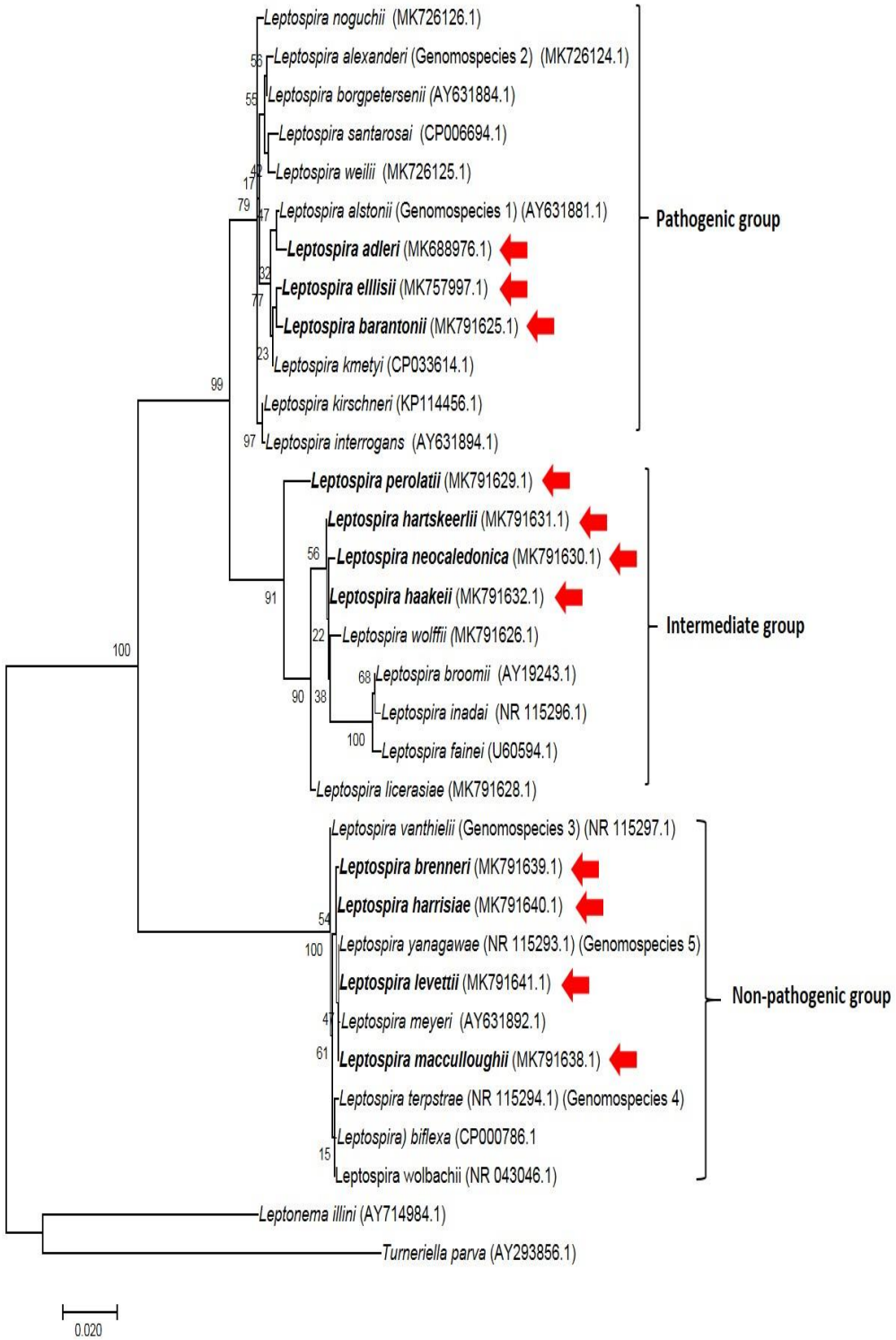


Figure 1.3: Leptospiraceae classification by 16S rRNA phylogenetic analysis.

The maximum-likelihood tree shows a relationship between each Leptospirocae representative species including novel species (shown with arrows), with the exception of L. saintgironsiae (belongs to intermediate group) (Thibeaux et al., 2018) based on ~1450 aligned based pairs constructed using MEGA7 (Tamura et al., 2013). Bootstrapping was performed 1000 times, and all positions containing gaps and missing data were eliminated.

Table 1.2: Distribution of serogroups in different Leptospira species. Table adapted from Zuerner, (2010) plus recent updates.

Species	Serogroup
L. interrogans	Australis, Autumnalis, Bataviae, Canicola, Djasiman, Grippotyphosa, Hedbomadis, Icterohemorrhagiae, Lousiana, Mini, Pomona, Pyrogenes, Ranarum, Sarmin, Sehgali, Sejroe
L. alexanderi (Genomospecies 2)	Hedbomadis, Javanica, Manhoa, Mini
L. biflexa	Semaranga
L. borgpetersenii	Australis, Autumnalis, Ballum, Bataviae, Celledoni, Hedbomadis, Javanica, Mini, Pyrogenes, Sejroe, Tarassovi
L. broomi	Undesignated
L. fainei	Hurstbridge
L. kirschneri	Australis, Autumnalis, Bataviae, Canicola, Cynopteri, Djasiman, Grippotyphosa,
L. licerasiae	Iquitos
L. meyeri	Javanica, Mini, Ranarum, Sejroe, Semaranga,
L. noguchi	Australis, Autumnalis, Bataviae, Djasiman, Lousiana, Panama, Pomona, Pyrogenes, Shermani, Tarassovi
L. santarosai	Autumnalis, Bataviae, Cynopteri, Grippotyphosa, Hedbomadis, Icterohemorrhagiae, Javanica, Mini, Pomona, Pyrogenes, Sarmin, Sejroe, Tarassovi
L. weilii	Celledoni, Hedbomadis, Icterohemorrhagiae, Javanica, Manhoa, Mini, Pyrogenes, Sarmin, Sejroe, Tarassovi
L. wolbachii	Codice
L. wolfii	Undesignated
L. alstonii (Genomospecies 1)	Ranarum
L. vanthielii (Genomospecies 3)	Holland
L. terpstrae (Genomospecies 4)	Icterohemorrhagiae
L. yanagawae (Genomospecies 5)	Semaranga
*L. adleri**	
*L. brantonii**	
*L. ellisii**	
*L. perolatii**	
*L. neocaledonia**	Undesignated
*L. saintgironsiae**	
*L. haakeii**	
*L. hartskeerlii**	
*L. harrisiae**	
*L. levetii**	
*L. brenneri**	
*L. macculloughii**	

**Novel Leptospira species recently discovered* (Thibeaux *et al.*, 2018)

1.4 Human leptospirosis

Transmission of *Leptospira* to man can be achieved by both direct and indirect ways; direct transmission is associated with direct contact of bodily fluids from carrier animals such as urine and vaginal fluid via cuts, and mucosal membranes (Director *et al.*, 2014; Loureiro *et al.*, 2017). Indirect transmission is via exposure to contaminated water and soil such as lakes, rivers, sewage and mud, which are favourable for leptospires survival (Fraga *et al.*, 2014). Human infection is primarily derived from the exposure of urine of the carrier animals or via urine-contaminated soil (Figure 1.4). Livestock farming is one route for contracting the disease directly from animals to humans and leptospirosis is primarily an occupational disease (Bharti *et al.*, 2003; Miyama *et al.*, 2018). Human-to-human transmission is possible but rare, although bacterial transmission via human milk and urine have been reported in the past (Bolin and Koellner, 1988; Chow *et al.*, 2012).

Human infection with pathogenic *Leptospira* may result in mild, self-limiting to a severe, life-threatening outcome. Man is an accidental host which does not maintain the bacteria in the body but may succumb to infection. When exposed, *Leptospira* rapidly disseminates in the blood within a few days and colonises multiple organs, such as liver, kidneys and lungs (Kishimoto *et al.*, 2004; Medeiros *et al.,* 2010; Tunjungputri *et al.*, 2017). The clinical features depend on the stage of infection, ranging from influenza-like symptoms characterised with sudden onset of fever, chills, headache and muscle pain (Ríos DI and Chaparro HM, 2015) followed by skin rashes (Iragorri and Tullus, 2009). The acute presentation is often confused with other febrile diseases that produce similar clinical signs such as dengue fever, malaria and influenza, which often causes misdiagnosis on the early stage of infection (Noor Rafizah *et al.*, 2012). The severe form of leptospirosis is known as Severe Pulmonary Haemorrhagic Syndrome (SPHS), is the fatal outcome of leptospirosis with a mortality rate of more than 50% (Gouveia *et al.,* 2008; Haake and Levett, 2015). Presently, human cases were reported across the world, notably in tropical countries (Figure 1.5). Cases have been reported in most of the tropical countries with highest rainfall, such as Thailand (Suwanpakdee *et al.,* 2015), the Philippines (Amilasan *et al.,* 2012) and Malaysia (Benacer *et al.,* 2016; Thayaparan *et al.,* 2013). These countries suffer major flood events as a result of severe rainfall with leptospirosis outbreaks ensuing from unavoidable human contact with leptospire contaminated water. For example, a spatial analysis carried out on pre-flood, during flood and post-flood events in north-eastern Malaysia between September 2014 to January 2015, resulted in 1229 human leptospirosis reported cases and highest cases were seen during the post flooding event (Mohd Radi *et al.,* 2018). High-density populations in an urban setting

promote *Leptospira* exposure especially in rat-infested areas lacking sanitary hygiene and/or poor drainage systems (Maciel *et al.*, 2008; Koizumi *et al.*, 2009a; Felzemburgh *et al.*, 2014). Human factors such as migration and travelling, enhance leptospirosis global spread (Bandara *et al.*, 2014). Another important human risk factor is water-based recreations, such as triathlons or open water swimming (Sejvar *et al.*, 2003; Brockmann *et al.*, 2010).

Diagnosis of leptospirosis is rather challenging, even though several diagnostic tools have been developed to enable detection in the early phase of infection (Budihal and Perwez, 2014). However, diagnosis is further complicated by concurrent illnesses with similar signs such as dengue fever (Bruce *et al.*, 2005; LaRocque *et al.*, 2005). Treatment of leptospirosis is effective with combinations of antimicrobial and symptomatic therapy during the acute and chronic stage (Kobayashi, 2005). Several licensed human vaccines are currently available (Xu and Ye, 2018) however, the majority of vaccines are derived from inactivated whole-cell leptospires, and therefore the protection is likely to be partial, with a lack of cross-protective immunity against heterologous serovars. Furthermore, these vaccines are manufactured in certain countries and may not work in a global setting due to different circulating *Leptospira* species/serovars.

Despite interest in leptospirosis growing significantly over the years, there is limited information on its pathogenesis, and this hampers efforts to control the disease.

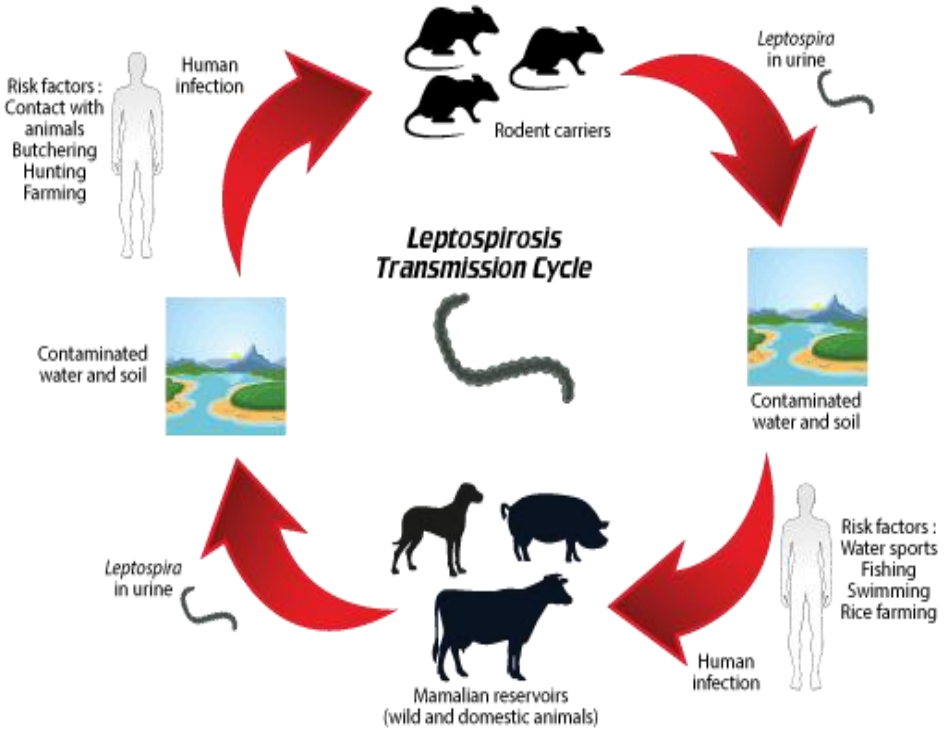


Figure 1.4: Transmission cycle showing leptospires can be transmitted from rodent carriers directly to the environment and indirectly to man with associated risk factors and animal hosts.

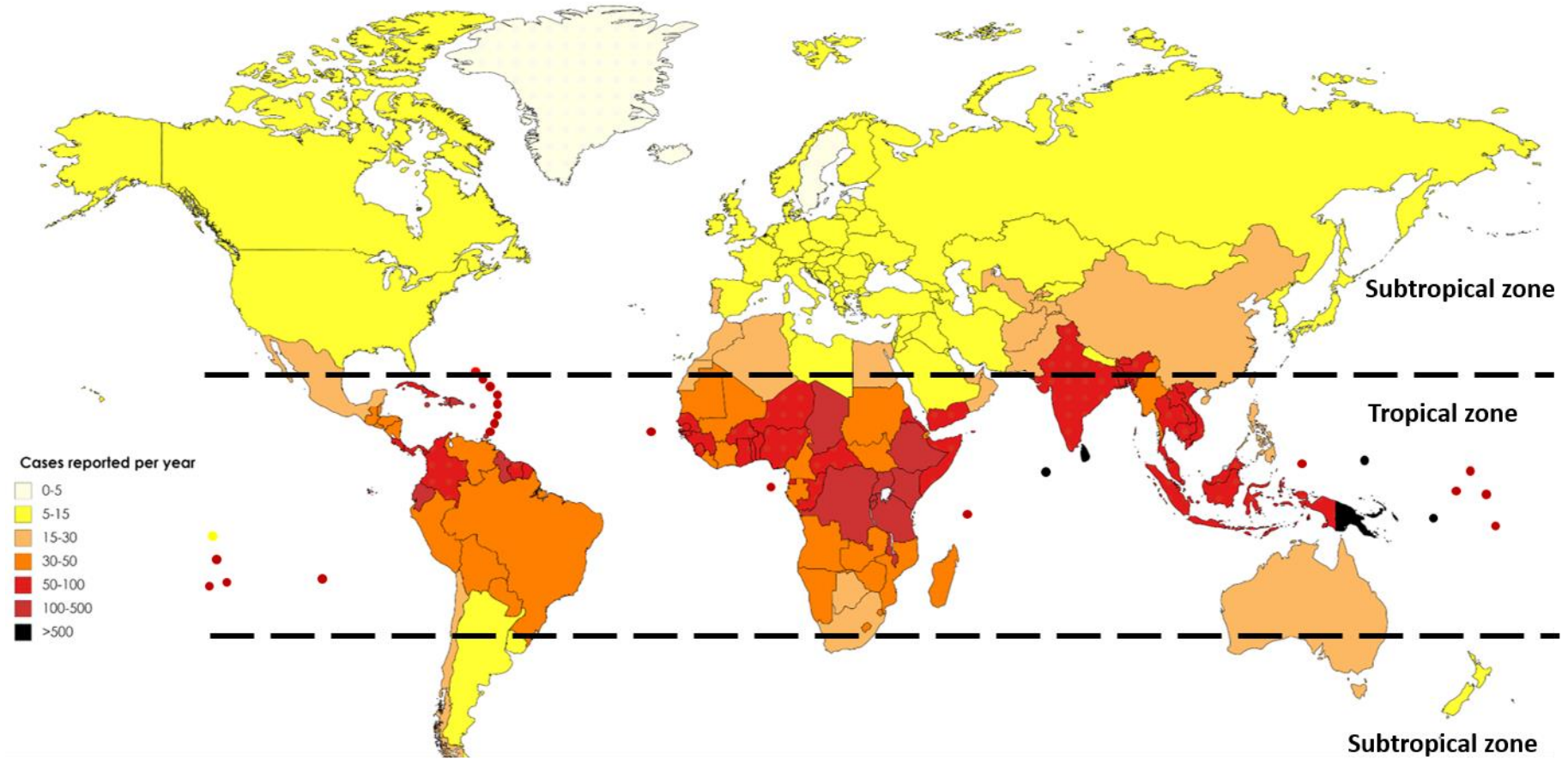


Figure 1.5: A world map showing the burden of human leptospirosis based on cases reported each year. Note that the majority of cases occur in tropical zone compared to subtropical zones. Figure regenerated and modified based on Torgerson et al. (2015).

1.5 Animal leptospirosis

Animals are the important hosts for *Leptospira*. Rats are the principal reservoir of *Leptospira*, and can carry and shed the bacteria throughout their lives (Ido *et al.*, 1917; Athanazio *et al.*, 2008; Costa *et al.*, 2015). Apart from rats, many animal species, including some marine mammals (Cameron *et al.,* 2008; Norman *et al.,* 2008) may also harbour the bacteria in their kidneys and spread them through renal excretion. While acting as both reservoir and carrier, vulnerable animals may also succumb to infection, especially when infected with non-native *Leptospira* species. Interaction between environment, maintenance hosts and *Leptospira* are pivotal for successful disease transmission. Numerous domestic species such as cattle, pigs, dogs, horses (Burriel *et al.,* 2003; Rocha *et al.*, 2004) and wild animals such as bats (Cox *et al.,* 2005) and wild boar (Koizumi, *et al.*, 2009b) are known to maintain several pathogenic leptospires serovars which can potentially spread to other animals and man (Table 1.1).

Table 1.3: Various animal hosts associated with Leptospira serovars.

Animal host	Serovars
Cattle	Hardjo, Pomona, Kennewicki, **Icterohaemorrhagiae*, Canicola*, Hebdomadis*, Sejroe*, Pyrogenes*, Autumnalis*, Australis*, Javanica*, Tarassovi*, Grippotyphosa***
Pigs	Australis, Pomona, Tarassovi**, Icterohaemorrhagiae*, Grippotyphosa* Canicola***
Dogs	Canicola, Icterohaemorrhagiae, **Grippotyphosa* Canicola***
Sheep	Hardjo, **Icterohaemorrhagiae*, Australis*, Grippotyphosa*, Sejroe***
Horses and donkeys	Bratislava, Kennewicki, **Grippotyphosa* Autumnalis*, Sejroe*, Canicola*, Ballum***
Rats	Icterohaemorrhagiae, Copenhageni
Mice	Ballum, Arborea, Bim
Raccoon	Grippotyphosa
Marsupials	Grippotyphosa
Bats	Cynopteri, Wolfii
Sea lion	Pomona

**Indicates accidental infection with non-native serovars. Table adapted from (Bharti et al., 2003; Ellis, 2015).*

Animals are likely to be exposed to *Leptospira* from birth, either from their mothers or directly acquired infection from other animals. Even so, animals are generally tolerant of leptospirosis due to their ability to harbour the bacteria in the kidneys. As with man, the transmission of disease between animal hosts may also occur through contact with a contaminated environment. Additionally, contact with infected wild animals and rats may result in incidental infection as is commonly seen in farm animals (Webster *et al.,* 1995; Burriel *et al.,* 2003). Even though wild mammals can be *Leptospira* carriers, leptospirosis may not have a detrimental effect on their health. Another potential leptospiral transmission route is oral (Luzzi *et al.,* 1987; Asoh *et al.*, 2014), although this route has not been widely studied. More details on this route can be found in Chapter 7 in this thesis. The interaction between man, animals and environment is associated with leptospirosis, and the disease possesses a significant public threat, especially in an endemic area where the disease is prevalent.

1.5.1 Impact of leptospirosis in animals

Leptospirosis is a significant disease primarily affecting the productivity and reproduction of livestock and causes substantial losses worldwide (Grooms, 2006). Reproduction losses such as low milk yield, abortion, reduced fertility, stillborn calves and the birth of weak calves/piglets are commonly reported following outbreaks. On the other hand, infection in companion animals such as dogs results in similar clinical outcomes to human cases (Goldstein *et al.*, 2006; Goldstein, 2010).

In this study, we will focus more on bovine leptospirosis as part of the research theme of the thesis. Bovine leptospirosis, including the economic impacts, pathogenesis, diagnosis, and treatment, will be described in more details in the following sections.

1.6 Introduction to bovine leptospirosis

Bovine leptospirosis (BL) is one of the leading causes of reproductive failures and milk drop syndrome in cattle herds (Ellis, 1984; Grooms, 2006). Cattle are known to maintain serovar Hardjo, which is pathogenic to both cattle and man. Studies on bovine leptospirosis have grown significantly over the years due to the awareness of public health relating to a human-cattle relationship (Agampodi *et al.*, 2010; Swai and Schoonman, 2012; Ndengu *et al.*, 2017). BL has a worldwide distribution, with substantial morbidity in tropical countries. However, the disease is very much underestimated due to subclinical signs that are difficult to

interpret, leading to misdiagnosis and making it one of the most neglected cattle diseases. Depending on the infective *Leptospira* serovars, BL causes moderate to severe reproductive losses, including reduced milk yield, abortion, premature birth of the weak calves and infertility (Ellis *et al.*, 1986). Although the economic loss is difficult to be determined, it has been reported that the estimated cost of output losses, treatment and prevention for bovine leptospirosis was £22.3 million in the UK (Bennett *et al.,* 1999) and USD $ 150,000 loss for calves mortality, vaccination and treatment in Argentina following an outbreak (Draghi *et al.*, 2011).

As previously described, several *Leptospira* serovars are known to be native to cattle, although other non-native serovars also may be able to cause incidental infection (Ellis, 2015) (Table 1.3). Transmission of bovine leptospirosis in cattle may be preceded by the presence of rodents on farm, contact with wild and other domestic animals known to be carriers for *Leptospira* such as dogs, pigs, horses and foxes and direct contact with a contaminated environment (Petrakovsky *et al.*, 2014; Fávero *et al.*, 2017). Other associated risk factors of BL including co-grazing with other ruminants, increase herd size, movement of cattle, access to contaminated water sources and poor biosecurity practices have previously identified as the primary source of infection in the herd-level (Table 1.4). The recent study by Yatbantoong and Chaiyarat (2019) highlighted farm management practice, such as keeping older cattle in the farm contributes to longer *Leptospira* exposure.

Leptospira serovars have been isolated from the urine and kidney from naturally infected cattle, indicating that cattle are a natural host (Prescott *et al.,* 1987; Pinna *et al.*, 2018). These serovars are maintained in the kidneys of the cattle and escape through the kidney tubules until eventually passed in the urine, contaminating the environment and exposing the naive herd or humans to leptospirosis (Table 1.4). Although no evidence exists as to whether leptospires can be sexually transmitted between cattle, this has been demonstrated in small ruminants (Director *et al.*, 2014). Moreover, recent reports highlighted that *Leptospira* could survive in milk. Therefore, the disease could be transmitted to the host via ingestion (Fratini *et al.*, 2016; Oliveira *et al.*, 2016).

Table 1.4: List of common risk factors of BL transmission in cattle farms.

Risk factor	References
Co-grazing with other species or contacts with other animals including rats	(Schoonman and Swai, 2010) (Gamage *et al.*, 2011) (Lilenbaum and Souza, 2003) (Subharat *et al.*, 2012)
Increase in herd size	(Leonard *et al.*, 2004) (O'Doherty, Sayers and O'Grady, 2013) (Ryan *et al.*, 2012) (Campos *et al.*, 2017) (Miyama *et al.*, 2018)
Access to contaminated water sources	(Campos *et al.*, 2017)
Age of cattle	(Schoonman and Swai, 2010) (Yatbantoong and Chaiyarat, 2019) (Suwancharoen *et al.*, 2013)
Natural calving	(Salgado *et al.*, 2014)
Movement of cattle Poor biosecurity	(van Schaik *et al.*, 2002)
Contact of adult animals with calves/grazing with calves	(O' Doherty *et al.*, 2014)
Other possible risk factors:	
Natural events (flooding)	(Chadsuthi *et al.*, 2018) (Ijaz *et al.*, 2018)
Infrequent vet visit to the farms	(Lilenbaum and Souza, 2003)
Lack of control programs	(Swai and Schoonman, 2012)

1.6.1 BL risk factors to human

As previously described, BL possesses a significant threat to humans, especially farmers. Serological studies on cattle farmers confirmed that humans are likely to be exposed to *Leptospira* serovars acquired during farming and butchering activities (Esmaeili *et al.*, 2016; Chadsuthi *et al.*, 2017; Daud *et al.*, 2018). Human infections derived from cattle have previously been reported, but not frequently (Davidson, 1971; Bolin and Koellner, 1988; Mclean *et al.*, 2014; Benschop *et al.*, 2017). This is because the mild infection is usually self-limiting, thus the symptoms are potentially overlooked. Additionally, the type of infective serovars may also influence *Leptospira* virulence that will determine the disease outcome. For instance, serovar Hardjo has been detected in several human cases (Mclean *et al.*, 2014; Benschop *et al.*, 2017). Human factors are also contributing to successful *Leptospira* transmission. Benschop *et al.* (2009) stated that *Leptospira* direct transmission could be enhanced through human contact with cattle, cuts on the body and contact with animal

effluents. Other human risk factors including immunosuppressive disease, poor hygiene, an unhygienic condition in an abattoir and lack of communication between human health inspector and veterinary services (Swai and Schoonman, 2012).

1.6.2 Epidemiology of bovine leptospirosis

Over the years, bovine leptospirosis has become a significant worldwide disease and a leading cause of reproductive failures in cattle. Several reports on leptospirosis epidemiology have been published to determine the disease prevalence of cattle and local abundance of *Leptospira* serovars in several countries (Table 1.5). Global prevalence varies depending on the area of study, types of cattle rearing and circulating leptospiral strains in the country. While higher BL seroprevalence is reported elsewhere across the world, the titre is relatively low in Scandivanian regions (Lindahl *et al.*, 2011) reflecting that the disease is probably not a major threat to local cattle herds.

In the UK, the prevalence of leptospirosis is thought to be higher in dairy cattle than beef cattle. Although the disease is not commonly encountered, seroprevalence studies showed the prevalence of serovar Hardjo infection in UK dairy cattle was more than 70% at the herd level (Bishop, Erkelens and Van Winden, 2010; Williams and Winden, 2014). Studies of BL in UK beef farms are limited, possibly due to lack of obvious clinical signs (e.g. abortion) seen by the farmers. The UK Animal and Plant Health Agency recorded 23 cases of cattle abortion in the UK from 2012-2018 related to leptospirosis (APHA, 2018). Additionally, a serological survey conducted by MSD Animal Health from 2013-2015 showed that 58% (dairy) and 33% (beef) farms tested positive for leptospirosis (Figure 1.6). These studies show that while BL is perceived as low risk in UK cattle farms, the serological evidence showed that these animals might have been exposed to the disease and need further investigations.

Despite recent available data, studies on BL epidemiology remain scarce, resulting in a poor understanding of disease dynamics and difficulties in disease control and in applying preventive actions. Most published studies are based on serological data, which has several limitations. The difficulty of isolating *Leptospira* strains is another pitfall in determining the actual disease prevalence.

1.6.3 Clinical manifestation of bovine leptospirosis

Infected cattle may show clinical signs which might be considered ranging from mild to severe manifestations. The clinical outcomes of the disease are largely dependent on the degree of

herd immunity, the physiological state of the animals and circulating serovars (Grooms, 2014). The chronic form is usually acquired from adapted serovars such as Hardjobovis, where animals may have been infected from a young age. Clinical signs associated with the chronic manifestation are mainly related to reproductive failure and likely present when the animal is pregnant. Infected animals may abort, or give birth to stillbirth or premature and weak infected calves (Bolin, 2005). Chronically infected animals tend to pass the disease to the surviving calves and remain infected throughout their entire life (Grooms, 2014).

The severe manifestation of leptospirosis in cattle is uncommon and is normally due to non-native serovars such as serovar Bratislava, Pomona, Grippotyphosa and Icterohaemorrhagiae infecting young animals (Ellis, 2015). These serovars normally originate from other carrier animals nearby the cattle farm. In dairy cattle, the severe and acute form presents as a marked drop in milk production or milk drop syndrome accompanied by fever, which lasts for two to ten days. The appearance of the milk is thick, yellow-stained and sometimes blood-tinged and the udder soft (Symington, 1957). Milk abnormalities and reduced milk yield have been similarly observed when serovar Hardjo infection presents acutely, and the animals typically recover after a few days without need for treatment (Higgins *et al.*, 1980). Acute infection also may cause immediate abortion in pregnant cows.

The subacute form of the disease causes similar clinical manifestations as in the acute form, only with milder symptoms. In this form, abortion is observed a couple of weeks after the infection. Very rarely, the severe acute form of leptospirosis in infected calves manifests as high fever, lethargy, haemoglobinuria, jaundice and death within a few days (Sutherland *et al.,* 1949; Guerra, 2009; Ellis, 2015).

Table 1.5: Prevalence studies of BL worldwide from 1998-2018.

Country	Region	Prevalence rate (%)	Samples	Method of diagnosis[a]	Dominant serogroups or serovars[b]	References
Asia						
India	Multiple region	70.5 (95% CI 65.0-75.0)	Serum	MAT	Hardjo, Pyrogenes*	(Balamurugan *et al.*, 2018)
	Konkan	35.5 (95% CI 37.0-45.2)	Serum	MAT	Australis, Hardjo*	(Balamurugan, 2016)
	Cauvery valley	87.0	Serum	MAT	Javanica, Autumnalis*	(Natarajaseenivasan *et al.*, 2011)
	Bihar	9.11 (95% CI 27.5-42.2)	Serum	ELISA	Hardjo**	(Pandian *et al.*, 2015)
	Odisha	42.5 (95% CI 34.0-51.4)	Serum	MAT	Australis, Hardjo*	(Balamurugan *et al.*, 2013)
	Gujarat	12.8	Serum	MAT	Pomona, Hardjo*	(Patel *et al.*, 2014)
Iran	Kerman	17.4	Serum	MAT	Pomona	(Khalili *et al.*, 2014)
	Shahrekord	18.8	Serum	MAT	Canicola, Grippotyphosa	(Ebrahimi *et al.,* 2004)
Japan	Hokkaido	12.8	Serum	MAT/ELISA	Hardjo	(Koizumi and Yasutomi, 2011)
Laos	Multiple region	3.0 (95% CI 1.9-4.2)	Serum	ELISA	N/D	(Vongxay *et al.*, 2012)
Malaysia	Kelantan	81.7 (95% CI 63.5-80.1)	Serum	MAT	Sarawak, Patoc*	(Daud *et al.*, 2018)
Mongolia	Multiple region	80.4, 28.0, 23.5 (three regions)	Serum	MAT/ELISA	Hardjo, Ruparupae*	(Odontsetseg *et al.,* 2005)
Pakistan	Punjab	56.3	Serum	ELISA	N/D	(Ijaz *et al.*, 2018)
Philippines	Luzon	7.80	Urine	PCR	*L. borgpetersenii/L. kirschneri*	(Villanueva *et al.*, 2016)
Sri Lanka	Gampaha	9.00	Urine	RT-PCR	N/D	(Denipitiya *et al.*, 2017)
	Kandy	38.8 (Urine), 20.3 (Serum)	Serum, Urine	MAT, PCR	Wolfii, Hebdomandis*	(Gamage *et al.*, 2011)
	Colombo	12.2	Kidney	PCR	*L. borgpetersenii*	(Gamage *et al.*, 2014)
Thailand	Multiple region	28.1 (95% CI 26.7-29.6)	Serum	MAT	Ranarum, Shermani*	(Chadsuthi *et al.*, 2017)
	Multiple region	9.9 (95% CI 9.3-10.5)	Serum	MAT	Ranarum, Sejroe*	(Suwancharoen *et al.*, 2013)
	Kanchanaburi	92.2	Serum	MAT	Tarassovi, Ranarum*	(Yatbantoong and Chaiyarat, 2019)
Turkey	Marmara	3.40	Serum	MAT	Hardjo	(Kocabiyik and Cetin, 2004)
Africa						
Cameroon	Adamawa	35.0 (95% CI 27.6-33.2)	Serum	ELISA	Hardjo**	(Scolamacchia *et al.*, 2010)

Morocco	El-Gharb	9.10	Serum	MAT, ELISA	Hardjo, Pomona**	(Lucchese *et al.*, 2016)
	Multiple region	15.0	Serum	MAT	Ballum, Sejroe*	(Benkirane *et al.*, 2016)
Nigeria	Kaduna	3.50	Serum	ELISA	Hardjo	(Ngbede *et al.*, 2012)
South Africa	KwaZulu-Natal	19.4 (95% CI 14.8-24.1)	Serum	MAT	Pomona, Tarassovi*	(Hesterberg *et al.*, 2008)
Tunisia	Mateur	75.0	Serum	MAT	Pomona, Autumnalis*	(Khbou *et al.,* 2017)
Tanzania	Tanga	51.0 (95% CI 44.1-57.9)	Serum	MAT	Hardjo, Tarassovi	(Swai and Schoonman, 2012)
	Tanga	58.5 (95% CI 9.5–67.1)	Serum	MAT	Hardjo, Tarassovi	(Schoonman and Swai, 2010)
Uganda	Kole, Mbale	19.3 (95% CI 14.9-24.5)	Serum	MAT	Pomona, Kenya*	(Dreyfus *et al.*, 2017)
	Southwest	27.1 (95% CI 26.1-28.3)	Serum	ELISA	Hardjo**	(Atherstone *et al.,* 2014)
Europe						
Greece	Multiple region	16.2	Serum	MAT	Bratislava, Copenhageni	(Burriel *et al.,* 2003)
Ireland	Multiple region	91.0	Serum	ELISA	Hardjo**	(Barrett *et al.*, 2018)
	Multiple region	41.8 (95% CI 41.0-41.3)	Milk	ELISA	Hardjo**	(Ryan *et al.*, 2012)
Italy	Multiple region	46.9	Serum	MAT	Sejroe, Bratislava*	(Tagliabue *et al.*, 2016)
Poland	Southwest	3.20	Milk	ELISA	Hardjo**	(Rypula *et al.*, 2014)
Portugal	Multiple region	15.3	Serum	MAT	Hardjo, Sejroe*	(Rocha, 1998)
Spain	Galicia	18.3	Serum	MAT	Bratislava, Grippotyphosa*	(Guitián *et al.*, 2001)
	Asturias	10.4	Serum	MAT	Pomona, Grippotyphosa*	(Espi *et al.*, 2000)
Sweden	Multiple region	<1.0	Serum	MAT	Sejroe (Mouse 2A)	(Lindahl *et al.*, 2011)
UK	Multiple region	71.9	Milk	ELISA	Hardjo**	(Williams and Winden, 2014)
	Multiple region	47.0 (95% CI 34.0-60.0)	Milk	ELISA	Hardjo**	(Velasova *et al.*, 2017)
	Wales	76.0	Milk	ELISA	Hardjo**	(Bishop *et al.,* 2010)
Americas						
Brazil	Teresina	50.1 (95% CI 49.5-61.6)	Serum	MAT	Hardjo, Icterohaemorrhagiae*	(Campos *et al.*, 2017)
	Santa Catarina	6.44	Serum	MAT	Pomona, Sejroe (serogroup)	(Fávero *et al.*, 2017)
	Rio de Janeiro	38.3	Serum	MAT	Sejroe (serogroup)	(Martins and Lilenbaum, 2013)
	Garanhuns	47.6	Serum	MAT	Hardjo, Bratislava*	(Oliveira *et al.*, 2001)
Canada	Multiple region	2.3[e]	Serum	MAT	Pomona, Grippotyphosa*	(Van De Weyer *et al.*, 2011)
Chile	De Los Ríos	75.0	Serum	MAT	Hardjo, Pomona*	(Salgado *et al.*, 2014)

Columbia	Córdoba	74.5	Serum	MAT	Hardjo, Saxkoebing*	(Ensuncho-Hoyos *et al.*, 2017)
Mexico	Toluca valley	10.3	Serum	MAT	Hardjo, Canicola*	(Leon *et al.*, 2008)
	Yucatan	62.8	Serum	MAT	Hardjo, Tarassovi*	(Segura-Correa *et al.,* 2003)
Trinidad	Multiple region	21.5	Serum	MAT	Icterohaemorrhagiae, Mankarso*	(Suepaul *et al.*, 2011)
Uruguay	Multiple region	77.0	Serum/Urine	MAT/PCR	Kennewicki, Hardjo	(Zarantonelli *et al.*, 2018)
USA	Texas	35.0	Urine	PCR	Pomona, Hardjo*	(Talpada *et al.*, 2003)
West Indies						
Saint Kitts	Saint Kitts	78.9 (95% CI 71.9–87.7)	Serum/kidney	MAT/PCR	Mankarso, Djasiman	(Shiokawa *et al.*, 2019)
Oceania						
Australia	Queensland	53.7	Serum	MAT	Hardjo, Tarassovi*	(Black *et al.,* 2001)
New Caledonia	New Caledonia	43.0	Serum	MAT	Hardjo, Copenhageni*	(Roqueplo *et al.*, 2013)
New Zealand	Multiple region	21 (95% CI 15.0–28.0)	Kidney	PCR	N/D	(Fang *et al.*, 2015)
	Multiple region	73 (95% CI 59.0 –83.0)	Serum	MAT	Hardjo, Pomona	
	Multiple region	53.7 (95% CI 51.4-56.0)	Serum	MAT	Hardjo, Pomona	(Dreyfus *et al.*, 2018)

[a]Method of diagnosis to determine BL prevalence. Abbreviations: ELISA; Enzyme-linked immunosorbent assay, MAT; Microscopic agglutination test, PCR: Polymerase chain reaction (See Section 1.8 for details)

[b] Positive antibodies detected to agglutinating Leptospira serogroups/serovars. N/D; Not determined

**Positive antibodies detected to more than five Leptospira serovars using MAT*

***Positive antibodies detected to serovar Hardjo using commercial ELISA test kit*

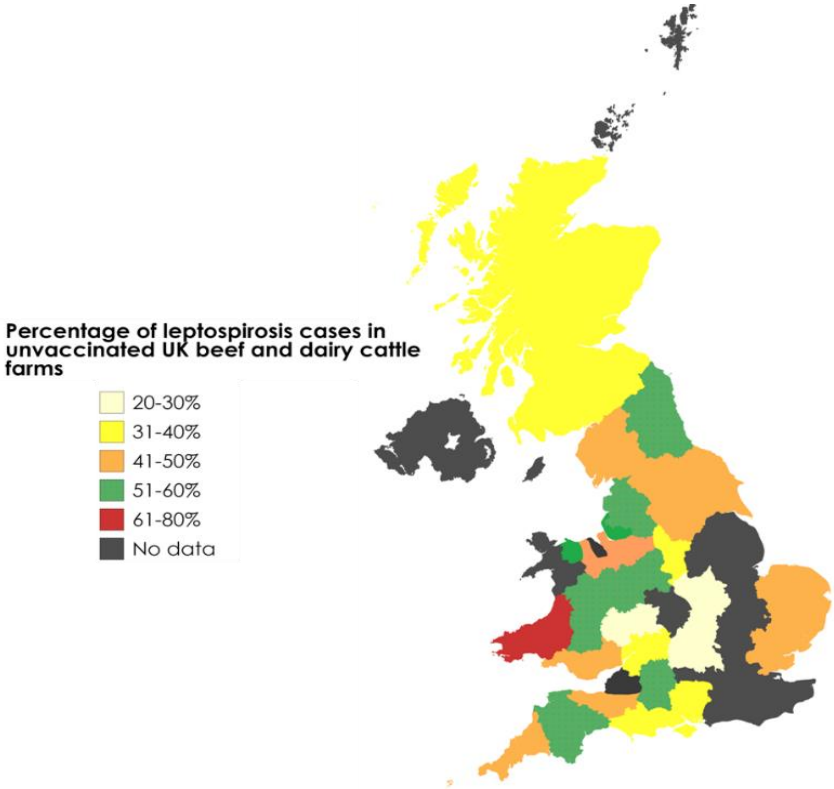


Figure 1.6: A map showing the percentage of positive BL in the UK from October 2013-2015.

Data and figure source from BeefCheck and DairyCheck data, MSD Animal Health (2018).

1.7 Pathogenesis of leptospirosis

The disease onset and progression are well-described in a clinical manner in both humans and animals. However, little is known about the mechanisms involved at both the cellular and molecular levels, and therefore, the pathophysiology of leptospirosis remains poorly understood. The main reason for this is due to the lack of advance genetic tools available to manipulate the genes (e.g. mutagenesis), which have been largely available in other bacterial species (Adler *et al.*, 2011; Adler, 2014). However, considerable recent progress has been made towards understanding the molecular pathogenesis aspects, prompting the discovery of various *Leptospira* virulence factors which may contribute to the disease mechanism such as LPS, hemolysins, OMPs and other surface proteins and adhesion molecules.

Leptospirosis pathogenesis involves several stages. The first stage is bacterial entry and dissemination. The entry of pathogenic *Leptospira* into the host proceeds through motility and chemotaxis. One theory is the attraction of *Leptospira* towards haemoglobin (Yuri *et al.*,

1993), which suggests that leptospires are attracted to sites of tissue injury. In the second stage, leptospires rapidly disseminate into the bloodstream and travel to adjacent organs. In order to establish a successful infection, leptospires must be able to adhere to host tissues to begin host colonisation. There is now a broad list of known leptospira surface adhesins that exhibit *in vitro* attachment to a range of host extracellular matrix (ECM) components (Table 1.6, Section 1.11).

Binding to host plasminogen and fibrinogen may result in disruption of haemostasis and wound repair. The binding to plasma proteins would facilitate inhibiting fibrin formation, causing degradations of ECM and fibrin clot leading to dissemination throughout the host (Vieira *et al.*, 2009; Oliveira *et al.*, 2013). As previously described, homeostatic imbalance in the infected host is clinically characterised by thrombocytopenia and haemorrhage, especially in the severe form of the disease (Edwards *et al.,* 1982). Moreover, adherence to plasminogen may interfere with the host complement system, therefore reducing opsonisation for phagocytosis and subsequently affecting the complement cascade at the cell surface by both the alternative and classical pathways (Vieira *et al.*, 2011).

The next stage is immune persistence. One important strategy for this stage is the ability to evade host immune response, and *Leptospira* possesses several known virulence factors that may enable them to escape host immune responses. Leptospires may interfere with the host complement cascade by binding to host regulatory proteins, such as factor H and C4 binding protein (C4BP) (Meri *et al.*, 2005; Barbosa *et al.*, 2009) in the serum and deactivate the complement pathways. Another important strategy is to evade from host phagocytic cells. Additonally, Leptospires may induce macrophage apoptosis (Merien *et al.,* 1997; Jin *et al.* 2009) and prevent the release of neutrophil phagosomes during cellular phagocytosis via inhibition of neutrophil myeloperoxidase that interferes with the innate immune response (Vieira *et al.* 2018). Collectively, both host immune reactions and leptospires responses can lead to damage to host tissues. As a result, the vulnerable host may progress to systemic inflammation, vascular damage, multiple organ failures and haemorrhage. In cattle, septicaemia may cause haemoglobinuria as a result of extensive intravascular haemolysis (Adugna, 2016). Surviving animals may progress to renal colonisation and persistence, which is vital for leptospires survival as the environment in the renal tubules is favourable for bacterial attachment and multiplication, and eventually, they will be passed into the urine to begin *Leptospira* transmission.

1.8 Diagnosis of bovine leptospirosis: Common techniques

Currently, there are no effective and ideal laboratory tests that can rapidly detect the organism during the early onset of disease despite a range of diagnostic tests being available (Musso and La Scola, 2013; Picardeau, 2013; Rajapakse *et al.*, 2015). The gold standard for leptospirosis diagnosis is the microscopic agglutination test (MAT) developed in France almost a hundred years ago (Martin and Pettit, 1918), which has since become the method of choice to determine leptospirosis seroprevalence in cattle worldwide (Table 1.5) and to identify *Leptospira* serovars. MAT is performed by incubating patient serum at various dilutions mixed with a large panel of live leptospires of various serovars in microtitre plates and the results are read under dark field microscopy. Observation of an agglutination reaction between a serovar and patient serum is indicated to be the infective serovar/serogroup (Chirathaworn *et al.*, 2014; Goris and Hartskeerl, 2014).

Even though MAT is highly specific towards *Leptospira* serogroups, the use of MAT is not always convenient because it requires locally circulating serovars or serovars belonging to all serogroups to be used as live targets to avoid false negative results (Turner, 1968). These serovars need to be maintained regularly in the laboratory, which is labour intensive and time-consuming. MAT also cannot be standardised because it uses live antigens and can only be performed in the laboratory with experienced laboratory personnel. Another disadvantage of MAT is that it cannot differentiate between post vaccination and natural infection (Smith *et al.*, 1994; Rajapakse *et al.*, 2015). Nevertheless, the use of a ELISA test was developed (Adler *et al.,* 1980) and applied in bovine studies (Adler *et al.*, 1982; Cousins, Robertson and Hustas, 1985; Bercovich *et al.,* 1990). The ELISA test is considered more sensitive and specific, and relatively rapid and easy to interpret compared to MAT. In a clinical setting where MAT is not available, ELISA is usually used and is mostly useful for acute infection, especially in human leptospirosis (Winslow *et al.*, 1997; Niloofa *et al.*, 2015).

The use of ELISA in cattle is unlikely to detect antibody in acute infection but can determine whether animals have been exposed to *Leptospira*. This is because the animal may have retained infection since it was young (e.g. chronically infected) and therefore the commercially available ELISA kits for cattle are based on IgG detection in either blood and/or milk (Yan *et al.*, 1999). However, like MAT, ELISA cannot differentiate antibody titer between infection and vaccinated animals (Smith *et al.*, 1994). When compared to MAT, ELISA has proven more sensitive for the diagnosis of BL (Cousins *et al.,* 1985; Sakhaee *et al.*, 2010).

Another advantage of using ELISA over MAT is that no live antigens are required and therefore, it is relatively safe to use in the laboratory.

Polymerase chain reaction (PCR) is another highly sensitive method to detect *Leptospira* in clinical samples and particularly in urine, tissues from aborted foetus and kidneys (Richtzenhain *et al.*, 2002; Director *et al.*, 2014; Shiokawa *et al.*, 2019) and is also useful to identify the carrier status of an animal (Pinna *et al.*, 2018). The most common PCR application in leptospirosis is the detection of *Leptospira* genes such as 16S rRNA (Shekatkar *et al.,* 2010), LipL32 (Stoddard, 2013) and OmpL1 (Reitstetter, 2006). However, PCR also has several limitations. For example, the target genes LipL32 and OmpL1 are only present in pathogenic leptospires (Haake *et al.*, 1993; Haake, 2001) not in non-pathogenic leptospires.

Lastly, isolation of the spirochetes by culturing is one of the conventional techniques but is not routinely carried out in the laboratory. Furthermore, the culture work is not always reliable as the bacteria may not be present if the animals were in a chronic state and were previously treated with antibiotics. The cultivation method is also often subjected to contamination, time-consuming, low sensitivity and requires serovar identification by reference laboratories (Smith *et al.*, 1994; OIE, 2008).

1.9 Treatment and prevention of bovine leptospirosis

For food-producing animals, leptospirosis has a major impact on cattle's health and productivity. The impact of cattle disease has a wide range of productivity losses affecting milk production and reproduction. In most tropical countries where BL is endemic, it is nearly impossible to eradicate the disease in the environment, especially during the wet season, and the animals are at the higher risk of contracting the infection. Therefore, controlling the infection becomes the main focus to minimise the transmission, reproduction losses and consequently, economic loses. In the case of acute leptospirosis, the use of antibiotics with supportive therapy is the treatment of choice (Mazzonelli, 1984). However, the owner needs to consider the withdrawal period of the antimicrobials and the cost of milk withdrawn during the period (Ellis, 2015). Due to this concern, treatment is not always reliable for bovine leptospirosis even though several studies have demonstrated the effectiveness of antibiotics for the elimination of *Leptospira* in the urine of infected animals (Alt *et al.,* 2001; Cortese *et al.*, 2007; Yupiana *et al.*, 2019). Therefore, disease prevention plays a significant role to combat infection at the herd level.

Vaccination is considered as a useful tool for controlling leptospirosis in many developed countries (Faine, 1982). Vaccines successfully limit the transmission of the bacteria by reducing the urinary shedding and urogenital tract colonisation and producing a strong antibody titre for longer immune protection (Zimmerman *et al.*, 2013; Balakrishnan and Roy, 2014). Very often, commercial cattle vaccines are formulated to contain more than one locally prevalent serovar or with the combination with chemically altered live strains of other pathogens causing reproductive failures in cattle. For example, Leptavoid®-H (MSD, Animal Health) containing two local strains (*L. interrogans* Hardjo and *L. borgpetersenii* Hardjo) is registered for use in the UK and Australia. Ultravac® 7 in 1, a combined 5 in 1/ Lepto Vaccine for Cattle (Pfizer, Australia) has been approved to be used for the prevention of clostridiosis and leptospirosis in cattle. However, as in man, vaccination in cattle has several limitations.

1.10 Limitation of cattle vaccines

In many developed countries where bovine leptospirosis is prevalent, access to vaccines is limited due to the cost and lack of knowledge of locally prevalent serovars. The commercially available cattle vaccines are generally prepared from the whole-killed cell so that the immunity is serovar-dependent and unable to confer protection against other pathogenic serovars which could be present in the herd. The majority of commercial cattle vaccines are short-lived and often require a booster to maintain the antibody titres (Balakrishnan and Roy, 2014). Most vaccines do not give full herd protection as minimal cross-protection occurs and some vaccinated animals can still shed the bacteria through urine (Bolin and Alt, 2001; Dib *et al.*, 2014). Consequently, there is a real need for universal, safer and generally longer protective cattle *Leptospira* vaccines to replace the early vaccines.

1.10.1 Bacterin vaccines

Generally, the immune response induced by bacterin (whole-killed) vaccine is serovar specific and humoral mediated, except in bovine species. In cattle, the type of immune reaction confered post-vaccination using a bacterin vaccine (containing serovar Hardjo) is primarily cell-mediated (CMI) (Naiman *et al.*, 2001) and generates good immunity compared to naïve animals. However, the problem with bacterin vaccines is the lack of efficacy spectrum of which it may confer protection against vaccine serovars and their closely related serovars (Levett, 2001; Adler, 2015b). Thus, given the lack of cross-protection between infecting *Leptospira* serovars, a good knowledge of circulating serovars is needed within a region and

formulating multivalent vaccines containing locally prevalent serovars. Additionally, the duration of immunity protection is another issue using these vaccines. Several authors have demonstrated that both monovalent and multi-valent bacterin vaccines only confer short term protection (<12 months) in calves and require additional boosters (Bolin, Zuerner and Trueba, 1989; Bolin and Alt, 2001; Zuerner *et al.*, 2011).

Furthermore, different vaccine formulations can affect the outcome of the immune response. For example, the pentavalent bacterin vaccine containing *L. interrogans* serovar Hardjopritno (LHP) failed to protect cattle infected with *L. interrogans* serovar Hardjobovis (LHB). Theoretically, LHP vaccine should be able to confer protection against LHB as they are serologically identical (Bolin, Zuerner and Trueba, 1989). A subsequent study by Rinehart *et al.* (2012) showed that a monovalent vaccine containing LHP was able to protect cattle challenged with LHB as well as prevention of renal colonisation and urinary shedding. This may be possible that serovars differences and vaccine formulation (such as types of adjuvant) used during preparation will affect the ability of the vaccine to induce a protective immune response.

1.10.2 Live (attenuated) and LPS-based vaccines

The variable immune responses and claimed efficacies of bacterin vaccines are a real issue, and it would be better if alternative vaccines could be identified. There are several reasons for this conclusion. One concern is the quality control of the immunogenicity of the strains. For example, the continuous passage of standard strains which may reduce the immunogenicity of strains (Hartskeerl and Smythe, 2015). Ultimately, other types of vaccines have been proposed as alternatives to bacterin vaccines such as live and LPS-based vaccines. An attenuated leptospiral live vaccine showed protection in animals (Stalheim, 1968) and storage of live vaccine containing serovar Pomona in liquid nitrogen could last up to six months (Stalheim, 1971). However, concerns about safety and method of attenuation were raised. Additionally, limited information on the leptospiral pathogenesis, lack of knowledge of serovars distribution and lack of genetic tools for gene manipulations have hampered the effort to develop such live vaccines; consequently, no live vaccines have been licensed for general use (Murray, 2015; Picardeau, 2015b; Bashiru and Bahaman, 2018).

An LPS-based vaccine was shown to induce protective immunity in animal models (Jost *et al.*, 1986; Schoone *et al.*, 1989); however, it was unsuccessful in cattle, and they are still vulnerable to serovar Hardjo infection (Bolin *et al.,* 1989). As the LPS-derived immunity is exclusively humoral, therefore it is possible that it may not elicit a protective immune

response in cattle via CMI activation. Furthermore, the LPS vaccines are allegedly serovar-specific, as similar to bacterin vaccines, and therefore may not be able to confer protection against heterologous challenge (Wang, Jin and Wegrzyn, 2007; Bashiru and Bahaman, 2018).

1.11 Outer membrane proteins as vaccine candidates

The outer membrane serves a vital role in the interaction of *Leptospira* and host tissues by enabling invasion and subsequent disease. As described earlier, the leptospiral outer membrane is generally similar to that of most spirochetes, composed of two main components which are the OMPs and an additional lipid component (LPS) (Cullen *et al.*, 2002; Kelesidis, 2014). Surfaced-exposed OMP plays an essential role for pathogenesis of *Leptospira* by mediating adherence to the host tissues (Matsunaga *et al.*, 2006) and pathogenic *Leptospira* species have relatively more LPS molecules which are also typically longer than the equivalent molecule in non-pathogenic species, thus making LPS an important putative virulent factor in this species. As previously mentioned, LPS was previously studied as a vaccine candidate. However, it was found that the antibody derived from LPS fractions is serovar-dependant and unable to induce protective immunity against strains from different serovars (Sonrier *et al.,* 2001).

Leptospiral OMPs are highly specialised proteins divided into three components; lipoproteins (attach lipid component on either side of the membrane), transmembrane protein (span across the membrane) and peripheral proteins (Cullen *et al.,* 2004) (Figure 1.7). To date, it is believed that there are more than 300 combined lipoprotein and transmembrane proteins waiting to be discovered and characterised in *Leptospira* (Haake and Matsunaga, 2010). Important examples of these proteins are provided in the following sections.

1.11.1 Lipoprotein OMPs

Some components of lipoproteins were identified as target vaccines candidates due to their ability to be expressed during natural infection and relative structural conservation among pathogenic species. One classic example is lipoprotein-32 kDa (denoted as LipL32), the most abundant lipoprotein on the bacterial surface, restricted to pathogenic *Leptospira* species, and extensively studied as a potential vaccine candidate (Cullen *et al.*, 2005; Vivian *et al.*, 2009; Murray, 2013). LipL32 is one of the dominant OMPs recognised by the immune system during the acute and chronic phase of leptospirosis in man (Guerreiro *et al.*, 2001), indicating that the protein is expressed during infection. As the LipL32 gene is highly conserved, and

the encoding receptor binds various ECM proteins (Hauk *et al.*, 2008; Hoke *et al.*, 2008; Tung *et al.*, 2010), it may play an essential role during pathogenesis and thus be an ideal vaccine candidate. However, the immunogenicity trials of recombinant LipL32 vaccine failed to confer sufficient protection in challenged animals and was only able to limit renal colonisation (Humphryes *et al.*, 2014). Furthermore, mutant LipL32 (by gene deletion) does not have any effect on leptospiral virulence, implying that it is not essential for infection (Murray *et al.*, 2009). Interestingly, when vaccinating with various recombinant LipL32 protein truncations in cattle, these proteins stimulated interferon gamma (IFN-γ) responses, a marker of cell-mediated immune responses (Lucas *et al.*, 2014). However, pentavalent vaccines containing five different pathogenic serovars, which all had LipL32, failed to confer protection against serovar Hardjobovis (Bolin *et al.*, 1989). Therefore, the role of LipL32 in protective immunity cattle is unproven.

Recently, multiple leptospiral lipoproteins (Table 1.6) were expressed and characterised as potential antigens, but no definitive vaccine trials have been reported (Adler, 2015b). Genetic diversity also influences lipoproteins protective capacity. For example, the Leptospiral immunoglobulin-like proteins (Ligs) are the major lipoproteins of the *Leptospira* surface and can induce a strong protective immunity in an animal model (Evangelista *et al.*, 2017). However, comparative genome analysis confirmed that the Ligs (LigA, LigB and LigC) are present in only a few pathogenic species and therefore would be poor vaccine candidates against various pathogenic species (Fouts *et al.*, 2016).

Another important consideration of leptospiral lipoproteins is the location of the proteins within the membrane. Lipoproteins are scattered everywhere within the cellular OM to one or more of four cellular compartments; the periplasmic leaflet of the inner membrane, the periplasmic or external leaflets of the outer membrane, and beyond the outer membrane in the surrounding cell (Haake, 2000; Cullen *et al.,* 2004). Although bioinformatic algorithm predictions such as LipoP (Juncker *et al.*, 2003) and SpLip (Setubal *et al.*, 2006) can identify lipoproteins, they are unable to determine their cellular localisation. Therefore, expressed lipoproteins may not be surface-exposed or involved during *Leptospira*-host interaction. Hence, more characterisation studies are needed to determine their usefulness as promising target antigens.

1.11.2 Transmembrane OMPs

Unlike lipoproteins, leptospiral transmembrane OMPs are integral proteins that span the entire lipid bilayer of the OM. They have significant roles for structural integrity and vital

physiological functions such as nutrient transport into the cell, adherence to host molecules for bacterial colonisation and removal of exogenous products, e.g. toxic waste/bactericidal properties (Patti and Höök, 1994; Pizarro-Cerdá and Cossart, 2006; Raja and Natarajaseenivasan, 2015). Similar to many Gram-negative bacteria, the classical structure of OMPs consists of 8-22 of densely packed antiparallel β-strands (Hong *et al.*, 2006; Misra, 2012). An example of a transmembrane OMP is provided in Figure 1.8. Leptospiral OMPs have been of great interest to many researchers as potential vaccinogens due to their surface-exposed loops that could stimulate host protective immune responses. One major leptospiral OMP is leptospiral 'porin' outer membrane protein (31 kDa), denoted as OmpL1, a transmembrane protein only present in pathogenic *Leptospira* spp*.,* which is thought to be a potential vaccine candidate as it is expressed naturally in mammalian infections (Barnett *et al.*, 1999) and is antigenically conserved among species (Dong *et al.*, 2008; Dezhbord *et al.*, 2014), and can induce cross-protection in heterologous infection (Maneewatch *et al.*, 2007).

OmpL1 binds host molecules such as laminin, fibronectin, fibrinogen and plasminogen (Vieira *et al.*, 2010; Fernandes *et al.*, 2012) which indicates the involvement of the protein in host invasion. Interestingly, while OmpL1 gene is highly conserved (~90% identity), however, its genetic diversity indicated that 20% of serovars possess mosaic OmpL1 genes on the major surface loops derived from horizontal DNA transfer and genetic recombination events (Haake *et al.*, 2004). Despite being well-characterised, its efficacy as a vaccine has never been published, although it was proven useful as a diagnostic antigen in man and animals (Subathra, Senthilkumar and Ramadass, 2013; Hernández-Rodríguez *et al.*, 2014).

A comparative genomic analysis by Nascimento *et al.* (2004) revealed 41% of the predicted *Leptospira* spp. proteins have at least one transmembrane OMP, relatively similar to those found in *Treponema pallidum* and *Borrelia burgdorferi.* This indicates that many more transmembrane OMPs are yet to be discovered. Previous methods of identifying leptospiral OMPs was through subcellular fractionation, detergent-phase partitioning and the isolation of OM vesicles. These methods were able to discriminate between OM from inner membrane lipoproteins, but not the transmembrane OMP (Haake *et al.*, 1991; Zuerner *et al.*, 1991; Haake and Matsunaga, 2002; Nally *et al.*, 2005).

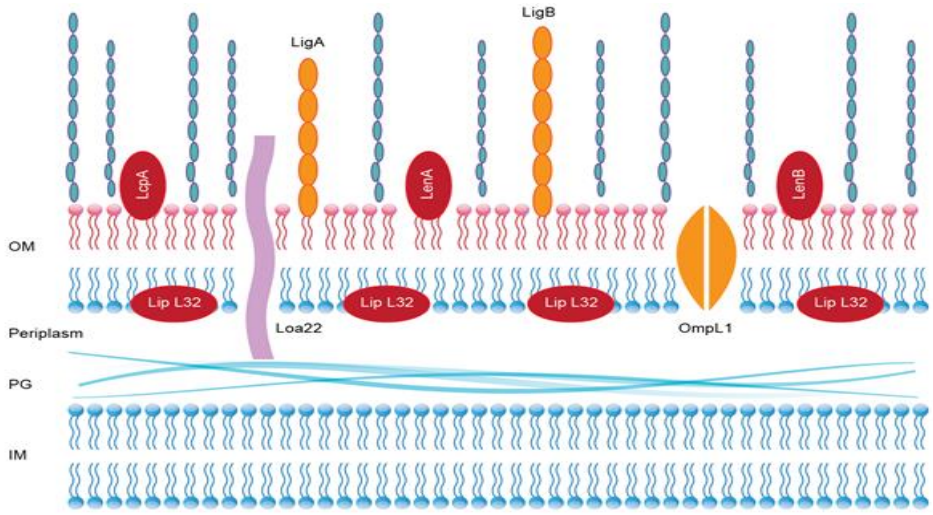


Figure 1.7: Schematic diagram of pathogenic Leptospira membrane architecture.

The inner membrane (IM) is closely associated with the peptidoglycan (PG) cell wall, which is overlaid by the outer membrane (OM). Surface-exposed lipoproteins (LipL32, LigA, LigB and Loa22), the transmembrane outer membrane protein porin (OmpL1), and lipopolysaccharide are among the main components of the outer membrane. Figure adapted and regenerated from (Fraga et al., 2011).

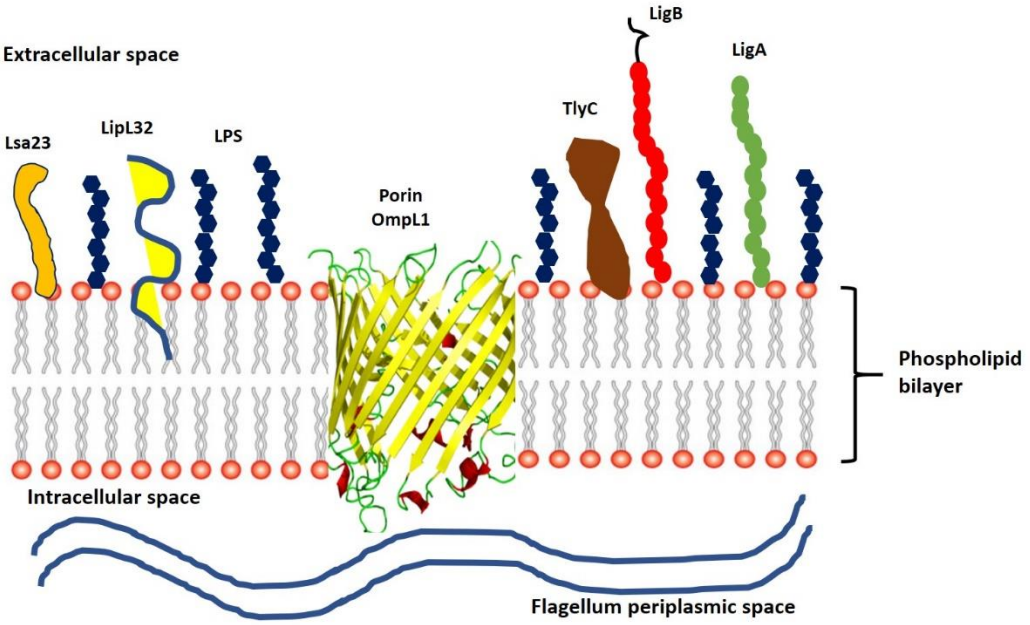


Figure 1.8: An example of leptospiral transmembrane OMP (porin OmpL1) topology expressed on the leptospiral outer membrane with other surface-exposed proteins (Lsa23, LipL32, TylC, LigA and LigB) including LPS. Porin β-strands are arranged in an anti-parallel fashion, resembling a barrel-like shape.

Leptospiral transmembrane OMPs can now be identified by screening a *Leptospira* whole genome by *in silico* analysis. Several transmembrane OMPs were identified (Pinne and Haake, 2009), and their functions subsequently defined. By combining both conventional and modern techniques, to date, several leptospiral transmembrane OMPs have been identified, and their functions are defined. The majority of OMPs were characterised in a single *Leptospira* species that is from a human/rats/dogs reference strain (*L. interrogans* serovar Copenhageni Fiocruz L1-130) (Table 1.6). There is a relative lack of studies investigating OMPs of other pathogenic *Leptospira* species. One species of interest, *L. borgpetersenii* serovar Hardjobovis is an important pathogen in cattle which shares about ~2708 genes with *L. interrogans,* but has more than 300 unique genes (Picardeau *et al.*, 2008; Adler *et al.*, 2011). On this basis, the cattle *Leptospira* strains (*L. borgpetersenii* serovar Hardjobovis L550 and JB197) were selected to screen for potential transmembrane OMPs, which will be further characterised for their novel functions. The identification of OMPs and their expressions will be carried on in line with reverse vaccinology methodology, using a genome-based approach which will be described in the following section.

Table 1.6: Characterised OMPs from L. interrogans serovar Copenhageni L1-130 with their functions.

Leptospiral OMP	Size (kDa)	Knockout virulence[a]	Putative function[b]	Other related information	References
Lipoprotein OM					
LenA (LfhA/Lsa24)	30.0	N/D	Binds to factor H (and its related proteins), fibronectin, plasminogen and laminin	Membrane-bound protein, may have functional redundancy	(Verma *et al.*, 2006, 2010)
LipL21	21.0	N/D	Inhibit neutrophil myeloperoxidase	Surface-exposed, elicit immune protection in guinea pigs against LipL21 DNA vaccine	(Cullen *et al.*, 2003; He *et al.*, 2008; Vieira *et al.*, 2018)
LipL32 (Hap 1)	32.0	Yes	Bind to laminin, collagen I, IV, plasminogen	Surface-exposed, not required during infection	(Hauk *et al.*, 2008; Hoke *et al.*, 2008; Murray *et al.*, 2009; Vieira *et al.*, 2010)
LipL36	36.0	N/D	-	Not surface-exposed, located at the inner membrane of OM	(Haake *et al.*, 1998)
LipL41	41.0	Yes	Binds to hemin	Surface-exposed, requires small chaperone protein for stable expression and exhibit synergistic immunoprotection with OmpL1	(Haake *et al.*, 1999; Asuthkar *et al.*, 2007; King *et al.*, 2013)
LipL46	46.0	N/D	-	Surface-exposed OMP	(Matsunaga *et al.*, 2006)
Ligs (A and B)	130-200	Yes	Binds to fibronectin, fibrinogen, collagen I, collagen IV and calcium	Surface-exposed, only present in several pathogenic *Leptospira* species	(Choy *et al.*, 2007; McBride *et al.*, 2009; Lucas *et al.*, 2011)
Loa22	22.0	Yes	Binds to peptidoglycan	Surface-exposed, confer partial immunoprotection in animal models	(Ristow *et al.*, 2007; Zhang *et al.*, 2010; Wu *et al.*, 2011)
Transmembrane OM					
BamA	113	N/D	OMP biogenesis	Involved in the folding, assembly and insertion of transmembrane OMPs in the OM	(Tommassen, 2007; Haake and Zückert, 2015)

CirA	92.0	N/D	Import channel	Siderophore receptor for iron uptake	(Haake and Zückert, 2015)
FecA	93.0	Yes	Import channel	TonB DR for ferric iron dicitrate	(Haake and Zückert, 2015)
FadL	52.0	N/D	Import channel	Fatty acid transporter	(Haake and Zückert, 2015)
GspD	113	N/D	Export channel	Type 2 secretion: Sec-dependent	(Haake and Zückert, 2015)
HbpA	80.0	Yes	TonB DR for hemin	Iron uptake	(Oke *et al.*, 2004; Marcsisin *et al.*, 2013)
OmpA (Lsa66)	68.0	N/D	Binds to laminin and plasma fibronectin	Partial inhibition on leptospiral adherence to immobilised extracellular matrix and plasminogen	(Oliveira *et al.*, 2011)
OmpL1	33.0	N/D	Binds to laminin, fibrinogen, fibronectin	Porin, immunoprotective antigen, recognised by host immune system	(Haake *et al.*, 1993; Shang *et al.*, 1995; Haake *et al.*, 1999; Fernandes *et al.*, 2012)
OmpL36	36.0	N/D	-	Recognised by host immune system	(Eshghi *et al.*, 2009; Pinne and Haake, 2009)
OmpL37	37.0	N/D	Binds to human elastin	Present in pathogenic species, recognised by host immune system	(Pinne and Haake, 2009; Pinne *et al.,* 2010)
OmpL47	47.0	Yes	Binds to Collagen III, IV, laminin fibronectin	Not recognised by host immune system	(Pinne and Haake, 2009; Pinne *et al.,* 2010)
OmpL54	54.0	N/D	-	-	(Pinne and Haake, 2009)
OstA	113	Yes	LPS assembly and transport	Translocating LPS to OM	(Sampson, Misra and Benson, 1989; Ruiz *et al.,* 2009; Sperandeo *et al.,* 2009)
TolC	60.0	N/D	Export channel	Type 1 secretion: Sec-independent	(Haake and Zückert, 2015)
TylC	50.4	N/D	Binds to fibronectin, laminin and collagen IV	Haemolysin-like protein but does not present haemolysin activity	(Carvalho *et al.*, 2009)

[a] *The knockout virulence to assess of OMP virulence in the host. Abbreviation: N/D; Not determined*

[b] *The primary function of an OMP. Abbreviation: TonB DR; TonB-dependent receptor*

1.12 Application of reverse vaccinology

Conventional vaccines have been developed over four centuries following the discovery of a small-pox vaccine, which was pioneered by Edward Jenner in the late 18th century (Plotkin, 2009). These vaccines were generally formulated using two methods; by attenuation of pathogens by continuous passage in the laboratory and secondly by the identification and purification of protective antigens to be used as potential vaccine candidates (Heinson, *et al.,* 2015). These approaches, however, are only useful for cultivable microorganisms, are time-consuming and only allow identification of the most abundant antigens to be purified in larger quantities for vaccine testing (Mora *et al.*, 2003). Furthermore, antigens that are not expressed *in vitro* cannot be identified (Rappuoli, 2000) and therefore, cannot be subjected to further characterisation. Although successful in many cases, conventional approaches take a long time to yield vaccines and are unable to provide a solution for those pathogens that do not have prominent immunodominant protective antigens (Rappuoli, 2000).

The arrival of the genomic era in the 21st century has led to substantial discoveries in both human and animal medicine. Ever since the first complete bacterial genome was sequenced, a barrage of emerging technologies has led to the ever more rapid sequencing of entire prokaryote genomes which has now become standard practice (Fleischmann *et al.*, 1995; Bambini and Rappuoli, 2009). The availability of the genome sequences of many pathogens allows discovery of more novel antigens that could be alternative vaccinogens to improve the present vaccines. The information present in the genome could be considered as a starting point for the identification of potential protein antigens. This approach, known as 'Reverse Vaccinology' (RV) utilises the genome sequence of the pathogen and identifies those antigens that are most likely to be candidates (Rappuoli, 2000). These antigens are identified by *in silico* analysis by screening the entire bacterial genome using various bioinformatics tools, and the selected candidates then undergo cloning and screening for expression in a heterologous system. After the purification of the recombinant proteins, the purified proteins are subjected to immunogenicity testing in an animal model to confirm the surface localisation and to analyse their ability to elicit an immune response (Mora *et al.*, 2003; Heinson *et al.,* 2015).

Moreover, the application of RV is not just to discover novel antigens, but this will lead to better understanding of pathogenesis on how these pathogens cause diseases to the host (Delany, Rappuoli and Seib, 2013). The first successful RV project targeted the serogroup B meningococcus (MenB) of *Neisseria meningitidis* (Pizza *et al.,* 2000). RV identified three main

antigens of MenB that are involved in meningococcal virulence and subsequently formulated the world's first subunit 4CMenB vaccine (Bexero) from these proteins (Heinson *et al.,* 2015. Since then, reverse vaccinology have been applied to a wide range of human and animal diseases, ranging from bacterial pathogens (Song *et al.*, 2009; Delany *et al.,* 2013), viruses (Bruno *et al.*, 2015) and to a lesser extent, parasitic pathogens (Lew-Tabor and Rodriguez Valle, 2016; Goodswen *et al.,* 2017).

Ever since the MenB vaccine was successfully developed and commercialised , RV was quickly adapted in vaccinology studies against many bacterial diseases, including spirochetal diseases such as bovine digital dermatitis (caused by *Treponema* spp.) (Staton, 2018), Lyme disease (Borreliosis) (Small *et al.*, 2016), and swine dysentery (caused by *Brachyspira hyodysenteriae*) (Song *et al.*, 2009). A similar approach was followed in several leptospirosis studies, although most of these studies only used *in silico* analysis (Maneewatch *et al.*, 2007; Pinne and Haake, 2009) to identify a small group of proteins rather than complete RV process. Some of these protein antigens showed significant protection in an experimental animal model, which reflect the possibility of protecting the host in a heterologous challenge. This shows that RV is one of the promising approaches to evaluate these candidates for future vaccine development. Furthermore, the lack of understanding on how identified antigens cause infection in the host triggered our interest to characterise their potential functions and to study their interaction with the host immune system to further comprehend their complex pathogenesis.

The availability of genome sequences for many of leptospiral strains on public databases provides an opportunity for the discovery of novel proteins with unknown functions. Despite the advancement in algorithms for assigning functions in unknown protein annotations, there is still a long list of leptospiral proteins with potential roles in pathogenicity and host adaptations that have not been previously studied and characterised (Dellagostin *et al.*, 2017). Therefore, RV is a promising approach to discover potential leptospiral antigens to study their functional and immunological properties to further understanding host-pathogen interaction and to be translated into competent vaccine candidates.

1.13 Aim and objectives

In this study, we aimed to identify novel putative surface-exposed outer membrane proteins from bovine leptospiral strains not previously identified using RV approach and to characterise these proteins function to further understand the interaction of these proteins with host molecules. Such studies should provide evidence towards their use in the development of vaccines and future diagnostic strategy. Furthermore, we also aimed to investigate the infection reservoirs and population biology of both pathogenic and non-pathogenic leptospires within dairy cattle and their environment in the United Kingdom farms. The objectives of this study are divided into five main themes;

(A) To identify novel leptospiral surface-exposed proteins from bovine leptospiral reference strains using a RV approach

- Analyse the entire *Leptospira borgpetersenii* serovar Hardjobovis L550 and JB197 genome for transmembrane OMPs using various bioinformatics prediction programs.
- Select unique L550/JB197 OMP encoding genes and compare with *Leptospira interrogans* orthologues.

(B) To clone, express and purify relevant surface-exposed leptospiral OMP in a heterologous expression system

- Clone and express selected OMP genes using *Escherichia coli* expression system as recombinant inclusion bodies.
- Isolate and solubilise the inclusion bodies from *Escherichia coli*.
- Refold and purify the recombinant proteins and determine their secondary structure through circular dichroism.

(C) To characterise the function of novel bovine leptospiral OMPs by assessing binding affinity to host molecules and the host immune response

- Analyse binding ability of each recombinant OMP to various host (ligand) molecules.
- Analyse the antibody titre association against recombinant leptospiral OMPs in cattle bulk milk.

(D) To investigate functional diversity of leptospiral OMP variants across pathogenic species

- Express and purify the selected OMP variants as recombinant proteins.
- Analyse binding ability of OMP variants to various host ligand.
- Evaluate the host immune response towards recombinant OMP variants using leptospires exposed cattle sera.

(E) To characterise *Leptospira* diversity in ruminant samples in United Kingdom farms through clinical/molecular detection.

- Detect leptospiral DNA in gastrointestinal samples (rectal and gingiva tissues) of ruminants through PCR detection assay.
- Identify the *Leptospira* species corresponding to any positive PCR samples and assess whether novel carriage sites have been identified.

Chapter 2: Materials and methods

This chapter describes the general materials and methods used throughout this thesis.

2.1 Bacterial strains

2.1.1 *Leptospira* spp. strains

The following *Leptospira* strains were used for cultivation, cloning and sequence of the primer design. All strains were obtained from Leptospirosis Reference Centre, Academic Medical Centre (AMC), Department of Medical Microbiology, the University of Amsterdam, Netherlands.

Table 2.1: List of Leptospira strains used in this thesis.

Code	Species	Serovar	Serogroup	Strain
KIT0215	*L. interrogans*	Copenhageni	Icterohaemorrhagiae	Fiocruz L1-130
KIT0243	*L. borgpetersenii*	Hardjobovis	Sejroe	JB197
KIT0242	*L. brogpetersenii*	Hardjobovis	Sejroe	L550
KIT0164	*L. biflexa*	Patoc	Semaranga	Patoc I

2.1.2 *Escherichia coli* strains

The following chemically competent *Escherichia coli* strains were used for cloning, transformation and expression in this project. All strains were obtained from Invitrogen™, Carlsbad, California, USA. Their genotypes are given below.

Table 2.2: List of E. coli strains used for cloning, transformation and expression in this thesis.

Strains	Purpose	Genotypes
One Shot™ TOP10	Cloning and transformation	*F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*
BL21-AI™	Large scale expression	*F- ompT hsdSB (rB-mB-) gal dcm araB: T7RNAPtetA*
One Shot™ BL21(DE3) pLysS	Large scale expression	*F- ompT hsdSB (rB-mB-) gal dcm (DE3) pLysS (CamR)*

2.2 Media and supplements for bacterial cultivation

Liquid medium was used to culture and to maintain leptospires weekly, while solid and liquid media were used to grow *E. coli* strains for cloning and expression purposes. Both media were supplemented with necessary chemical reagents to enhance bacterial growth. The list of media and supplements used throughout this thesis are listed in Table 2.3, along with their method of preparation.

Table 2.3: Media and supplement used for both leptospires and E. coli cultures.

Media/Supplement	Preparation
Ampicillin (10 mg/ml)	0.1 g of Ampicillin sodium salt (Sigma-Aldrich, Dorset, UK) was dissolved into distilled water and filter sterilised using 0.22 µm pore syringe filter (Appleton woods, Birmingham, UK) and stored at 4°C for up to one week. Ampicillin stock solution was freshly prepared before LB broth preparation, and LB solid media with ampicillin addition was kept at 4°C for up to one week.
Fluorouracil (5-FU) (10 mg/ml)	0.1 g of 5-FU was dissolved in 10 ml of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Dorset, UK), filter sterilised using a 0.22 µm pore syringe filter and stored in 500 µl aliquots at -20°C up to four months.
Growth medium for *E. coli* strains	For liquid and solid media: Lauria Broth Base (Miller's LB Broth Base)™ (Invitrogen™, Carlsbad, California, USA) was prepared according to the manufacturer's instruction (Final pH 7.5 ± 2). Formulation per one litre: Bacto tryptone 10 g Bacto yeast extract 5 g Sodium Chloride (NaCL) 10 g

	Media were prepared using distilled water and sterile by autoclaving at 121°C (15 psi) for 20 minutes. For solid media, 16 g/litre of Select Agar™ (Invitrogen™, Carlsbad, California, USA) was added. Molten agar was cooled at 55°C before antibiotic addition. ~25 ml of molten agar was plated in a sterile 90 mm triple vent petri dish (Appleton woods, Birmingham, UK). Media preparation was carried out in a laminar flow under standard aseptic conditions.
Liquid medium for Leptospiral strains (Ellinghausen, McCullough, Johnson and Harris- EMJH) (Johnson and Harris, 1967; Wuthiekanun *et al.*, 2014)	For EMJH medium base preparation: 2.3 g of Difco™ Leptospira Medium Base EMJH (Becton-Dickinson, Detroit, USA) was dissolved in 900 mL of distilled water and sterile autoclaved at 121°C (15 psi) for 15 minutes. The EMJH medium base solution was kept in room temperature, and 4.5 ml of medium was aliquoted into sterile 15 ml culture tubes and kept at 4°C prior cultivation (final pH 7.5 ± 2). Approximate formula per litre: Disodium Phosphate 1.0 g Monopotassium Phosphate 0.3 g Sodium Chloride 0.25 g Ammonium Chloride 0.005 g Thiamine 0.005 g For *Leptospira* culture: 10% (v/v) of Difco™ Leptospira Enrichment EMJH (Becton-Dickinson, Detroit, USA) containing a solution of albumin, polysorbate 80 and additional growth factors for *Leptospira* was added into EMJH liquid medium base prior *Leptospira* inoculation. Media preparation was carried out in a laminar flow under standard aseptic conditions.
Sodium pyruvate (10 mg/ml)	0.1 g of sodium pyruvate (Sigma-Aldrich, Dorset, UK) was dissolved into 10 ml distilled water, filter sterilised using a 0.22 µm pore syringe filter and stored in 500 µl aliquots at -20°C.

2.3 Buffer and reagents

Standard buffers and reagents used throughout the project are listed in Table 2.4, along with their preparation.

Table 2.4: Buffers and reagents used in various studies in this thesis.

Buffer/reagents	Preparation
Ampicillin (10 mg/ml)	See details in Table 2.3
Acrylamide solution 30% (w/w)	A 30% (w/w) Acrylamide solution was obtained from Severn Biotech Limited, Kidderminster, United Kingdom.
Agarose gel 1% (w/v)	1.0 g of agarose powder (Bio-Rad, Hemel Hempstead, UK) was dissolved in 100 ml of 1X TAE buffer by heating and allowed to set. Agarose gel preparation was carried out in a fume chamber.
Ammonium persulphate 10% (w/v) (APS)	1.0 g of APS (Sigma-Aldrich, Dorset, UK) was dissolved in 10 ml of distilled water and stored at 4°C. APS stock was replenished every 2-3 weeks as APS will decay slowly in the solution.
Chelex-100 resin 5% (w/v)	5.0 g of Chelex-resin (Bio-Rad, Hemel Hempstead, UK) was dissolved in 10 ml distilled water and stored in at 4°C.
Cell lysozyme buffer 0.0025% (w/v)	0.05 g of chicken lysozyme powder was mixed with 2.5 ml of Tris-HCl (0.5M, pH 7.9) buffer and distilled water was added up to 20 ml and stored at 4°C.
Colour prestained protein marker	Colour prestained protein standard (0.2 mg/ml), broad range 11-245 kDa were obtained from NEB, Hertfordshire, UK.
Clonase II enzyme	Clonase II enzyme mix (20 reactions) was obtained from Invitrogen™, Carlsbad, California, USA.
dNTPs	5.0 mM of dATP, dTTP, dCTP and dGTP (in 20 mM stock) were obtained from Thermo Fisher Scientific (Hemel Hempstead, UK) and stored in 100 µl aliquots at -20°C.
Dialysis buffer	Protein dialysis buffer preparation (1 litre); 50 ml of 5.0 M NaCl 40 ml of 0.5 M Tris HCL pH 7.9 (see details) 3.3 ml 0.01% (v/v) of LDAO (see details) Distilled water up to 1 litre

	Protein dialysis buffer preparation (6 litre); 86.7 g NaCl (250 mM) 300 ml of 0.5 M Tris HCL pH 7.9 (see details) 20 ml of 0.1% (v/v) of LDAO (see details) Distilled water up to 6 litre These buffers were prepared fresh prior use.
Dithiothreitol (DTT)	1 M DTT (Sigma-Aldrich, Dorset, UK) was prepared by dissolving 3.09 g of DTT in 20 ml distilled water, and filter sterilised using 0.22 µm pore syringe filter and stored at -20°C in 1.0 ml aliquots.
Ethidium Bromide (EtBr)	10 mg/ml of EtBr in ethanol was obtained from Bio-Rad, Hemel Hempstead, UK.
Glycerol	10 ml aliquots of glycerol (BDH, Dorset, UK) was sterilised by autoclaving.
Imidazole stock solution (4M)	17 g of imidazole powder (Sigma-Aldrich, Dorset, UK) was dissolved in 50 ml distilled water.
Isopropyl β-D-1-thiogalactopyranoside (IPTG) 100 mM	100 mM of IPTG (Sigma-Aldrich, Dorset, UK) was prepared by dissolving 0.238 g of IPTG in 10 ml distilled water and filter sterilised using 0.22 µm pore syringe filter and stored at -20°C.
Isobutanol	Isobutanol (2-propanol) was obtained from Biorad, Hemel Hempstead, UK.
Kanamycin (10 mg/ml)	Kanamycin sulphate solution (10 mg/ml) was obtained from Invitrogen™, Carlsbad, California, USA.
Skimmed milk 2% (w/v)	5.0 g of dried skimmed milk (Marvel, Chivers, Dublin, Republic of Ireland) mixed in 100 PBST and freshly prepared before plate coating.
L-arabinose 0.1-0.2% (w/v)	1.0-2.0 g of L-arabinose (Sigma-Aldrich, Dorset, UK) was dissolved in 10 ml distilled water to give a concentration of 0.1 -0.2% (w/v) respectively, and filter sterilised using 0.22 µm pore syringe filter and stored at room temperature.
N, N-Dimethyldodecylamine N-oxide (LDAO) 33% (v/v)	LDAO solution was obtained from (Sigma-Aldrich, Dorset, UK) and concentration provided at 33% (v/v).

Nickel agarose beads (Ni-NTA)	Ni-NTA agarose beads (50% slurry in 30% ethanol) were purchased from Thermo Fisher Scientific, Hemel Hempstead, UK.
PageBlue protein staining solution	PageBlue™ Protein staining solution was obtained from Thermo Fisher Scientific, Hemel Hempstead, UK.
Phosphate buffered saline with Tween® 20 (PBST) pH 7.4	Preparation of phosphate buffered saline (PBS) per litre: Five tablets (200 mg/ml) of PBS (Sigma-Aldrich, Dorset, UK) was dissolved in 1.0 litre of distilled water and autoclaved at 121°C (15 psi) for 20 minutes. PBS was stored at room temperature. Preparation of 0.05% (v/v) PBST per litre: 500 µl of Tween® 20 (Sigma-Aldrich, Dorset, UK) solution was dissolved in 1.0 litre of prepared PBS and stored at room temperature.
Protein blank solution	Protein blank preparation (10 ml) 5.0 ml of 5 M NaCl 4.0 ml of 0.5 M Tris HCL pH 7.9 (see details) 33 µl 0.01% (v/v) of LDAO (see details) Distilled water up to 10 ml
Proteinase K solution 20 mg/ml	Proteinase K solution containing 50 mM Tris, pH 8.3 mM $CaCl_2$, 50% (v/v) Glycerol (stock of 20 mg/ml) was obtained from Invitrogen™, Carlsbad, California, USA.
Protein standard molecular-weight maker	SigmaMarker™, wide range 6.5-200 kDa (Sigma-Aldrich, Dorset, UK) was dissolved in distilled water and stored at -20°C in 10 µl aliquots.
Protein solubilisation buffer	Recipe of protein solubilisation buffer (50 ml): 28.5 g Guanidine HCL (Sigma-Aldrich, Dorset, UK) 2.5 ml Tris HCl (0.5 M, pH 7.9) 100 µl EDTA (Sigma-Aldrich, Dorset, UK) Distilled water up to 50 ml
Ponceau S, acid red	Ponceau S solution 0.1% (v/v) was obtained from Sigma-Aldrich, Dorset, UK. The solution was stored at room temperature.
Refolding buffer	Protein refolding buffer preparation (250 ml) 25 ml of 0.5 M Tris HCL pH 7.9 (50 mM) 12.5 ml 5.0 M NaCl (250 mM)

	41.7 ml LDAO 5.0% (v/v) Distilled water up to 250 ml
Sample buffer (Loading buffer)	Preparation of 1X sample buffer (10 ml): 50 mM Tris HCL pH 6.8 (see details below) SDS 2% (v/v) (see details below) Glycerol 10% (v/v) 50 mM DTT Bromophenol blue 0.02% (v/w) Distilled water up to 10 ml Preparation of 5X sample buffer (10 mL): 250 mM Tris HCL pH 6.8 (see details below) SDS 10% (v/v) (see details below) Glycerol 50% (v/v) 50 Mm DTT Bromophenol blue 0.25% (v/w) Distilled water up to 10 ml Sample buffer was prepared fresh due to DTT degradation after one week and stored at room temperature.
Super optimal broth (S.O.C) medium	S.O.C medium containing 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose was obtained from Invitrogen™, Carlsbad, California, USA.
Sodium chloride (NaCl) 5 M solution	146.1 g of NaCl (Sigma-Aldrich, Dorset, UK) was dissolved in 500 ml of distilled water and stored in room temperature.
Sodium dodecyl sulfate (SDS) 10% (w/v) stock solution	10 g of SDS (Sigma-Aldrich, Dorset, UK) was dissolved in 80 ml of distilled water and heated for few seconds and added more distilled water up to 100 ml. SDS solution was stored at room temperature and dissolved by heating prior usage.
Stopping solution	25 ml of concentrated hydrochloric acid (analytical grade (36.46 g/mol) Sigma-Aldrich, Dorset, UK) was added to 475 ml distilled water and stored at room temperature.
Tergitol solution 4% (v/v)	Tergitol solution was obtained from Sigma-Aldrich, Dorset, UK.

Tris-acetate-EDTA- TAE (1X) electrophoresis buffer	100 ml of TAE (40X) molecular grade (Sigma-Aldrich, Dorset, UK) was added to 3900 ml of distilled water to give a working solution of 1X TAE. TAE buffer was continuously used until the change of colour is noticed.
Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) resolving gel 12% (v/v)	Preparation of SDS-PAGE resolving gel (10 ml): 3.3 ml distilled water 4.0 ml 30% (w/w) acrylamide mix 2.5 ml Tris HCL (1.5 M, pH 8.8) 100 µl SDS 10% (v/w) 100 µl APS 10% (v/w) 4.0 µl TEMED ~99% (v/v) The gel solution was mixed thoroughly and pipetted carefully into the 2/3 Mini- Protean® (Bio-Rad, Hemel Hempstead, UK) SDS-PAGE casting plates. 100 µl of absolute 2-propanol was added onto the remaining 1/4 space to remove air bubbles. After resolving gels were set, the excess 2-propanol was rinsed off using distilled water. SDS-PAGE resolving gels were kept at 4°C up to one week. Distilled water was used to prevent dehydration of the gels.
Tris-glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS page) stacking gel 4% (v/v)	Preparation of SDS-PAGE stacking gel (4 ml) 2.7 ml distilled water 670 µl 30% acrylamide mix 500 µl Tris-HCL (1.5 M, pH 8.8) 40 µl SDS 10% (v/w) 40 µl APS 10% (v/w) 4.0 µl TEMED ~99% (v/v) The gel solution was mixed thoroughly and pipetted carefully on the top of the set resolving gels. 10 or 15 Mini- Protean® combs (Bio-Rad, Hemel Hempstead, UK) was used to make the wells. After stacking gels were properly set, the combs were removed slowly, and excess gel solution was rinsed off using distilled water. SDS-PAGE stacking gels were kept at 4°C up to one week. Distilled water was used to prevent dehydration of the gels.
Tetramethylethylenediamine (TEMED)	TEMED (electrophoresis grade) was obtained from Sigma-Aldrich, Dorset, UK and stored at 4°C. The concentration provided is ~99% (v/v).

Transfer buffer	Transfer buffer recipe (1 litre): 3.03 g Trizma base (Sigma-Aldrich, Dorset, UK) 14.4 g Glycine (Sigma-Aldrich, Dorset, UK) 200 ml Methanol (analytical grade) (Thermo Scientific, Hemel Hempstead, UK) Distilled water up to 1.0 litre The transfer buffer solution was kept refrigerated at 4°C prior use.
Tris HCL (1.0 M, pH 6.8), (1.5 M, pH 8.8) and (0.5 M pH 7.9)	To prepare a 1 M solution, 121 g of Tris base was dissolved in 800 ml of distilled water. The pH was adjusted to the desired value by adding concentrated HCl. The volume of the solution was adjusted to 1 or 2 litre with distilled water and stored at room temperature.
Tris-glycine electrophoresis SDS running buffer (1X)	1X SDS running buffer recipe (500 ml): 1.51 g of Trizma base 9.4 g Glycine 5.0 ml of SDS 10% (w/v) Distilled water up to 500 ml. 1x running buffer was stored at room temperature.

2.4 Vectors selection for cloning and transformation

The following vectors are selected for cloning and transformation of *Leptospira* genes of interest into *E. coli* TOP10 strain.

Table 2.5: List of vectors used throughout this thesis.

Vectors	Purpose	Bacterial resistance	Fusion tag	Size (kb)
pENTR (Invitrogen™)	Entry vector (cloning)	Kanamycin	-	2580
pDEST17 (Invitrogen™)	Destination vector (transformation)	Ampicillin	N-terminal	6354
pET_21a+ (EMD Bioscience)	Destination vector (transformation)	Ampicillin	N-terminal	5443

2.5 Bacterial culture

2.5.1 *Leptospira* spp. culture

Leptospira culture method and maintenance of strains were applied according to a standard laboratory protocol for both pathogenic and saprophytic leptospires (Johnson and Harris, 1967; Zuerner, 2005) with a slight modification.

Pathogenic *Leptospira* cultures (*L. borgpetersenii* serovar Hardjobovis strain L550 and JB197, and *L. interrrogans* serovar Copenhageni strain Fiocruz L1-130) from a reference lab were provided in 500 µl semi-solid EMJH media. From the stock cultures, a stab culture was performed by perforating a sterile plastic loop into the stock culture semi-solid medium and was slightly twisted to collect some medium on the loop. The loopful medium was then transferred to a fresh 4.5 ml of EMJH liquid medium containing EMJH base medium and 10% (v/v) of Difco™ *Leptospira* Enrichment EMJH supplemented with 45 µl of sodium pyruvate solution for bacterial enhancement (Johnson *et al.*, 1973; Wuthiekanun *et al.*, 2013). The primary *Leptospira* cultures were incubated at 30°C for 2-3 weeks and weekly checked under a phase-contrast microscope (Leitz Diaplan, Leica Microsystems, Wetzlar, Germany) for observation of growth. In the other hand, a saprophytic *Leptospira* strain (*Leptospira biflexa* serovar Patoc 1) which originally arrived in liquid EMJH stock culture was cultured using a similar manner as described above. All procedures were carried out in a category 2 biosafety cabinet (Thermo Fisher Scientific, Horsham, UK) under aseptic condition.

2.5.2 *Escherichia coli* culture

All *E. coli* strains were grown on solid LB media supplemented with kanamycin (50 µg/ml) and ampicillin (100 µg/ml) for cloning and transformation purposes, respectively. For the growth of *E. coli* in LB liquid cultures, Erlenmeyer flasks were incubated in an orbital shaking

incubator at 225 rpm. For working cultures of *E. coli,* plates were sealed with parafilm to prevent evaporation and stored at 4°C. For maintenance of *E. coli,* strains were grown at 30°C overnight in 5.0 ml LB liquid supplemented with described antibiotics, and 100 µl of the culture of each strain was inoculated in 10% (v/v) sterile glycerol aliquots and stored at -80°C.

2.5.3 Leptospires observation under a phase-contrast microscope

To observe leptospiral growth, two drops of EMJH liquid medium were put on a clean microscope glass slide (thickness 0.95 -1.05 mm) (VWR, Leicestershire, UK) and covered with 22 mm coverslip (VWR, Leicestershire, UK). The slide was viewed under a phase-contrast microscope on the area of a higher power at x100 magnification with focus adjustment. Leptospiral growth were score based on an arbitrary scale; 0 = no growth; ++++ = growth (Vogel, 1961). The score given was in accordance with Table 2.6 below;

Table 2.6: An arbitrary scale used to describe leptospires growth under the phase-contrast microscope at 100x magnification.

Growth assessment	Arbitrary scale
No growth	0
Presence of 1 leptospire per view	+
Presence of leptospires covering 25% per view	++
Presence of leptospires covering 50% per view	+++
Presence of Leptospires covering 75% or more per view	++++

2.6 Maintenance and storage of *Leptospira*

The maintenance of both pathogenic and saprophytic *Leptospira* strains was maintained on EMJH liquid cultures weekly as previously described. When contamination was detected, 5-FU antibiotic (50-250 µg/ml) was added into the medium to minimise bacterial contamination. 5-FU, which is an analogue of uracil, was selected as an ideal antimicrobial agent, which can be used as a standalone compound or combination with other antimicrobials in many of past leptospiral isolation and growth studies (Johnson and Rogers, 1964; Chakraborty *et al.*, 2011; Saito *et al.*, 2013). Although all leptospires strains can 5-FU it indirectly inhibits contaminants growth, this may also suppress leptospires growth. Therefore 5-FU dosage was adjusted from 50-250 µg/ml depending on the growth assessment as shown in Table 2.6.

For storage, 1.0 ml of the cultures from the EMJH liquid medium was inoculated and mixed thoroughly with 10% (v/v) sterile glycerol stock and were stored at -80°C. Glycerol is a cryoprotective agent which is able to protect cells in the phase of freezing and thawing, and 10% (v/v) stock was thought to be an ideal concentration for the leptospires recovery after thawing (Alexander *et al.*, 1972). Leptospires viability in glycerol stock was assessed monthly by re-inoculating 500 µl of the leptospires glycerol stock into a fresh EMJH liquid medium and incubate at 30°C for 14-28 days. Additionally, some of the *Leptospira* glycerol stocks were kept in liquid nitrogen freezing for long-term preservation (Rossetti and Auteri, 2008).

2.7 *Leptospira* DNA extraction

DNA extraction from bacterial culture was performed according to a protocol by de Lamballerie *et al.* (1992), which was adapted from the extraction of human DNA from clinical samples for PCR amplification with some modification (Walsh, Metzger and Higuchi, 1991).

0.5 g of Chelex® 100 resin was weighed and suspended in 10 ml distilled water and vortexed for 10 seconds to make 0.05% (w/v) stock solution. 250 µl of Chelex® 100 solution were pipetted carefully to mix the resins and transferred to 1.5 ml locked Eppendorf microcentrifuge tubes (Starlab, Milton Keynes, UK). 250 µl of fresh leptospires of each strain were mixed with the solution by pipetting up and down. The mixed solution was then boiled in hot water for 10 minutes. Chelex® 100 works by preventing DNA nucleases from degrading DNA/RNA, which was subsequently released after cell rupture during boiling (Walsh, Metzger and Higuchi, 1991). After boiling, the mixed solution was centrifuged at 13,000 *g* using a benchtop microcentrifuge (Prism™, Labnet, USA) for 10 minutes. The centrifugation force will separate the beads that are bound to metal ions and other potential impurities and retain pure DNA in the supernatant solution. A supernatant solution containing pure *Leptospira* DNA was carefully pipetted and transferred to 1.5 ml microcentrifuge tubes whilst residual Chelex® 100 resin was discarded. Leptospiral genomic DNA solutions were stored at -20°C for downstream application.

2.8 Designing PCR primers for cloning

For selected OMP genes (Chapter 3), the forward and reverse primers were designed for PCR prior entry cloning. All primers were designed (using PCR Primer Design Tool, an online software by Eurofins Genomic, Germany) containing the following characteristics; 1) an overhang sequence (CACC) at the beginning (5') of a forward primer. 2) a sequence contains

18-22 nucleotides in length. 3) preferably not contain more than 4 or more of one base or dinucleotides repeats in a sequence. 4) should have guanine-cytosine (GC) content between 40-55%. 5) preferably contain GC clamp in the last five bases at the 3' end of the primer and 6) Average melting temperature (T_m °C) should be around 50°C and 65°C (Lorenz, 2012). All primers were synthesised by Eurofins Genomic. The primers for each of OMP gene is shown in Table 4.1.

The PCR forward primer was designed by inserting the sequence, CACC, at the end 5' end of the primer to enable directional cloning. These nucleotides will be paired with the overhang sequence, GTGG, which is present in the entry vector. The PCR reverse primer was based on reverse complement 3' sequence end from each gene. The list of primers can be found in Table 2.7. All primers were synthesised commercially by Eurofins Genomic (Ebersberg, Germany) and provided in lyophilised form. Primers were re-suspended with nuclease-free water to make a stock concentration of 100 pmol, diluted to 1:100 and kept in 50 µl aliquots in -20°C for downstream applications.

Table 2.7: List of designed primers used in this study.

Gene	Primers sequence (Forward/Reverse)	Size (kb)	Melting temperature Tm (°C)	GC content (%)
LBL_2618	F: 5' **cacc**GAAAGGATCAGTATCGATGC 3'	1.35	F: 55.2	F: 50.0
			R: 54.5	R: 47.4
	R: 3' TCAGAGATCATCACTGACG 5'			
LBL_2510	F: 5' **cacc**AAATCATACGCAATTGTAGGA 3'	0.89	F: 52.0	F: 40.0
			R: 52.8	R: 31.8
	R: 3' TTAGAGTTCGTATTTATAGCCA 5'			
LBL_2925	F: 5' **cacc**GCTGAAAAAAAAGAGGAATCTGC 3'	0.59	F: 57.1	F: 44.4
			R: 55.2	R: 45.0
	R: 3' TTATTGTTGTGGAGCGGAAG 5'			
LBL_1054	F: 5' **cacc**GAACAAGTTGTAACCACGAAA 3'	1.39	F: 54.0	F: 44.0
			R: 52.8	R: 31.8
	R: 3' TTAAAACTCTATTGTGGTTCTC 5'			
LBL_1341	F: 5' **cacc**CAACTTTGGACGCCGC 3'	1.57	F: 54.3	F: 65.0
			R: 54.0	R: 38.1
	R: 3' TTAAAAACTTAAACCGCCCGA 5'			
LBL_0972	F: 5' **cacc**AACGATGGAAACGAAAATTCTTC 3'	1.03	F: 55.5	F: 40.7
			R: 56.0	R: 55.6
	R: 3' TTACGGGTTACAAGGCGC 5'			
LBL_0375	F: 5' **cacc**CAAGAAGATTTGGATGAAAATCC 3'	1.02	F: 55.3	F: 45.0
			R: 55.2	R: 45.0
	R: 3' TTATTTCTTGGCTGGAGGAG 5'			
LIC_10973	F:5' **cacc**AAAACATATGCAATTGTAGGATTTG 3'	0.89	F: 54.8	F: 34.5
			R: 54.7	R: 36.4
	R: 3' TTAGAGTTCGTGTTTATAACCG 5'			

*The **cacc** short overhang sequence was added at the beginning of 5' sequence to pair with the overhang sequence gtgg in the cloning entry vector (section 4.0.1). GC: Guanine-cytosine*

2.9 Polymerase chain reaction (PCR)

There two types of PCR carried out in this thesis, which is PCR used for cloning (Chapter 4), and PCR used as assays for the diagnostic purposes (Chapter 7). Details on both PCR methods are as described in Section 2.9.1 and 2.9.2.

2.9.1 PCR amplification of leptospiral DNA using Phusion High-fidelity DNA polymerase for cloning

Leptospiral DNA from each strain was subjected to PCR to amplify genes of interest ready for cloning purposes (see details in Chapter 4). PCR was performed using High-Fidelity DNA polymerases (Thermo Fisher Scientific, Horsham, UK), which entails DNA amplification with minimal error in the newly synthesised DNA strand through extremely low misincorporation

of base pairs together with proofreading activity. The PCR master mix was performed according to the following protocol in Table 2.8

Table 2.8: The PCR master mix components for Phusion High-fidelity DNA polymerase reaction.

PCR component	20 µl reaction	Final concentration
5x Phusion high-fidelity buffer	5.0 µl	1X
10 mM dNTPs	0.4 µl	200 µM each
Forward primer	0.2 µl	0.5 µM
Reverse primer	0.2 µl	0.5 µM
DMSO	0.6 µl	3%
Phusion High-fidelity DNA Polymerase	0.2 µl	0.02 U/ µl
Leptospiral DNA	1.0 µl	-
Nuclease-free water	Up to 20 µl	-

The master mix was equally distributed, consisting of 19 µl in each 200 µl PCR domed-shaped tubes (Starlab, Milton Keynes, UK), the PCR was set up in a thermocycler (Biometra, TRIO thermocycler, Glasgow, UK) to run for 35 cycles using the 3-step protocol as instructed by manufacture in Table 2.9.

Table 2.9: The 3-step protocol PCR cycle condition for Phusion High-fidelity DNA polymerase reaction for leptospiral DNA amplification.

Cycle step (3-step protocol)	Temperature	Time (s)	Cycles
Initial denaturation	98 °C	30	1x
Denaturation	98 °C	10	35x
Annealing*	X °C	30	
Extension	72 °C	30	
Final extension	72 °C	600	1x

**The annealing temperature is based on the calculated temperature gradient for both forward and reverse primers.*

2.9.2 Diagnostic PCR assay

Standard PCR protocols for diagnostic PCR assays used Taq polymerase for 25 µl reactions as follows (Table 2.10):

Table 2.10: The PCR master mix components for diagnostic PCR using DNA Taq polymerase reaction.

PCR component	25 µl reaction	Final concentration
10x reaction buffer	5.0 µl	1X
Q-solution	5.0 µl	
1.5 mM MgCl2	1.5 µl	
10 mM dNTPs	0.4 µl	200 µM each
Leptospira forward primer*	1.0 µl	0.5 µM
Leptospira reverse primer*	1.0 µl	0.5 µM
Taq DNA Polymerase	0.25 µl	0.02 U/ µl
Sample DNA	1.0 µl	-
Nuclease-free water	Up to 25 µl	-

**Leptospira forward and reverse primers are based on various studies. Refer to Section 7.2 (Chapter 7) for details.*

Leptospira genomic DNA (Section 2.7) was used as a positive control, and deionised water was included as a negative control for each PCR master mix. PCR cycling condition and annealing temperature were optimised according to standard protocols from various studies (Section 7.2). For each optimal diagnostic PCR assay, condition cycle consisted of the steps described in Table 2.11. PCR products were analysed by agarose gel electrophoresis (Section 2.10).

Table 2.11: 3-step protocol PCR cycle condition for Taq DNA polymerase reaction for leptospiral DNA amplification.

Cycle step	Temperature	Time (s)	Cycles
Initial denaturation	98 °C	30	1x
Denaturation	98 °C	10	35x
Annealing*	X °C	30	
Extension	72 °C	30	
Final extension	72 °C	600	1x

**Based on annealing optimisations in this study. Refer to Section 7.2 (Chapter 7) for details.*

2.10 Agarose gel electrophoresis

PCR products from various PCR methods (Section 2.9.1 and 2.9.2) were subjected to agarose gel electrophoresis to visualise the correct size of DNA fragments corresponding to the DNA ladders. A 1% (w/v) agarose gel was immersed in 1X TAE electrophoresis buffer in a geneflow electrophoresis tank (GeneFlow Ltd, Staffordshire, UK). 5.0 µl of 1 kb and 100 bp 6X DNA ladders (Thermo Fisher Scientific, Hemel Hempstead, UK) were loaded at the opposite ends of each well of the gel and 5.0 µl of PCR product was mixed with 6X Orange DNA loading dye (Thermo Fisher Scientific, Hemel Hempstead, UK) in 5:1 ratio and loaded into each well, between the two ladders. The loaded gel was run at 120 V using Bio-Rad PowerPac 300 (Bio-rad, Watford, UK). DNA fragments/bands on the gel were visualised using ultraviolet light via gel imaging system Geldoc gel documentation instrument, (Bio-Rad, Hemel Hempstead, UK).

2.11 DNA purification

DNA purification was carried out using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and the procedure was according to the manufacturer instructions. PCR product was added with buffer PB in 1:5 ratio and mixed well. The solution was transferred to the provided 2 ml collection tube and centrifuged at 13,000 *g* at RT for 1 minute (with this speed, temperature used in subsequent steps). The flow-through was discarded, and 750 µl of buffer PE was added to the column and centrifuged for a further 1 minute to wash the DNA product. The tube was re-centrifuged for an additional 1 minute to completely remove the ethanol residual from Buffer PE. DNA was eluted with adding 50 µl Buffer EB (10 mM Tris.Cl pH 8.5) to the column and left to stand for 5 minutes before centrifuged to collect purified DNA in a clean Eppendorf microcentrifuge tube. Purified DNA was stored at -20°C for future downstream application.

2.12 pENTR directional TOPO cloning of leptospiral genes

Directional TOPO cloning is a simple and popular cloning choice. When a blunt end DNA fragment (of interest) is added into the reaction containing pENTR™/D-TOPO®, the enzyme specifically recognises the nucleotides at 5'-(CACC---)3' that are specifically designed at one end of the DNA fragment and anneals to the overhang GTCC on the 5' end and a blunt end on the 3' end of the vector (Figure 2.1). TOPO I will then ligate the DNA in the correct orientation. Advantages of using directional TOPO cloning include fast reaction, efficient recombinant cloning results and able to obtain high-level expression.

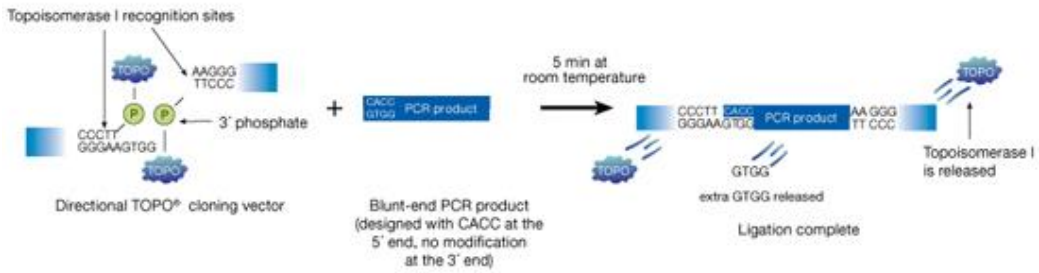


Figure 2.1: The schematic diagram on how directional TOPO cloning reaction works using blunt-end of a DNA fragment. Figure adapted from Directional TOPO Cloning, 2018.

PCR products which had been produced as described in Section 2.9.1 were cloned into a pENTR™/D-TOPO® vector according to manufacturer's instruction using a total volume 6.0 µl reaction mix as according to manufacturer's instruction. The reaction was mixed gently by pipetting up and down and incubated for 5 minutes at room temperature. The reaction was placed on ice, and 2.0 µl of the reaction was used to transform the *E. coli* TOP10 cells, as described in Section 2.13.

2.13 Transformation of *E. coli* TOP10 cells with an entry clone

A vial of 50 µl of One Shot® TOP10 Chemically Competent *E. coli* cells was thawed on ice for 10 minutes. 2.0 µl of the TOPO cloning reaction was added to the cells and mixed gently by pipetting up and down, and the solution was incubated in ice for 30 minutes. Cells were heat-shocked for 30 seconds in a 42°C and immediately transferred to the ice for 2 minutes. 250 µl of S.O.C medium was added to the solution and cells incubated at 37°C in a shaking incubator (225 rpm) for one hour. The transformation mixture was spread on prewarmed LB agar plates containing 50 µg/ml of kanamycin and incubated overnight at 37°C.

2.13.1 Analysing transformed *E. coli* colonies containing entry clones via colony PCR

E. coli colonies were screened to analyse positive transformation from the cloning procedure as described in Section 2.12. Five to ten colonies were randomly selected as a DNA template for PCR. The master mix PCR for 25 µl reaction was performed according to the description in Table 2.12.

Table 2.12: The PCR master mix components for analysing the positive transformation of E. coli.

PCR component	25 µl reaction
10x reaction buffer	5.0 µl
10 mM dNTPs	1.0 µl
Forward primer (0.5 µM) (M13 within plasmid)	1.0 µl
Reverse primer (0.5 µM) (within OMP genes)	1.0 µl
E. coli colony	-
Phusion Taq polymerase (2 U/µL)	0.25 µl
Nuclease-free water	Up to 25 µl

The master mix reaction was equally distributed in PCR tubes, and each *E. coli* transformation colony were picked up by stabbing at the edge of the colony and using the tip and mixed with individual PCR components. The reaction mix was incubated in a thermocycler for 40 cycles. The cycling condition was programmed as described in Table 2.13.

Table 2.13: Cycling condition for colony PCR for analysing the positive transformation of E. coli.

Cycle step	Temperature	Time	Cycles
Initial denaturation	98 °C	30 s	1x
Denaturation	98 °C	10 s	40x
Annealing*	X °C	30 s	
Extension	72 °C	30 s	
Final extension	72 °C	10 min	1x

**The annealing temperature is based on the calculated temperature gradient for both forward and reverse primers.*

For each set of primers, Phusion Taq PCR was performed using the cycling method described in Section 2.9.1. The appropriate annealing temperature for colony PCR for each leptospiral gene was calculated using the online Thermofisher Tm calculator (Thermo Fisher Scientific) which uses the modified Allawi and Santa Lucia's thermodynamics method (Allawi and Santalucia, 1997). All annealing temperatures of each gene are shown in Table 2.14.

Table 2.14: Phusion Taq annealing temperatures for leptospiral OMP genes in this study.

Leptospiral OMP gene	Phusion Taq annealing temperature (°C)
LBL_2618	57.8
LBL_2510	58.6
LBL_2925	63.0
LBL_1054	57.9
LBL_1034	64.4
LBL_0972	64.0
LBL_0375	52.1
LIC_10973	61.0

The PCR product from each randomly selected colony was run on 1% (w/v) agarose gel in an electrophoresis tank (Section 2.10) to visualised DNA bands under a gel reader to confirm the presence of the each OMP gene within the *E.coli* TOP 10 cells which indicates successful transformation.

2.13.2 Plasmid DNA purification from *E. coli* cells

Five colonies from successfully transformed *E. coli* cells (Section 2.13.1) were inoculated individually in 5.0 ml LB liquid medium containing 50 µg/ml kanamycin and incubated overnight at 30°C in an orbital shaker incubator (200 rpm). *E. coli* cultures from each colony were transferred into a 15 ml falcon tube and the cells were centrifuged at 5000 rpm for 10 minutes at 10°C. The supernatant was discarded, and the pellet was subjected to plasmid

DNA purification using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Plasmid DNA was obtained by adding 50 µl of EB buffer to the centre of the column, the column was left standing for 5 minutes and centrifuged for 1 minute at 13,000 rpm to elute the plasmid DNA. The purified DNA containing entry clones were stored at -20°C for future downstream applications.

2.13.3 Analysing transformation of entry clones by restriction enzyme

The purified DNA plasmid was subjected to restriction enzyme analysis to determine whether the plasmid contains the correct sized gene by adding a specific restriction enzyme, which recognises specific nucleotides sequence and cuts DNA molecules at their recognition site. The procedure was carried out using Thermo Scientific™ Fast digest EcoRV (Eco321)* (Thermo Fisher Scientific, Hemel Hempstead, UK). Two 200 µl of PCR dome-shaped tubes were prepared, and each was labelled 'cut' and 'uncut' respectively. 12.0 µl of nuclease-free water was added into both tubes, and 2.0 µl of 10X FastDigest Green buffer (Thermo Fisher Scientific, Hemel Hempstead, UK) mixed into both tubes. 5.0 µl of purified plasmid DNA was transferred into two tubes, and 1.0 µl of EcoRV (Eco321) enzyme was added into the cut-labelled tube and mixed gently, while the uncut tube was left without enzyme addition. Both sample solutions were incubated at 37°C for 10 minutes into a heat block, and the products were run side by side (cut/uncut) on 1% (w/v) and visualised the band size in a gel-doc reader. The plasmid sizes were then compared with the size estimated calculated using pENTR-D-TOPO selection within the Clone Manager 7 (Sci-ED Central, Denver, USA) to validate the actual band size(s) after enzyme cleavage.

2.13.4 Sequencing of entry clones

After the entry clone's sizes were correctly determined, the entry DNA plasmid was sent for sequencing to confirm that the insert genes are cloned in the correct orientation and to ensure no mutation occured within the nucleotide sequence. A reverse primer (Table 4.1, Chapter 4), of each gene were sent alongside the plasmid DNA to Source Bioscience (Cambridge, UK) that sequenced both DNA strands using the primer we supplied and M13 primer. The outcome of the entry clone sequences was analysed using ChromasPro version 2.1.8 software (Technelysium Pty Ltd, South Brisbane, Australia) to check that the gene was inserted in the correct orientation and was complete and without error.

2.13.5 The attL and attR (LR) recombination reaction of entry clones

After confirming the gene of interest was inserted in the correct orientation within the entry vector by sequencing, the LR recombination reaction was performed to transfer each leptospire gene from the entry vector (Section 2.13.2) into a destination vector to generate an expression clone. The LR recombinant reaction was performed according to the Gateway® Technology with Clonase® II protocol, and 7.0 µl of an entry clone plasmid was mixed with 1.0 µl of pDEST17 destination vector.2.0 µl of LR Clonase® II Enzyme Mix, which was briefly thawed on ice for 2 minutes, was added into the sample and mixed well by pipetting up and down. The reactions were incubated at 25°C for an hour. 1.0 µl of Proteinase K was added into the sample to stop the reaction, and the sample was further incubated for 10 minutes at 37°C.

2.14 Transformation of *E. coli* TOP10 cells from the LR recombination reaction

A vial of 50 µl of One Shot® TOP10 Chemically Competent *E. coli* cells was thawed on ice for 10 minutes. 2.0 µl of the LR recombinant reactions (Section 2.13.5) was added to the cells and mixed gently by pipetting up and down, and the solution was incubated on ice for 30 minutes. The cells were heat-shocked for 30 seconds in a 42°C and immediately transferred to the ice for 2 minutes. 250 µl of S.O.C medium cells were added to the solution and cells incubated at 37°C in an orbital shaking incubator (200 rpm) for one hour. The transformation mixture was spread on prewarmed LB agar plates containing 100 µg/ml of ampicillin solution and incubated overnight at 37°C. Plasmid DNA purification containing destination clones were obtained as described in Section 2.13.2.

2.14.1 Analysing transformation of *E. coli* colonies containing destination clones via colony PCR, restriction enzyme analysis and sequencing

The following procedures to analyse transformation containing destination clones are as described in Section 2.13 of this thesis except for a few exceptions. Here, for analysing transformation via colony PCR, the forward primer was with the T7 primer (again supplied by the sequencing service), and the annealing temperature was calculated accordingly (Table 2.15) while the cycling condition was maintained (Table 2.13). For analysing *E. coli* TOP10 transformation by restriction enzyme analysis, the *ecoRV* enzyme was changed to *ecoR1* enzyme and the predicted band size was calculated using the pDEST17 selection within the

Clone Manager 7 to estimate the size after enzyme cleavage. The sequencing results of destination clones were analysed similarly, as described in Section 2.13.4.

Table 2.15: The annealing temperature of each leptospiral gene PCR when used in colony-based Taq polymerase PCR with the respective OMP primer and either M13 forward primer (pENTR) or T7 reverse primer (pDEST17).

Leptospiral OMP gene	Annealing temperature (°C) for pENTR PCR(M13)[a]	Annealing temperature (°C) for pDEST17 PCR (T7)[b]
LBL_2618	55.8	55.8
LBL_2510	55.0	55.0
LBL_2925	55.8	43.2
LBL_1054	43.5	43.2
LBL_1034	47.1	43.2
LBL_0972	47.1	44.0
LBL_0375	43.2	43.2
LIC_10973	55.0	43.2

[a] *Annealing temperatures of PCR assay used for analysing pENTR transformation*

[b] *Annealing temperatures of PCR assay used for analysing pDEST17 transformation*

2.14.2 Transformation of *E. coli* BL21-AI/ BL21 (DE3) pLysS cells

A vial of 50 µl of competent BL21-AI or BL21 (DE3) pLysS *E. coli* cells were thawed on ice. 5.0 µl of purified DNA plasmid containing a cloned gene was added to the cells and pipetted up and down, and the cell solution was kept in ice for 30 minutes. The cells were then heat-shocked in a 42°C water bath for 30 seconds and immediately transferred on ice for 2 minutes. 250 µl of S.O.C medium was added to the cells and the mixture incubated in an orbital shaker incubator at 37°C for an hour (200 rpm). The transformation mixture (50 µl and 80 µl) was spread onto the LB agar plates containing 100 µg/ml ampicillin solution and incubated overnight at 37°C.

2.15 Pilot expression of recombinant leptospiral OMP genes

The following protein expression protocol was adapted from Staton, (2018) with relevant optimisation. A single colony from a transformation agar plate (Section 2.14.2) was inoculated in 20 ml LB liquid medium containing 100 µg/ml and grown overnight at 30°C in an orbital shaker (200 rpm). 20 ml of LB liquid medium containing 100 µg/ml ampicillin was inoculated with 100 µl of the overnight culture and grown for 1.5 hours in an orbital shaker incubator (200 rpm) at 37°C until the optical density reaches mid-log phase OD_{600} 0.6 measured using Ultrospec 2000 UV-Visible Spectrophotometer (Pharmacia Biotech Inc, USA) at 600nm absorbance.

500 µl of the culture was collected as uninduced culture, and 0.1% (w/v) of L-arabinose (for BL21-AI strain) and 1 mM of IPTG (for BL21 (DE3) pLysS strain was added into the remaining cultures for induction and the cells were further grown for 4 hours. 500 µl of induced culture was collected, and the previous collected uninduced culture was centrifuged for 1 minute at 13, 000 g at 4°C and the supernatant was discarded. Pellets from both uninduced and induced cultures were stored in -20°C. To observed expression, both pellets were re-suspended with 50 µl of 1x SDS-PAGE sample buffer, heated in a heat block at 100°C for 10 minutes and analysed by SDS-PAGE as described in Section 2.17.

2.16 Large-scale expression of leptospiral recombinant OMP genes

The large-scale expression was subsequently carried out after the identification of a protein expression band of the correct size was observed using the SDS-PAGE analysis of the pilot expression. A single colony from a transformation agar plate (Section 2.14.2) was inoculated in 5.0 ml of LB liquid medium containing 100 µg/ml ampicillin solution and grown overnight in an orbital shaker incubator (200 rpm) at 30°C. 500 ml of prewarmed LB liquid medium containing 100 µg/ml ampicillin solution was inoculated with 5.0 ml of overnight culture and grown for 2 hours. The OD was checked after 2 hours and the cells were continued to grow until it reaches OD_{600} between 0.6-0.8. 1.0 ml of culture (labelled as uninduced) was taken, centrifuged at 13,000 *g* to remove the supernatant, and the pellet was kept at -20°C. The remaining cells were induced using 0.1% (w/v) of L-arabinose, or 1.0 mM of IPTG (depending on selected expression strains) was added to the cells and allowed to grow for 4 hours. 1.0 ml of culture (labelled as induced) was taken, centrifuged at 13,000 *g* to remove the supernatant, and the pellet was kept at -20°C. The cells were then centrifuged at 5,000 rpm for 30 minutes. The supernatant was removed, and the pellet was kept at -20°C. Both uninduced and induced pellets were re-suspended with 50 µl and 100 µl of 1x SDS-PAGE sample buffer, heated in a heat block at 100°C for 10 minutes and analysed by SDS-PAGE as described in Section 2.17.

2.17 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Protein electrophoresis was carried out based on the original described method (Laemmli, 1970). The method employed for protein visualisation is as described in the later chapter of the thesis. For this purpose, a 12% (v/v) SDS-polyacrylamide resolving gel and 4% (v/v) of SDS-polyacrylamide stacking gel were used, and solutions for making these gels are in Table 2.4. Before gel preparation, a gel cast was set-up in a mini-gel system (Mini-Protean electrophoresis system, Biorad, Hemel Hempstead, UK) using gel cassettes and glass plates (0.75 mm short plates and spacer plates) (Biorad, Hemel Hempstead, UK). Acrylamide resolving gel solution was dispensed carefully using a pipette in between the glass plates to fill ¾ of the space and overlaid carefully with absolute ethanol to remove bubbles and allows equal distribution of the gel. After the polymerisation was complete for about 25 minutes, the overlay was washed off several times with distilled water to remove any unpolymerised acrylamide. SDS-polyacrylamide stacking gel solution was added on top of polyacrylamide resolving gels, and 15 lane Teflon combs (mini protean combs 0.75 mm) (Biorad, Hemel Hempstead, UK) were placed immediately to produce the wells of the gel. After the polymerisation was complete for about 25 minutes, Teflon combs were removed gently as not to cause damage to the wells, and the wells were washed with distilled water to remove any unpolymerised acrylamide.

The set gels were transferred to a gel gasket and inserted into a vertical electrophoresis tank. Gel gasket was then immersed in 500 ml Tris-glycine electrophoresis running buffer covering the tank. Protein samples were re-suspended either in 1x of 5x SDS gel-loading sample buffer (Table 2.4) and denatured by heating at 100°C for 5 minutes using a heat block prior loading into wells. 10 µl of protein standard molecular-weight maker was added on the first well, and 20 µl of each protein samples were added across remaining wells. The electrophoresis tank containing the loaded SDS-PAGE gels was run at 180 V for 50 minutes using a Biorad Powerpac 300 (Biorad, Hemel Hempstead, UK). The gels were then removed carefully from the plates and transferred to a heat-durable plastic box containing 100 ml of distilled water and then heated for 40 seconds using high power. The distilled water was discarded, and the step was repeated three times. The PageBlue protein staining solution was used to stain the SDS-PAGE gels, and this was added to the gels and heated again for another 30 seconds. The stained gels were placed on a rocking platform (ProBlot 35 deluxe rocking platform) (Appleton Woods, Birmingham, UK) for 10 minutes. The gel was rinsed with water three times to remove the stain and then placed back to the rocking platform for another 10 minutes for further de-stain before assessment of protein bands. The application of heat

post-gel run in stain, and de-stain protocol is aim to achieve better protein resolution, therefore increasing the sensitivity for protein which helps for visualisation while indirectly reduces the staining and de-staining time (Kurien and Scofield, 2012).

2.18 Far-western blotting

The far-western blot was used to detect bacterial OMP-ligand interaction *in vitro*.

A litre of transfer buffer (Table 2.4) was prepared and pre-chilled at 4°C for 1 to 2 hours before the procedure. 100 µg/ml of selected ECM protein host ligand (1.0 mg/ml stock) was diluted in 350 µl of 1x SDS-PAGE loading buffer with the addition of 200 mM of DTT. The solution was mixed by 5-second vortex and briefly heated in a heat block at 100°C for 5 minutes. Samples were loaded into wells of a 4-20% precast polyacrylamide gel, 8.6 x 6.7 cm Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad, Hemel Hempstead, UK) and 10 µl of Color Prestained Protein Standard, broad range 11-245 kDa was used as a protein marker. Gel was run in the electrophoresis tank with 1x SDS-PAGE running buffer as described (Section 2.17) to resolve the protein. Far-western blot apparatus were prepared by soaking sponges, filter papers, and a 0.2 µm nitrocellulose membrane sheet (NCS) (Bio-Rad, Hemel Hempstead, UK) in pre-chilled transfer buffer temporarily. These components were cut resembling the gradient gel dimension (8.6 x 6.7 cm). After the gel migration was complete, the gel was electrophoretically transferred to the nitrocellulose membrane sheet by carefully removing the stacking part of the gel. A straight line was drawn using a pen on the top of the membrane to mark the upright position of the protein marker on the membrane. A Mini Trans-Blot Module transfer cassette was used to hold the components. Components were arranged by the following order;

- The black side of transfer cassette
- A sponge
- 2x Filter paper
- 4-20% Protein Gel
- NCS membrane sheet
- 2x Filter paper
- A sponge
- White of the transfer cassette

A pipette was used to roll in between layers to remove air bubbles. Once layered, the cassette was locked and placed in the transfer holder (black side of the cassette against the black side of the holder). Another transfer cassette with components (without protein gel and membrane) was placed in the same transfer holder side-to-side with gel-clad cassette and tank with an ice pack. A magnetic flea was added, and the tank was placed on a magnetic stirrer. Transfer buffer was added into the tank covering the holder. The transfers were run at 110 V, 240 mA for 2 hours.

The transferred membranes were removed and washed three times in PBST for 5 minutes on a rocking platform, and then were stained with 10 ml Ponceau S solution and placed on the rocking platform for 5 minutes to allow membrane staining. Membranes were cut into strips, and all strips washed three times for 5 minutes in PBST. Next, membranes were blocked overnight in 5% (w/v) dried-skimmed milk in PBST and incubated at 4°C on a rocking platform. After incubation, membrane strips were washed three times for 5 minutes on a rocking platform. Strips were placed in individual 15 ml falcon tube and 30 micrograms of recombinant OMP solution was added on each strip and incubated at room temperature for 90 minutes. After washing with PBST, strips were incubated in monoclonal anti-his tag antibody (Sigma-Aldrich, Dorset, UK) (dilution factor 1:2000 in PBST) and shaken gently on a rocking platform at room temperature for 1 hour. One strip served as a negative control; therefore, it was incubated only in PBST.

Membrane strips were washed three times for 5 minutes in PBST to remove unbound anti-his antibody. Conjugate antibody (Anti-mouse polyvalent immunoglobulins) was added to the strips (dilution factor 1:10,000) and incubated at room temperature for 1 hour with rocking and membrane strips further washed three times in PBST with rocking. All strips were placed in clear, white plastic and 2.0 ml of TMB blotting solution (3, 3', 5, 5'-tetramethylbenzidine and hydrogen peroxide) (Uptima-Interchim, Montlucon, France) was added into each strip, and their top was covered using another a clear, white plastic. Strips were incubated in a dark room for 5-15 minutes and checked occasionally for a change, denoted by presence of bands on the membrane. Lastly, strips were dried, and photos of all strips taken using white light in a gel-documentation system.

2.19 Inclusion body extraction of leptospiral OMP

After obtaining *E. coli* pellets from Section 2.16, cells were subjected to inclusion body preparation for recombinant protein extraction. The pellets were homogenised with 0.25 %

(w/v) lysozyme buffer (Table 2.4), and the solution was incubated on ice for 30 minutes to detach the cell wall. To release the protein component of the cell wall, the cell suspension was subjected to sonication using a cell sonicator (Bendelin, Berlin, Germany) and the pulse mode was set for 5 minutes operation (burst cycle; 20 seconds off, 10 seconds off) (20-50 kHz). After sonication, the cell suspension was centrifuged at 13,000 *g* at 4°C for 20 minutes, and the supernatant was discarded. The insoluble pellet was re-suspended with 4% (v/v) tergitol solution (Table 2.4), and the mixed solution was stirred continuously for ~2 hours to remove any protein contaminant originated from broken *E. coli* cell wall and other outer membrane components in the inclusion body pellet.

The inclusion body solution was then centrifuged at 13,000 *g* for 20 minutes, and the supernatant discarded. The pellet was re-suspended in 50 mM Tris HCl pH 7.9 washing buffer and again centrifuged in a similar manner to remove the contaminants and excess tergitol solution. The step was repeated for 2-3 times until a pure white inclusion body pellet was obtained. After the last wash, 1.0 ml of inclusion body suspension was collected, centrifuged at 13,000 rpm for 1 minute to remove the supernatant and run on the SDS-PAGE gel as described in Section 2.17 to observe the inclusion body contents. Clean inclusion bodies pellets were kept in -20°C until use.

2.20 Preparation of protein refolding

A protein refolding protocol was adapted from Staton, (2018) and the principle of the method is described further in Chapter 4. Clean and white inclusion bodies (Section 2.19) containing misfolded protein was solubilised in 50 ml solubilisation buffer containing a high concentration of guanidine hydrochloride (GnHCL) (Table 2.4), and the mixed solution was placed on a rocking platform at room temperature for one hour. To separate the insoluble materials, the solution was centrifuged at 13,000 *g* for 30 minutes at 4°C. Solubilised protein was transferred into a clean 50 ml falcon tube and slowly dripped into refolding buffer (Table 2.4) under normal gravity. The refolding buffer containing drip fed solubilised protein solution was rapidly stirred using a magnetic flea for 4 hours to prevent protein-protein stickiness that would affect protein refolding to their native state.

2.21 Overnight dialysis of refolded recombinant OMPs

Refolded protein solution was subjected to overnight dialysis for removal of excess detergent and contaminants that may present in the refolding solution by selective and passive diffusion through a semi-permeable membrane. Refolded protein solution (Section 2.10) was transferred to 40 cm dialysis tubing cellulose membrane (average flat width 10 mm) with capacity to retain proteins of molecular weight at least 12 kDa (Sigma-Aldrich, Dorset, UK) and tied at both ends. The tube was immersed overnight in a six-litre dialysis buffer (Table 2.4) and placed in a cold room (4°C).

2.22 Protein affinity purification

The following method describes the purification of recombinant protein in a native state.

Here, 2.5 ml of nickel agarose beads (Qiagen, Hilden, Germany) was transferred to an Econo-Pac® chromatography column (1.5 x 12 cm, polypropylene columns, 14 cm, 20 ml bed volume) (Biorad, Hemel Hempstead, UK), held upright using a clamp stand. Dialysed protein was mixed with slurry beads (20 ml at a time), and the solution passed through the beads under gravitational force. The step was repeated until all dialysed proteins had passage through the column (Figure 2.2). The flow-through was discharged, and the column was washed with 50 ml wash buffer (Table 2.4). The flow-through from wash buffer was collected, the column was rewashed with four fractions (5 ml each) of elution buffer (Table 2.4) into five separate tubes (labelled E1-E4).

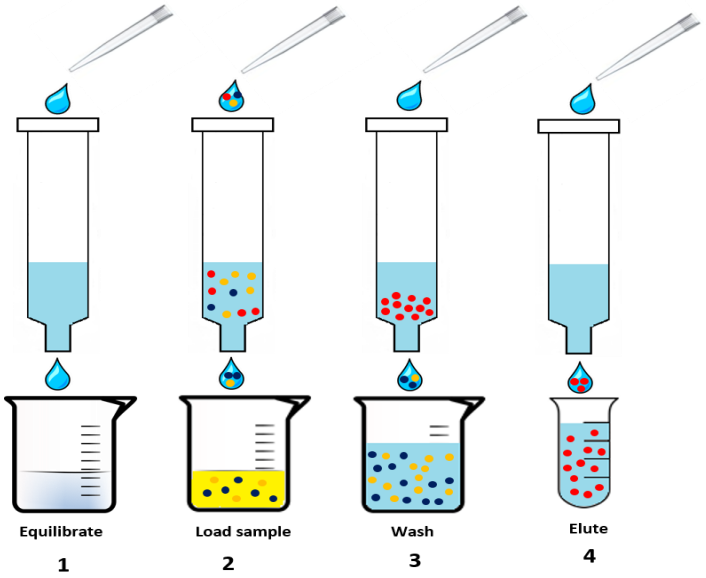


Figure 2.2: Schematic image showing the example of His-tagged protein purification protein workflow using an affinity chromatography column.

Recombinant protein purification using an affinity chromatography column involves four stages; 1 Equilibrate (loading of nickel-charged affinity resin into the column until the equilibrium level is achieved), 2) Loading of protein solution, 3) Washing of resin using washing buffer, 4) Loading of elution buffer per 5.0 ml fractions as described in Section 2.22.

Samples from each fraction including wash buffer were analysed on the 1D SDS-PAGE (Section 2.17) by mixing 16 µl of protein samples with 4.0 µl of 5X SDS-PAGE loading buffer (Table 2.4) with additional of a reducing agent (DTT/2ME) (see detail in Chapter 4) and heated on a heat block for 5 minutes before loading them into the gel wells. One fraction from E1 was duplicated and unheated. All protein fractions were run in the electrophoresis tank, and the gel was washed and stained as described in Section 2.17.

2.23 Overnight dialysis of concentrated protein solution

E1 fraction from Section 2.22, which contains the highest protein portion was subjected to overnight dialysis to remove imidazole and other possible impurities. Protein fraction was transferred to 10 cm dialysis tubing cellulose membrane (average flat width 10 mm) (Sigma-Aldrich, Dorset, UK) and both ends were tied. The tube was immersed in 1.0 ml dialysis buffer overnight at 4°C. After dialysis, the protein fraction was transferred to a sterile tube, and the concentration was quantified using NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Massachusettes, USA). Using this instrument, the purified proteins were

measured by using direct UV absorbance at 280 nm (A280), and the protein concentration is automatically calculated using the Beer-Lambert equation A280 = c * ε * b where ε is the wavelength-dependent protein extinction coefficient, b is the pathlength. The concentration of each protein is shown using two units, mg/ml or µg/ml.

2.24 Amicon concentration

Protein samples were further concentrated using Amicon Ultra centrifugal filter units MWCO 30 kDa (Sigma-Alrich, Dorset, UK) when the concentration from nanodrop was below 200 µg/ml. Samples were transferred to the device and concentrated by centrifuging at 3000 *g* for 3-5 minutes, and the supernatant was collected, and its concentration was re-analysed using the nanodrop.

2.25 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed to determine the secondary structure of the expressed recombinant OMPs. This procedure was carried out using a Jasco-1100 spectropolarimeter equipped with a Peltier unit for temperature control (Jasco, Easton, MD). The purified recombinant proteins were dialysed against a blank buffer containing 40 mM Tris-HCl, 50 mol NaCl and 0.05% (v/v) LDAO. The spectra reading was taken an average of three times, from 180 to 260 nm. Spectrum data were analysed with BeStSel (http://bestsel.elte.hu/index.php) and CAPITO (http://capito.nmr.leibniz-fli.de/plot.py) to obtain secondary-structure data.

2.26 Enzyme-linked immunosorbent assay (ELISA)

An ELISA method was employed for functional assays and binding saturation studies of leptospiral recombinant OMPs (Chapter 5 and 6), and detection of anti-leptospiral antibody in milk and serology immunological assays in two chapters of this thesis.

2.26.1 Functional assays and binding saturation studies

For host molecules and protein-binding saturation studies, non-activated, 96-well microtitre ELISA plates (Microplate Immunlon 2HB 96 well 128 mm x 86 mm- 0.330 ml well volume 2.37 cm^2 per well) (Thermo Fisher Scientific, Horsham, UK) were coated with 5.0 µg/ml of ECM proteins with bovine serum albumin (BSA) as a control, which was diluted in 1X PBS, pH 7.2.

Plates were incubated for 1 hour at 37°C and overnight at 4°C. Unbound protein was rinsed off by washing the plates three times (5 minutes apart) with PBST. Next, 0.1 % (w/v) of BSA (Sigma-Aldrich, Dorset, UK) was applied to the wells, to serve as a blocking buffer to prevent non-specific binding of antigens to the microtitre well (Hornbeck, 1991). Leptospiral recombinant OMPs were diluted according to the concentration as described (Chapter 5 and 6) and 100 µl was added into ELISA plate wells in duplicate.

Plates were then incubated for 1.5 hours at 37°C and subsequently washed six times with 5 minutes apart with PBST. Monoclonal anti-his tag antibody (Sigma-Aldrich, Dorset, UK) was diluted (1:2000) with PBST, and 100 µl of the diluted solution was added to plate wells. Plates were incubated for 1 hour at 37°C, and subsequently washed three times with 5 minutes apart with PBST. Anti-mouse polyvalent immunoglobulins (Sigma-Aldrich, Dorset, UK) served as a conjugate antibody for binding with anti-his antibody was diluted (1:10,000) with PBST and 100 µl of this solution was added to plate wells. Plates were incubated for 1 hour at 37°C and subsequently washed three times with 5 minutes apart with PBST. 100 µl of 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) liquid substrate was added across plate wells, and the antibody-conjugated reaction (measurement by the blue appearance of the wells) was visualised by incubating the plates in a dark room at room temperature for 15 minutes.

The reaction was stopped by adding a stopping solution (Table 2.4), which produces a colour reaction from blue to yellow. The colour absorbance of each well was measured by a microtitre (Multiskan EX) plate reader (Thermo Fisher Scientific, Delaware, USA) using a standard 450 nm filter. Each plate was measured three times, and the mean values were averaged as a representative for further analysis. Both functional and binding saturation ELISA assays were repeated as three independent experiments, and the results are averaged for statistical analysis. Figure 2.3 and Figure 2.4show the ELISA plate layout used for both functional assays and binding saturation studies.

2.26.2 Serology and milk immunological ELISA

For the serology and milk immunological assay, the ELISA protocol was implemented differently. Figure 2.5 and Figure 2.6 show the ELISA layout for milk and serum samples, respectively.

ELISA plates were coated with 5.0 µg/ml of leptospiral recombinant OMP as antigen and incubated at 37°C for 1 hour and overnight at 4°C. Plates were washed three times with 5 minutes apart to remove access unbound antigens. For bulk milk samples, 100 µl of 0.2%

(w/v) of milk powder (2.0 g in 100 ml PBST) was added into each well as a blocking agent and plates were incubated at 37°C for 1 hour. For serology ELISA, no blocking agent was used. After washing three times with 5 minutes apart, 100 µl of bulk milk samples with fat layer initially removed by centrifugation (preparation details in Chapter 5) was added to the wells in duplicate and plates were incubated at 37°C for 1 hour. For serology, individual serum sample was diluted (1:100) in PSBT and 100 µl was added to the wells (except milk blank wells-replaced with 100 µl PBST) and plates were incubated at 37°C for 1 hour. 100 µl of positive, weak positive and negative sera (supplemented in Priocheck™ *L. Hardjo* Ab Strip Kit) (Thermo Fisher Scientific, Horsham, UK), diluted 1: 200 in PBST were included in the study as sera controls.

Plates (containing milk/serum samples) were washed three times to remove unbound antibodies. 100 µl of Mouse Anti-Bovine Immunoglobulin class G subclass 1 (IgG1) or 100 µl of Mouse Anti-Bovine Immunoglobulin class G subclass 1 (IgG2) (Biorad, Hemel Hempstead, UK) was added to each well at a 1: 1000 dilution in PBST. 100 µl of diluted monoclonal anti-his tag antibody was added to His-tag wells which served as an antibody control.

Plates were incubated again at 37°C for 1 hour, and after washing, the conjugate antibody, substrate and stopping solution was added according to Section 2.26.1. Conjugate and substrate solution were not added to the control wells, and these wells were covered with 100 µl of PBST. Similarly, ELISA plates were analysed in a microtitre plate reader using a standard 450 nm filter the results were read three times, and the mean absorbance was averaged. Milk and serology ELISA assays were repeated as two independent experiments, and the results were averaged for statistical analysis.

	OMP 1				OMP 2				OMP 3			
	0 µg/ml		10.0 µg/ml		0 µg/ml		10.0 µg/ml		0 µg/ml		10.0 µg/ml	
	1	2	3	4	5	6	7	8	9	10	11	12
A	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA
B	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1
C	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2
D	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3
E	ECM 4	ECM 4	ECM 4	ECM 4	ECM 4	ECM 4	ECM 4	ECM 4	ECM 4	ECM 4	ECM 4	ECM 4
F	ECM 5	ECM 5	ECM 5	ECM 5	ECM 5	ECM 5	ECM 5	ECM 5	ECM 5	ECM 5	ECM 5	ECM 5
G	ECM 6	ECM 6	ECM 6	ECM 6	ECM 6	ECM 6	ECM 6	ECM 6	ECM 6	ECM 6	ECM 6	ECM 6
H	ECM 7	ECM 7	ECM 7	ECM 7	ECM 7	ECM 7	ECM 7	ECM 7	ECM 7	ECM 7	ECM 7	ECM 7

Figure 2.3: ELISA plate layout for functional study (host molecules screening) of leptospiral recombinant OMPs.

Binding of an OMP against a library of host molecules (including BSA as a control) is analysed in duplicate using two OMP concentrations, 0 µg/ml and 10.0 µg/ml. Each ELISA plate may analyse up to three OMPs separately (discriminated by colour separation as shown).

	1	2	3	4	5	6	7	8	9	10	11	12
A	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA
B	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA
C	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1
D	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1	ECM 1
E	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2
F	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2	ECM 2
G	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3
H	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3	ECM 3

Figure 2.4: ELISA plate layout for binding saturation assay. From host molecules screening results, ECM with significant binding affinity was subjected to ligand-saturation assay.

Binding of an OMP against selected host molecules (including BSA as a control) was analysed in duplicate in its parallel column using concentrations ranging from 0.0 µM-6.0 µM.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	9	9	17	17	25	25	33	33	**CB**	**CB**
B	2	2	10	10	18	18	26	26	34	34	**CB**	**CB**
C	3	3	11	11	19	19	27	27	35	35	**SB**	**SB**
D	4	4	12	12	20	20	28	28			**SB**	**SB**
E	5	5	13	13	21	21	29	29				
F	6	6	14	14	22	22	30	30	**++**	**++**		
G	7	7	15	15	23	23	31	31	**+**	**+**	**HIS**	**HIS**
H	8	8	16	16	24	24	32	32	**-**	**-**	**HIS**	**HIS**

Figure 2.5: Serology ELISA plate layout. Each serum sample is analysed in duplicate shown by the duplication of each number in its parallel column.

Abbreviations: CB (conjugate blank), SB (substrate blanks), HIS (His-tag blank). (++) indicates strong positive serum control, (+) indicates weak serum control and (-) indicates negative serum control

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	9	9	17	17	25	25	33	33	**MB**	**MB**
B	2	2	10	10	18	18	26	26	34	34	**MB**	**MB**
C	3	3	11	11	19	19	27	27	35	35	**CB**	**CB**
D	4	4	12	12	20	20	28	28	36	36	**CB**	**CB**
E	5	5	13	13	21	21	29	29	37	37	**SB**	**SB**
F	6	6	14	14	22	22	30	30	38	38	**SB**	**SB**
G	7	7	15	15	23	23	31	31	39	39	**HIS**	**HIS**
H	8	8	16	16	24	24	32	32	40	40	**HIS**	**HIS**

Figure 2.6: Milk ELISA plate layout. Each bulk milk sample is analysed in duplicate shown by the duplication of each number in its parallel column.

Abbreviations: MB (Milk blank), CB (conjugate blank), SB (substrate blanks), HIS (His-tag blank)

2.27 Ethical approval and project licensing

All experiments were approved by the University of Liverpool ethical review board which includes *Leptospira* related project (Application number: VREC578).

2.28 Statistical analysis

2.28.1 Non-parametric statistical test

Non-parametric statistical tests were used to determine possible significant different(s) between one or more groups. These tests were selected throughout the project due to two reasons; 1) Unknown distribution of a population data, and 2) Small-sample size. Non-parametrical statistical tests used in this thesis are explained below.

2.28.1.1 Comparison of data between two groups

Mann-Whitney U-test (non-parametric-unpaired t-test) was used to determine possible significant differences between the means of two groups of the same population (sample).

Mann-Whitney U-test is designated as U and the formula used is given as:

$$U = n_1 \cdot n_2 + \frac{n_1 \cdot (n_1 + 1)}{2} - T_1$$

Whereby:

U = Mann-Whitney calculation for sample X

n_1 = Sample size of group 1

n_2 = Sample size of group 2

T_1 = Sum of ranks of group 1

Mann-Whitney test was calculated as a two-tailed test and performed using GraphPad version 7.02 (GraphPad Software, California, USA). A *P-v*alue less than (≤0.05) was considered statistically significant, which indicates there is a difference between the means; thus, the significant difference enables rejection of the null hypothesis.

2.28.1.2. Comparison of data between several groups

Dunnett's multiple comparison test was selected to compare the variation of means of three or more groups in a sample using a single negative control, performed by Graphpad version 7.02. A *P*-value less than (≤0.05) was considered statistically significant, and therefore, the null hypothesis can be rejected.

2.28.2 Regression analysis

Regression analysis was used for estimating the relationships between a dependent (target) and independent variable (predictor). The types of regression analysis chosen in this thesis are explained below.

2.28.2.1 Linear regression

Linear regression was selected to study the relationship between the dependent variable and one or more independent variable using regression (a best fit straight line). The analysis is represented by an equation which is able to predict the value of the target variable based on a given predictor variable. The linear equation is given by; Y= a+b*Y + e where a is intercept, b is the slope of the line and e is error term. To get the best fit straight line, the line was calculated using the least square method for fitting a regression line. The coefficient of determination of the data points scattered around the fitted regression line is calculated by R-squared (R^2).

R^2 is the percentage (from 0% to 100%) of the dependent variable that linear model given by;

$$R^2 = \frac{\text{Variance explained by model}}{\text{Total variance}}$$

The larger R^2, the better regression model the fit the observation. Linear regression analysis was modelled using GraphPad (Prism) version 7.02.

2.28.2.2 Nonlinear regression

Nonlinear regression was used to measure the interaction between a host ligand binding to another protein to determine its binding affinity. Using ELISA data, the strength of binding interaction between protein-ligand binding is measured by the equilibrium dissociation constant (K_d) based on a method described by Lin *et al.*, (2009) which using the following equation;

$$K_d = \left(\frac{[A_{max}][Protein]}{[A]} \right) - [Protein]$$

where [A] is the absorbance at a given protein concentration, [Amax] is the maximum absorbance for the ELISA plate reader (OD 450 nm), [protein] is the protein concentration and K_d is the equilibrium dissociation constant for a given protein concentration. The K_d is calculated at micromolar unit (μM), which corresponds to ligand concentration of which half of the proteins are bound at equilibrium. This also refers to the concentration of reactant protein to achieve a half-maximum binding at equilibrium. Thus, the lower the value of K_d, the stronger the binding reaction given by its unit, Moles per litre (M) (Pollard, 2010). Equilibrium binding assays for protein-protein interaction graph were plotted, and the K_d values were calculated using a curve fit, nonlinear regression (one-site total) equation performed by GraphPad (Prism) version 7.0.

Chapter 3: Identification of putative outer membrane proteins from pathogenic bovine *Leptospira*

3.1 Introduction

3.1.1 *Leptospira* outer membrane components

Spirochetal outer membranes are considered to have the same structure as other Gram-negative bacteria. The outer membrane has several essential functions for the bacterial cell, and these roles are regulated by highly specialised groups of proteins. As briefly described in Chapter 1, the *Leptospira* outer membrane consists of two major protein components, which are located within the lipid bilayer with the exterior facing lipid layer consisting of both lipid and carbohydrate O-antigens described as Lip-O-polysaccharides (LPS). LPS is the major outer membrane component and localised abundantly on the surface of the outer cell membrane and serves as a determinant of *Leptospira* serovars (Bulach *et al.*, 2000).

In most other Gram-negative bacteria, LPS is an endotoxin source, and the toxin can be associated with septicaemia caused by the gradual release of this cell molecule after cell death (Leeson *et al.,* 1994). This same principle was considered to apply to leptospiral LPS, however, reports have shown that LPS toxin extracted from *Leptospira* is less potent compared with other Gram-negative bacteria (Isogai *et al.*, 1986; Shimizu *et al.*, 1987) and their roles in pathogenicity are considered more secondary. Host immune responses elicited towards leptospiral LPS are specific, and thus, this results in selective pressure for genetic change to produce O variations. Such variations result in the hundreds of leptospiral serovars that have been differentiated based on distinctive reactivity with antibodies or antisera in the microscopic agglutination test (MAT) (Haake and Zückert, 2015) (Table 1.2, Chapter 1).

The two major protein components within the OM are outer membrane lipoproteins and integral/transmembrane OMPs. As previously mentioned in Chapter 1 of the thesis, several lipoproteins have been studied as potential vaccine candidates, however, their roles in pathogenesis remain undisclosed, and their lack of immune efficacies against heterologous infections require further evaluations. Our interest here is directed towards the transmembrane OMPs due to their surface-exposed loops that are likely in contact with the host cell's receptors, which represent a potential target of a protective immune response.

3.1.2 *Leptospira* transmembrane outer membrane proteins

The transmembrane OMPs are made up of eight to 24 antiparallel β-sheet strands as observed in most of the Gram-negative bacterial species (Fairman *et al.,* 2011) with the larger transmembrane OMPs in other bacteria acting as autotransporters or adhesins. The first transmembrane OMP from *Leptospira* to be characterised was identified as forming a porin channel (Shang *et al.*, 1995) and determined as only being present in pathogenic species. Such porin channels exhibit the shape of a cylinder or barrels as a result of transmembrane β-strands that continuously thread their way back and forth across the lipid bilayer, thus the formed proteins are called "beta-barrels" (Haake and Zückert, 2015).

As previously noted, OmpL1 was the first transmembrane OMP that was discovered for *Leptospira* with a series of porin-like transmembrane using surface immunoprecipitation method (Haake *et al.*, 1991; Haake and Champion, *et al.*, 1993). Further work on this demonstrated that OmpL1 not only form a porin channel but also possess other typical porin characteristics such as heat-modifiable electrophoretic mobility, cross-linkable trimers and ability to form a channel in bilayer assay (Shang *et al.*, 1995). After successfully identifying the first *Leptospira* transmembrane OMP, investigators have successfully identified several other OMPs from the most widely studied genomospecies *L. interrogans*. However, little has been done in other pathogenic genomospecies, such as *L. borgpetersenii* which may have diverse OMPs that are different from *L. interrogans*. Hence, the following work was primarily directed towards exploring other potential leptospiral OMPs specifically focusing on those considered unique to *L. borgpetersenii* and used a mixture of advanced bioinformatics tools, which will be discussed in the next section.

3.1.3 Identification of surface-exposed proteins through *in silico* analysis

The first goal towards identifying the potential vaccine candidates as part of a reverse vaccinology approach is to identify all surface-exposed proteins encoded in the bacterial genome via bioinformatics analysis. The initial screening is aimed to reduce the number of targets genes from thousands to hundreds, and screening using specific *in vitro* analyses will further reduce the number of vaccine candidates that will undergo laboratory testing (Grassmann *et al.*, 2017).

For vaccine target such as transmembrane OMPs, the first step to identify such encoded proteins in a bacterial genome is to determine the presence of amino-terminal signal peptides by sequence analysis. This short string of amino acids containing 20-25 amino acid residues is synthesized from an mRNA-ribosome complex together with the proteins of which

after they are later released and facilitate the directing of these newly synthesised proteins to their destination, mainly within the cellular membrane. The transmembrane OMPs are transported across the inner membrane to the periplasm by the Sec translocase complex where the signal peptides will be cleaved off by signal peptidase I. The remaining mature protein are then shuttled across the periplasm to the OM by chaperone SurA (Sklar *et al.*, 2007). The importance of the ability to recognise signal peptides has prompted the development of several computational methods for identifying and differentiating signal peptides from non-signal peptides and for determining the signal cleavage sites. These programs were designed based on protein annotations from public databases and had a range of accuracies (Zhang and Henzel, 2009).

After the genes with signal peptides are identified, the second step is to further identify and clarify the locations of where the proteins (encoded from these genes) would be delivered. Gram-negative bacteria possess five different major subcellular localisation sites which include the cytoplasm, the inner membrane, the outer membrane, the periplasm and the extracellular space. By predicting the location of these proteins, it may provide an insight into their biological roles within the membrane (Jensen *et al.*, 2002). A number of bacterial subcellular localisation programs (BSL) have been developed for this purpose, such as Subcellular Localisation Prediction Tool (PSORTb) (Gardy *et al.*, 2005), and Subcellular Localisation Predictive System (CELLO) (Yu *et al.,* 2004) which will be explained further in discussion section.

The use of BSL tools, however, have typically been somewhat limited as they only predict the possible location of genes within the membrane, but cannot predict the protein topology (Gardy *et al.*, 2005). Therefore, to increase a chance of finding the transmembrane OMPs within the membrane, it is crucial to expand the search by including algorithms that specifically predict for β-barrel proteins. Multiple algorithm servers are available online and have been developed and improvised over time for maximum performance. Some of the popular β-barrel tools with high accuracy of prediction have been suggested in the literature across Gram-negative bacteria including β-barrel Outer Membrane protein Predictor (BOMP), Prediction of Transmembrane Beta-Barrel Proteins (PRED-TMBB) (Bagos *et al.*, 2004b), Markov Chain Model for Beta Barrels (MCMBB) (Bagos *et al.*, 2004b; Bagos *et al.,* 2004c; Berven *et al.*, 2004) which will be discussed in detail in both methodology and discussion sections.

Domain identification prediction is a useful method to gain information with regards to protein's family/domain and annotations (Mulder and Apweiler, 2007; Punta *et al.*, 2012) and is recommended to be used alongside other protein prediction tools. Tools such as Pfam (Finn *et al.*, 2016) and Interpro (Finn *et al.*, 2017) are two of the most popular search engines for the prediction of protein family and structural domains for this purpose. Moreover, adding this method as a part bioinformatics framework in reverse vaccinology pipeline will not only provide valuable information on the protein's function, this also will increase confidence that the selected genes could probably be the true targets as vaccine candidates.

For a past decade, bioinformatics is a useful platform for identifying and selecting desired antigens from whole genomes based on a reverse vaccinology approach in leptospirosis research (Yang *et al.*, 2006; Dellagostin *et al.*, 2017). Antigen selection strategy is based on the multiple user-friendly web tools, publicly available to predict a specific signature of genome sequences. With the increasing number of prediction tools available, one can do multiple analyses and compare the outcomes from one tool to one another, and therefore may increase confidence to select the most likely genes of interest based on desired criteria. Given the dearth of available information on *L. borgpetersenii* OMPs, here we attempt to carry out the first stage of a reverse vaccinology pathway, by which to identify unique β-barrel encoding genes from entire genomes for subsequent analyses.

3.2 Materials and Method

3.2.1 Selection criteria for *L. borgpetersenii* OMP encoding genes to be used in expression studies

The selection for *L. borgpetersenii* OMP candidate genes was based on the several criteria of *L. interrogans* transmembrane OMP in a study by Pinne and Haake, (2009). The selected criteria including; 1) Presence of signal peptides cleavage sites in protein without lipoprotein signal peptidase, 2) Absence of inner membrane α-helices, 3) Prediction of at least six membrane-spanning β-strands using the β-barrel prediction tools. Additionally, 4) The prediction of cellular localisation sites of selected OMP encoding genes were added into the list of criteria to further verifying the protein location on the leptospires outer membrane.

3.2.2 Identification of genes encoding putative β-barrel of *L. borgpetersenii* L550

Two complete *L. borgpetersenii* serovar Hardjobovis strain L550 (with accession numbers chromosome 1 (NC_008508) and chromosome 2 (NC_0085509) and JB197 genomes with accession numbers chromosome 1 (NC_008510) and chromosome 2: (NC_008511) were extracted from National Center for Biotechnology Information (NCBI) database (Bulach *et al.*, 2006). Genome annotations of each strain were submitted to Rapid Annotation using Subsystem Technology (RAST) available online (http://rast.nmpdr.org/rast.cgi) (Aziz *et al.*, 2008).

The following algorithm programs were employed to predict the genes within the outer membrane; SignalP version 3.0 to discriminate between signal peptides of secretory proteins and transmembrane regions (Petersen *et al.*, 2011), TMHMM version 2.0 for the prediction of transmembrane helices (Krogh *et al.*, 2001), PSORTb version 3.0 and CELLO version 2.5 for the prediction of subcellular localisation (Yu *et al.*, 2006, 2010). The probable β-barrel proteins were predicted by using three β-barrel prediction programs: PRED-TMBB (Bagos *et al.*, 2004c), BOMP (Berven *et al.*, 2004) and MCMBB (Bagos *et al.,* 2004b). Lastly, Pfam, Interpro and BLASTp were used to identify the putative function of protein's domain (Altschul *et al.*, 1990; Finn *et al.*, 2016, 2017). The list of selected bioinformatics tools is shown in Table 3.1 and summary of reverse vaccinology-based bioinformatics workflow for the identification of novel OMP is shown in Figure 3.1.

Table 3.1 : List of algorithm programs used for the selection of L. borgpetersenii transmembrane OMP candidates.

***In silico* predictor**	**Server**	**URL**	**References**
Signal peptide prediction	SignalP v 3.0	http://www.cbs.dtu.dk/services/SignalP3.0/	(Petersen *et al.*, 2011)
Prediction of bacterial protein subcellular localisation	PSORTb v 3.0 CELLO v 2.5	http://www.psort.org/psortb http://cello.life.nctu.edu.tw/	(Yu *et al.,* 2004; Yu *et al.*, 2010)
Prediction of transmembrane Helices	TMHMM v 2.0	http://www.cbs.dtu.dk/services/TMHMM/	(Krogh *et al.*, 2001)
β- barrel OMP prediction	PRED-TMBB BOMP MCMBB	http://bioinformatics.biol.uoa.gr/PREDTMBB/ http://services.cbu.uib.no/tools/bomp http://athina.biol.uoa.gr/bioinformatics/mcmbb/	(Bagos *et al.*, 2004b; Bagos *et al.,* 2004c; Berven *et al.*, 2004)
Domain identification	Pfam Interpro BLASTp	https://pfam.xfam.org/ https://www.ebi.ac.uk/interpro/ https://blast.ncbi.nlm.nih.gov	(Altschul *et al.*, 1990; Finn *et al.*, 2016, 2017)

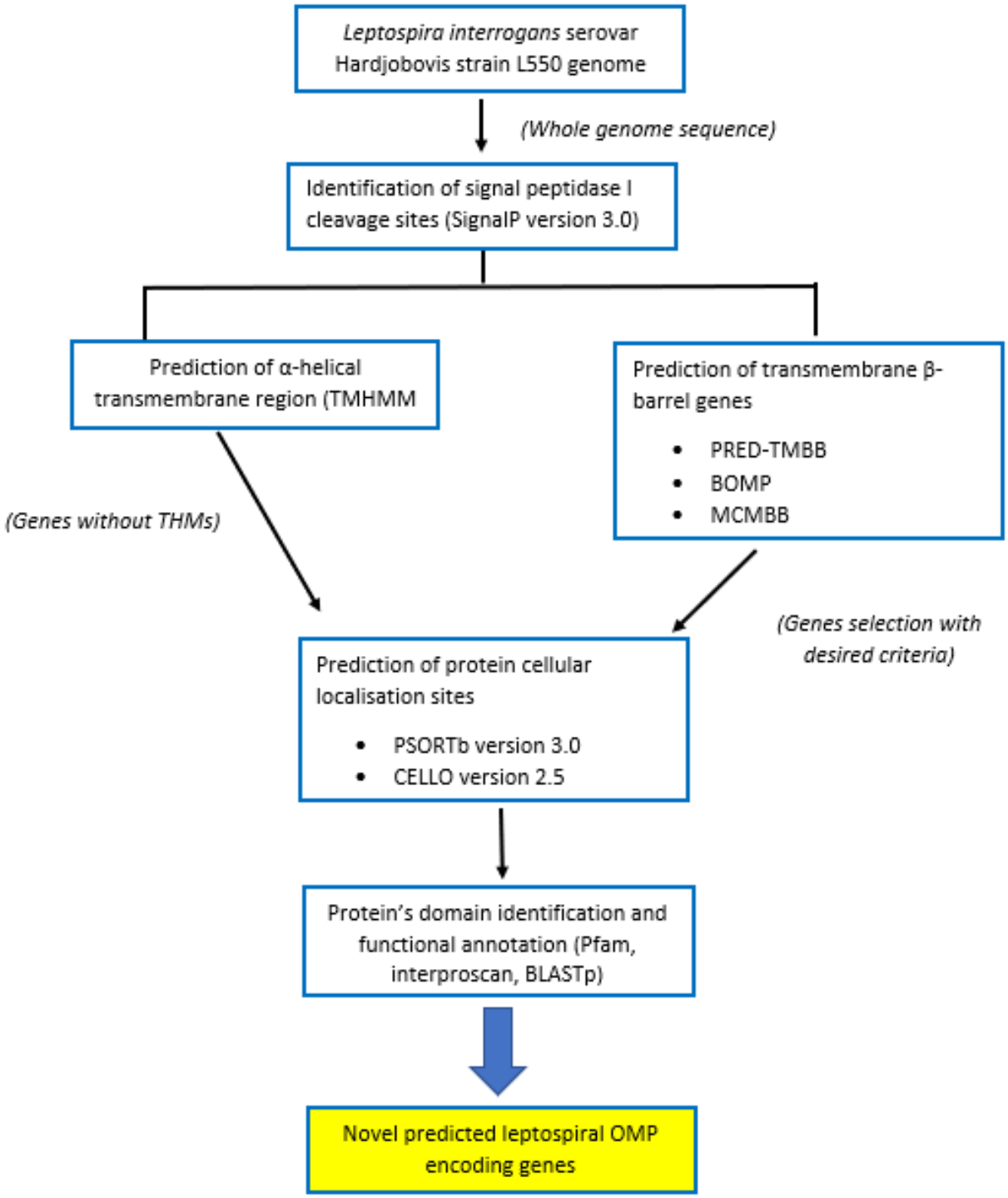


Figure 3.1: Summary of reverse vaccinology-based bioinformatics workflow for the identification of novel Leptospira borgpetersenii serovar Hardjobovis L550 OMPs in this study.

3.3 Results

3.3.1 *Leptospira* genome annotation

A downloaded version of Artemis genome browser and annotation tool (Wellcome Trust Sanger Institute, Hinxton, UK) was used for visualising, browsing and analysing *Leptospira* sequence data directly from the GeneBank. Each strain consists of two chromosomes; large chromosomes (CI) of about ~3.6 kbp and one small chromosome (CII) of about 0.3 kbp. Based on RAST annotation outcomes, both strains appear to have similar number coding regions on their genomes that codes for proteins. Interestingly, when comparing with other *Leptospira* pathogenic reference strain, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (Accession numbers: NC_005823 and NC_005824) and non-pathogenic species, *L. biflexa* serovar Patoc strain Patoc 1 (Ames) (Accession numbers CP_000777 and CP_000778), *L. borgpetersenii* has a genetic size reduction of about ~700 kb than that of *L. interrogans* species as reported by Murray *et al.* (2013). The coding sequence (CDS) of both *L. borgpetersenii* strains have also reduced by 5-7% compared with the pathogenic *interrogans* species. The features of various *Leptospira* species are tabulated in Table 3.2.

3.3.2 Outcomes from the prediction tools

Through bioinformatics analysis as described in the methodology section, from whole *L. borgpetersenii* serovar Hardjobovis (L550 and JB197) genomes, 104 genes were predicted to have signal peptide cleavage sites (signal peptidase I), and these genes were screened for all OMP criteria with relevant prediction tools as described in Section 3.2.1 and 3.2.2. Due to close identical genetic content of both *L. borgpetersenii* strains, only the L550 genome was selected in this study and subsequently throughout this thesis. The list of L550 genes with predicted signal peptide cleavage site I and OMP features can be found in Appendix A of the thesis. Overall, 94 genes were predicted without inner membrane α-helices via TMHMM. The total of 45, eight and five genes were predicted as β-barrel OMP from PRED-TMBB, BOMP and MCMBB respectively. A total of 48 and 33 genes were predicted as proteins located at the cellular membrane by PSORTb and CELLO, respectively.

From the list of genes outputted, 17 genes were identified as present in *L. borgpetersenii* and absent from *L. interrogans* (Table 3.3). A total of five genes, were specifically selected as a final choice based on;1) Having predicted β-barrels by at least one of the three β-barrel predictor programs, 2) Encoding a calculated protein molecular weight between 20-65 kDa, and 3) Containing an OMP domain that has not yet been characterised. As OmpL1 from *L. interrogans* species was known to be recognised by the respective hosts (human and canine) immune systems (Guerreiro *et al.*, 2001; Dong *et al.*, 2008; Fernandes *et al.*, 2012; Subathra *et al.,* 2013), here we included the OmpL1 gene from *L. borgpetersenii* L550, to evaluate any relevant host immune response in cattle milk and serum samples (Chapter 5 and 6). Additionally, OmpL1 (LIC_10973) from *L. interrogans* serovar Copenhageni Fioncruz strain L1-130 was selected as an OMP positive control for biochemical function in this study (Shang *et al.*, 1995; Fernandes *et al.*, 2012). The final L550 genome selection with their prediction outcome is shown in Table 3.4.

Table 3.2: The features of several leptospiral strains from RAST annotation outcomes.

Strain	Genebank accession	Size (bp)	GC content (%)	No of CDS
***L. borgpetersenii* serovar Hardjobovis strain L550**				
L550 (CI)	NC_008508	3,614,446	40.2	3966
L550 (CII)	NC_008509	317,336	40.2	362
***L. borgpetersenii* serovar Hardjobovis strain JB197**				
JB197 (CI)	NC_008510	3,576,473	40.2	3905
JB197 (CII)	NC_008511	299,762	40.4	335
***L. interrogans* serovar Copenhageni Fiocruz strain L1-130**				
L1-130 (CI)	NC_005823	4,277,185	35.0	4117
L1-130 (CII)	NC_005824	350,081	35.0	329
***L. biflexa* serovar Patoc strain Patoc 1 (Ames)**				
Patoc 1 (CI)	CP_000777	3,603,977	38.9	3556
Patoc 1 (CII)	CP_000778	277,995	39.3	284

CI and CII: Chromosomes I and Chromosomes II; CDS: Coding sequence; GC: Guanine-cytosine content (%)

Table 3.3: List of unique L. borgpetersenii serovar Hardjobovis L550 genes and their domain annotations comparing to L. interrogans.

Locus Tag	Mol Size	Pfam	InterProScan	BLAST	Characterised?	Homologues to *L. interrogans*[a]
LBL_0238	29.5	None Predicted	None Predicted	Hypothetical protein	No	-
LBL_0353	20.0	None Predicted	None Predicted	Hypothetical protein	No	-
LBL_0375	39.1	None Predicted	None Predicted	Hypothetical protein	No	
LBL_0972	37.5	DUF	Pectin lyase fold	Hypothetical protein	No	-
LBL_0976	37.3	Phenol-MetA	MetA-pathway phenol degradation	Hypothetical protein	No	-
LBL_1054	54.3	None Predicted	None Predicted	Hypothetical protein	No	
LBL_1341	63.0	None Predicted	None Predicted	Hypothetical protein	No	-
LBL_1344	31.9	None Predicted	None Predicted	Hypothetical protein	No	-
LBL_1930	58.4	Bacterial surface	Bacterial surface	Peptide binding protein	Yes	-
LBL_2155	26.9	OMP lolA-like	OMP lolA-like	OMP (*L. interrogans*)	No	-
LBL_2510	33.7	Porin OmpL1	Porin OmpL1	OMPL1	Yes	LIC_10973*
LBL_2618	51.6	DUF	None Predicted	Hypothetical protein	No	-
LBL_2800	13.0	Fibronectin-binding protein	None Predicted	Fibronectin-binding protein	Yes	-
LBL_2925	20.7	OMPA	OMP A Family Protein	OMP A Family Protein	No	-

Selected genes are highlighted in the table. (-) denotes as not known

Abbreviations: Pfam: Protein families, InterProScan: Protein sequence analysis and classification, BLAST: Basic local alignment search tool, DUF: Domain of an unknown function, OMP: Outer membrane protein

[a]Proteins shared the same homologues with L. interrogans but included in the research

**Positive control included in the study*

Table 3.4: List of selected L550 OMP encoding genes in respect to desired molecular weight, presence of signal peptides, β-barrel prediction scores and number of predicted α-helices within the genes.

Locus Tag	Molecular weight	Signal peptide	β-barrel prediction score			Number of predicted α-helices (TMH)	
	Size (kDa)	Cleavage site	BOMP[a]	PRED-TMBB[b] (Yes/No) with scores		MCMBB[c]	TMHMM[d]
LBL_2510 (OmpL1)	31	LSA-KS	0	Yes	2.91	0.024	0
LBL_1341	61	IQA-QL	1	Yes	2.94	0.015	1
LBL_0972	36	AGA-ND	0	Yes	2.88	0.024	0
LBL_1054	50	THA-EQ	0	Yes	2.95	0.025	0
LBL_2618	49	SQA-ER	0	No	3.02	0.008	0
LBL_2925	20	SSA-EK	0	Yes	2.94	0.00	0
LBL_0375	37	LVA-QE	0	Yes	2.92	0.016	0
LIC_10973* (OmpL1)	31	LSA-KT	1	Yes	2.90	0.024	1

[a] BOMP: The output format for proteins predicted to be integral OMPs are classified 0 to 5 where 0 means that the predictor did not find the protein to be an integral OMP whilst 1 to 5 is for proteins predicted to be integral outer membrane proteins, where 1 is the least reliable prediction, and 5 is the most reliable.

[b] PRED-TMBB: The output format for proteins predicted to be transmembrane OMPs are based on the threshold level. Threshold level below than 3.00 indicates that the protein is likely to be β-barrel.

[c] MCMBB: The output format is based on a 1st order Markov Chain model, which captures the alternating pattern of hydrophilic-hydrophobic residues occurring in the membrane-spanning β-strands of β-barrel OMPs. A score higher than 0, indicates that the protein is more likely to be a beta-barrel OMP, whereas a score lower than 0, indicates that the protein is probably not a β-barrel.

[d] TMHMM: The format output predicted the number of transmembrane helices (TMH) within a protein sequence

** Positive control used in this study.*

LBL_2618, which scored higher than a fixed score threshold of 2.965, indicating that the sequence does not belong to an outer membrane protein (Bagos *et al.*, 2004c). However, BOMP predictor did not identify the majority of selected sequences to be being integral outer membrane proteins except two proteins, LBL_1341 and LBL_1054. MCMBB predictor showed all but one of the proteins have a score of at least 0, indicating the proteins are most likely to be β-barrels. Only two proteins (LBL_1341 and LIC_10973) contain a THM are included in the list of selected sequences due to a strong correlation of being outer membrane proteins as predicted on β-barrel programs.

BLS tools further validate the localisation site of each protein (Table 3.5). CELLO predicted that most of the proteins are located either extracellular or on the outer membrane with the respective scores. PSORTb however, was unable to predict four proteins (LBL_2510, LBL_2925, LBL_0375 and LIC_10973), although other proteins share similar predictions as CELLO. The unknown prediction is due to the multiple localisation sites share the same distribution scores, or two sites have about similar high scores indicating a particular protein may have multiple localisation sites within the bacterial cell membrane (Gardy *et al.,* 2005). Each protein was then submitted to domain identification tools (Pfam, Interproscan, and BLAST) to further identify the domain of their functional annotations. Based on the findings, three proteins are predicted to be OMPs or have an OMP domain. Most of the proteins are hypotheticals and not characterised, and some proteins are classified in the domain of the unknown function (DUF). The finding from each protein domain is listed in Table 3.6.

Table 3.5: Outcomes from CELLO and PSORTb 3.0 predicting protein's localisation site from selected proteins in this study.

Locus tag	CELLO		PSORTb v 3.0	
	Localisation site	**Score**	**Localisation site**	**Score**
LBL_2510	Extracellular	2.640	Unknown	-
LBL_1341	Outer membrane	3.933	Outer membrane	9.49
LBL_0972	Extracellular	4.557	Extracellular	9.65
LBL_1054	Outer membrane	4.633	Outer membrane	9.49
LBL_2618	Extracellular	3.774	Extracellular	9.64
LBL_2925	Periplasmic	3.606	Unknown	-
LBL_0375	Extracellular	2.732	Unknown	-
LIC_10973*	Extracellular	2.759	Unknown	-

** Positive control used in this stu*dy, *(-) denotes as no score*

Each localisation site shows the highest score from multiple membrane component, thus indicating the most probable position within the leptospiral cell membrane.

Table 3.6: Domain identification results from selected L550 OMP genes.

Locus tag	Pfam	Interproscan	Blast-p	Nearest functional *Leptospira* homologues		References
				Identity (%)	E-value	
LBL_2510 (OmpL1)	Porin	Porin OmpL1	OmpL1	100	0.0	(Haake and Matsunaga, 2002)
LBL_1341	NP	NP	HP	97	0.0	-
LBL_0972	DUF 1565	Pectin lyase fold	HP	96	0.0	(Jenkins, Mayans and Pickersgill, 1998)
LBL_1054	NP	NP	HP	94	0.0	-
LBL_2618	DUF 1566	NP	Adhesin	77	0.0	Unpublished
LBL_2925	OmpA	OmpA Family Protein	OmpA Family Protein	100	5e-139	(Mot *et al.*, 1992)
LBL_0375	NP	Immunoglobulin-like fold	HP	99	0.0	(Potapov *et al.*, 2004)
LIC_10973* (OmpL1)	Porin	Porin OmpL1	OmpL1	100	0.0	(Haake and Matsunaga, 2002)

**Positive control used in this study*

The identity percentage is the measurement of the similarity between L550 and other Leptospira homologues on BLAST. Expect (E) value is a BLAST parameter that describes the number of hits that can be expected when searching a database of a particular size. DUF: Domain of unknown function, HP: Hypothetical protein, NP: None predicted, OMP: Outer membrane protein (-) denotes as not known

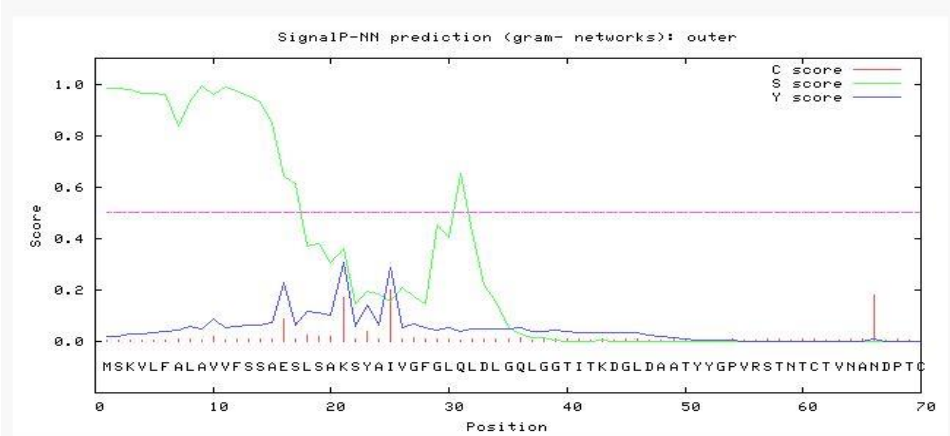
3.4 Discussion

Before the arrival of the genomic era, the outer membrane proteins were identified through subcellular fractionation. The process was highly complex, involving several steps to separate the largely insoluble components of the outer membrane (Smither *et al.*, 2007). Subcellular fractionation has successfully discriminated several proteins of the OM from the IM for many Gram-negative bacteria including some leptospiral studies (Zuerner *et al.*, 1991; Haake and Matsunaga, 2002; Nally *et al.*, 2005). However, such fractionation of whole cell bacteria has been reported to ineffectively recognise the fraction of leptospiral transmembrane OMPs such as the channel-forming porin and therefore is not a comprehensive suitable method for the studies of spirochete OMPs (Pinne and Haake, 2009). Such limitations could explain why many other leptospiral OMPs were overlooked and therefore unreported, likely due to their poor solubility, low abundance and inability to be easily analysed and visualised by SDS-PAGE.

The genomic era began in the early 2000s where whole genome sequencing finally became available for the identification of vaccine candidates using the reverse vaccinology strategy. The presence of available sequence data in the public databases has driven the focus towards *in silico* antigen predictions. *In silico* prediction of antigens for vaccine candidates is determined by screening against various bioinformatics tools that can identify specific signatures of the genes associated with surface-exposed or secreted proteins. The method is broadly applied to a wide range of pathogens and indirectly discovering novel antigens that were not characterised previously (Talukdar *et al.*, 2014; Meunier *et al.*, 2016). A similar method had been applied to many *L. interrogans* studies (Gamberini *et al.*, 2005; Pinne and Haake, 2009; Murray *et al.*, 2013) and which has led to the discovery of several new OMPs for further functional and immunological evaluations.

In this study, we applied *in silico* identification methods to determine putative OMPs in a bovine species *Leptospira borgpetersenii* serovar Hardjobovis L550. The first step towards the identification of OMPs is to identify the signal peptide that is targeted to the secretory pathway in prokaryote cells and identify their corresponding cleavage sites. There are many computational signal peptide prediction algorithms available to identify these, such as PrediSi (Jahn *et al.*, 2004), Phobius (Käll *et al.,* 2004), SPEPlip (Fariselli *et al.,* 2003) and SignalP which have reported different prediction performances. We chose SignalP version 3.0 as recommended by Petersen *et al.* (2011), which has been reported as the best performing method compared with similar algorithms of discriminatory function. SignalP version 3.0 is a

web-based tool based on an artificial neural network method, and the output scores are determined within the position in the input sequence; raw cleavage site score (C-score), signal peptide score (S-score) and combined cleavage site score (Y-score). The overall discrimination score between signal peptides and non-signal peptides is calculated by a weighted average of the mean S and maximal Y score, and this will determine the most likely cleavage site within an amino acid sequence. The outcome of each sequence is supplemented in a graphical form. Figure 3.2 shows the three different scores (C, S and Y) for each position in the sequence.




```
# data


>outer                 length = 70
# Measure  Position  Value  Cutoff  signal peptide?
  max. C    25       0.197   0.52   NO
  max. Y    21       0.306   0.33   NO
  max. S     9       0.991   0.92   YES
  mean S     1-20    0.828   0.49   YES
       D     1-20    0.567   0.44   YES
# Most likely cleavage site between pos. 20 and 21: LSA-KS
```

Figure 3.2: SignalP version 3.0 prediction output.

Signal peptide prediction output by the neural network method is determined by the discrimination scores of raw cleavage site score (C-score), signal peptide (S-score) and combined cleavage site score (Y-score). The final discrimination score (Average mean S and maximal Y score) will determine the most likely cleavage site position within an amino acid.

PRED-TMBB is a web-server based on a Hidden Markov Model method that is capable of predicting the topology and discriminating beta-barrel OMPs from alpha-helical proteins (Bagos *et al.*, 2004a,c). The program is trained to maximise the probability of the correct prediction of transmembrane proteins using 16 of known OMP's that were previously characterised. The program consists of several states connected using the transition probabilities. The decoding method gives the prediction of the transmembrane strands in both statistical and 2D graphical output. The example of PRED-TMBB output is shown in Figure 3.3. In this study, we generated a list of potential OMPs genes with more than eight transmembrane strands and comparing these genes using other beta-barrel prediction programs. Using this collection of software, we selected 45 out of 104 proteins from *L. borgpetersenii* serovar Hardjobovis L550 with a signal peptide that demonstrates at least eight strands within the outer membrane. PRED-TMBB has been shown as a useful tool to predict the beta-barrel OMPs across the Gram-negative bacteria including spirochetes such as *Treponema* spp. (Desrosiers *et al.*, 2012; Staton, 2018) and *Borrelia* spp. (Dyer *et al.*, 2015).

The BOMP program allows prediction of integral β-barrel OMP from a polypeptide sequence from Gram-negative bacteria. This method is based on two independent methods; C-terminal recognition on the last ten amino acids and the second method is based on the integral β-barrel score calculated from the abundance of amino acids that highly match the membrane-spanning arrangement of transmembrane β-strands. The outcome of BOMP is classified from number 1 to 5 category, and higher category indicates the probability that the query sequences are being β-barrel proteins (Berven *et al.*, 2004). The accuracy of the prediction was 80% with a recall of 88% when tested in *Escherichia coli* K 12 and *Salmonella* Typhimurium (Berven *et al.*, 2004). BOMP has been widely applied in many of reverse vaccinology studies, including in some *Leptospira* studies (Grassmann *et al.*, 2017; Zeng *et al.*, 2017). In our observation, BOMP showed a direct hit of 8 out of 104 of proteins in L550 genome with the presence of a signal peptide in the preliminary list of OMP candidates.

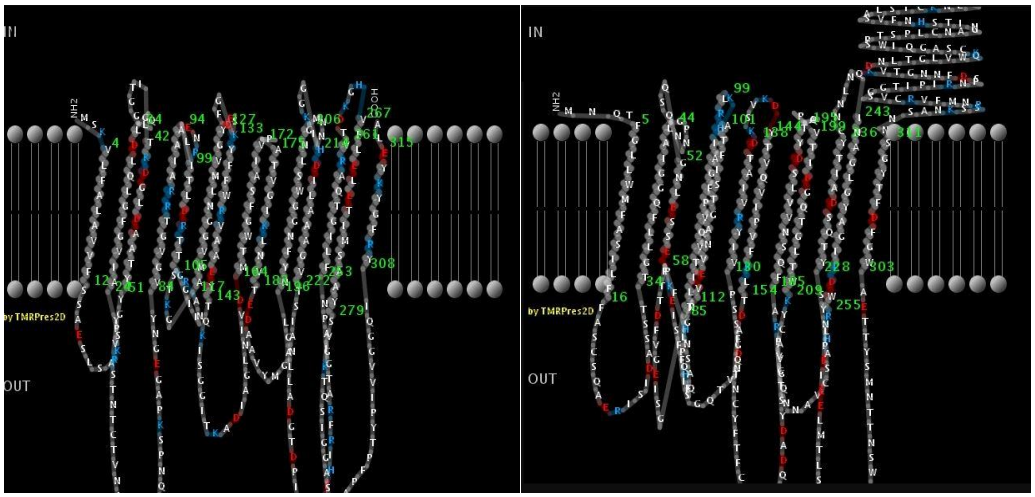


Figure 3.3 : The 2D representative hydrophobic models of two target genes.

A: LBL_2510, B: LBL_2618 of the putative leptospiral OMPs incorporated within the lipid bilayer predicted by PRED-TMBB.

MCMBB is a simple, readily available online algorithm which is able to discriminate β-barrel OMPs from globular protein and alpha-helical membrane proteins. The prediction is based on a 1st order Markov Chain model, which acquires the differences of hydrophilic-hydrophobic residues in the transmembrane β-strands of the outer membrane (Bagos *et al.,* 2004b). The outcome of MCMBB prediction is provided by Markov Chain calculation which is simplified by an overall score where greater than 0 indicates the probability that a protein in an OMP. MCMBB predicted 16 proteins across L550 genome which scored 0 or higher, to be included in our list of probable OMP.

To our knowledge, MCMBB is not widely used in spirochetes studies. However, it has been used in several OMPs studies in other Gram-negative bacteria with higher accuracy up to 90% for β-barrel proteins prediction (E-komon *et al.*, 2012; Samaniego-Barrón *et al.*, 2016). In a recent study, MCMBB was employed to identify leptospiral vaccine candidate and led to 26 new potential vaccine candidates in *L. interrogans* serovar Copenhageni Fiocruz L1-130 (Grassmann *et al.*, 2017). Thus, MCMBB appears practically useful as an OMP search tool and reverse vaccinology pipeline for spirochete studies.

In addition to this, we applied a transmembrane α-helix (THM) predictor to exclude proteins with α-helix topology. This step is crucial as to discriminate between the transmembrane and inner membrane protein location's as proteins with transmembrane α-helix tend to be localised to the inner membrane and potentially not surface-exposed. TMHMM software uses a Hidden Markov Model (HMM) to differentiate between soluble and membrane proteins with a high degree of accuracy (Krogh *et al.*, 2001). Using this software, we predicted 94 genes that contain at least a THM, and therefore, they are unlikely to be β-barrel proteins. We selected the remaining genes that are not predicted by TMHMM as to assume they contain β-barrels topology and compared with the rest of transmembrane β-barrel predictors.

The localisation of a protein within a bacteria gives essential information to understanding its possible interaction, function and help to identify drug and vaccine targets (Imai *et al.*, 2013). There is a selection of bacterial subcellular localisation prediction software that applies to Gram-negative bacteria such as PSORTb (Gardy *et al.*, 2003), CELLO (Yu *et al.,* 2004), SubcellPredict and HensBC (Bulashevska and Eils, 2006) and SLP-Local (Matsuda *et al.*, 2005). Among all of the software, we selected two programs based on the higher accuracy localisation prediction and are available as an online server.

We selected PSORTb and CELLO software to validate the localisation site of our selected OMP proteins, and we referred both programs to discriminate between the proteins located within the inner or outer membrane layer. The PSORT program utilises different computation techniques as initiated by Nakai and Kanehisa (1991) by analysing several sequence features to influence its location within the Gram-negative bacteria. The PSORTb works by combining six different analytical algorithm programs to generate an overall prediction of localisation site following an amino acid sequence. A score for each possible localisation sites is calculated, and a higher score indicates high confidence that the query protein is located in the subcellular location (Gardy *et al.*, 2003). During the study, we used the updated version of PSORTb (PSROTb version 3.0) with additional computational features to give a more precise prediction of about 80-95% on protein's specific localisation sites of most bacterial proteomes (Yu *et al.*, 2010).

Concurrently, we applied CELLO to compare the results with PSORTb. CELLO works by using support vector machines (SVM), and the coding scheme is determined based on the n-peptide composition of amino acid sequences to predict the protein's classification. The predictive performance is measured using Matthew's correlation coefficient (MCC), and the

perfect prediction is donated with a score of one and zero for a variable protein's prediction (Yu *et al.,* 2004; Yu *et al.*, 2006). The accurate prediction of CELLO is topped at 95%-97% in both Gram-positive and Gram-negative bacteria comparing to other bacterial subcellular localisation prediction software (Yu *et al.*, 2010).

Based on results in Table 3.5, CELLO and PSORTb shared similar predictions on the localisation sites of almost all the selected proteins and this show that both programs are reliable to use concurrently. Moreover, the likelihood of getting a similar prediction is higher and therefore, may increase confidence in the true identification of relevant outer membrane protein candidates of Gram-negative bacteria.

We improved our bioinformatics network by adding domain identification tools to analyse the uncharacterised and novel OMP sequences predicted from β-barrel and SCL tools. Pfam is a database source that contains a large collection of protein families database available via an online server (Finn *et al.*, 2014). Each Pfam family (referred to as Pfam-A) is generated from a seed alignment derived from a subset of matching sequences, and this will be used to construct a HMM profile. The HMM profile is then used to generate a full alignment that contains protein sequences belonging to the family that aligned to profile HMM from the primary sequence database (Finn *et al.*, 2016).

Interpro database is another useful source of protein domain identification software. The program provides protein classification with an annotation describing the domain/family (known as signatures) by integrating multiple protein databases in order to add information on the biological annotation and cross-references to diverse data sources (Mulder and Apweiler, 2007). The outcome allows the user to gain a better overview of the protein's putative function (Jones *et al.*, 2014; Finn *et al.*, 2017).

In addition to domain identification tools, we also analysed all our predicted OMP sequences using BLAST® search. Similar to both Pfam and Interpro, BLAST is a powerful bioinformatics search tool that enables comparison of any protein against a sequence library database, therefore, identifies sequences that resemble the query sequence (Altschul *et al.*, 1990). It also provides information on protein's annotation, locating known domains that exist in a large library database and identify if the query protein sequence has been previously characterised. Using these domain tools, several selected genes (LBL_2510, LBL_2925 and LIC_10973, LBL_0972 and LBL2618) belong to at least one protein domain, whereas two genes (LBL_1341 and LBL_1054) do not belong to any of the known protein domains.

Using both protein domain tools and BLAST search, we further narrowed down the list of selected probable OMPs into eight final candidates based on three criteria; i) Genes with uncharacterised function, ii) Has an OMP domain and iii) 'None predicted' domain but appear in at least in one of the BSL and β-barrel search tools.

Identification of OMP genes through *in silico* analysis has numerous advantages as it saves time and laboratory resources. While most studies thus far identifying *Leptospira* OMPs through this route are leaning toward *L. interrogans* (Gamberini *et al.*, 2005; Yang *et al.*, 2006; Yan *et al.*, 2010), less attention has been given to other pathogenic species. Given the abundance of genomic data now available, it is now possible to explore the potential virulence factors in other *Leptospira* species which can be identified using various bioinformatics tools based on RV. Furthermore, many *Leptospira* genome annotations have no known orthologues, prompting RV as a promising approach for the identification of novel immunogens that lead to the development of leptospire recombinant vaccines.

3.5 Conclusion

In silico analysis is the first and crucial step in reverse vaccinology pipeline in identifying potential genes from a genome. This method allows the discovery of both previously identified proteins and those uncharacterised genes with the aid of various bioinformatics tools. Identification of such targets may enable new targets for vaccine development or diagnostic components.

Using a whole genome sequence from a bovine strain reference *L. borgpetersenii* serovar Hardjobovis L550, 104 out of ~4000 genes were predicted to have signal peptide cleavage site, and the list was narrowed down using several prediction tools for identifying genes with desirable transmembrane β-barrel criteria and also the protein most likely localisation site within the bacterial outer membrane. A total of 14 genes were initially chosen using this process and finally, seven unique and uncharacterised L550 OMP genes selected (including two OmpL1 positive controls) that meet the designated criteria of this study. These genes are the key focus of this thesis. Next, the candidate genes will be subjected to cloning, expression and purification and further characterised to explore their function(s) and immunological roles.

Chapter 4: Overexpression, refolding and purification of putative leptospiral outer membrane proteins

4.1 Introduction

Recent advances in genomics, coupled with established molecular biology methods have enabled substantial protein characterisation studies, especially the analysis of structure and function. Using these complementary disciplines, it is now possible to easily generate large quantities of pure proteins as recombinants, which are a useful resource for such functional studies. The availability of pathogenic leptospire genomes deposited in sequence repositories enabled the selection of genes encoding unique *L. borgpetersenii* serovar Hardjobovis L550 OMPs in Chapter 3 which here, together with access to the respective culture collection reference strain, enables cloning, overexpression, refolding and purification of these proteins ready for characterisation.

4.1.1 Directional topoisomerase I cloning for protein expression

In order to study the encoded protein from a DNA sequence of interest, the first step is to apply a cloning method where the DNA sequence of interest from an organism is combined into an entry vector, which is genetically designed to propagate genes into high-number of identical copies from a single recombinant vector. Molecular cloning was first introduced in the early 1970s following the discovery of restriction enzymes that cuts DNA molecules at specific sites (Jackson *et al.,* 1972; Cohen *et al.*, 1973) and has since become a standard protocol for DNA manipulation to further understanding of gene function in a particular organism. Traditional cloning methods involve four basic steps; 1) Isolation of a target DNA fragment (insert), 2) Ligation of the insert into an appropriate cloning vector (plasmid), 3) Transformation of recombinant plasmids into a competent host for propagation and 4) Screening of hosts containing the positive recombinant gene. Whilst the restriction enzyme-based cloning has successfully enabled the cloning of many genes, it is laborious due to the selection of restriction sites and commercial enzyme availability (Chee and Chin, 2015).

Modern recombinant cloning technologies offer flexible, straightforward methods, in which the gene cloning can be done in as short as 5 minutes without the use of DNA ligases. Directional topoisomerase I (TOPO I) cloning (Appendix B Figure B.1), a recent cloning technique developed by Invitrogen (Thermo Fisher Scientific, Massachusetts, USA) utilises DNA topoisomerase I derived from Vaccinia virus, which serves dual functions; as restriction and ligase.

4.1.2 Bacterial transformation of recombinant gene

After an entry clone containing the recombinant gene is generated, the next step is to transform this clone into a competent cell in order to enable propagation of the clone/gene prior to creating an expression clone. Bacterial strains are the most common host used for this purpose due to their ability to take up exogenous DNA for replication using their DNA replication machinery. Several genetically modified, competent *E. coli* strains (commercially available) are used for this purpose. One Shot™ TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific, Massachusetts, USA) was selected to carry out the transformation steps in this study due to its stability in replication of high-copy number plasmids and higher transformation efficiency (1×10^9 cfu/µg) plasmid DNA, which are ideal for both maximum cloning efficiency and plasmid propagation (Ganoza *et al.*, 2006; Evangelista *et al.*, 2014). The entry of plasmid containing cloned DNA into the cell is facilitated via chemical transformation or electroporation in which the cell membrane is compromised by reduced membrane potential and formation of pores on the cell surface as a result of lipid detachment (Panja *et al.*, 2006, 2008).

The transformed cells are cultured in antibiotic-free medium for a short period to allow expression of antibiotic resistance genes acquired from the inserted plasmid. Plasmids are purified and ready to be shuttled to a destination vector to create an expression clone.

4.1.3 Gene reassembly by LR recombination reaction

For the gene of interest to be translated into protein, the gene needs to be subcloned into a destination vector that has all the necessary elements for expression. The process is catalysed using a specific enzyme that integrates the *attL* site from the entry clone and *attR* of the destination vector. This reaction will generate an expression vector, which will undergo another bacterial transformation (in *E. coli* TOP10 cells) similar to Section 4.1.2, to propagate more plasmid copies before being used for protein expression. Ideal destination vector for protein overexpression should contain the following elements; a replicon, a (T7) promoter, a

suitable selection (antibiotic) marker and an affinity tag for purification (Rosano and Ceccarelli, 2014). Gateway™ pDEST™17, is an expression vector available from Gateway Technologies (Thermo Fisher Scientific, Massachusetts, USA) was selected to express the leptospiral OMP genes in this study. The pDEST17 plasmid (Appendix B Figure B.2) includes a T7 promoter and N-terminal 6xHis-tag and is derived from pBR322, a low-copy plasmid which is able to maintain the stability of the plasmid and enables expression of toxic proteins (Lin-Chao and Bremer, 1986; Howe, 2007).

4.1.4 Recombinant protein expression using an *Escherichia coli* expression system

After an expression clone is created and purified, the construct is ready to be expressed in a compatible host carrying T7 RNA polymerase gene (tRNAs), which is controlled by a lac promoter. *E. coli* strains are the most widely used host for large scale protein expression owing to their rapid growth, ease of culture and low cost. The *E. coli* BL21 strain and its derivatives are the most popular choice of host and been routinely used in the laboratory for high-level expression and both T7 and non-T7 expression (Jeong *et al.,* 2015).

E. coli strain BL21-AI is a T7-based expression host that is designed to express toxic proteins under the control of the araBAD (arabinose) promoter. This has been shown to have low basal levels of expression enabling toxic protein expression and to promote strong expression of heterologous genes (Chen *et al.*, 2009; Yao *et al.*, 2009).

4.1.5 Inclusion bodies, refolding and protein purification

High-level expression of *E. coli* carrying heterologous genes often leads to protein aggregation, also known as inclusion bodies. When protein molecules aggregate as inclusion bodies, they are not biologically active. Hence, to restore their biological functions, several strategies are required to revert the protein aggregates to their native conformation form by solubilisation, refolding and purification. Solubilisation of inclusion bodies requires a high concentration of denaturants and chaotropic salts, such as guanidine HCl or urea which results in complete denaturation of protein structure and therefore increase the solubility of hydrophobic molecules (Robinson and Jencks, 1965). Furthermore, reducing agents such as DTT or 2-ME are often added into the solubilisation buffer for proteins containing cysteines residues (Cleland, 1964; Stevens *et al.,* 1983). Cysteines may form incorrect disulphide bonds due to random oxidation, which can interrupt the correct refolding formation and cause the protein to be misfolded (Burgess, 2009).

After solubilisation is completed, the next step is to refold the solubilised proteins to their native-like state using several methods, such as dialysis (Sørensen *et al.,* 2003), reverse dialysis (Vinogradov *et al.*, 2003), rapid and slow dilution (Tsumoto *et al.*, 2003). To refold membrane proteins, the refolding buffer is usually added with the presence of detergent to mimic the hydrophobic condition of the protein for the refolding process. Several classes of detergent are widely used for membrane protein extractions and LDAO (a zwitterionic detergent) was chosen as a detergent of choice in this research due to the relatively small size micelle size, able to maintain the protein neutral charge and low cost. Furthermore, LDAO was successfully used in some OMP crystallisation studies (Pautsch *et al.*, 1999; Pautsch and Schulz, 2000) including in spirochetal OMPs studies (Dyer, 2013; Staton, 2018). A drip (rapid) dilution refolding technique was applied as it was used in many OMP studies (Ye *et al.*, 2013; Gessmann *et al.*, 2014; Staton, 2018). The advantage of the drip refold technique is it minimises protein misfolding and precipitation (Cabrita *et al.,* 2004).

Dialysis is used after the refolding step to remove denaturants. A standard protocol for refolded protein dialysis is between 16-24 hours and recommended dialysate buffer is 100:1 to samples volume ratio (Graewert, 2016) and should enable a clean protein solution before final purification step.

Affinity chromatography purification is the process by which a protein of interest (typically fused to a protein tag) is separated from other proteins (without the tag) based on their affinity for specific molecules, which the column matrix is consists of, and results in the protein being retained by the column. Commonly used fused protein tags for recombinant proteins are polyhistidine (His) and glutathione-S-transferase (GST) containing peptide sequences. In this study, pDEST17 expression vector was selected, which consists of six histidine residues (6XHis) that allows purification of recombinant tagged protein using a nickel-chelating resin such as Ni-NTA in denaturing conditions. This pre-charged resin is able to bind up to 50 mg of recombinant protein per 1 ml of resin slurry. Immobilised metal ion affinity chromatography (IMAC) is a purification method for His-tagged recombinant protein, which separates chelating compounds on a column to trap metal ions to which the target protein is adsorbed. Following adsorption, the bound proteins are released by the action of protonation, ligand exchange or extraction of the metal ions using a strong chelating agent such as EDTA or proton pump effect by imidazole (Gaberc-porekar and Menart, 2001). The eluted protein solution should contain highly purified protein and can be visualised by SDS-PAGE and western blotting and can now be subjected to a range of characterisations.

4.1.6 Determination of the secondary structure of the recombinant OMP

Circular dichroism (CD) spectroscopy is a routine method for determining the folding, conformational changes and the secondary structure of proteins (Kelly, Jess and Price, 2005). CD spectroscopy has been used for many years firstly on soluble proteins and later was subsequently refined to analyse membrane proteins as well (Wallace and Mao, 1984; Sreerama and Woody, 2004). This method measures differences in absorption of left-handed circularly polarised light (L-CPL) and right-handed (R-CPL) polarised light by molecules which contain a chiral centre or have a three-dimensional structure that provides a chiral background (Miles and Wallace, 2016). In this study, the protein secondary structure information was derived from CD signals in the far ultraviolet (UV) wavelength region between ~260 to 190 nm which can be used to predict the percentage of each structural element in the membrane proteins structures. CD spec has been widely used to analyse bacterial OMP secondary structures, and the spectra can be processed using analysis online software such as Beta Structure Selection (BeStSel) and CD analysis and Plotting Tool (CAPITO) (Wiedemann *et al.,* 2013; Micsonai *et al.*, 2018).

In this chapter, we aim to clone the genes identified as encoding putative leptospiral outer membrane proteins from Chapter 3 of this thesis and subsequently overexpress, refold, purify the encoded proteins which together with preliminary structural analyses enable subsequent studies to dissect their role in disease pathogenesis.

4.2 Materials and methods

Full description for gene cloning, expression and protein purification including determination of protein secondary structure are detailed in Chapter 2 (Section 2.12-2.25).

4.3 Results

4.3.1 Validation of PCR primers for leptospiral OMP genes

The designed PCR primers for all selected leptospiral OMP genes were shown to be successful (Figure 4.1) in that bands of predicted size resulted.

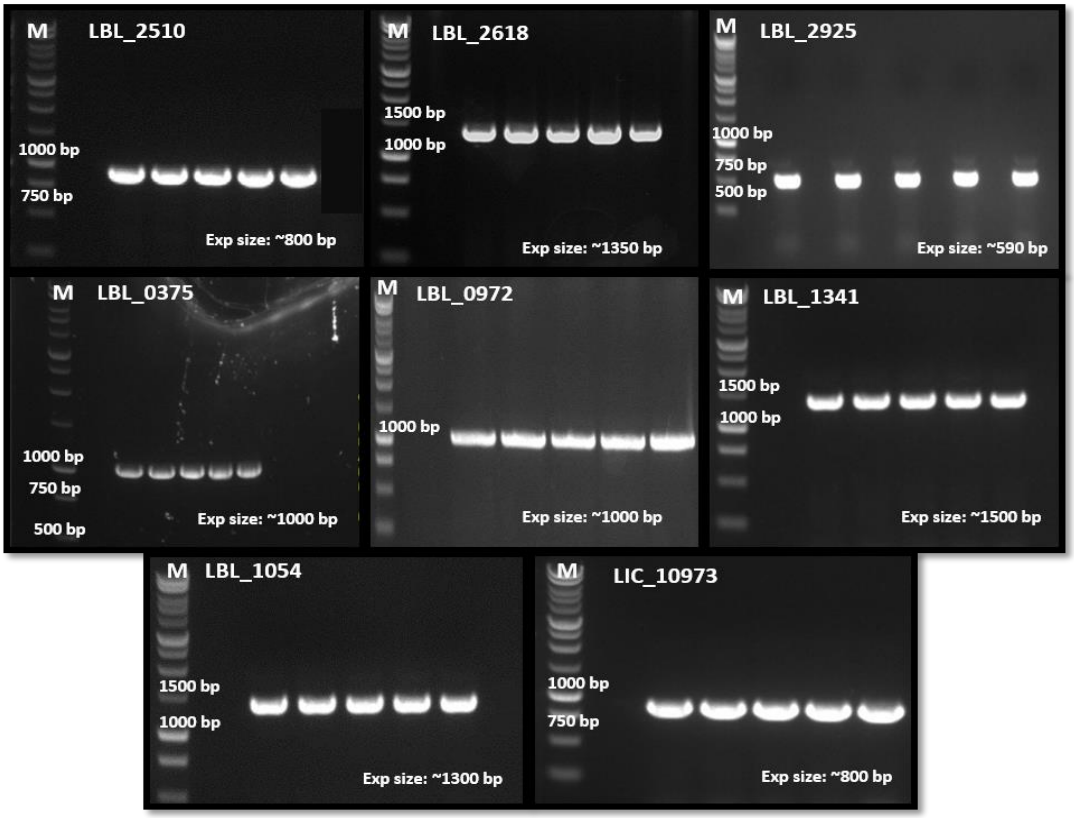


Figure 4.1: PCR amplification product bands on 1 % (w/v) agarose gels viewed under the UV light.

The Phusion Taq PCR of each of the leptospiral OMP gene and positive control (LIC_10973) and their expectant sizes repeated in five replicates are denoted from A-H. The marker (M) sizes are 500, 750, 1000, 1500 bp upwards.

4.3.2 Cloning, transformation and restriction analysis of the OMP gene in the entry vector

All genes were successfully cloned in the pENTR vector and transformed into *E. coli* Top10 cells. Several *E. coli* colonies (from 2-50 colonies per plate) were analysed via Taq polymerase PCR using M13 forward primer to determine the cloning efficiency, with genes smaller than 1 kb exhibiting better cloning efficiency than larger size genes. Restriction analysis by enzyme digestion showed that nearly all DNA sequences contained recognition sites (*Not I* and *Not II*) as the ecoRV enzyme cuts the fragments in single or multiple sizes. Sequencing results further confirmed that the inserts were in-frame with the construct entry vector and no mutations detected. Results of the cloning, transformation and restriction analysis of pENTR are shown in Table 4.1.

Table 4.1: Cloning, transformation, restriction analysis and sequencing results of all the leptospiral candidate genes in pENTR with the positive control (LIC_10973).

Gene	Colonies per plate (pENTR transformation)	Colony PCR size (kbp)	Fragment sizes cut by EcoRV (kbp)	Mutation detection?
LBL_2510	>50	0.8	3.43	No
LBL_2618	~20	1.35	3.89	No
LBL_0972	>50	1.03	3.58	No
LBL_0375	~15	1.02	3.60	No
LBL_2925	<15	0.59	0.50, 2.56	No
LBL_1341	>30	1.57	0.20, 0.54, 2.97	No
LBL_1054	~10	1.39	3.90	No
LIC_10973	>50	0.8	3.50	No

Note that the smaller the gene size, the better cloning/transformation efficiency as observed in the number of colonies per plate.

4.3.3 Subcloning of leptospiral genes from entry vector into destination vector (pDEST17)

From a total of eight genes, only six genes were successfully subcloned in pDEST17 (via clonase reaction), transformed into *E. coli* TOP10 cells and subjected to restriction analysis by EcoR1 enzyme and followed by expression trial. Subcloning and transformation of the two unsuccessful genes were repeated twice; however, this repeatedly failed, and these genes were excluded from this experiment. Sequencing results for the remaining six proteins confirmed that the inserts had correct orientation within the destination vector and no mutations observed. Results of the cloning, transformation and restriction analysis of pDEST17 are shown in Table 4.2.

Table 4.2: Subcloning, transformation, restriction analysis and sequencing results of all the leptospiral candidate genes in pDEST17.

Gene	Colonies per plate (pDEST17 transformation)	Colony PCR size (kbp)	Fragment sizes cuts by EcoR1 (kbp)	Mutation detection?
LBL_2510	~10	0.8	7.24	No
LBL_2618	<10	1.35	0.11, 1.19. 4.93	No
LBL_0972	~30	1.03	0.21, 0.72, 5.07	No
LBL_0375	>100	1.02	6.00	No
LBL_2925	~20	0.59	6.30	No
LBL_1341*	-	1.57	-	-
LBL_1054*	-	1.39	-	-
LIC_10973	>50	0.8	5.87	No

**Two genes (LBL_1341 and LBL_1054) failed to be subcloned and transformed into the E. coli TOP10 cells, therefore excluded from this further experiment.*

4.3.4 *E. coli* BL21-AI strain transformation, protein expression, refolding and purification

All transformed genes were successfully transformed in *E. coli* BL21-AI and subjected to both pilot and large-scale expression, protein refolding and purification. All were successfully expressed as insoluble proteins, except LBL_2925 which failed to form inclusion bodies, most probably due to gene toxicity that killed the *E. coli* cells during expression. Therefore, this protein was excluded from further investigation. An example of an expression result of a protein is shown in Figure 4.2. In total, five proteins were successfully purified as recombinant proteins and were subjected to further investigations (Figure 4.3).

Due to the presence of an additional band shift of a different molecular size (~25 kDa) on both OmpL1s (Figure 4.3 A and E), an experiment was performed to see whether this protein is heat-modifiable and its ability to withstand vigorous treatments for denaturation as described previously by Shang *et al.* (1995) with a minor modification. Both recombinant OmpL1s samples were subjected to five different treatment; 1) unheated sample at 25°C, 2) heated samples at 100°C for 10 minutes with 10 mM DTT, 3) heated samples at 100°C (10 minutes) with 10 mM DTT and 8 M urea, 4) heated samples at 100°C (40 minutes) with 10 mM and lastly, 5) heated samples at 100°C (40 minutes) with 10 mM DTT and 8 M urea. An example of OmpL1 denaturation result is shown in Figure 4.4.

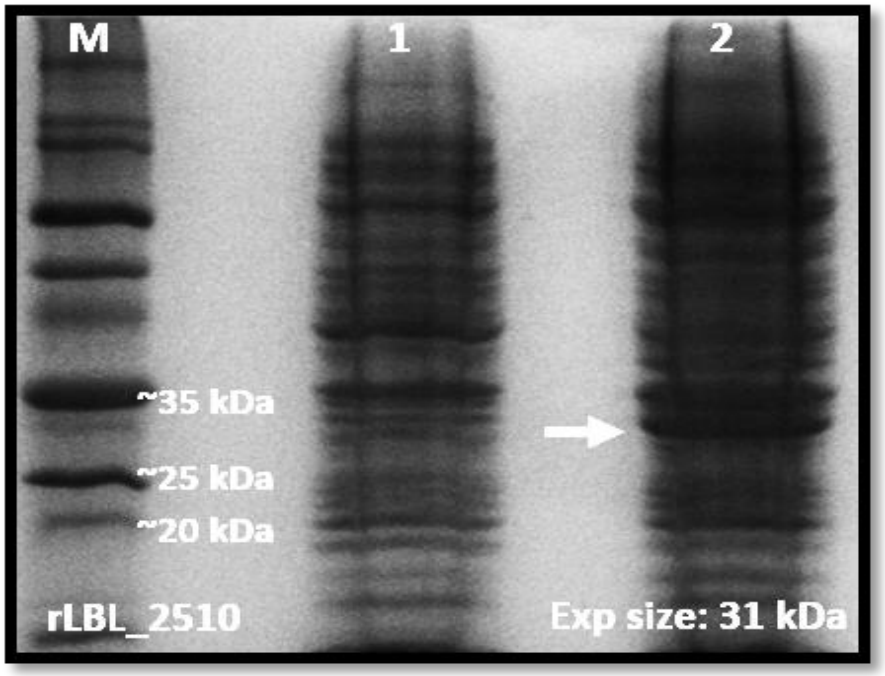


Figure 4.2: Example of a success expression of a leptospiral recombinant OMP (rLBL_2510) view on a 12% (v/v) SDS-PAGE gel.

Lane 1 marked as uninduced protein and lane 2 marked as expressed protein with a present of a clear, visible protein band of expected size (pointed by an arrow) which indicates successfully expression after induction with 0.1% (w/v) L-arabinose or 100 mM IPTG.

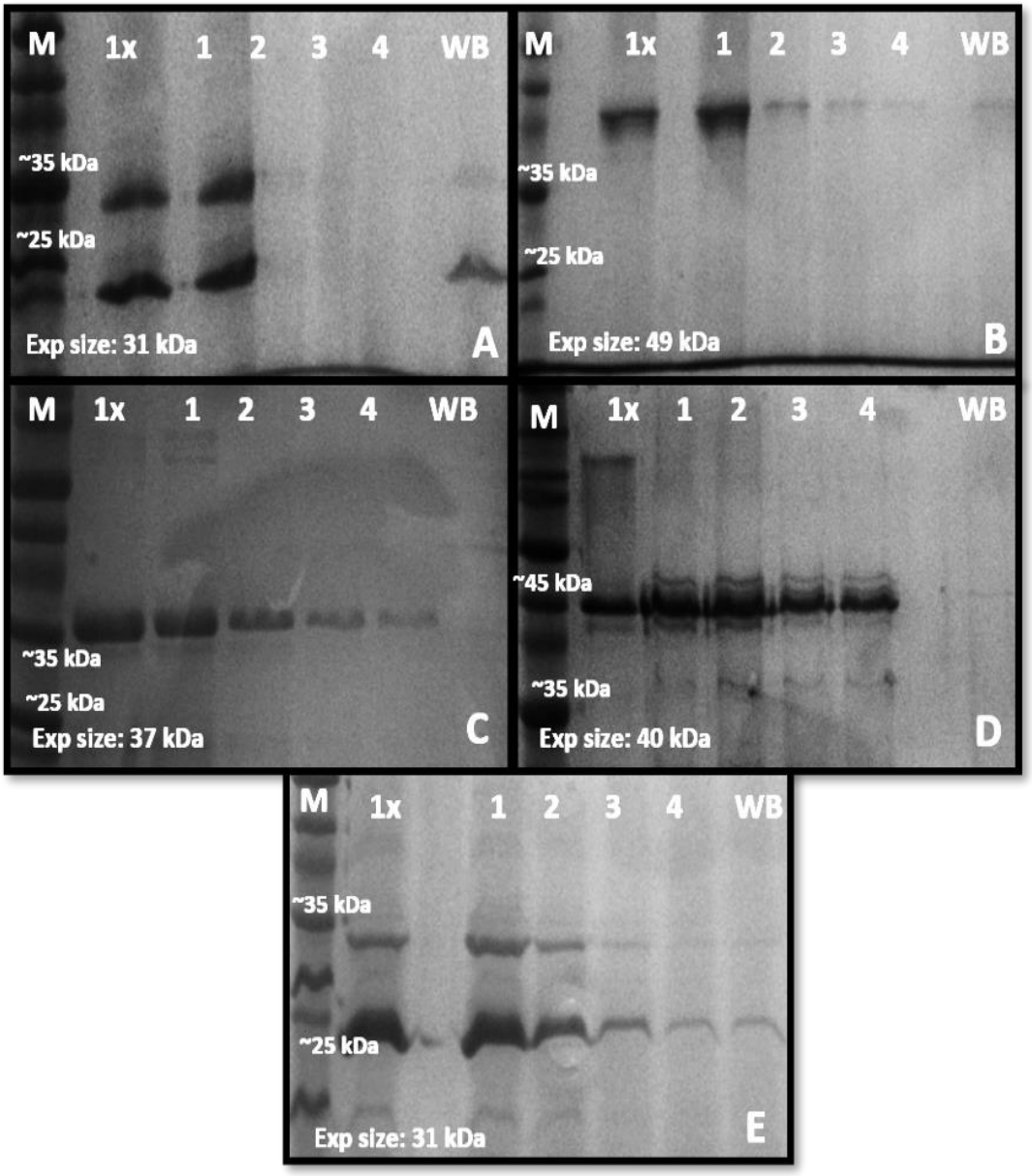


Figure 4.3 (A-E): Leptospiral recombinant OMPs purification (A-E) analysed on 12% (v/v) SDS-PAGE gel under denaturing conditions.

Samples from each eluted protein fraction (A: rLBL2510, B: rLBL2618, C: rLBL0972, D: rLBL0375 and E: rLIC10973) were heated at 100°C for 5 minutes and an addition one sample from first protein fraction (1x) before loading on the gel. M: 6.5-200 kDa protein marker Lane 1x: Unheated protein sample from first elution fraction. Lane 1: Protein sample from first eluted fraction, Lane 2: Protein sample from second eluted fraction, Lane 3: Protein sample from third eluted fraction, Lane 4: Protein sample from fourth eluted fraction, WB: Wash buffer fraction.

4.3.5 Assessment on the effect of heat, reduction and urea on OmpL1 denaturation

Treatment with heat, DTT and urea on OmpL1 showed that the lower band of 25 kDa migrated to its denatured form at 31 kDa at 100°C boiling temperature as the time increases and with the addition of the denaturing agent (8 M urea). Boiling at 10 and 40 minutes with only DTT did not affect the electrophoretic mobility of the undenatured OmpL1 form (Figure 4.4 no 2 and 4). However, with additional of 8 M urea in 10 minutes boiling time caused a partial denaturation (Figure 4.4 no 3) and almost complete denaturation is achieved when the heated samples were left boiling at 40 minutes with both DTT and urea (Figure 4.4 no 5). This showed that OmpL1 is a heat resistant protein and further treatment with both reducing and denaturing agents resulted in unfolded form and allowed to migrate to its predicted molecular mass.

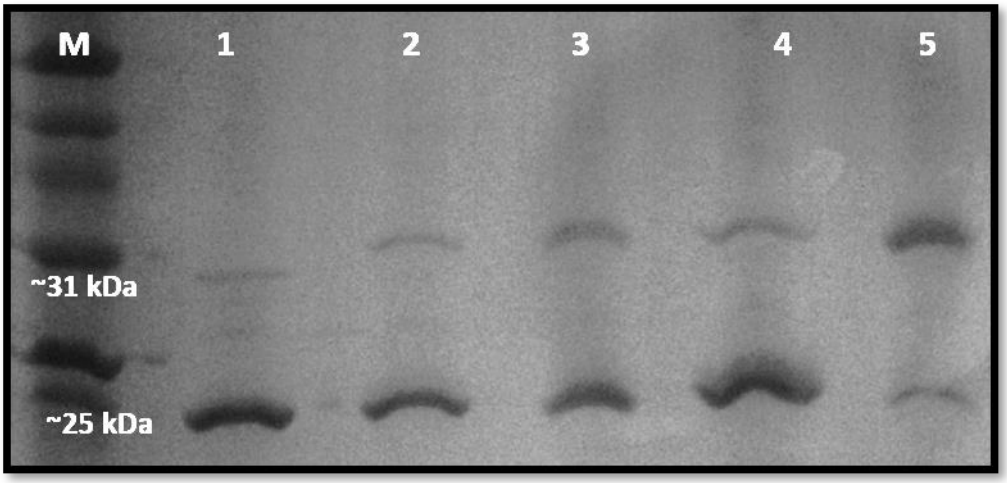


Figure 4.4: The effect of heat, DTT and urea on a recombinant OmpL1 (rLIC10973) in this study.

Both denatured and undenatured molecular mass is 31 kDa and 25 kDa, respectively. The OmpL1 samples were subjected to five different treatment; Lane 1: Unheated sample at 25°C, Lane 2: Heated samples at 100°C for 10 minutes with 10 mM DTT, Lane 3: heated samples at 100°C (10 minutes) with 10 mM DTT and 8 M urea, Lane 4: heated samples at 100°C (40 minutes) with 10 mM DTT and Lane 5: heated samples at 100°C (40 minutes) with 10 mM DTT and 8 M urea. M: 6.5-200 kDa protein marker.

4.3.6 Secondary structure determination of recombinant OMPs by circular dichroism

Far-UV CD spectra data of all the purified recombinant OMPs demonstrated that four out of five proteins had predominantly β-barrel spectra in their secondary structure, as shown inFigure 4.5. The range of spectra was taken between 180-260 nm, with spectra exhibiting a minima trough at about 215 nm and the maximum peak at approximately ~195 nm, which is distinctive structure of the β-strand secondary structure. Only one recombinant protein, rLBL_0375 demonstrated a different spectrum (minimum trough at ~208 nm, and positive maximum at 260 nm), which suggested that this protein protein contains a mixture of α-helical and β-barrel structure. Results of both programs are shown in Table 4.3 and Figure 4.5.

Table 4.3: Analysis of the circular dichroism spectra using two online servers; BeStSel and CAPITO show components of each recombinant OMP by their secondary structure.

Protein	Helix	Anti-parallel	Parallel	Turn	Others	Helix 1 (Regular)	Helix 2 (distorted)	Anti 1 (Left-twisted)	Anti 2 (relaxed)	Anti 3 (Right twisted)	Conc (mg/ml)	Molar conc (µM)	Predominant spectra by BeStSel	Predominant spectra by CAPITO
rLBL2510	0.0	44.6	0.0	13.6	41.8	0.0	0.0	3.8	21.2	16.6	2.3	72.7	β-sheet	β-sheet
rLBL2618	0.0	43.8	0.0	13.7	42.5	0.0	0.0	4.6	19.5	19.6	0.3	6.12	β-sheet	β-sheet
rLBL0375	69.0	31.0	0.0	0.0	0.0	69.0	0.0	0.0	0.0	31.0	1.3	35.1	α-helix	Irregular
rLBL0972	0.0	42.8	0.0	14.1	43.1	0.0	0.0	4.1	19.6	19.1	0.7	19.4	β-sheet	β-sheet
rLIC10973	0.0	44.8	0.0	13.8	41.4	0.0	0.0	4.1	21.3	19.4	1.3	41.9	β-sheet	β-sheet

The overall score of α-helix and β-sheet are defined as 'Helix' and 'Anti-parallel', respectively. The α-helix scores are based on a regular part of the helix (regular); The middle part of α-helices and the Helix 2 (distorted ends); 2-2 residues at the ends of α-helices. The β-sheet scores are based on antiparallel β-sheets which are divided into three subclasses: Anti 1; Left-handed twisted, Anti 2; relaxed (slightly right-handed twisted) and Anti 3; right-hand twisted. The definition of 'Turn' is the turn and bend segment longer than one residue. 'Others' are described any additional features present within a protein such as 33,,-helix, π-helix, β-bridge, bend, loop/irregular and invisible region of the structure. Most proteins show β-barrel structures denoted by predominance anti-parallel score, except for rLBL0375. The secondary structure determination of most recombinant OMPs is in agreement with the β-barrel prediction made using bioinformatics.

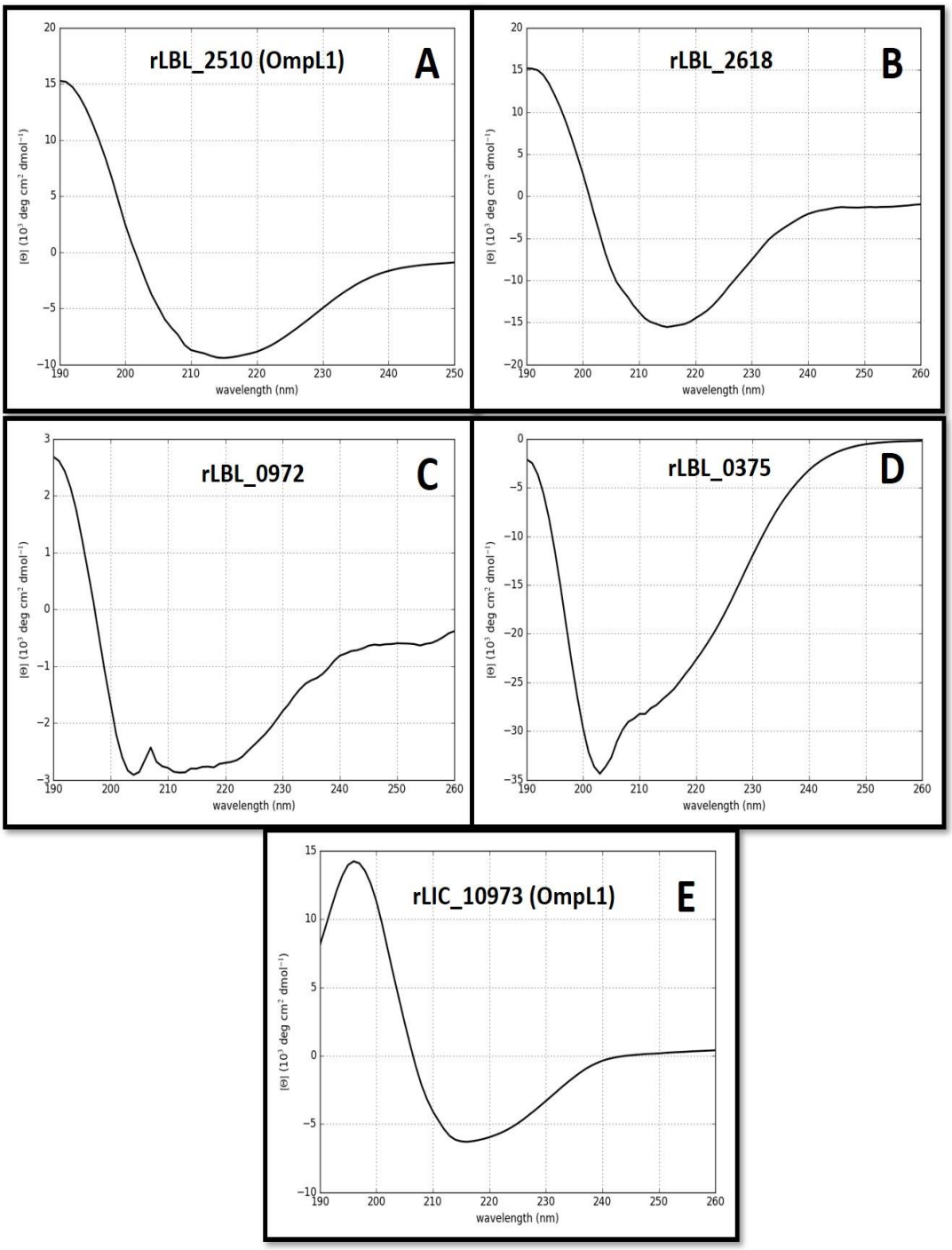


Figure 4.5 (A-E): Circular dichroism (CD) spectra of recombinant OMPs depicting the predominance of β-sheets in secondary structure.

Majority of recombinant OMPs are showing a β-sheet spectrum of a minima band between 210 nm – 215 nm and a maxima band between 195 nm – 200 nm (A, B, C, and E). Recombinant protein rLBL0375 (Figure D) is showing an ambiguous result of neither a random or mixed spectrum which has a minimum band around ~200 nm and 208 nm. The CD spectrum is presented as an average of three scans recorded from 190 to 260 nm. All graphs were plotted using CAPITO software.

4.4 Summary of success of overexpression, refolding and purification of selected leptospiral recombinant OMP

An overall summary of recombinant protein expression, refolding, and purification methodology can be found in Appendix B Figure B.3 and B.4 of the thesis. The summary of each leptospiral OMP gene cloning, expression, refolding, and purification with secondary structure determinant is tabulated in Table 4.4.

Table 4.4: Overview of overexpression, refolding and purification of selected leptospiral recombinant protein in this chapter.

Gene	Cloning to pENTR	Transformation to pDEST17	Expression	Inclusion body formation	Refolding	Purification	CD Spectra
LBL_2510	✓	✓	✓	✓	✓	✓	β-barrel
LBL_2618	✓	✓	✓	✓	✓	✓	β-barrel
LBL_0972	✓	✓	✓	✓	✓	✓	β-barrel
LBL_0375	✓	✓	✓	✓	✓	✓	Mixed/Irregular
LBL_2925	✓	✓	✗	✗	✗	✗	-
LBL_1341	✓	✗	✗	✗	✗	✗	-
LBL_1054	✓	✗	✗	✗	✗	✗	-
LIC_10973	✓	✓	✓	✓	✓	✓	β-barrel

✓Indicates success and ✗ indicates failure, (-) denotes unsuccessful expression.

4.5 Discussion

An *E. coli* expression system is a convenient method to produce a large amount of target proteins for further characterisation studies. Although more advanced expression methods have been developed over the recent years, *E. coli* expression is still a method of choice as it is relatively inexpensive, easy to work with, and able to generate high protein yields. Protein solubility can be a major issue when using this system as over-expression of many recombinant proteins may lead to protein aggregations, commonly known as inclusion bodies, which may require additional solubilisation, refolding and purifying steps to recover the protein in its native state. Strategies to recover inclusion bodies are continuously being optimised and improved, making *E. coli* the most popular bacterial expression system still today (Wingfield *et al.,* 2001; Palmer and Wingfield, 2004; Simpson, 2010; Mohammadian *et al.*, 2018).

Recombinant cloning of the gene of interest is the first step towards gene expression. Choosing an appropriate cloning vector is crucial to ensure it is stable to carrying and propagating the gene of interest without harming the bacterial host. The efficacy of gene cloning can be justified by the successful transformation of an entry vector containing the gene of interest into competent cells, growing them under a laboratory condition and subsequently analysing the number colonies that are successfully carrying the gene. Here, we successfully cloned all the candidate leptospiral genes into the entry vector, although the efficacy of cloning is generally better with target inserts with sizes less than 1.0 kb. Three leptospiral genes (LBL_1054, LBL_1341 and LBL_2618) with sizes more than 1kb were poorly transformed into *E. coli* TOP10 cells. Many recombinant cloning techniques demonstrate better cloning efficiency using smaller DNA fragments to insert into the vector as demonstrated in studies by several authors (Gibson, 2011; Zhang *et al.,* 2012; Koskela and Frey, 2015).

On the other hand, inefficient cloning and transformation could also be due to the inappropriate insert-to-vector molar ratio during cloning reaction, for example, a higher concentration of DNA insert comparing to TOPO® vector. We used a ratio of 1:1 for this purpose; however, due to the large size (> 1kb) of several DNA inserts, it is recommended to use 2:1 instead. This theory is supported by a transformation efficacy study using *E. coli* DH5α strain which suggested that a lower insert DNA concentration compared to vector concentration yields a better transformation effect (Kostylev *et al.*, 2015). Sanger sequencing

of DNA in successfully transformed colonies confirmed the correct coding sequences without the presence of any gene mutations. This shows that using high-fidelity Phusion Taq DNA polymerase had successfully generated accurate DNA template replication, which is key to enabling subsequent effective cloning and expression of complete recombinant proteins without premature stop codons or changes to the protein primary structure.

Although all genes were successfully cloned into the primary TOPO vector, subcloning these genes into the destination vector, pDEST17 was not as successful. Two genes (LBL_1054 and LBL_1341), with sizes more than 1 kb, failed to incorporate into pDEST17 via the LR clonase reaction. This could be due to a lower clonase reaction against these entry clones. Future studies might improve the effectiveness of the recombinant reaction by increasing the volume of clonase II or using a more efficient transformation technique such as ultrahigh competent cells or electroporation to successfully sub-clone these genes (Reece-Hoyes and Walhout, 2018).

From the six successfully transformed genes in pDEST17, only five were successfully expressed and isolated as recombinant proteins in 500 ml culture volumes. Initially, the *E. coli* BL21-DE3 strain was used for expression, however, the expression yields were poor, and we moved to *E. coli* BL21-AI for tighter expression, and the yield was significantly improved. One gene (LBL_2925) failed to be expressed in both *E. coli* strain BL21-DE3 and BL21-AI. Expression failure is defined by the inability to achieve optimum growth (measured by optical density at OD_{600}) after induction and the absence of the correct size expression band in SDS-PAGE. The reason for the failure of expression of this gene in all expression strains is not fully understood, and several factors might link to the outer membrane expression problem such as toxicity due to limited capacity of translocon protein complexes to cross membranes and cellular metabolic stress and changes of pH homeostasis during protein overexpression (Wagner *et al.*, 2007; Gubellini *et al.*, 2011).

Additionally, we attempted transformation of this gene into another toxic-friendly expression strain, *E. coli* strain BL21-DE3 (pLysS), as this strain is recommended for the expression of toxic proteins (Montigny *et al.*, 2004; Rosano and Ceccarelli, 2014) because the pLysS plasmid can reduce basal level expression of the toxic gene that may result in tolerance of toxic proteins. However, we failed to achieve protein expression using this strain. Due to the difficulty in expressing this gene following several unsuccessful expression trials, we decided to exclude this gene from this study. Whilst beyond the remit of the current study,

future attempts might achieve greater success by using different expression strategies such as using other toxic-tolerant strains such as Walker strains C41 (DE3), C43 (DE3) and other BL21 (DE3) derived expression strains that may improve expression of toxic membrane proteins (Miroux and Walker, 1996; Wagner *et al.*, 2008; Meuskens *et al.*, 2017).

All successful genes were expressed as insoluble proteins in this experiment and formed inclusion bodies due to protein aggregation. Protein aggregation normally occurs as a result of signal peptides removal, and the gene overexpression causes newly synthesised proteins to be accumulated in the cytoplasmic space leading to protein aggregation. Inclusion bodies are typically colourless insoluble masses separated by low centrifugation force from cell lysate and here were washed with detergent to remove the contaminants originating from protein components of the *E. coli* outer membrane and cell wall materials during overexpression (Palmer and Wingfield, 2004). Inclusion bodies can be solubilised using a high concentration of guanidine HCl or urea as strong chaotropic denaturants which resulted in complete denaturation of secondary structures preventing aggregation of protein molecules during the refolding process (Singh *et al.*, 2015). Guanidine HCl was used here in the protein solubilisation step instead of urea due to; 1) urea may potentially be contaminated by cyanate ions that may cause carbamylation of the amino groups blocking the N-terminus of a protein (Cole and Mecham, 1966) and 2) and a high concentration of urea in nonionic or zwitterionic detergent solution may form inclusion compounds that are less soluble and cause precipitation (Rabilloud, 2009). On the other hand, the use of guanidine HCL is recommended as it was reportedly exhibiting 1.5 to 2.5 times stronger per mole than urea (Pace, 1986).

As in many protein solubilisation techniques, sulfhydryl reagent or reducing agents such as DTT or 2-Mercaptoethanol (2-ME) are usually added within the solubilisation buffer to maintain cysteine residues in the reduced state and prevent disulphide bond formation (Wingfield, 2016). In our experiments, we selected DTT to extract inclusion bodies during the solubilisation step, mainly because of the toxicity risk of 2-ME. Additionally, we added a chemical chelating agent, 0.5 M EDTA to prevent metal-catalysed air oxidation of cysteines (Singh and Panda, 2005).

The solubilised protein solution, which now contains denatured proteins, will undergo *in vitro* refolding procedure to restore its biological activity. Several refolding strategies have been developed to recover inclusion bodies to refold at their native (active) state (Basu, Li and

Leong, 2011; Yamaguchi and Miyazaki, 2014). Our refolding strategy is based on a dilution method by Burgess, (2009) which uses a low concentration of zwitterionic detergent, LDAO in the refolding buffer. However, in some cases, protein aggregation may form during the refolding process which may significantly reduce protein yield especially if the protein concentration is high (Song *et al.*, 2011). Application of rapid/flash dilution method is considered simple and may have the tendency to lower the formation of protein aggregation (Harrison *et al.*, 2015). Overnight protein dialysis was performed after refolding step to remove excess guanidine HCL and possible unwanted macromolecules or contaminant compounds, leaving only the recombinant proteins in a clear, colourless solution. Temperature also plays a significant factor contributing to the dialysis performance. Generally, the rate of diffusion is faster when protein being dialysed at room temperature. However, this may also tend to cause rapid precipitation due to protein instability (Pohl, 1990). Therefore, due to this reason, all our proteins were dialysed at 4°C overnight to minimise precipitation and to reducing the concentration of small contaminants within protein solutions before purification.

Protein purification of all expressed recombinant proteins in this study was a success, and the protein concentrations were varied from 0.3-2.0 mg/ml. Proteins with molecular weight less than 35 kDa produced high protein concentration (~1.0 mg/ml). Most of the proteins remained stable in the solution and did not precipitate. However, one protein, rLBL_2618 (49 kDa) yielded less than ~0.5 mg/ml and this protein also precipitated easily when kept at -80°C. Multiple factors interplay with protein stability, such as temperature, pH, salt concentration and buffer types (Pesarrodona *et al.,* 2015) could cause precipitation and the most relevant explanation to the precipitation problem in our study is that the protein has a higher degree of hydrophobicity and high salt concentration decreases the protein's solubility, therefore causing precipitation (Duong-Ly and Gabelli, 2015). To mitigate this problem, we reduced the salt concentration to 300 mM in dialysis buffer and 20 mM in protein elution buffer to minimise precipitation in the solution. Another suggested method, which we did not attempt in this study is to store purified proteins with the addition of 5-10% (v/v) glycerol in the buffer because glycerol helps to limit protein precipitation for longer storage (Bondos and Bicknell, 2003; Vagenende *et al.,* 2009).

Protein analysis on SDS-PAGE showed that all proteins migrated in accordance with their respective sizes. We demonstrated two recombinant OmpL1 proteins (rLBL_2510 and rLIC_10973) which each appears to have a secondary band which is identified as an

undenatured form of OmpL1 (~25 kDa) (Figure 4.3 A and E). We tested one recombinant OmpL1 sample (rLIC_10973), by treating it with a reducing agent, heat and urea to observe if these factors help to denature the protein on the SDS-PAGE gel. Our result showed that boiling of OmpL1 samples at 100°C for 40 minutes with the addition of 10 mM DTT and 8 M urea resulted in further loss of the secondary structure (~25 kDa) and increased band intensity of the 31 kDa form (Figure 4.4). This confirms that OmpL1 is a heat-modifiable protein, and their compact structure needs intensive treatment to completely unfold the protein to allow migration at their original molecular mass (Shang *et al.*, 1995). In doing so, we successfully demonstrated that our predicted gene (LBL_2510) is an OMP that is heat stable and possibly forming a porin channel on the leptospiral outer membrane. To further investigate whether this lower band is specific to OmpL1, we performed a western blot. We found two bands of similar molecular weights reacted against anti-his antibody, and thus we confirmed that the lower molecular band specifically belongs to OmpL1, as they are recognised by the anti-his antibody probing (Results not shown).

Interestingly, another recombinant protein (rLBL_0375) did not migrate following its expected molecular weight (~40 kDa) and appeared to be slightly larger ~55 kDa (Figure 4.4 D). This anomaly, often observed in membrane proteins, is referred as 'gel-shifting' and the occurrence is thought due to the alteration of detergent binding and protein helical conformation of the protein causing reduce gel mobility (Rath *et al.*, 2009). The remaining two proteins (rLBL0972 and rLBL2618) did not show heat modification or reduced mobility.

As shown in Figure 4.5, most of the recombinant proteins show a predominantly β-barrel secondary structure when analysed by far-UV CD spectrophotometer. However, the percentage proportion between α-helix, β-sheet (or anti-parallel) and mixed structure vary among the recombinant proteins. One recombinant protein (rLBL_0375) was originally predicted to be a β-barrel OMP, and the result of the spectra suggested that this protein may have a mixture of α-helices. Even though the result on BeStSel predicted the likelihood of this protein to be a β-barrel based on the given spectra of two different peaks at 205 nm and 215 nm respectively (Results not shown). We speculated that this protein contains a mix of α-helix and β-sheet. However, CD spectra could not provide more details about the structure of a protein and further investigations are warranted in the future to investigate the protein's structure closely using crystallography or nuclear magnetic resonance (NMR) method (Corrêa and Ramos, 2009). Whilst the rLBL0375 result shows ambiguity, we decided to further analyse this protein to determine its possible binding and interactions with host cells

(Chapter 5). The CD analysis of the remaining recombinant proteins (except rLBL_0375) predicted high percentages of mixed structures and these refolded proteins have been shown to have acquired a secondary structure which is consistent with previous studies of OmpL1 (Fernandes *et al.*, 2012, 2017), and their stability in solution further evidence that these proteins likely have refolded close to their native structure.

4.6 Conclusion

In this study, we applied both cloning and expression methods using Gateway™ Technologies to selected unique leptospiral predicted genes. Although most genes were successfully cloned, expressed as recombinant proteins and subsequently purified, some genes failed to progress at key steps and were excluded from this study. Several recommendations and suggestions are discussed in detail to further improve cloning and expression efficiency of these genes for future studies. Generally, most purified target proteins produced good yields, although the yield reduces with increasing protein molecular weight. The SDS-PAGE profiles of the recombinant *Leptospira* OMPs showed that some but not all proteins were heat-modifiable, which is in line with reports that some but not all β-barrel proteins are heat-modifiable (Verhoeven *et al.*, 2009). CD spec analysis identified that the majority of the target proteins adopted predominantly β-sheet secondary structures, which increases our confidence that these proteins may have correctly refolded close to their native structure. Overall, we successfully produced five recombinant OMP proteins out of seven predicted genes using a heterologous (*E. coli*) expression system and these proteins will be further investigated for their functional and immunological evaluations in subsequent chapters.

Chapter 5: Functional and immunological studies of *Leptospira* recombinant OMPs

5.1 Introduction

5.1.1 Bacterial invasion and colonisation to the host cell: General pathophysiology

In order to establish successful colonisation of a host, all pathogens must first overcome the innate immunity of a host. The innate immunity system is divided into two subclasses; 1) First line of defence which consists of physical and chemical barriers such as intact skin, mucous membrane and their secretions, microbial bacteria living in the gastrointestinal system and antibacterial enzymes such as those in tears and saliva. 2) Second line of defence is the non-specific interaction between a pathogen and the host immunity system after the first defence is compromised. Before the second line of innate immunity is triggered, the pathogen must first be able to adhere to the surface of the host cell. Adherence of the pathogen to the host's cell is the first step towards invading their immune system to establish infection (Paulsson and Riesbeck, 2018). Generally, bacterial adhesion to the cells involves several strategies which depend on the types of bacteria (e.g. Gram-positive versus Gram-negative, intracellular versus extracellular). The diversity of mechanisms is reviewed by Pizarro-Cerdá and Cossart (2006), and in this thesis, we will focus specifically on *Leptospira* adherence to host cells.

5.1.2 The *Leptospira* OMP

Like other Gram-negative bacteria, spirochetal bacteria possess an outer membrane which serves as biointerface between the cell and its external environment. As previously described in Chapter 1, the outer membrane of spirochetes is made up of several components that vary from one species to one another. Unlike *Treponema* and *Borrelia* genera, *Leptospira* outer membrane consists largely of LPS, which is important for serovar identification and antigenic properties, roles that are similar to typical Gram-negative bacteria, such as *Salmonella* and *Pasteurella* (Harper *et al.*, 2012; Ryan *et al.,* 2017). This LPS, as well as being the major outer membrane component of leptospires, also covers the entire cell surface (Cullen *et al.,* 2004). It was generally thought that leptospiral LPS is a key virulence factor during host colonisation and bacterial dissemination; however, some studies have suggested that leptospiral LPS has no apparent pathological effect on host tissues (Murray *et al.*, 2010; Srikram *et al.*, 2011). Furthermore, their endotoxic activity is lower than that of typical Gram-negative bacteria,

owing to the unique structure of their Lipid A component (Que-gewirth *et al.*, 2004). LPS appears to have no direct attachment to the host cell surface, where they would expect to be recognised by the host Toll-like receptors such as TLR2 and TLR4 (Werts *et al.*, 2001; Nahori *et al.*, 2005). LPS has been widely studied both as a potential vaccine candidate (Section 1.10.1, Chapter 1) and biomarker for diagnosis; however, due to their inability to confer cross-protection against multiple serovars, vaccine formulation based on the whole-cell or LPS derivatives are considered limited.

Leptospiral cell-surface OMPs have become a subject of interest to study how these proteins interact with the host cells. The OMPs are classified into three types; 1) lipoproteins, which adhere to the side of the membrane, 2) transmembrane proteins/integral OMPs and 3) the peripheral proteins (Cullen *et al.,* 2004). While a small number of proteins have a defined purpose such as cell maintenance (e.g. OMP biogenesis and import/export channels) (Section 1.11, Chapter 1), the majority of leptospiral cell-surface OMP roles are still elusive. However, *in vitro* studies showed that most of these proteins are likely to have adhesin function adhering directly to host cells, although this role needed further investigation. Several leptospiral OMPs have shown to be expressed and recognised in host immune cells during infection, further highlighting their possible roles in leptospirosis pathogenesis.

5.1.3 Adherence to host molecules

The ECM comprises non-cellular macromolecules made up from proteoglycans and fibrous proteins and is present within many tissues and organs. The ECM provides mechanical support to cells and also regulates numerous essential biochemical and biomechanical cellular functions such as tissue morphogenesis, homeostasis and differentiation (Frantz *et al.,* 2010). Major common ECM components include collagen, proteoglycans, laminin and fibronectin and elastin. These proteins have multiple binding sites for cell surface receptors that mediate ECM-bacterial interactions. Bacterial adhesion of ECM components is mediated by a group of adhesive proteins known as microbial surface components recognising adhesive matrix molecules or MSCRAMM. These proteins are secreted at the cell surface of a bacterial cell and initiate binding towards the ECM components (Patti and Höök, 1994). Several leptospiral cell surface proteins are thought to be MSCRAMM, due to their ability to adhere to the ECM components *in vitro* and might be linked in pathogenicity, although the role of each MSCRAMM may not be essential in natural infection due to frequent functional redundancy across OMPs.

Further assessment of protein functions can be determined by assays of biochemical properties. A conventional method to analyse the protein-protein binding interaction is by an adherence assay, a modified-ELISA based technique where an OMP of a known concentration is allowed to bind to immobilised host proteins, and the binding background is measured by ELISA. The binding of antigen-host proteins will then be compared to same antigen binding to a negative control using a host protein that is known not to bind to any antigen (e.g. BSA, gelatin, fetuin). The method had successfully demonstrated in many spirochetes recombinant antigens, including *Leptospira* proteins (Cameron, 2003; Barbosa *et al.*, 2006; Verma *et al.*, 2009).

Additionally, once the OMP binding to the host is established, the next step is to determine the strength of the OMP adherence in a dose-dependent manner. The process is rather similar to the modified-ELISA described previously, except the protein host ligand interaction is tested with increasing antigen concentration until saturation binding is achieved, fulfilling a specific interaction of a typical receptor-ligand interaction through inhibitory effect (Fernandes *et al.*, 2012). The outcome from the study is crucial to determine a possible interaction between *Leptospira* and host ligand in natural infection which to demonstrate the role of of particular leptospiral antigens as adhesins for cellular attachment. For example, several leptospiral transmembrane and surface-exposed recombinant OMPs such as LigA and LigB (Choy *et al.*, 2007), OmpL1 (Fernandes *et al.*, 2012), OmpL37 (Pinne, Choy and Haake, 2010) along with multiple leptospiral adhesins (Lsa20, Lsa25 and Lsa33) (Mendes *et al.*, 2011; Domingos *et al.*, 2012) previously showed specific attachment of various host ligand molecules *in vitro*, that indicates that these proteins may be expressed to carry out binding functions during leptospirosis pathogenesis.

Such binding diversities of various leptospiral recombinant proteins with multiple host ligands prompted the present study to determine binding interactions of novel leptospiral recombinant proteins (Chapter 4) towards various ECM molecules.

5.1.4 Leptospiral OMPs as potential diagnostic antigens

Historically, the MAT has been widely used in sero-epidemiological studies to screen for leptospirosis in both human and animals to determine infective serovars/serogroup, also considered as the *Leptospira* gold standard diagnostic method. However, this method is not reliable to detect acute infection and requires a panel of live antigens representative to all serogroups that are geographically prevalent in a given area. Moreover, MAT is rather

difficult to interpret, and interlaboratory variation is high; therefore, it needs skilled users to interpret (Bharti *et al.*, 2003).

Alternatively, ELISA emerges as a popular choice for screening of leptospirosis in both human and animal studies. Besides being rapid, easy to use and relatively inexpensive, this method has a higher sensitivity and has been widely applied in many leptospirosis seroprevalence studies worldwide. Principally, ELISA works by detecting anti-leptospiral antibodies using different antigen preparations, assay protocols and assay platforms (OIE, 2008). Most commercially available leptospiral test kits antigen targets are derived from whole-cell preparations, which are specific to certain serovars and may give non-specific cross-reactivity between different serovars. Moreover, there are several reports highlighting the lack of specificity of some commercial *Leptospira* kits (Reller *et al.*, 2011; Rao *et al.,* 2019), which need definitive confirmation by MAT.

Recently, recombinant antigens have been considered as an alternative to replace whole cell preparations as potential antigens. Studies on recombinant leptospiral proteins such as LipL21, LipL32, LipL41 and Loa22 demonstrated significant immunoreactivity when tested against positive human serum samples (Chalayon *et al.*, 2011). Another potential recombinant protein, OmpL1, has also been demonstrated to react with antibodies (IgG present in canine serum samples, and reported to have 100% sensitivity when compared to MAT (Subathra *et al.*, 2013). Both studies showed that leptospiral recombinant OMPs could be utilised as potential tools for the serodiagnosis of both human and canine leptospirosis using ELISA-based methods. Apart from serology findings, bulk milk screening using ELISA-based kits is routinely applied to dairy cattle samples and has been used to in several leptospirosis prevalence studies in a dairy herd (Tabatabaeizadeh *et al*., 2011; Ryan *et al.*, 2012). Similar to serology studies, these kits are also developed using whole-cell lysate, which may be only restricted to certain leptospiral serovars, which eventually reduces the kit's specificity and may give rise to false negative results. Hence, leptospiral recombinant OMPs may be an improvement as an alternative antigen for this problem. However, there are no studies to support this hypothesis, and therefore, the present study was designed to; 1) Identify and characterise novel adhesins (produced in Chapter 4), and 2) To determine the interaction of the OMPs with the host immune system to underpin future vaccine and diagnostic development.

5.2 Materials and Methods

5.2.1 Host ligand target molecules

For the screening of leptospiral recombinant protein binding with selected host molecules, eight different ECM macromolecules were purchased from Sigma-Aldrich, Dorset, UK. Fibronectin (Cat no: F1141) derived from bovine plasma, collagen I (Cat no: C9879) from bovine skin, heparin sulphate sodium salt (Cat no: H7640) isolated from bovine kidney, laminin (Cat no: L2020) from Engelbreth Holm-Swarm murine sarcoma basement membrane, elastin (Cat no: E6527G) isolated from bovine neck ligament, fibrinogen (Cat no: F8630) derived from bovine plasma and chondroitin sulphate sodium salt (Cat no: 6737) was from bovine cartilage. Bovine serum albumin (Cat no: A9418) was used as a control. All macromolecules were prepared in PBS as a 1 mg/ml stock and were further diluted to 5.0 µg/ml to coat ELISA microtitre plates.

5.2.2 Recombinant leptospiral OMP preparations

For host molecule binding surveys, all leptospiral recombinant OMPs were prepared as described in the previous chapter. The OmpL1 (rLIC10973) from *L. interrogans* serovar Copenhageni Fiocruz L1-130 was prepared for use as a positive control for several experiments based on a study demonstrating its ability to bind to multiple host ligands (Fernandes *et al.*, 2012). All recombinant OMPs were diluted in PBS to 10 µg/ml as testing concentration, and PBS was used as a control. For binding saturation assay analysis, the OMPs were analysed using various micromolar concentration from zero to 6.0 µM against selected host molecules with statistically significant binding rated as $P < 0.05$. BSA was used as a control.

5.2.3 Cattle bulk milk preparation

A total of 30 bulk milk samples (of variable volumes) from dairy cattle consisting of *Leptospira* antibody positive and negative samples were kindly supplied from Cattle Information System (CIS, UK). Milk samples were preserved with sodium azide tablets prior to transportation. The *Leptospira* status of milk samples was pre-determined from the routine *Leptospira* test (Linnodee *Leptospira Hardjo* ELISA Kit™) from the collection centre which can be found in Appendix C (Figure C.1). The test kit utilises LPS fractions from serovar Hardjo from *L. interrogans* and *L. borgpetersenii* species as coating antigens, and has been previously used to determine leptospirosis status in cattle milk samples (Yan *et al.*, 1999; Lewis *et al.*, 2009; Ryan *et al.*, 2012). Milk samples were centrifuged at 1000 *g* for 20 minutes, and the

supernatant fat layer was removed. Samples were immediately tested upon arrival, and the remaining amounts were kept at -80°C for future use.

5.2.4 Binding of leptospiral recombinant OMPs to host ligand molecules

Screening of leptospiral recombinant OMPs to individual host molecules were carried out according to described ELISA protocols (Barbosa *et al.*, 2006; Staton, 2018), which can be found in Section 2.26.1. Statistical analysis was performed to compare the binding of the recombinant proteins to host molecules with negative control (BSA) using Dunnett's multiple comparisons test by GraphPad (Prism) version 7.02. Each experiment was repeated three times, and the means of optical density were averaged for statistical analysis.

5.2.5 Binding saturation curve of leptospiral recombinant OMPs to selected host ligand molecules

Host molecules with significant binding from Section 5.2.4 were selected to determine the strength of recombinant OMP binding to the host molecules. Each microplate was coated with the selected host molecules, and BSA as a negative control (Section 2.26.1). Each recombinant protein (0.0-5.0 µM/ml in 100 µl PBS tween) were added to the specific host molecules coated wells. The ELISA method was as described in Section 2.26.1. Statistical analysis using nonlinear regression was performed to measure the equilibrium dissociation constant (K_d) as previously described by (Lin *et al.*, 2009). Each experiment was repeated three times, and the means of optical density were averaged for statistical analysis.

5.2.6 Determination of fibrinogen-binding proteins by a far-western blot

Recombinant OMPs with significant binding affinities with fibrinogen from Section 5.2.5 were subjected to far-western blot to confirm the protein-protein interactions and identify specific binding sites for the interaction between the OMP and fibrinogen's chain structures. A standard far-western blot using 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, (15-track) was employed to separate fibrinogen protein components as described (Staton, 2018) and his-tagged OMPs were used to a probe and detect target proteins on the membrane. The procedure was carried out according to Section 2.18.

5.2.7 Host immune response against leptospiral recombinant OMPs in cattle milk

Detection of specific anti-leptospiral antibodies against leptospiral recombinant OMP as antigens in cattle milk was carried out based on the original serology ELISA method with slight modification (Yan *et al.*, 1999) as described in Section 2.26.2. Statistical analysis between

positive and negative titres was calculated using Mann-Whitney U test, and linear regression tests determined the association between IgG1 and IgG2 titres for each recombinant protein in GraphPad (Prism) version 7.02. Each experiment was repeated twice, and the means of optical density were averaged for statistical analysis.

Additionally, both results from the test kit (milk reference) and IgG1 and IgG2 binding to leptospiral recombinant OMPs in this study were compared to determine the association between two assays as to whether both assays are related.

5.3 Results

5.3.1 Binding of leptospiral recombinant OMPs to host ligand molecules

All leptospiral recombinant OMPs were tested against selected host molecules using an ELISA based method as described in Section 5.2.4. The results are shown in Figure 5.2 and Table 5.1, respectively. Significant binding of the recombinant OMPs to host ligand molecules was observed for fibrinogen, laminin and fibronectin, in comparison with BSA control, whereas no statistical adhesiveness (*P*-value >0.05) was observed against chondroitin, heparan sulphate, and collagen.

5.3.2 Binding saturation curve of leptospiral recombinant OMPs to selected host molecules

All the host molecules that showed significant binding during the ligand screen were subjected to a dose-dependent (binding saturation curve) experiment. Binding affinity was measured by an equilibrium dissociation constant (K_d) using one site-total binding equation (Qiu *et al.,* 1996; Pathirana *et al.*, 2006; Lin *et al.*, 2009) as previously described in Chapter 2. Most recombinant OMPs showed binding saturation for fibrinogen, fibronectin, and laminin (Figure 5.3). However, rLBL0375 failed to achieve binding saturation to both BSA and fibrinogen (Figure 5.3 D). Therefore, this ligand binding interactions for this protein is considered non-specific. Additionally, rLBL2618 showed non-specific high OD binding to BSA at increasing concentration from 0.0 µM to 1.0 µM and gradually decreased at the highest concentration (Figure 5.3 B). The same phenomenon was also observed in rLBL0972 binding to fibronectin (Figure 5.3 C). Table 5.2 show the binding affinity results of each OMPs to selected ECM molecules.

5.3.3 Recombinant leptospiral OMP binding to fibrinogen components (far-western blotting)

Further investigation of fibrinogen binding to OMPs were carried out to identify specific fibrinogen binding sites. From the result in Figure 5.1, all proteins bound to at least to one fibrinogen protein chains (β-chain) and stronger binding was observed based on the bright intensity of the visible band rLBL2618> rLBL0972> rLIC10973 and lastly rLBL0375. All three chains (α, β, γ) were bound to rLBL2618 and rLBL0972, respectively, and weaker band intensity was observed in rLIC10973 (bound to both β and γ chains), and lastly, rLBL0375 was only bound to β chain.

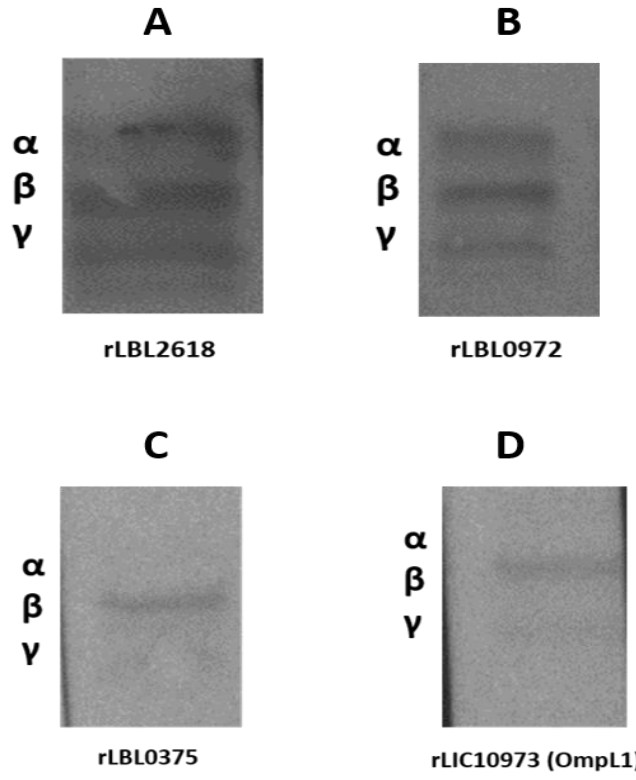


Figure 5.1 (A-D): Far-western blot to detect binding of His-tagged OMPs to α-, β- and γ-chains of fibrinogen.

The molecular weight of each fibrinogen chain is α (64 kDa), β (59 kDa) and γ (48 kDa), respectively (McDonagh *et al.*, 1971).

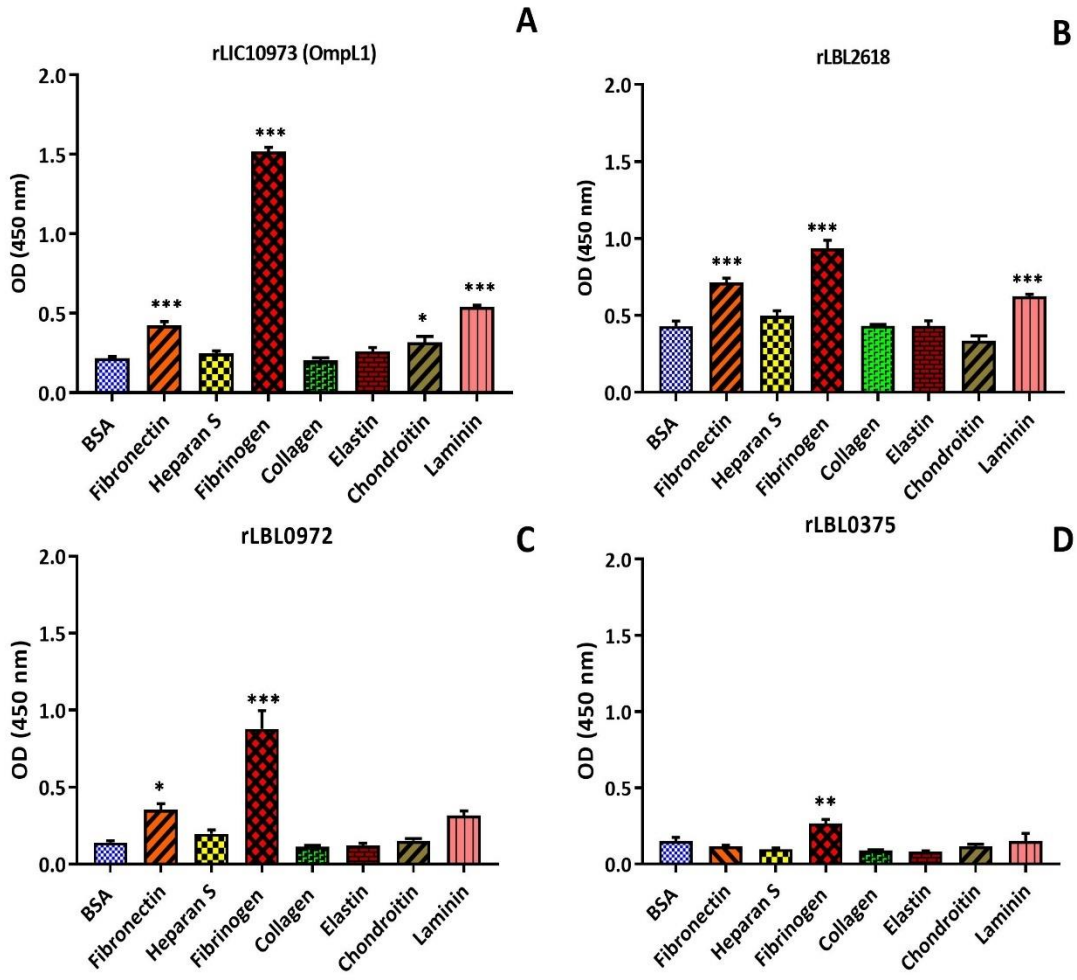


Figure 5.2 (A-D): Binding of novel leptospiral recombinant OMPs and positive control (OmpL1 of L. interrogans serovar Copenhageni L1-130) (A) to host ligand molecules.

*Data represent the mean absorbance at 450 nm ± the standard error and the mean (SEM) of three independent experiments. The binding of leptospiral recombinant OMPs to host ligand molecules was compared to their binding to BSA by Dunnett's multiple comparison test (*** P <0.001, ** P <0.01, * P <0.05).*

Table 5.1: Binding of leptospiral OMPs to various host ligand molecules.

Recombinant OMP	Binding to host ligand molecules						
	Fibrinogen	Fibronectin	Heparan sulphate	Laminin	Chondroitin	Collagen	Elastin
rLBL2618	Yes ***	Yes ***	NS	Yes***	NS	NS	NS
rLBL0972	Yes ***	Yes *	NS	NS	NS	NS	NS
rLBL0375	Yes *	NS	NS	NS	NS	NS	NS
rLIC10973 (OmpL1)	Yes ***	Yes ***	NS	Yes *	Yes *	NS	NS

*Binding significance is denoted with asterisk sign * (P <0.05), ** (P <0.01) and *** (P <0.001)*
Abbreviations: BSA: Bovine serum albumin NS: Not significant

Table 5.2: The apparent K_d (estimated at micromolar concentration) for saturating binding with 95% confident interval was calculated as the mean concentration of recombinant OMP at the half –maximal binding ± standard deviation.

Recombinant OMP	Host ligand molecules [Mean K_d (µM) ± SEM]						
	Fibronectin	Laminin	Heparan sulphate	Chondroitin	Fibrinogen	Collagen	Elastin
rLBL2618	0.10 ± 0.02	0.14 ± 0.01	ND	ND	0.05 ± 0.01	ND	ND
rLBL0972	0.44 ± 0.21	ND	ND	ND	0.20 ± 0.06	ND	ND
rLBL0375	ND	ND	ND	ND	NS	ND	ND
rLIC10973 (OmpL1)	0.81 ± 1.30	1.46 ± 5.44	ND	NS	0.92 ± 1.38	ND	ND

Abbreviations: ND: Not determined, NS: Not saturated

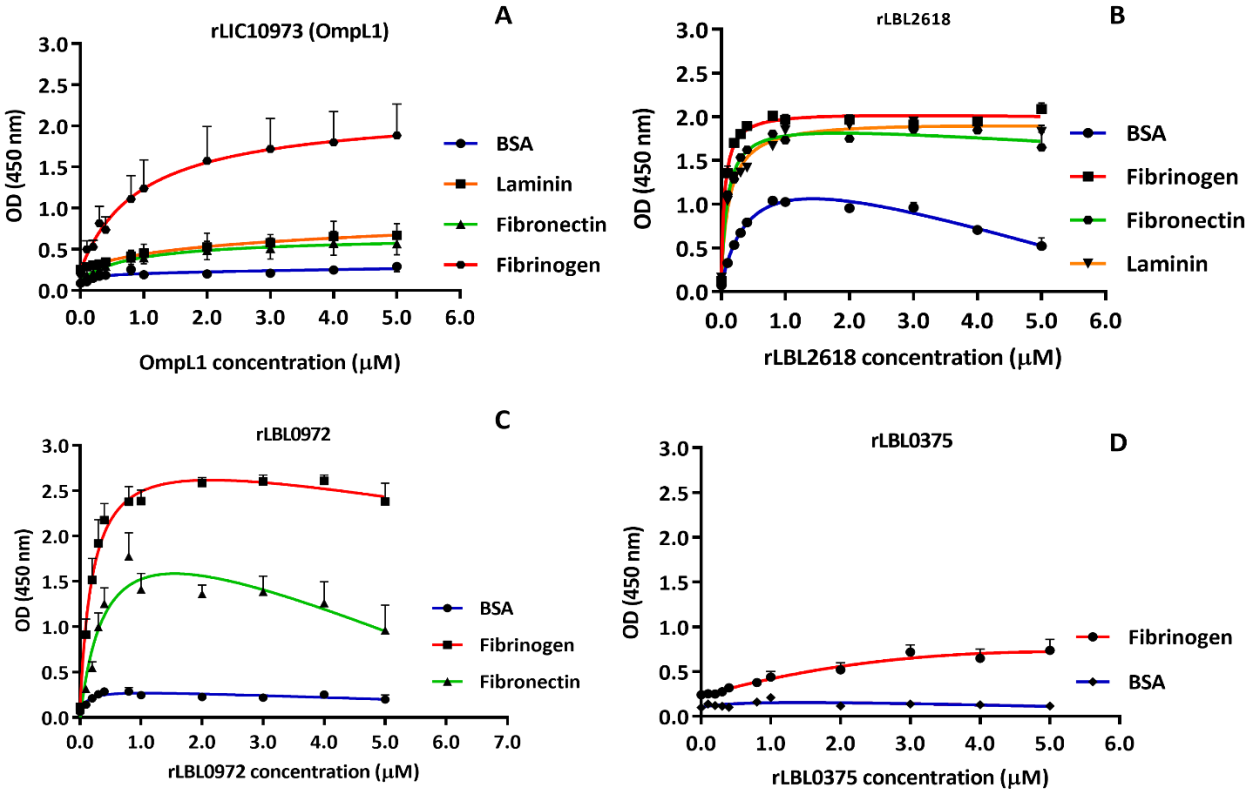


Figure 5.3 (A-D): Binding saturation curves of recombinant leptospiral OMPs to selected host ligand molecules.

Data represent the mean absorbance at 450 nm ± the standard error of the mean (SEM) of three independent experiments. The binding of leptospiral recombinant proteins to host ligand molecules was compared to their binding to BSA by non-linear regression. The equilibrium dissociation constant (K_d) of each protein was estimated at half-maximal binding (see Table 5.2).

5.3.4 Detection of anti-leptospiral immunoglobulins in cattle bulk milk samples

The leptospirosis status (positive or negative) of cattle bulk milk (*n*= 30) was previously determined by Cattle Information System (CIS, UK) by using a commercial *Leptospira* antibody test kit Linnodee *Leptospira* Hardjo ELISA Kit® (Linnodee Animal Care, Ballyclare, Northern Ireland). The test kit has a sensitivity and specificity of 94.1% and 94.8%, respectively. These samples were then tested by IgG-based ELISA against all of the leptospiral recombinant OMPs for the presence of specific bovine IgG antibodies (subclass IgG1 and IgG2) within the samples (Figure 5.4). From the results, significant titres were observed for IgG1, compared to IgG2, against all OMPs. However, only rOmpL1 (LBL_2510) of *L. borgpetersenii* serovar Hardjobovis L550 and rLBL_0375 showed statistically significant of IgG1 titres (P <0.05, P <0.001) between positive and control bulk milk samples. The results of the OMP antibody titre were used to study the titres association which was determined using Pearson's coefficient correlation measured by simple linear regression to compare the relationship between the optical densities produced by the two assay systems. From the results obtained in Figure 5.5, no correlation was observed in the antibody responses to the individual OMPs compared to the test kit.

In addition to this, the IgG1 titres between OMPs were compared to determine their association. Overall, the results showed the majority of OMPs showed a moderate to very strong, positive correlation between one another (r values ranging from +0.35-0.80, P <0.001, P <0.0001), which indicates the two variables are related. However, no linear association (r = 0.00, P >0.05) was found when comparing between OMPs titres against both OmpL1. Conversely, titres of both OmpL1 showed a strong, positive correlation against one another (r = 0.41, P <0.0001). Figure 5.6 and Table 5.3 are showing the results of IgG1 association of recombinant OMPs against one another.

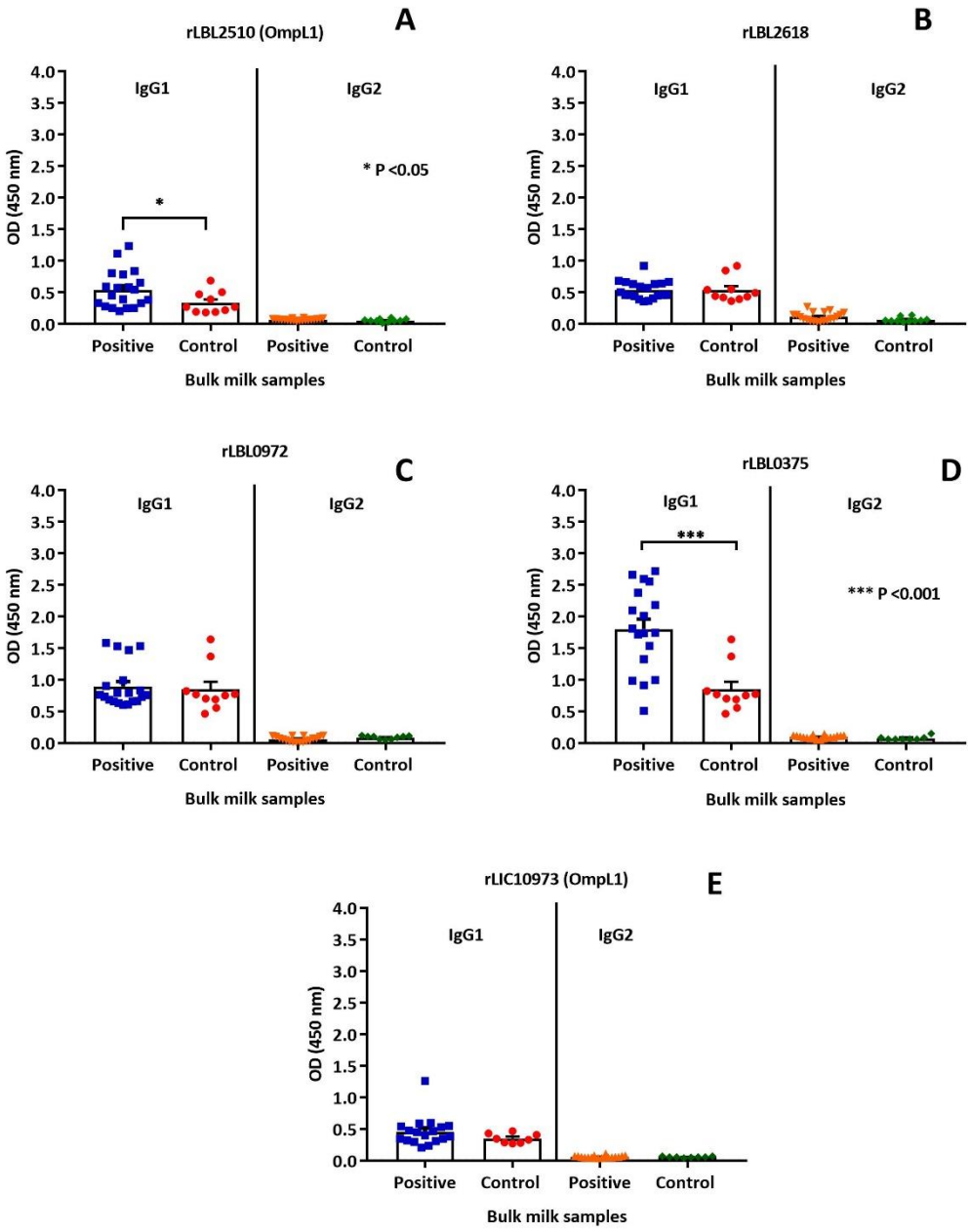


Figure 5.4 (A-D): Host immune response assessment to Leptospira OMPs in cattle bulk milk samples represented in scatter dot-plot graphs.

*Data represent the mean absorbance of secondary antibody concentration (mouse anti-bovine IgG1 and IgG2) at 450 nm ± the standard error of the mean (SEM) of two independent experiments. The antibody titre against each OMP was compared between Leptospira positive and negative samples by Mann-Whitney U test (*** P <0.001, * P <0.05).*

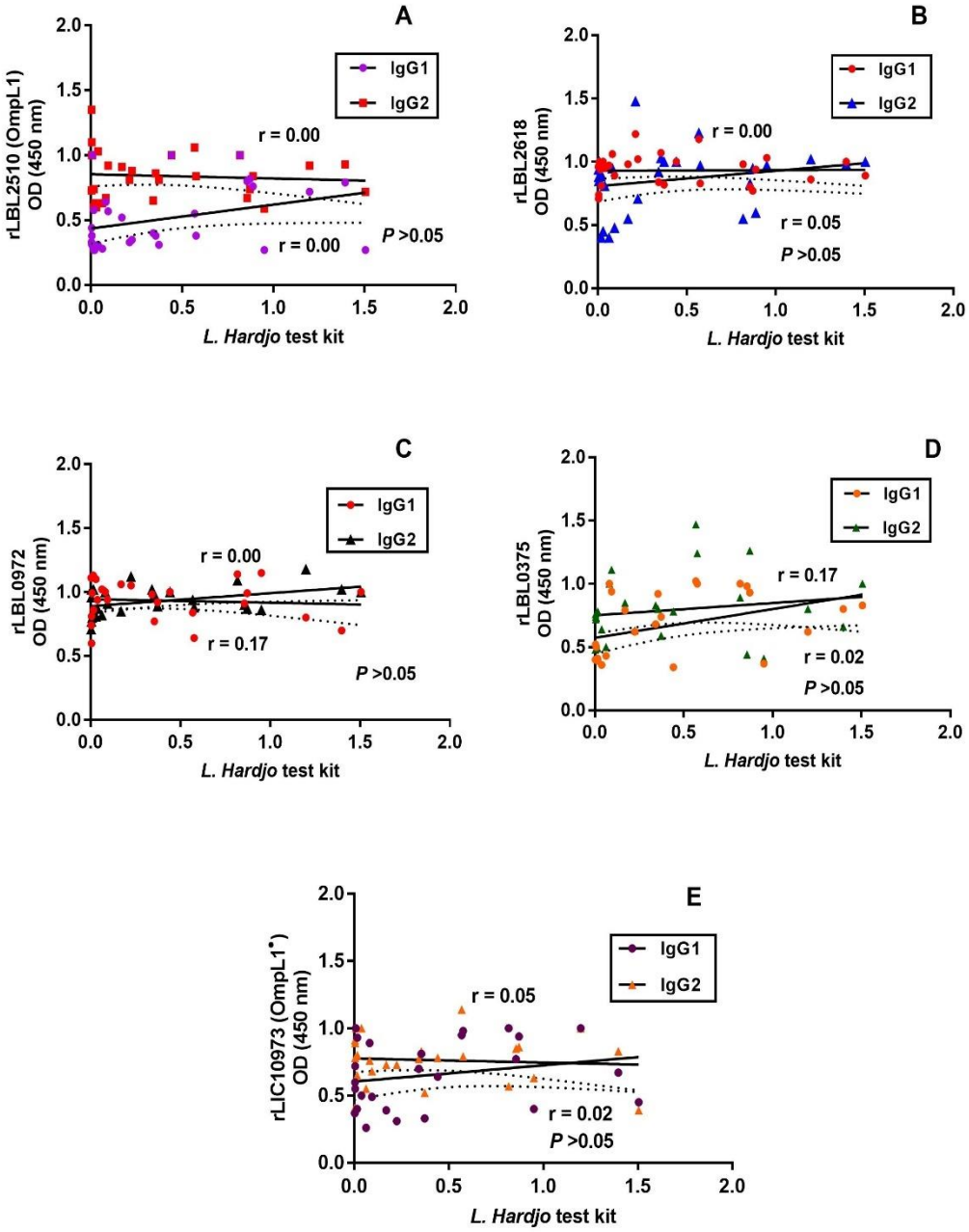
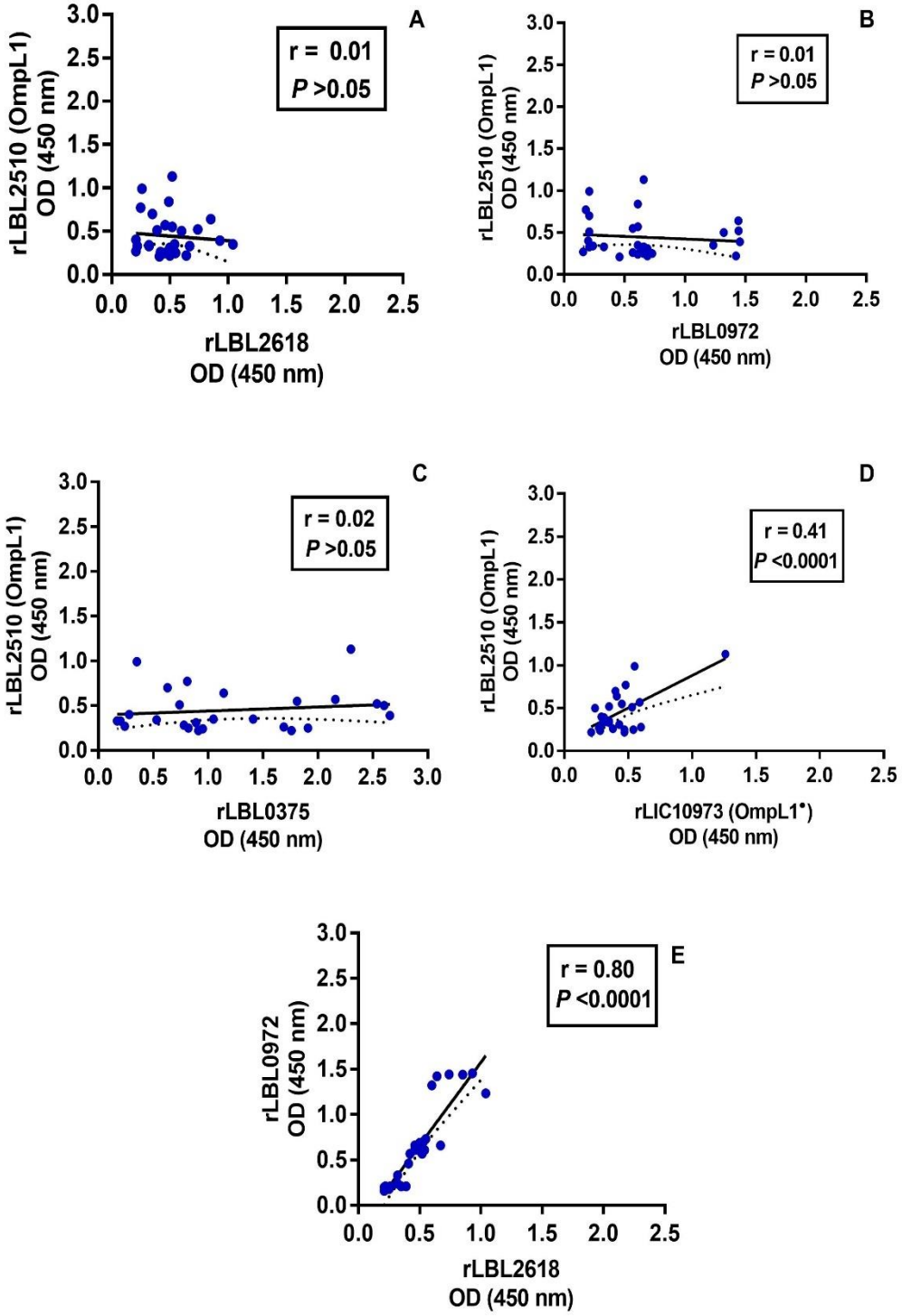


Figure 5.5 (A-E): The association between milk reference obtained from Leptospira Hardjo (Linnodee) test kit anti-leptospiral antibodies and antibodies (IgG1 and IgG2) binding to leptospiral recombinant OMPs including OmpL1* positive control from L. interrogans serovar Copenhageni L1-130 tested in cattle bulk milk samples.

The trend line represents the correlation coefficient*, denoted as (r values), which estimates the relationship between the OD (450 nm) of both results. P-values determine the significant level of association between two assays of which P*-value of at least *<0.05 is considered significant.*


A
r = 0.01
P >0.05
rLBL2510 (OmpL1) OD (450 nm)
rLBL2618 OD (450 nm)
B
r = 0.01
P >0.05
rLBL2510 (OmpL1) OD (450 nm)
rLBL0972 OD (450 nm)
C
r = 0.02
P >0.05
rLBL2510 (OmpL1) OD (450 nm)
rLBL0375 OD (450 nm)
D
r = 0.41
P <0.0001
rLBL2510 (OmpL1) OD (450 nm)
rLIC10973 (OmpL1*) OD (450 nm)
E
r = 0.80
P <0.0001
rLBL0972 OD (450 nm)
rLBL2618 OD (450 nm)

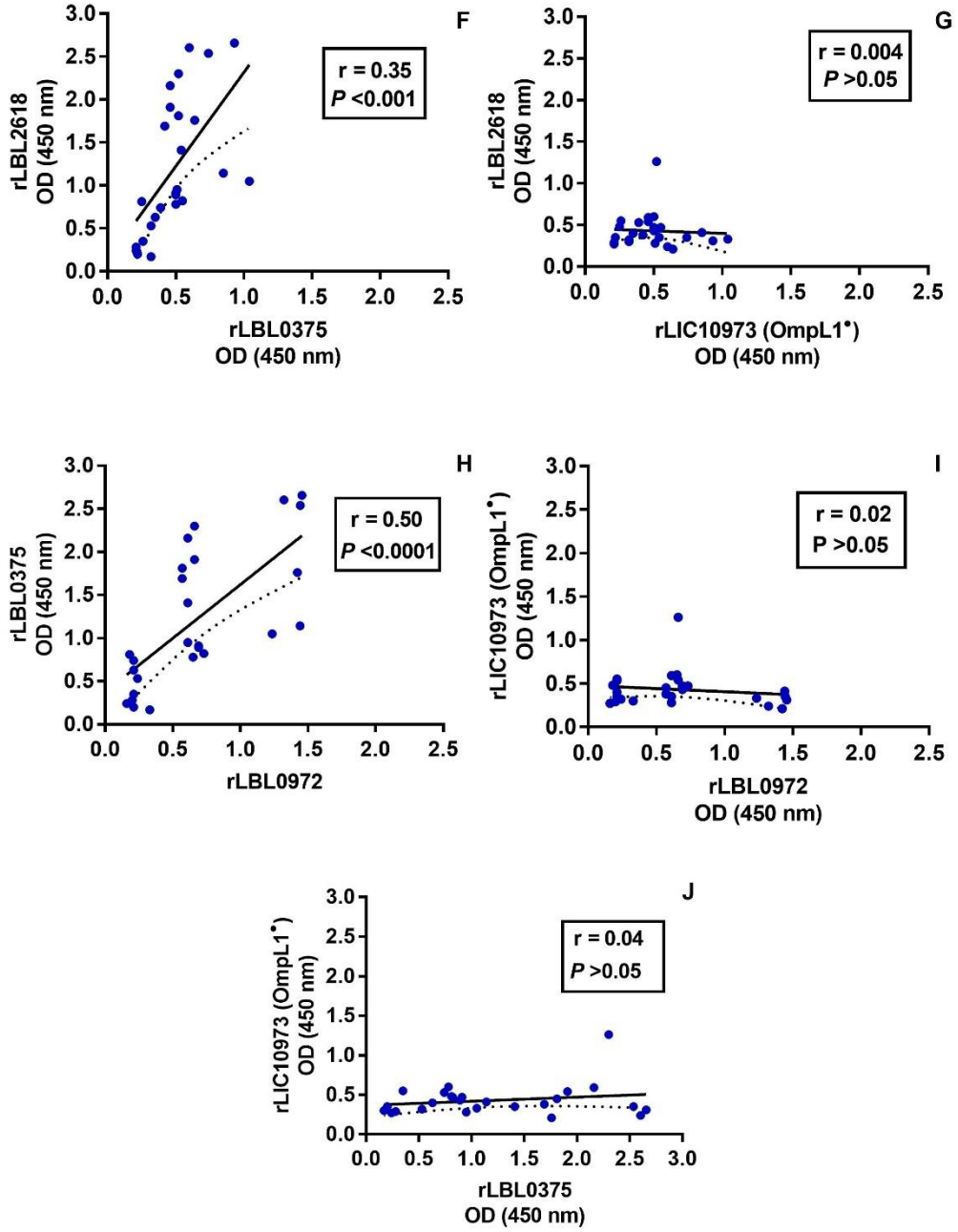


Figure 5.6 (A-J): The association of IgG1 titres of recombinant proteins against each other represented including OmpL1• positive control from L. interrogans serovar Copenhageni L1-130 using Pearson's correlation coefficient measured by the linear correlation between optical densities of tested proteins.

The trend line represents the correlation coefficient denoted as (r values), which estimates the relationship between the OD (450 nm) of both results. P-values determine the significant level of association between two assays of which P-value of at least <0.05 is considered significant.

Table 5.3: The summary matrix of r values of IgG1 titres of all recombinant OMPs against one another with their corresponding P values.

Recombinant OMPs/r values	rLBL2510	rLBL2618	rLBL0972	rLBL0375	rLIC10973
rLBL2510 (OmpL1)		r = 0.01 NS	r = 0.01 NS	r = 0.02 NS	**r = 0.41 (*P* <0.0001)**
rLBL2618			**r = 0.80 (*P* <0.0001)**	**r = 0.35 (*P* <0.001)**	r = 0.004 NS
rLBL0972				**r = 0.50 (*P* <0.0001**)	r = 0.02 NS
rLBL0375					r = 0.04 NS
rLIC10973 (OmpL1•)					

•*The OmpL1 (L. interrogans serovar Copenhageni L1-130) used as a positive control in this study.*
NS: Not significant.

5.4 Discussion

5.4.1 Leptospiral OMPs binding to host ligand molecules

Adherence of pathogens to the host cells and tissues is a critical step to establish successful infection through colonisation and dissemination. Like most bacteria, the leptospiral cell surface plays an important role in this infection process. For instance, LPS is a major cell wall structure in Gram-negative bacteria that responsible for bacterial attachment, aggregation and biofilm formation in host ECM. Another important biochemical property for adherence to the host cell is the special group of proteins known as MSCRAMM (or adhesins), which are secreted at the cell surface to interact with host cells and proteins that lead to bacterial accumulation and aggregation.

In this study, we studied the binding ability of four leptospiral OMPs, plus a positive control from *L. interrogans* serovar Copenhageni L1-130 (rLIC10973/OmpL1) that were previously identified by bioinformatics as potential adhesins towards several host ligands (Chapter 3) and produced as recombinant proteins (Chapter 4). From the host ligand screening binding, it was found that all proteins bound significantly to host fibrinogen. This finding is unsurprising because several known *L. interrogans* proteins have been characterised to bind with fibrinogen demonstrated *in vitro* experiments (Murray, 2015). Fibrinogen is a plasma protein that is essential for haemostasis and wound repair. Binding of a bacterial cell to fibrinogen may lead to disruption of blood coagulation, causing haemorrhage, which a common feature of severe leptospirosis manifestation in man (Dall'Antonia *et al.*, 2008).

Several known surface-exposed proteins of *L. interrogans* demonstrate fibrinogen-binding function including OmpL1 (Oliveira *et al.*, 2013), LigA and LigB (Stevenson *et al.*, 2007; Castiblanco-Valencia *et al.*, 2012) and several surface adhesins such as Lsa25, Lsa30 and Lsa33 (Oliveira *et al.*, 2013). As well as bacterial surface proteins binding to fibrinogen in *Leptospira,* the same fibrinogen-binding phenomena are observed by proteins found in other spirochetes, such as in *Treponema* recombinant OMPs (Staton, 2018). Interestingly, there are a limited number of studies linked to fibrinogen-binding by *Borrelia* proteins, although one *Borrelia* protein (BBA70) is able to degrade fibrinogen by activation of plasmin via plasminogen binding (Koenigs *et al.*, 2013). In this study, one of the tested *Leptospira* proteins rLBL0375 showed ambiguous dose-dependent binding to fibrinogen, and therefore, the binding is considered as non-specific.

Far-western blot is a routine molecular technique to assess protein-protein interaction (Wu, Li and Chen, 2007) and the method was employed in this study to assess the interaction between fibrinogen and recombinant *Leptospira* proteins. Interestingly, all proteins bound to β-chains, and two proteins (rLBL2618 and rLBL0972) showed binding to all fibrinogen chains. Attachment of proteins to either α and β chains (which make up the thrombin binding site) inhibit thrombin cleavage from releasing fibrinopeptides A and B, a component essential for fibrin formation during blood clotting (Madrazo *et al.*, 2001). Binding to the fibrinogen γ chain may also have effects in haemostasis and other blood-related mechanisms (de Willige *et al.*, 2009). These findings show that our recombinant proteins may be able to cause the haemorrhagic syndrome, a role that needs to be translated into an animal host for future investigations.

Fibronectin is an ECM component which is found as insoluble cellular fibronectin and also exists in the soluble form (soluble plasma fibronectin). Both forms play important roles for cellular attachment, growth, migration and differentiation, as well as in wound repair and embryonic development. In this study, most of the tested proteins exhibited significant binding to cellular fibronectin. The finding parallels that of more than 30 leptospiral proteins that were previously characterised (Murray, 2015). Fibronectin-binding proteins are also an important feature of *Treponema pallidum* (Tp0155 and Tp0483) and *Borrelia burgdorferi* (RevA and RevB) in allowing pathogen colonisation of the host (Cameron *et al.*, 2004; Brissette *et al.*, 2009). Additionally, our fibronectin-binding proteins exhibited stronger affinity binding at lower protein concentrations (rLBL_2618: K_d 0.10 ± 0.02 and rLBL0972: K_d 0.43 ± 0.20) compared to rLIC10973 (OmpL1) (K_d 0.81 ± 1.30) and saturated at an increasing

concentration in dose-dependent saturation studies reflecting the strong fibronectin binding function and eliminating the possibility of non-specific protein-ligand interaction.

Laminin is a protein component of ECM found abundantly on the surface of endothelium, mesothelium and endothelium layers of most cells and organs. Attachment of pathogens to laminin enhances invasion and dissemination to organs. Binding to host laminin is a crucial function for spirochetes to begin bacterial colonisation of the host and is mediated by several identified adhesins such as Tp0751 and BmpA (Cameron, 2003; Verma *et al.*, 2009). Interaction of *L. interrogans* proteins with laminin has been reported, and it was shown that a large number of leptospiral proteins had been found to manifest this function *in vitro* assays (Murray, 2015). In this study, positive control OmpL1 of *L. interrogans* (rLIC10973) showed significant binding to laminin (P <0.01), which is supported by the previous finding by Fernandes *et al.* (2012). Interestingly, we found that one OMP of *L. borgpetersenii* (rLBL2618) had statistically significant binding (P <0.001) to host laminin compared to BSA. The finding is interesting because it shows that laminin-binding function is not only restricted to a particular protein family domain (e.g. OmpL1, OmpL37 and OmpL47) within a particular species (*L. interrogans*) (Pinne *et al.,* 2010; Fernandes *et al.*, 2012), the result suggest that *L. borgpetersenii* acquire the laminin-binding gene through genetic expansion resulting in several surface-exposed proteins have the similar ability to interact with host laminin.

Based on our findings, it is quite clear that two these bovine OMPs, rLBL2618 and rLBL0972 (but not rLBL0375) are considered novel adhesins, therefore, we designate them as '**L**epto**s**piral **a**dhesin' (denoted by an abbreviation 'Lsa') followed by their respective molecular sizes; rLBL2618 (49 kDa) as **Lsa49** and rLBL0972 (37 kDa) as **Lsa37**. The term will be used throughout the thesis otherwise stated. However, it is difficult to interpret the non-specific high titre of Lsa49 binding saturation curve to BSA and Lsa37 to fibronectin. One possible reason is due to the prozone (or hook) effect due to competitive binding between both antibody (his-tagged) and conjugate antibody against antigen (OMP) and preventing the sandwich formation (Roy *et al.,* 2017). As a result, antigen bound with conjugate antibody will be rinsed off and giving a false 'low' signal which will be picked up by ELISA plate reader. This will create a formation of a 'hook'like effect when ELISA data is plotted as OD versus antigen concentration.

The overall binding results suggest that these proteins possess a high degree of functional redundancy. This is not surprising as many leptospiral proteins have paralogs which overlap

in functional binding to various host ECM as a result of genomic expansion through genetic duplication (Murray, 2015). The emphasis of functional redundancy is intricate as the proteins may work in different stages, either during different infection stages, or different tissues or may even work simultaneously. It may be possible that the loss of one putative function of one protein is covered with another protein of similar function (Adler *et al.*, 2011).

5.4.2 Reactivity of leptospiral recombinant proteins in cattle bulk milk samples

The ELISA-based method was performed to analyse the immune reaction of cattle IgGs to novel recombinant leptospira OMPs in cattle bulk milk, of which the leptospirosis status was pre-determined using the commercial *Leptospira* test kit. IgG is the dominant antibody in cattle milk and mediates pathogen opsonisation, which promotes their destruction via phagocytosis by polymorphonuclear cells (PMNs). In this study, OmpL1 (rLBL2510) of *L. borgpetersenii* serovar Hardjobovis L550 and Lsa37 showed a statistically significant immune IgG1 titre between positive and negative samples. The finding is interesting because OmpL1 was previously demonstrated as a serological antigen for the diagnosis of human and canine leptospirosis (Flannery *et al.*, 2001; Okuda *et al.*, 2005; AiHua *et al.*, 2011) and has never been tested in cattle milk samples. In contrast to this, OmpL1 (rLBL10973) of *L. interrogans* serovar Copenhageni Fiocruz L1-130 in this study did not have a significant immune reactivity titre. The differences between these two OmpL1s are clear given there is only 85% of amino acids shared and substantial diversity in predicted loop regions (Haake *et al.*, 2004). We considered these differences meant that specific *Leptospira* antibodies produced in cattle might not recognise *Leptospira* proteins from non-bovine *Leptospira* strains. Thus, further investigation is warranted to determine whether OmpL1 obtained from various *Leptospira* strains can be recognised in cattle milk samples.

Additionally, Lsa37 was recognised by cattle humoral immune system, demonstrated by a high IgG1 titre in *Leptospira* positive samples, although this protein has no apparent binding functions as previously described in the result section. This suggested that this protein is likely to be expressed in cow's milk during infection, however, may not be required in actual infection. We suggest that based on our data that this protein does not act as an adhesin or that it is likely to have another function apart from binding to host molecules which we have not tested for. The finding is similar to one leptospiral OMP (LipL41) that has no binding function, but largely recognised by host immunity during convalescent-phase and not necessary for *Leptospira* virulence (Guerreiro *et al.*, 2001; King *et al.*, 2013). Additionally, a high antibody titre detected in this study using Lsa37 suggests that this protein is recognised

by the immune system and can be considered as a potential diagnostic assay to detect specific anti-leptospiral immunoglobulin presence in cow's milk. No significant reaction was observed against IgG2 to all tested OMPs. This is likely due to the low concentration of IgG2 comparing to IgG1 in cattle milk caused by preferential uptake of IgG2 back to the extracellular fluid, and not passed on the alveolar lumen and into the mammary secretion (Butler, 1969; Hurley and Theil, 2013). Due to this reason, the IgG2 titres in milk samples are expected to be low and concurred with the findings in this study.

The investigation to determine any association between a standard *Leptospira* diagnostic assay and recombinant OMPs in cattle bulk milk in this study showed no correlation. This was probably due to different antigens in ELISA assays used e.g. LPS versus OMP. It is known that LPS (contained within the Linnodee *L. Hardjo* ELISA test kit) is an immunodominant antigen and demonstrates high sensitively to both infection and vaccination (Chapman *et al.,* 1988; Gitton *et al.*, 1994). This is most likely due to the ability of LPS to activate polyclonal B cells, causing cell proliferation and generates mounted antibodies against LPS, contributed to a high anti-LPS immune response (Parekh *et al.*, 2003). Additionally, lack of correlation observed between OMP and LPS in this study indicates that the antibody induced by the OMPs may be lesser in quantity due to poor surface exposure, low expression or temporal expression limited to an infection phase that does not stimulate a large immune response. For example, LPS is also termed endotoxin because it is a toxin released on bacterial death where the host might produce a large antibody and inflammatory response against the LPS (Sweet and Hume, 1996). In contrast, the OMPs may interact with the immune system early during infection during the colonisation phase using their adhesin functions reported here and not be expressed during cell death.

When comparing the immune titres between each OMPs, Lsa37, Lsa49 and rLBL0375 were positively correlated to one another but not to OmpL1. Conversely, OmpL1 from both species (rLBL2510 and rLIC10973) demonstrated a strong correlation to one another. The finding is interesting, because this may indicate that these OMPs could be expressed in the milk and recognised by the cattle immune system. A moderate to strong correlation represented in positive linear regression (r values between +0.35-0.80, P <0.0001) between OMPs, except to both OmpL1s in this study indicates that these proteins are likely to have similar immune responses. It may be possible Lsa49, Lsa37 and rLBL0375 shared similar antigenic epitopes that are identical and equally recognised by host immunity, resulting in strong titre compared to OmpL1. Additionally, these OMPs may have a large surface-exposed structure that

potentially allows more antibody binding reflecting the significant immune response. A strong correlation between the two OmpL1s (r = + 0.4, *P* <0.0001) suggests conserved immunogenicity given they are the same OMP from different pathogenic *Leptospira* species as previously reported (Dong *et al.*, 2008; Dezhbord *et al.*, 2014; Muthiah *et al.,* 2015) especially given there is >85% amino acids conserved regions between *L. interrogans* serovar Copenhageni Fiocruz L1-130 and *L. borgpetersenii*. Furthermore, *Leptospira* was previously demonstrated in mammary tissues of both human and animal host (Bolin and Koellner, 1988; Oliveira *et al.*, 2016) that leads to vertical transmission, therefore, it may be possible that OmpL1 may be directly involved in *Leptospira* interaction in the host mammary tissues for colonisation. However, it is difficult to intricate the lack of correlation between OmpL1 and all OMPs seen in this study. We suggest that this is could be due to OMP structural diversity and evidence of reciprocal expression. The structural difference of OmpL1 and other OMPs in this study could affect the outcome of the host immune response. As previously noted, OmpL1 may not share similar antigenic epitopes compared with the rest of OMPs, and therefore, this may trigger a distinct host immune response to produce different between antibody titres measured between two types of OMPs. This is interesting findings; therefore, a further study is warranted to identify the epitope structures of each OMPs and compare to one another to determine the conserved amino acid regions that allow antibody binding.

Secondly, the difference in antibody titres also could be due to reciprocal expression during stages of infection. As seen in other pathogenic spirochetes (e.g. *Borrelia burgdorferi*) the expression of *Borrelia* outer surface proteins occurs in different infection stages between tick reservoir and definitive host. For instance, expression of outer surface protein A (*ospA*) is induced by an unfed tick and the expression maintained until tick feeds on mammal blood, marking the transmission of bacteria to the host (Schwan *et al.*, 1995). At this stage (host invasion), *ospA* expression is greatly reduced and switched to outer surface protein C (*ospC*) expression which is essential for bacterial survival in the bloodstream of the host (Tilly *et al.*, 2006; Caine and Coburna, 2015). In this study, it may be possible that Lsa37, Lsa49 and rLBL0375 are likely to be expressed in milk at certain disease stage, whilst OmpL1 expression is not prominent during this time. It should be noted that anti-OmpL1 is consistently detected in the serum of infected patients during from both acute and convalescent phase (Guerreiro *et al.*, 2001; Fernandes *et al.*, 2012) reflecting that OmpL1 expression may be more significant in the blood rather than in milk.

5.4.3 Limitations of the study

Several limitations were noted in this study. For instance, BSA was used as a sole negative protein control in binding studies, which may give non-specific binding interactions with host ligands and therefore reduce the statistical significance of comparator ligands in turn. Although BSA is widely used as a sole negative control in host-ligand studies, future studies might include other negative controls such, as fetuin or gelatine in parallel to BSA in a single experiment (Pinne *et al.,* 2010; Siqueira *et al.*, 2013). Moreover, our *in vitro*-characterised proteins require further investigation in animal models, and that any artefactual findings need to be identified and eliminated. Nevertheless, our findings provide useful information on the possible pathophysiology interactions of these novel antigens during infection. Additionally, several considerations need to be taken into an account when interpreting the results in this study. As we do not know the true infection status of the animal (e.g. by using experimentally infected cattle), it is not clear if the expression of these OMPs is reflective of true disease progression. These OMPs may be expressed and detected by the host immune system as a result of leptospires, but this may not necessarily be due to acute disease and could reflect subclinical infection with little clinical signs. Therefore, the antibody titres measured by ELISA in this study may represent a chronic or recovered phase of the disease, as frequently seen in the longitudinal antibody responses exhibited during human infection (Picardeau *et al.*, 2014).

5.5 Conclusion

The present study has opened up a broad perspective towards understanding the *L. borgpetersenii* -host interactions. More studies are warranted to further characterise the role of these leptospiral OMPs as potential virulence factors in *Leptospira* pathogenesis, which may possibly be identified via future mutagenesis and *in vivo* analyses. In this study, we have characterised novel bovine *Leptospira* OMPs from *L. borgpetersenii* serovar Hardjobovis L550 through binding studies using various host ligands and evaluated the cattle's immune response in bulk milk samples using these recombinant proteins as test antigens. Two OMPs showed an adhesive property towards several host components and therefore, they are designated as Lsa49 and Lsa37 according to their function and molecular sizes. Taken together, we consider that these proteins might have a significant role during the leptospiral invasion of the host.

Chapter 6: Functional and immunological characteristics of OMP variants from different pathogenic *Leptospira* species

6.1 Introduction

6.1.1 The genetic evolution of *Leptospira* species

The *Leptospira* genus is one of the most genetically diverse organisms due to their ability to infect a large spectrum of mammalian hosts. The phylogenetic comparative analysis of 16S rRNA sequences showed the *Leptospiracae* emerged from the deepest branch of their phylogenetic tree, which diverged into *L. biflexa* (saprophytic) and *L. interrogans* (pathogenic), which resulted from a single evolutional event (Schwan *et al.*, 1991). To date, there are more than 250 recognised pathogenic serovars, of which 24 of them are antigenically defined as serogroups (Cerqueira and Picardeau, 2009; Adler and de la Peña Moctezuma, 2010). The classification of leptospires is further complicated as there are now 32 genomospecies recognised during more recent taxonomic reassignment and reappraisal of the genus (Figure 1.3, Chapter 1).

The diversity of *Leptospiracae* is attributed to both reduction and addition of genes, as well as the variation of genetic determinants that may contribute to their ability to infect mammalian hosts and survive in host or environment. The availability of genome sequence allows the study of the evolution of a pathogen and can provide insights into how the gene encoding a particular function evolved, and associated outcomes. For example, a detailed genomic study on a non-pathogenic strain of *L. biflexa* (which has 2/3 of orthologue genes in both *L. interrogans* and *L. borgpetersenii* strains) revealed that survival of *L. biflexa* within the environment is due to the presence of tertiary circular replicon (p74) that is not present in all pathogenic species (Picardeau *et al.*, 2008). Other genetic determinants are also thought to influence the ability of *L. biflexa* to survive to the environment with an abundant number of signal transduction and exopolysaccharide genes compared to pathogenic species, which helps to enhanced metabolic capability, contributing to fast growth rate and ability to form a biofilm reflecting their ability to survive in aquatic environment (Picardeau *et al.*, 2008).

Additionally, differences in genome size appear to contribute to the ability of *Leptospira* to survive within the host. Genome reduction, observed in *L. borgpetersenii* and *L. biflexa* compared to *L. interrogans,* is thought to limit survival of the microbe to either the host or

environment, respectively. For example, there are 16% of genes absent in *L. borgpetersenii* serovar Hardjobovis strains L550 and JB197, in comparison to *L. interrogans*. This difference is attributed to a process of insertion sequence (IS) mediated genome reduction resulting in host-to-host transmission restriction due to loss of several genes essential for broad survival (Bulach *et al.*, 2006). However, the same study revealed that 31% of *L. borgpetersenii* genes encode predicted OMPs and lipoproteins that includes the transmembrane porin (OmpL1) and TonB/TonC related proteins similar to *L. interrogans* genomes. Identification of leptospiral OMPs is essential for the functional and immunological studies that lead to potential vaccine development, and therefore, it is important to evaluate the variability of these proteins at the molecular level and identify any functional differences.

6.1.2 Outer membrane protein variation in pathogenic *Leptospira* species

Comparative sequence analysis of OMP genes can provide information on molecular evolution in pathogenic bacteria. An excellent example of this is the variation of leptospiral OMP genes as reported by Haake *et al.* (2004) for several leptospiral antigenic OMPs (OmpL1, LipL41 and LipL32). This study revealed highly variable DNA and amino acid sequences for OmpL1 followed by LipL41, whilst LipL32 and 16S rRNA genes were the most conserved. The evolutionary mechanism for this diversity was both horizontal DNA transfer across *Leptospira* species as well as single-nucleotide mutation. For OmpL1, when multiple *Leptospira* species were compared, there was evidence of both positive selection, as well as a gene segment being identified as undergoing genetic recombination including the acquisition of sequence from a peregrine allele of unknown origin (Haake *et al.*, 2004). Historically, several Gram-negative bacterial OMPs have undergone similar genetic recombination, including WSP of *Wolbachia* spp. (Baldo *et al.,* 2005; Desjardins *et al.*, 2010), PorA and PorB of *Neisseria meningitis* (Feavers *et al.*, 1992; Bash *et al.*, 1995; Dyet and Martin, 2005) and OspA and OspC of *Borrelia* spp. (Marconi *et al.*, 1994; Jauris-Heipke *et al.*, 1995).

The molecular evolution of OMPs within species and exchange of genetic materials between species raise a critical question; ***Does interspecies gene variation of OMPs affect functional capability?*** Vedhagiri *et al.* (2009) suggested variability of leptospiral OMP genes acquired through a slow evolutionary process may have affected the antigenicity and pathogenic characteristics of serovar-specific strains. Nevertheless, a study on OmpL1 immunogenicity across pathogenic species revealed that variations in OmpL1 sequences did not affect the protein's immunogenicity, although optimal vaccine protection rates were only observed when the OmpL1 vaccine allele matched the allele within the infecting leptospire from the

challenge model (Dong *et al.*, 2008). Little is known about whether variation in individual genetic loci affects the functional ability of *Leptospira* to infect host tissues. Therefore, the present study aimed to determine whether the variation of two key OMPs across pathogenic *Leptospira* genomospecies, resulted in any phenotypic divergence, namely in host molecule adhesion and immunogenicity.

6.2 Materials and methods

6.2.1 Identification of OmpL1 and Lsa49 variants across *Leptospira* pathogenic species

A BLAST protein search (BLASTp) was carried out to identify OMP variants across all leptospiral pathogenic genomospecies for OmpL1 and Lsa49 using query gene sequences from *L. borgpetersenii.* The resulting output list of sequences was compiled into a sequence alignment using Bioedit and was filtered by carefully removing duplications of identical genomospecies sequences and examining the locus tags for correct annotation and analysing sequence identity matrix. Based on available annotated OmpL1 sequences, all sequences shared at least >85% amino acid sequence identity in line with a previous study (Haake *et al.*, 2004). As Lsa49 was not already annotated on line, we included all *Leptospira* genes containing DUF1566 across genomospecies, which resulted in minimum >65% amino acid sequence identity. A phylogenetic tree of each OMP variants collection was constructed using the maximum-likelihood method (Tamura *et al.*, 2013). The tree, together with a generated sequence identity matrix was analysed for both proteins, so that a total of five sequences for each OMP (and including the original *L. borgpetersenii* serovar Hardjobovis L550 sequence) were selected from different separated deep branches.

6.2.2 Construction of expression vector

Signal peptides from each selected amino acid sequence were removed using the prediction from SignalP 3.0 as previously described (Chapter 3), and all sequences were submitted to GeneMill (University of Liverpool, UK) to synthesise expression constructs containing DNA of interest within the pET system via modular cloning. The synthesised plasmids were propagated in *E. coli* Top10 cells and were grown on LB plates containing 100 µg/ml ampicillin overnight. Five to ten colonies were selected and grown in LB broth containing 100 µg/ml of ampicillin solution overnight, and plasmids were harvested using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Plasmid preps were stored at -20°C for further downstream applications.

6.2.3 Protein expression and purification of *Leptospira* recombinant OMP variants

All recombinant protein expression was performed according to Chapter 2 and 4, with minor modifications. In large scale expression, the transformed *E. coli* BL21-AI cultures were induced using a combination of 0.2% (w/v) L-arabinose and 1 mM IPTG at a final concentration as recommended by the manufacturer. Inclusion body extraction and protein purification were carried out as described in Section 2.19-2.22, and purified proteins were concentrated when the yield was below 200 µg/ml. All proteins were stored at -80°C for further application.

6.2.4 Circular dichroism spectroscopy of *Leptospira* recombinant OMP variants

Circular dichroism (CD) spectrometry measurements were performed on all recombinant OMP variants by using a Jasco-1100 spectropolarimeter equipped with a Peltier unit for temperature control (Jasco, Easton, MD). Far-UV CD spectra were measured in a 0.1-mm-path-length cell at 0.5 nm intervals. The spectra reading was taken as an average of three readings from 190 to 260 nm. Spectrum data were analysed similarly as described in Section 4.2.5.

6.2.5 Screening and binding saturation evaluation of *Leptospira* recombinant OMP variants to host molecules

Binding of leptospiral recombinant OMP variants to multiple host molecules via an ELISA-assay was performed similarly to Chapter 5 (Section 5.2.5), and ligands with statistical significance were selected for dose-response saturation binding to determine the binding affinity of each OMPs similar as described in Section 5.2.6 in this thesis.

6.2.6 Immunological evaluation of leptospiral OMP variants in cattle's sera

Thirty cattle sera were collected from a national diagnostic lab (Scottish Rural College, UK). Leptospirosis status of each serum was determined in the lab using a commercial *Leptospira* detection kit PrioCHECK™ *L. hardjo* Ab Strip Kit (Bercovich *et al.*, 1990). The kit utilises whole-killed cells from *L. interrogans* serovar Hardjo as a coating antigen, and the procedure was carried out according to manufacturer's instruction which can be found in Appendix C (Figure C.2). Sera were kept at -20°C in 100 µl aliquots and diluted as required. Detection of anti-leptospiral antibodies against leptospiral recombinant OMPs in serum samples was carried out based on the ELISA method as described in Section 2.26.2.

6.3 Results

6.3.1 Selection of OmpL1 and Lsa49 across pathogenic genomospecies

Five of each OmpL1 and Lsa49 representing the diversity across all pathogenic *Leptospira* genomospecies were selected from different deep branches of the phylogenic tree Figure 6.1 and Figure 6.2) and included OmpL1 (LBL2510) and Lsa49 (LBL2618) previously described in Chapter 3. The sequence analysis matrix determined the shared amino acid sequence identity of these sequences is at least 85% (for OmpL1) and 65% (for Lsa49) (Results not shown). Lsa49 protein was identified by using the presence of the Domain of Unknown Function 1566 (DUF1566) protein group or belonging to family protein 07603 (FP07603), of which can be found in *L. interrogans*, has previously been hypothesised as playing a role in host-pathogen interactions (Atzingen *et al.*, 2012; Domingos *et al.*, 2012). The DUF1566 is located in two different locations within Lsa49 amino acid sequence of all genomospecies, which are between 176-305 and 320-345 of the mature peptide, respectively (Figure 6.14). Table 6.1 shows the selected OmpL1 and Lsa49 variants across genomospecies in this study.

Table 6.1: Selection of OMP variants from phylogenetic analysis.

NCBI Accession No./ Locus tag	*Leptospira* species	Protein domain[a]	Identity to OmpL1 or Lsa49[b]	Expession vector[c]
LIC_10973*	*L. interrogans* svr Copenhageni L1-130	OmpL1	100%	-
LBL_2510*	*L. borgpetersenii* svr Hardjobovis L550	OmpL1	87%	-
WP_061249915.1	*L. alstonii*	OmpL1	86%	pGM176_8
WP_046692058.1	*L. santarosai*	OmpL1	94%	pGM176_7
WP_004450512.1	*L. noguchii*	OmpL1	88%	pGM176_3
AAT_48513.1	*L. interrogans* svr Pyrogenes	OmpL1	86%	pGM176_1
LBL_2618*	*L. borgpetersenii* svr Hardjobovis L550	DUF1566/Lsa49	100%	-
WP_061250085.1	*L. alstonii*	DUF1566/Lsa49	69%	pGM176_9
WP_036069232.1	*L. noguchii*	DUF1566/Lsa49	65%	pGM176_6
WP_020778757.1	*L. kirschnerii*	DUF1566/Lsa49	65%	pGM176_5
WP_004492537.1	*L. santarosai*	DUF1566/Lsa49	77%	pGM176_4

[a] The protein domain for each OMP were identified by a database of protein families (Pfam) (Finn, 2006).

[b] The amino acid identity of each OMP versus OmpL1 or Lsa49 (DUF1566) were determined through sequence identity matrix aligned in Bioedit. DUF; Domain of unknown function.

[c] The expression vector code for each OMP variants.

**OMP included from the previous studies (Chapter 3-5).*

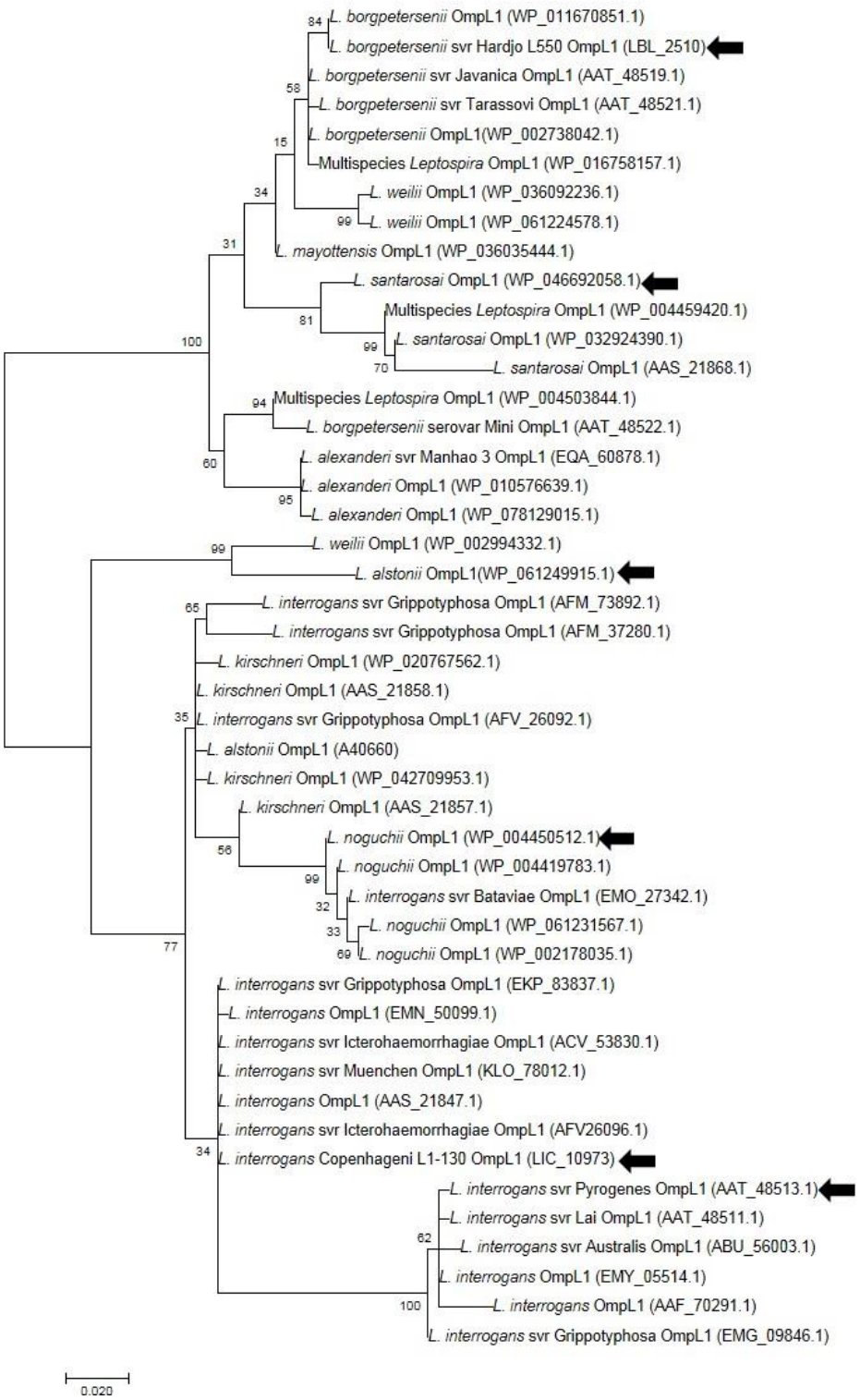


Figure 6.1: The phylogenetic analysis of the amino acid sequence of OmpL1 across various pathogenic Leptospira genomospecies and serovars by maximum likelihood method.

Six OmpL1 genes; L. interrogans serovar Hardjobovis L550 (LBL_2510), L. interrogans serovar Copenhageni L1-130, L. interrogans serovar Pyrogenes (ATT_48513.1), L. santarosai (WP_046692058.1), L. alstonii (WP_061249915.1) and L. noguchii (WP_004450512.1) from the each deepest branch (indicates by arrows) were selected for functional and immunological evaluation in this study (Table 6.1).

***Figure 6.2: The phylogenetic analysis of Lsa49 amino acid sequences across pathogenic Leptospira genomospecies by maximum likelihood method and rooted by a distantly related 49 kDa protein (Lp49) from L. interrogans (denoted by a circle)* (Giuseppe *et al.*, 2008).**

Five Lsa49 genes, indicated by arrows; L. borgpetersenii serovar Hardjobovis L550 (LBL2618), L. kirshneri (WP_020778757.1), L. santarosai (WP_004492537.1), L. alstonii (WP_061250085.1) and L. noguchii (WP_036069232.1) were randomly selected for functional evaluation in this study (Table 6.1).

6.3.2 Protein overexpression and purification of selected OMP genes

The majority of selected OMP genes were successfully expressed in *E. coli* and purified with the concentration of each protein varying from 0.2-1.7 mg/ml. However, only one OMP (Lsa49 of *L. santarosai*) had poor expression despite several expression attempts and was excluded from this study. Additionally, all Lsa49 variants required centricon concentration due to poor yield. All expressed proteins were analysed on 12% (v/v) SDS-PAGE gels.

6.3.3 Secondary structures of OMP variant recombinant proteins

Far-UV CD spectra data of all purified recombinant OMPs demonstrated all OmpL1 preparations had predominantly β-sheet spectra in their secondary structure, which agrees with the OmpL1 data in this study (Chapter 4) and from the previous study (Fernandes *et al.*, 2012). All Lsa49 protein also manifested a similar spectrum, which is indicative of β-sheet secondary structure, with some additional protein features as previously discussed in Chapter 5. The CD spectrum of all recombinant OMP variants was analysed using online tools; BestSel and CAPITO as described previously. Spectra results of all OMP variants are shown in Figure 6.3 and Figure 6.4, and the detailed analysis of each OMP can be found be Appendix C (Table C.4 and C.5).

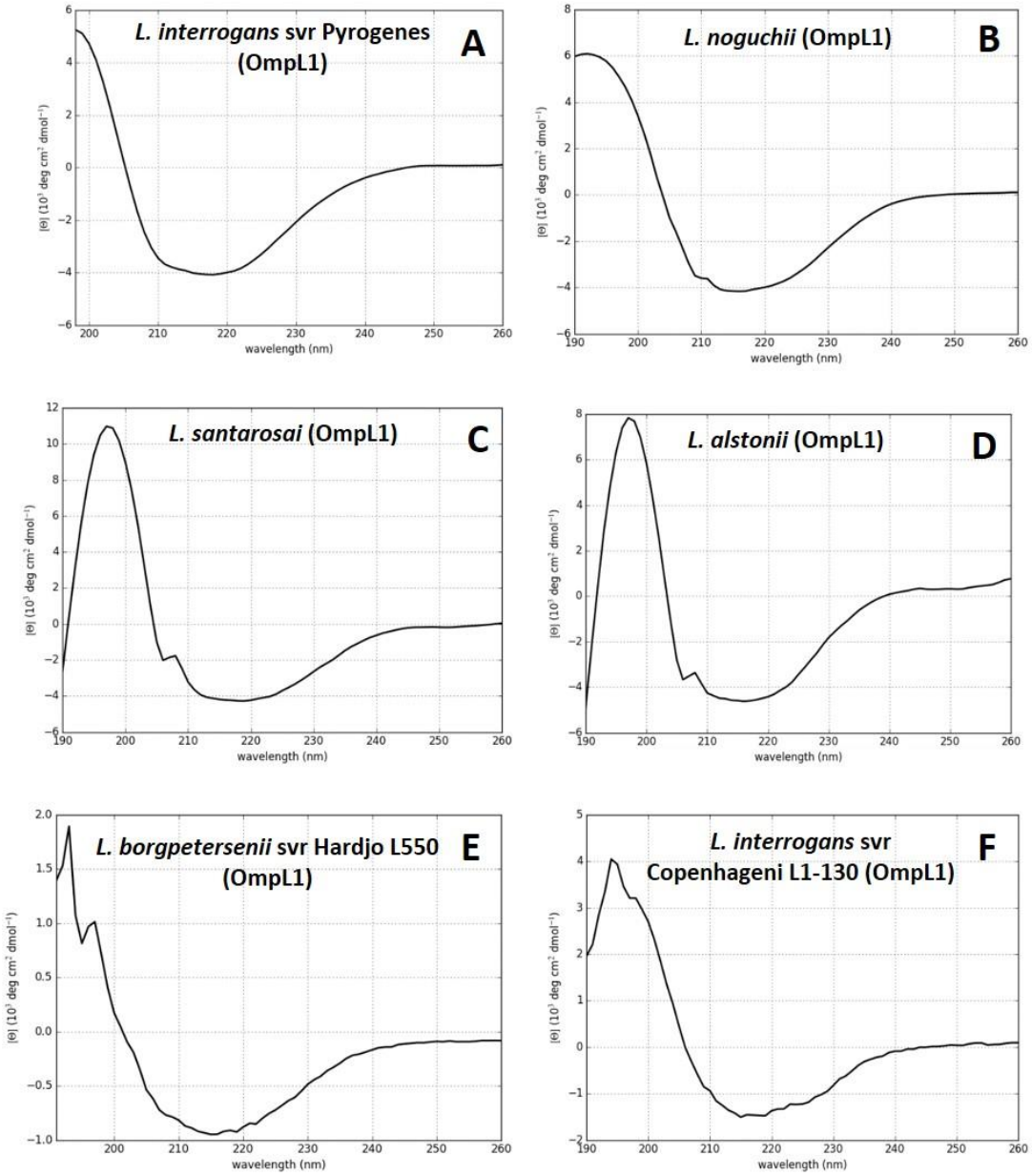


Figure 6.3 (A-F): Circular dichroism (CD) spectra of OmpL1 variants across selected genomospecies.

All OmpL1 variants are showing a β-sheet profile with a minima between 210 nm – 215 nm and a maxima between 195 nm – 200 nm. The CD spectrum is presented as an average of three scans recorded from 190 to 260 nm. All graphs were plotted using CAPITO software.

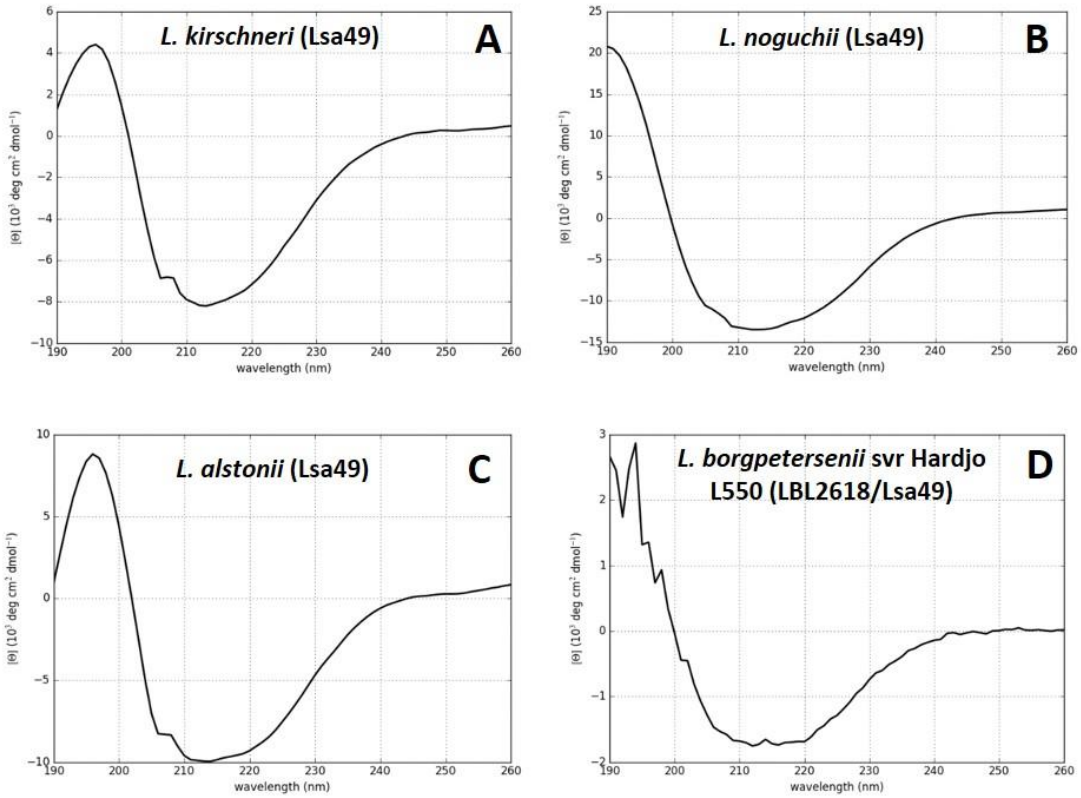


Figure 6.4 (A-D): Circular dichroism (CD) spectra of Lsa49 variants across selected Leptospira genemospecies.

All Lsa49 variants are showing a β-sheet profile with a minima between 210 nm – 215 nm and a maxima between 195 nm – 200 nm. The CD spectrum is presented as an average of three scans recorded from 190 to 260 nm. All graphs were plotted using CAPITO software.

6.3.4 Binding of leptospiral OMP variants to host molecules

All recombinant OMP variants were subjected to functional host molecules binding by an ELISA-based assay as described briefly in Section 6.2.5. For OmpL1, binding variation was observed between *L. borgspetersenii* serovar Hadjo L550 and *L. santarosai* both bound significantly to fibrinogen (P <0.001), and interestingly *L. borgpetersenii* serovar Hadjo L550 and *L. santarosai* showed remarkable adherence towards bovine elastin (P <0.001). The OmpL1 of *L. santarosai* also bound to laminin (P <0.001) and chondroitin (P <0.05). The OmpL1 from both *L. interrogans* also showed significant host molecule binding variation; *L. interrogans* serovar Copenhageni L1-130 has additional binding preference to fibronectin (P <0.001) and laminin (P <0.001) and also chondroitin (P <0.05) compared to *L. interrogans* serovar Pyrogenes that bound only to fibrinogen (P <0.001). Furthermore, no significant binding was observed to collagen and heparan sulphate for all OmpL1s (Figure 6.5).

Due to the significant elastin (bovine skin) binding of OmpL1 observed in *L. borgpetersenii* serovar Hadjo L550 and *L. santarosai*, an additional elastin binding study was carried out using human skin and aorta elastin based on the previous experiment by Pinne, Choy and Haake (2010). Interestingly, OmpL1 of *L. borgpetersenii* showed a statistically significant level of adherence to both aorta and skin elastin (P <0.001), whereas OmpL1 from *L. santarosai* showed binding preference to only aorta elastin (P <0.01) (Figure 6.9).

For Lsa49 variants, there was also some variation in host ligand binding between species. In general, all Lsa49 proteins bound to fibrinogen (with P <0.001, to P <0.05). Only *L. alstonii* and *L. borgpetersenii* serovar Hardjobovis L550 exhibited additional bindings to laminin and fibronectin (P >0.001) (Figure 6.6). Additionally, no significant binding was observed to heparan sulphate, chondroitin, collagen, and elastin, respectively. Table 6.2 shows the binding results of all recombinant OMP variants.

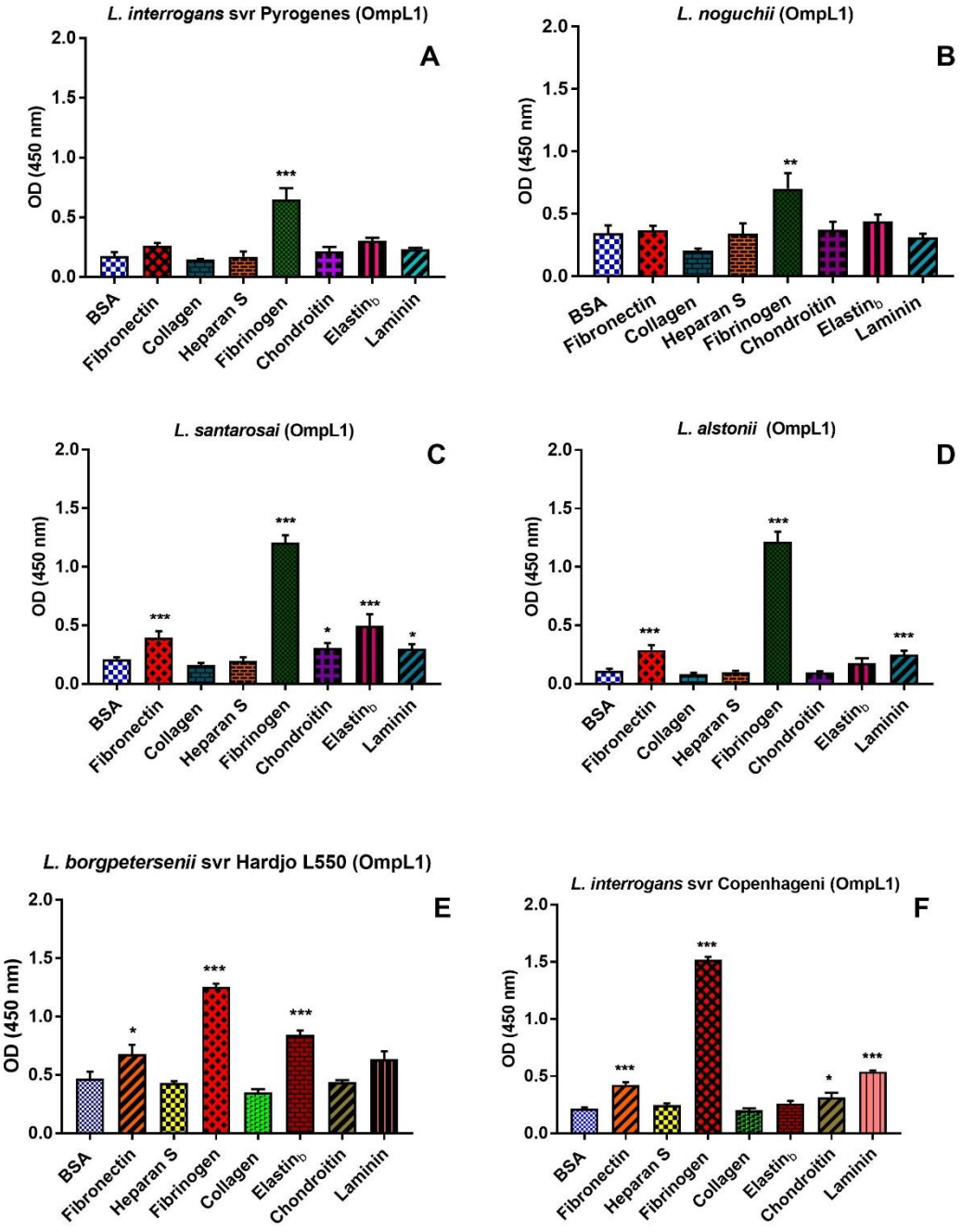


Figure 6.5 (A-F): Binding to host components including bovine elastin (denoted by subscript b) of leptospiral recombinant OmpL1 variants across various Leptospira pathogenic genomospecies.

*Data represent the mean absorbance at 450 nm ± the standard error and the mean (SEM) of three independent experiments. The binding of recombinant proteins to tissue components was compared to their binding to BSA by Dunnett's multiple comparison test (*** P <0.001, ** P <0.01, * P <0.05).*

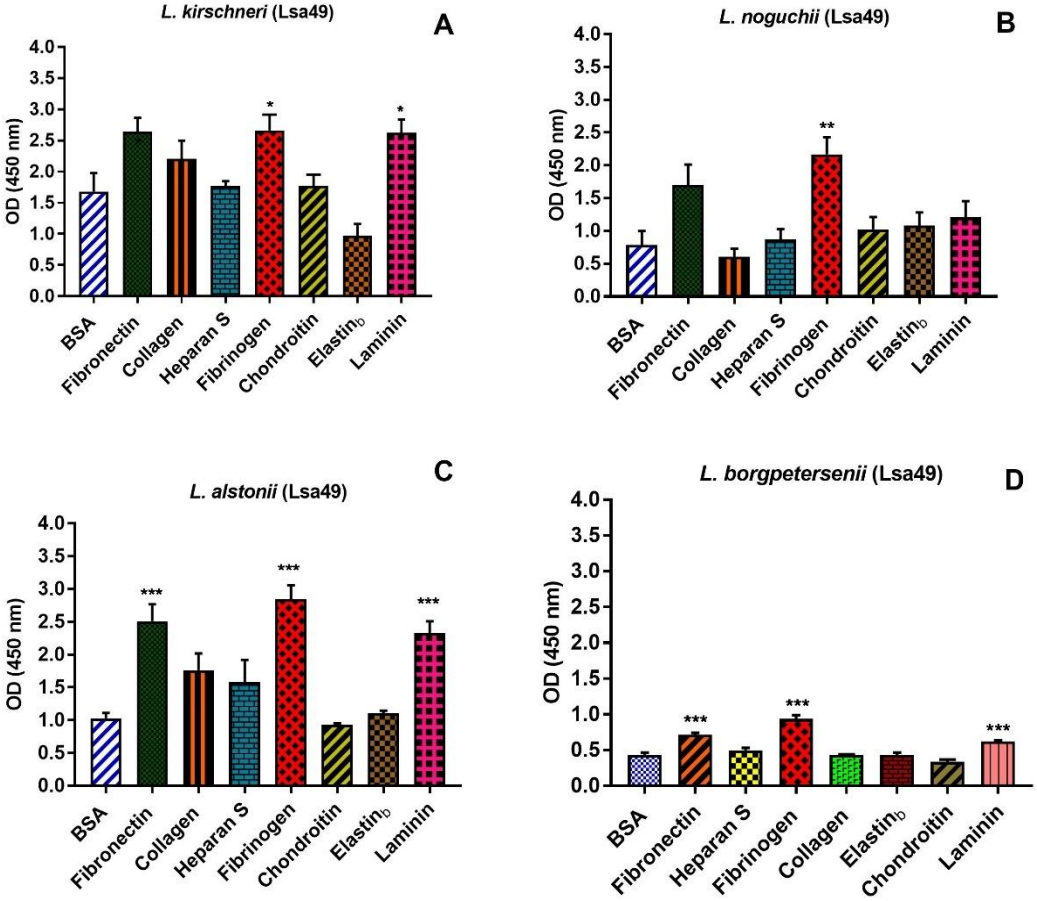


Figure 6.6 (A-D): Binding to host components including bovine elastin (denoted by subscript b) of recombinant Lsa49 variants across various Leptospira pathogenic genomospecies.

Data represent the mean absorbance at 450 nm ± the standard error and the mean (SEM) of three independent experiments. The binding of recombinant proteins to tissue components was compared to their binding to BSA by Dunnett's multiple comparison test (P <0.001, ** P <0.01, * P <0.05).***

6.3.5 Binding saturation curves of leptospiral recombinant OMP variants to selected host molecules

All host molecules for which significant OMP binding occurred were selected for dose-dependent experiment binding using a ELISA-based assay similarly as described in Section 6.2.5. For OmpL1, all species showed a nearly saturated curve for fibrinogen, and the strong binding association was observed equally for both *L. santarosai* and *L. alstonii* with K_d of 0.29 ± 0.08 µM and 0.21 ± 0.12 µM, respectively. The OmpL1 from *L. borgpetersenii* serovar Hardjobovis L550 showed a stronger affinity to bovine elastin (K_d 0.83 ± 0.42 µM) compared with *L. santarosai* (K_d 1.83 ± 1.27 µM) (Figure 6.7). Additionally, binding saturation of OmpL1 of *L. borgpetersenii* was achieved to both human aorta (K_d 0.75 ± 0.22 µM) and skin elastin (K_d 2.28 ± 1.07 µM), respectively (Figure 6.10). However, the binding saturation of OmpL1 of *L. santarosai* to aorta elastin was not determined due to limited protein stock.

Binding affinities of both fibronectin and laminin for OmpL1 of *L. santarosai*, *L. borgpetersenii* and *L. interrogans* serovar Copenhageni L1-130 were weak as the K_d micromolar concentration showed saturation at high concentration (~1.0 µM). Binding saturation to chondroitin by both *L. santarosai* and *L. interrogans* serovar Copenhageni L1-130 was not achieved, therefore the binding interactions were considered non-specific (Results not shown). For all Lsa49 variants, binding variations were observed among species, with *L. borgpetersenii* serovar Hardjobovis L550 and *L. alstonii* sharing a similar binding profile to several host molecules (fibrinogen, laminin and fibronectin, P <0.001). Most genomospecies achieved binding saturation curve to the tested host molecules with significant adherence selected from a binding profile (Figure 6.8). Interestingly, all Lsa49 variants demonstrated a hook-like effect to the BSA control suggesting for binding interference. The comparison of apparent K_d of multiple OmpL1 and Lsa49 proteins are shown in Table 6.3.

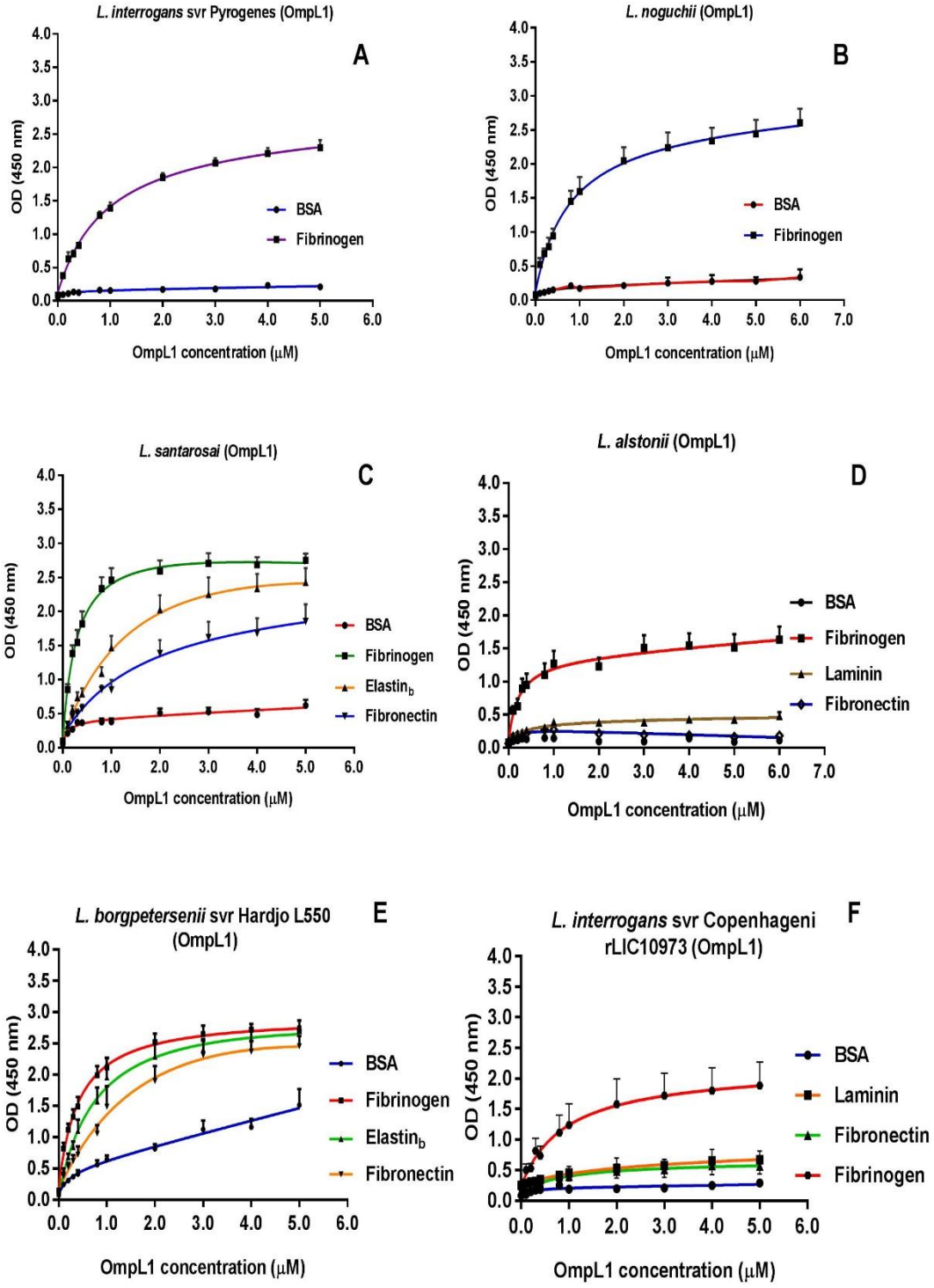


Figure 6.7 (A-F): Binding saturation curves of recombinant OmpL1 variants across pathogenic genomospecies to selected host components.

Data represent the mean absorbance at 450 nm ± the standard error of the mean (SEM) of three independent experiments. The equilibrium association constant (K_d) of each protein was estimated at half-maximal binding (see Table 6.3).

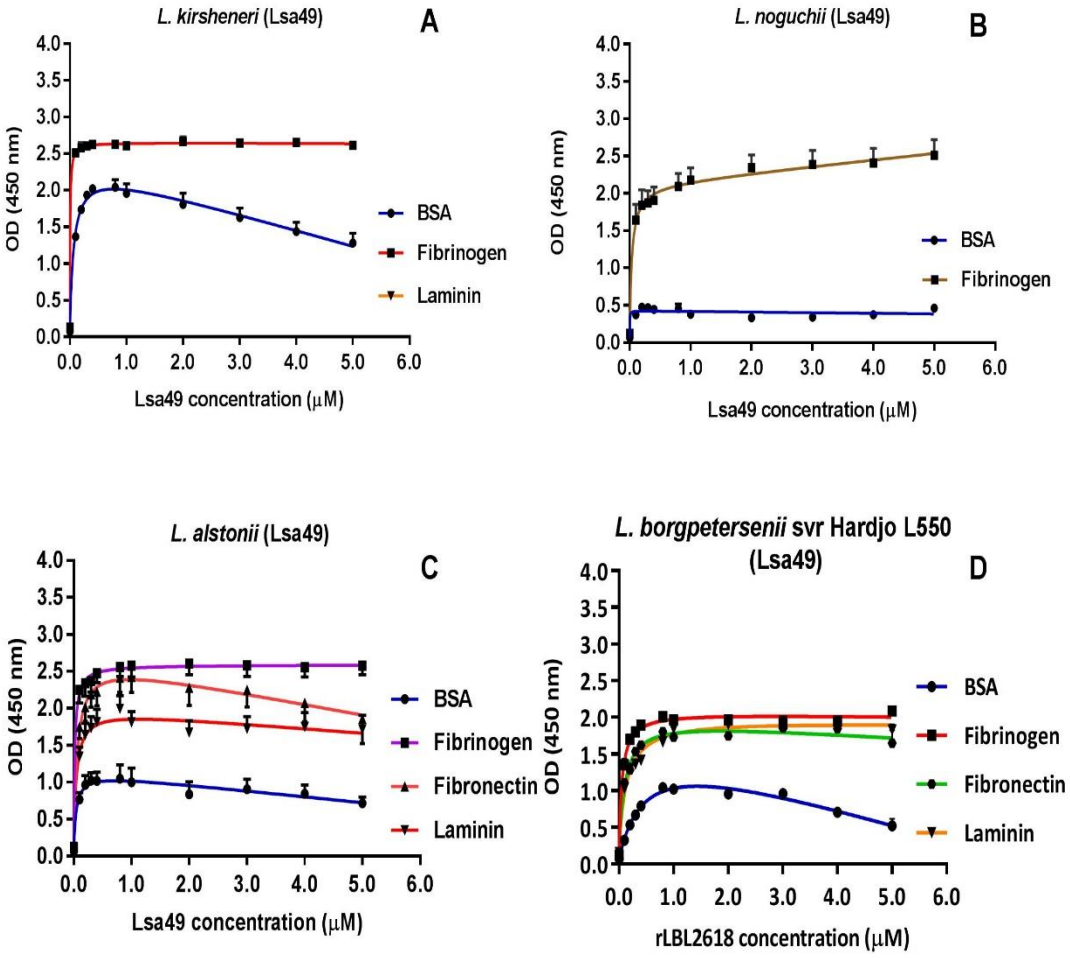


Figure 6.8 (A-D): Binding saturation curves to selected host components of recombinant Lsa49 variants across pathogenic genomospecies.

Data represent the mean absorbance at 450 nm ± the standard error of the mean (SEM) of three independent experiments. The equilibrium association constant (K_d) of each protein was estimated at half-maximal binding (see Table 6.3).

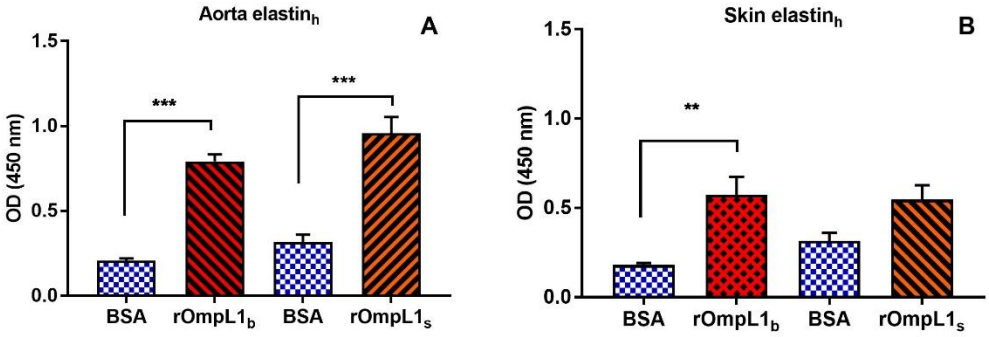


Figure 6.9 (A and B): Binding of OmpL1 of L. borgpetersenii serovar Hardjobovis L550 (OmpL1$_b$) and OmpL1 of L. santarosai (OmpL1$_s$) to both human aorta (A) and skin (B).

*Data represent the mean absorbance at 450 nm ± the standard error of the mean of three independent experiments. The binding of OmpL1 to both elastin was compared to their binding to BSA by Dunnett's multiple comparison test (*** P <0.001, ** P <0.01).*

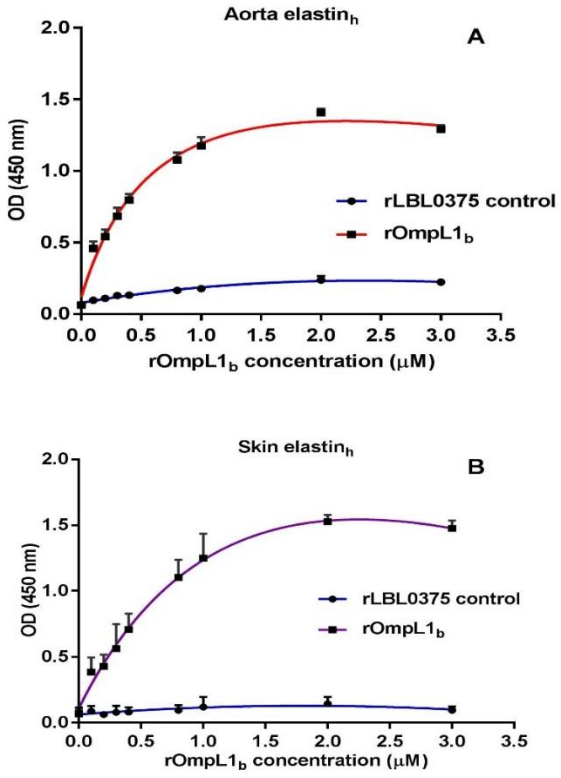


Figure 6.10 (A and B): Dose-dependent binding of recombinant OmpL1 of L. borgpetersenii serovar Hardjobovis L550 (rOmpL1$_b$) to human aorta elastin (A) and aorta (B) elastin, indicates by subscript 'h'. Recombinant rLBL0375 from the previous chapter (Chapter 5) was included as a negative control for this experiment.

Data represent the mean absorbance at 450 nm ± the standard error of the mean (SEM) of three independent experiments.

Table 6.2: The binding of leptospiral OMP variants to various host molecules, including BSA as a negative control.

***Leptospira* species**		**Binding to host molecules**								
	Annotation	**Fibronectin**	**Laminin**	**Heparan sulphate**	**Chondroitin**	**Fibrinogen**	**Collagen**	**Elastin$_b$**	**Aorta elastin$_h$**	**Skin elastin$_h$**
L. interrogans svr Pyrogenes	OmpL1	NS	NS	NS	NS	**Yes *****	NS	NS	NS	NS
L. noguchii	OmpL1	NS	NS	NS	NS	**Yes ****	NS	NS	NS	NS
L. santarosai	OmpL1	**Yes *****	**Yes ***	NS	**Yes ***	**Yes *****	NS	**Yes *****	**Yes *****	NS
L. alstonii	OmpL1	**Yes *****	**Yes *****	NS	NS	**Yes *****	NS	NS	NS	NS
L. borgpetersenii L550	OmpL1	**Yes ***	NS	NS	NS	**Yes *****	NS	**Yes *****	**Yes *****	**Yes *****
L. interrogans svr Copenhageni L1-130	OmpL1	**Yes *****	**Yes *****	NS	**Yes ***	**Yes *****	NS	NS	NS	NS
L. kirschneri	Lsa49	NS	NS	NS	NS	**Yes ***	NS	NS	NS	NS
L. noguchii	Lsa49	NS	NS	NS	NS	**Yes ****	NS	NS	NS	NS
L. alstonii	Lsa49	**Yes *****	**Yes *****	NS	NS	**Yes *****	NS	NS	NS	NS
L. borgpetersenii svr Hardjobovis L550	Lsa49	**Yes *****	**Yes *****	NS	NS	**Yes *****	NS	NS	NS	NS

[b] Bovine elastin, [h] Human elastin

*Significant binding is denoted with asterisk sign * (P <0.05), ** (P <0.01) and *** (P <0.001).*

Abbreviation: NS: Not significant.

Table 6.3: The apparent K_d (estimated at micromolar concentration) for saturating binding with 95% confidence interval was calculated at half-maximal binding.

***Leptospira* species**	**Binding to host molecules (K_d) (μM)**						
	Annotation	**Fibronectin**	**Laminin**	**Fibrinogen**	**Elastin$_b$**	**Aorta elastin$_h$**	**Skin elastin$_h$**
L. interrogans svr Pyrogenes	OmpL1	ND	ND	0.92 ± 0.27	ND	ND	ND
L. noguchii	OmpL1	ND	ND	0.88 ± 0.21	ND	ND	ND
L. santarosai	OmpL1	1.79 ± 2.21	ND	0.29 ± 0.08	1.83 ± 1.27	S-ND	ND
L. alstonii	OmpL1	0.21 ± 0.12	0.54 ± 0.37	0.14 ± 0.08	ND	ND	ND
L. borgpetersenii svr L550	OmpL1	2.60 ± 2.46	NS	0.43 ± 0.11	0.82 ± 0.42	0.75 ± 0.22	0.69 ± 0.01
L. interrogans svr Copenhageni L1-130	OmpL1	0.81 ± 1.30	1.46 ± 5.44	0.92 ± 1.38	NS	NS	NS
L. kirschneri	Lsa49	ND	ND	0.01 ± 0.00	ND	ND	ND
L. noguchii	Lsa49	ND	ND	0.04 ± 0.02	ND	ND	ND
L. alstonii	Lsa49	0.06 ± 0.03	0.05 ± 0.02	0.02 ± 0.01	ND	ND	ND
L. borgpetersenii svr Hardjobovis L550	Lsa49	0.10 ± 0.02	0.14 ± 0.02	0.05 ± 0.01	ND	ND	ND

[b] Bovine elastin, [h] Human elastin

Abbreviation: ND: Not determined, S-ND: Significant, but not determined.

6.3.6 Identification of the amino acid binding site of OmpL1 and Lsa49 variants

As both OmpL1 and Lsa49 showed variable host ligand-binding profiles across respective variants, a comparative amino acid analysis was performed to determine the amino acid binding sites that are most likely to adhere to the corresponding host molecules. For OmpL1, the analysis was made based on the conserved amino acid sites of both transmembrane segments and surface-loop regions of both proteins (Figure 6.11-6.13). Leucine located on surface-loop region (**SL 4**, amino acid site 249) corresponding to fibronectin binding and for *L. interrogans* serovar Copenhageni L1-130, *L. borgpetersenii* serovar Hardjobovis L550, *L. alstonii* and *L. santarosai*, whereas leucine located on surface-loop region (**SL 1**, amino acid site 88) appears associated with laminin-binding in *L. interrogans* Copenhageni, *L. alstonii* and *L. santarosai* respectively. Additionally, several amino acids groups were identified in both transmembrane and surface-exposed loops corresponding to elastin-binding for both *L. borgpetersenii* L550 and *L. santarosai,* respectively (Table 6.4). However, the specific fibrinogen-binding site of OmpL1 could not be identified as all alleles encode protein variants that bind to this molecule.

For the Lsa49 amino acid sequences, the predicted topology protein model by I-TASSER (Figure 6.14) exhibited 20 beta-barrel strand, of which the amino acids variability appears to be both across strands and loop regions. Similarly, in this analysis, we could not determine a specific binding site for fibrinogen, as all Lsa49 variants bound to this molecule. However, from the same analysis, we found a large number of multiple conserved amino acid sites responsible for laminin and fibronectin-binding, and a list of all Lsa49 binding sites can be found in Appendix C (Table C.3).

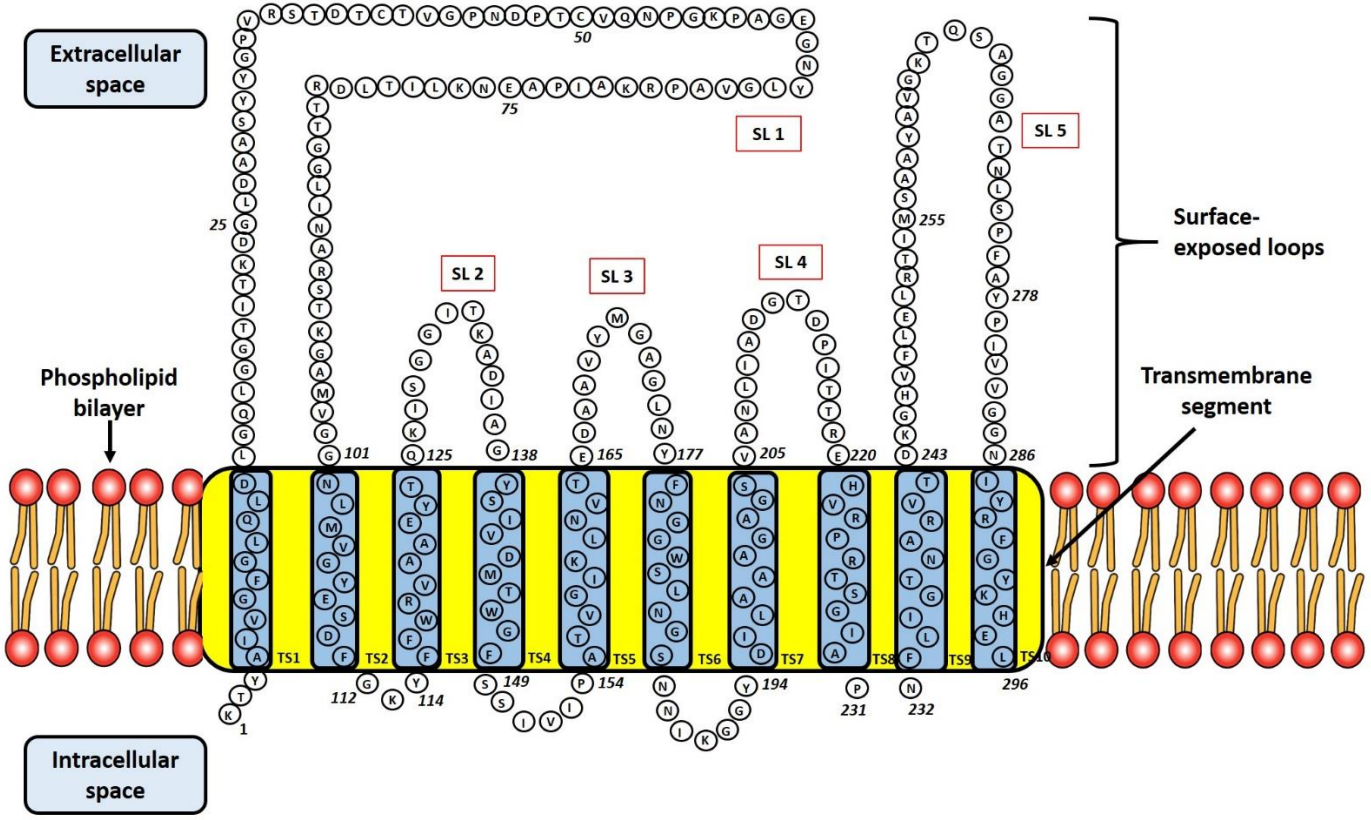


Figure 6.11*: *OmpL1 topology model. The transmembrane contains 10 segments (TS 1-10) containing an alternating hydrophobic amino acid pattern and five surface-exposed loops (SL 1-5). The figure was reconstructed based on the original model proposed by Haake et al. (1993).

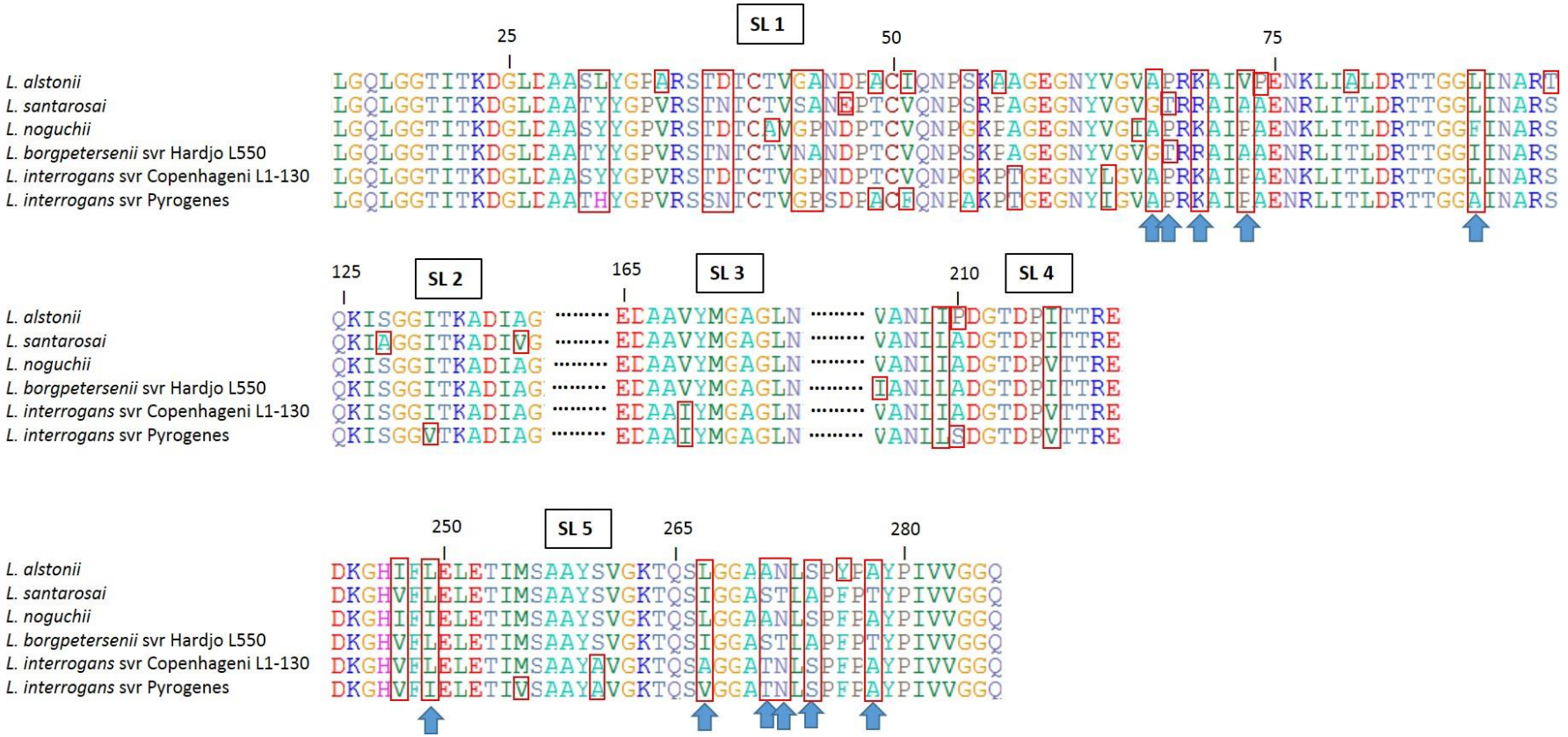


Figure 6.12: Amino acid sequence alignments of OmpL1 in six Leptospira genomospecies split into five surface-exposed loops (SL 1-5) based a topological model by Haake et al. (2004) shown in Figure 6.11. Locations of variable amino acids are indicated by red boxes and are numbered starting from mature peptide, and small arrows indicate amino acid differences between Leptospira species exhibiting specific host-binding properties (Table 6.4).

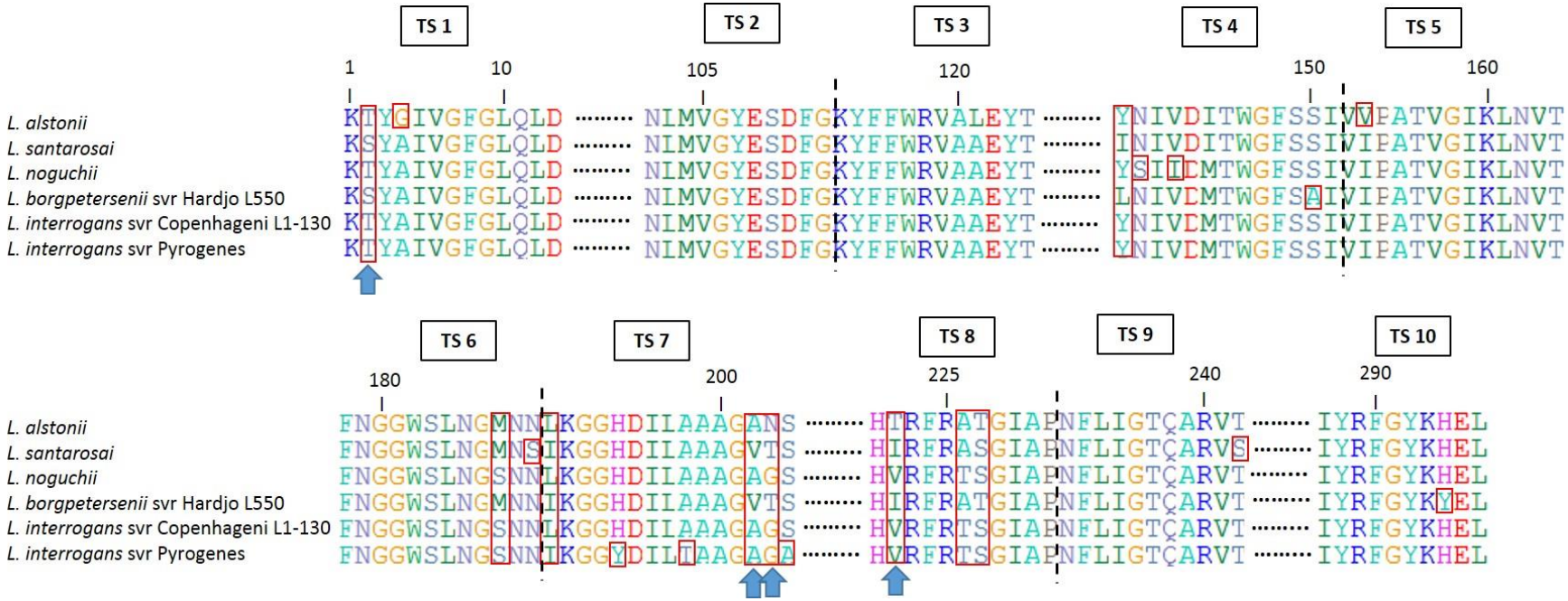


Figure 6.13: Amino acid sequence alignments of transmembrane OmpL1 which are divided into ten segments (TS 1-10) divided by a longitudinal dash line into six Leptospira genomospecies based on a topological model by Haake et al. (2004) shown in Figure 6.11. Location of variable amino acids are indicated by red boxes are numbered starting from mature peptide, and small arrows indicate amino acid differences between Leptospira species which exhibited specific host molecules binding properties (Table 6.4).

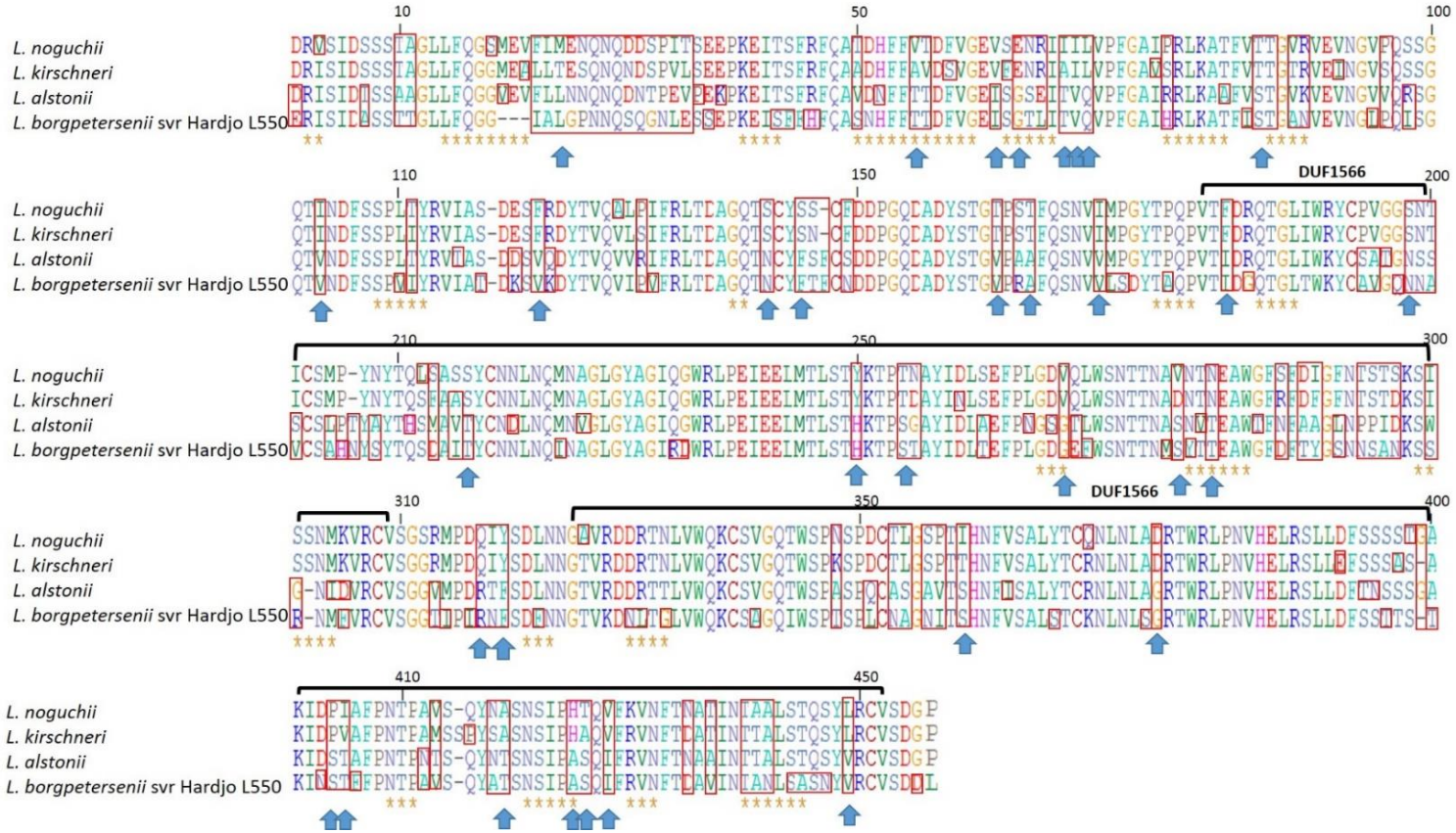


Figure 6.14: Predicted topology of Lsa49 variant generated from I-TASSER software represented in amino acid sequence alignment* (Yang and Zhang, 2015). *The asterisk sign indicates the predicted strand region and location of variable amino acids are indicated by red boxes. Sequence are numbered starting from mature peptide. The location of DUF1566 (between site 176-319 and site 325-452) is marked by square brackets above sequences, and small arrows indicate amino acid differences between Leptospira genomospecies which exhibited specific host molecules binding properties (Appendix C.3).

Table 6.4: The predicted site of conserved amino acids corresponding to host molecules binding that is present in OmpL1's variable regions of both transmembrane segments and surface-loop regions of selected Leptospira species in this study.

Host molecules	*Leptospira* species bind to corresponding host molecules	The conserved amino acid in OmpL1 transmembrane segment (TS)	The conserved amino acid in OmpL1 surface-exposed loop region (SL)
Fibronectin	*L. interrogans* svr Copenhageni L1-130 *L. borgpetersenii* svr Hardjobovis L550 *L. alstonii* *L. santarosai*	-	SL4: 249 L
Elastin	*L. borgpetersenii* svr Hardjobovis L550 *L. santarosai*	TS1: 2 S TS7: 202 V, 203 T TS8: 222 I	SL1: 67 G, 68 T, 70 R, 73 A SL4: 267 I, 271 S, 272 T, 274 A, 278 T
Laminin	*L. interrogans* svr Copenhageni L1-130 *L. alstonii* *L. santarosai*	-	SL1: 88 L

Abbreviations: A: alanine, G: guanine, I: isoleucine, L: leucine, S: serine, R: arginine, T: threonine, V: valine,

The transmembrane is divided into ten segments (TS1-10) and surface-exposed loop region consists of four major regions (SL1-4) (Figure 6.13).

6.3.8 Detection of anti-leptospiral antibodies in cattle sera

Results from PrioCHECK™ *L. hardjo* kit was used as a reference to determine the *Leptospira* status from each serum sample. The test kit for sera gives three categories for sample interpretations; positive (percentage positivity >45%), negative (percentage positivity <20%) and inconclusive (percentage positivity between 20%-45%) (Appendix C.2). For this study, it was recommended to include the inconclusive category within the positive group, as it was recommended by Lewis *et al.* (2009) for a practical statistical analysis, which previously used in disease prevention investigation. From 30 individual cattle serum, 21 animals were tested positive, and nine were negative, respectively.

6.3.9 Antibodies to OMP variants in cattle serum samples

The IgG-based ELISA assay was employed to investigate the presence of specific bovine immunoglobulin subclass IgG1 and IgG2 present in the cattle sera and determined that these classes of antibodies did bind to all recombinant OMP variants surveyed in this study. From the results shown in Figure 6.15, only OmpL1 of *L. borgpetersenii* serovar Hardjobovis L550 demonstrated a significant IgG1 titre (P <0.05) with the *Leptospira* positive and negative samples (as determined by the commercial test kit). No significant results were identified for any of the Lsa49 variants (Figure 6.16). Additionally, no significant results for IgG2 were observed against all OMPs variants.

6.3.10 Comparison of IgG1 titres in cattle sera against recombinant OMPs variants.

Linear regression was performed to determine the association of bovine IgG1 titres against each variant of similar OMP annotation (OmpL1 vs OmpL1; Lsa49 vs Lsa49). Moderate to strong IgG1 antibody response correlations were observed between OmpL1 titres and Lsa49 titres across *Leptospira* species with R values ranging from +0.2 to +0.8 with an associated probability (P- value) of <0.05 was considered significant (Figure 6.17). However, the correlation of OmpL1 of the majority of the species (except *L. borgpeterseni* serovar Hardjobovis L550) was reduced to non-significant when comparing with *L. interrogans* serovar Copenhageni. For Lsa49, though the IgG1 responses were strongly correlated across species, no significant correlation was observed between *L. alstonii* and *L. borgpetersenii* serovar Hardjobovis L550 (rLBL2618) (Figure 6.18). Additionally, a linear regression comparison of bovine IgG1 titres of all OMP variants with PrioCHECK™ *L. hardjo* kit was performed, however, no significant correlations with OMP responses were observed. The results can be found in Appendix C (Figure C.3).

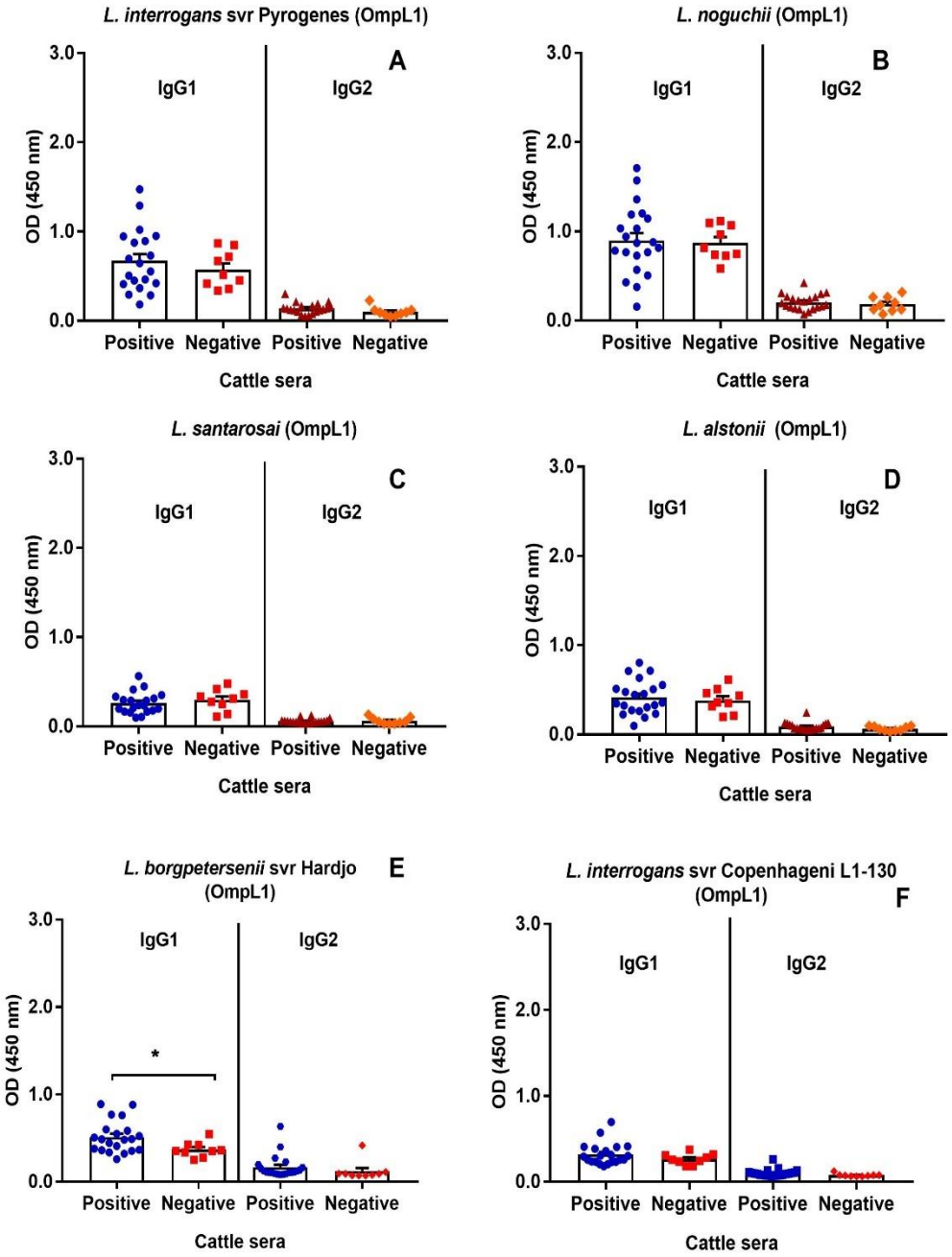


Figure 6.15 (A-F): Cattle sera antibody responses to all OmpL1 variants across selected Leptospira genomospecies represented in scatter dot-plot graphs.

Data represent the mean absorbance of secondary antibody concentration (mouse anti-bovine IgG1 and IgG2) at 450 nm ± the standard error of the mean (SEM) of two independent experiments. of two independent experiments. Both IgG1 and IgG2 titres were compared between positive and negative samples by Mann-Whitney U test with a P-value of < 0.05 was considered significant.

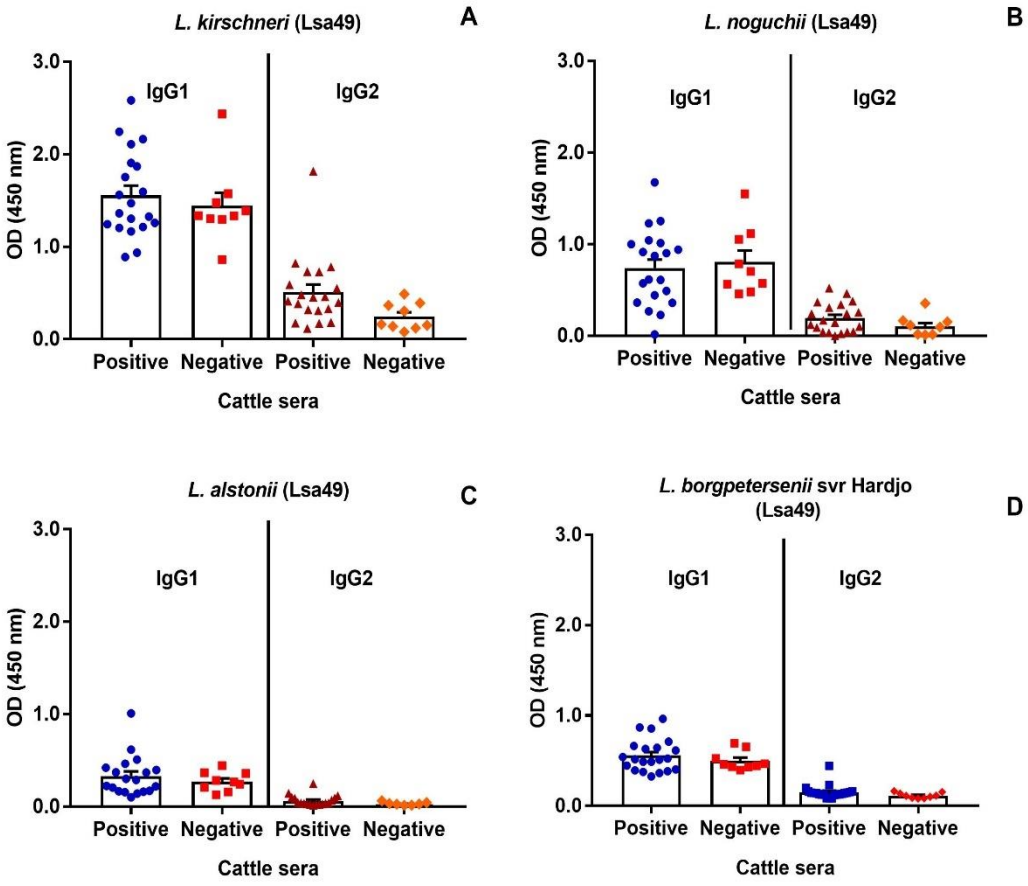
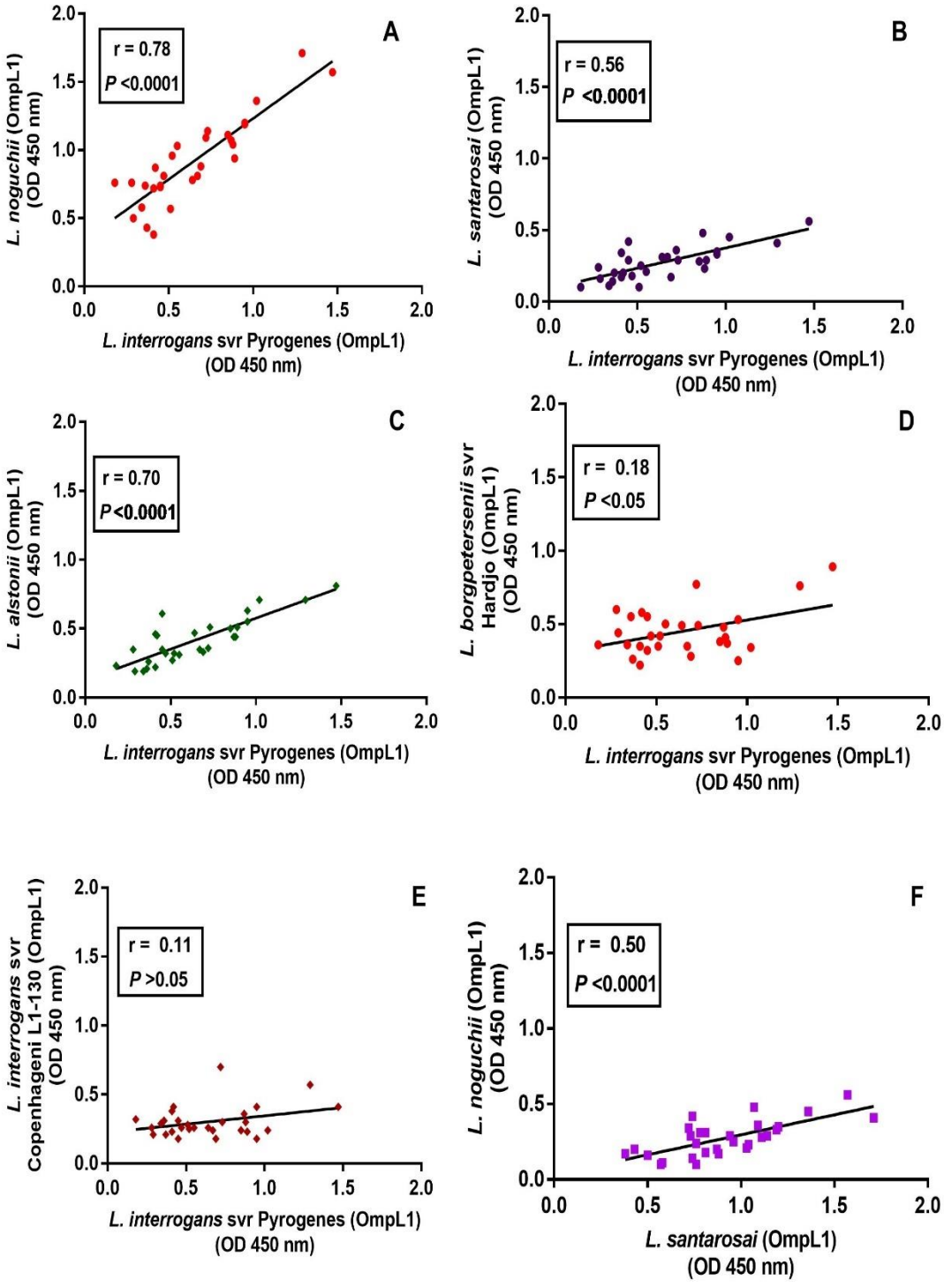
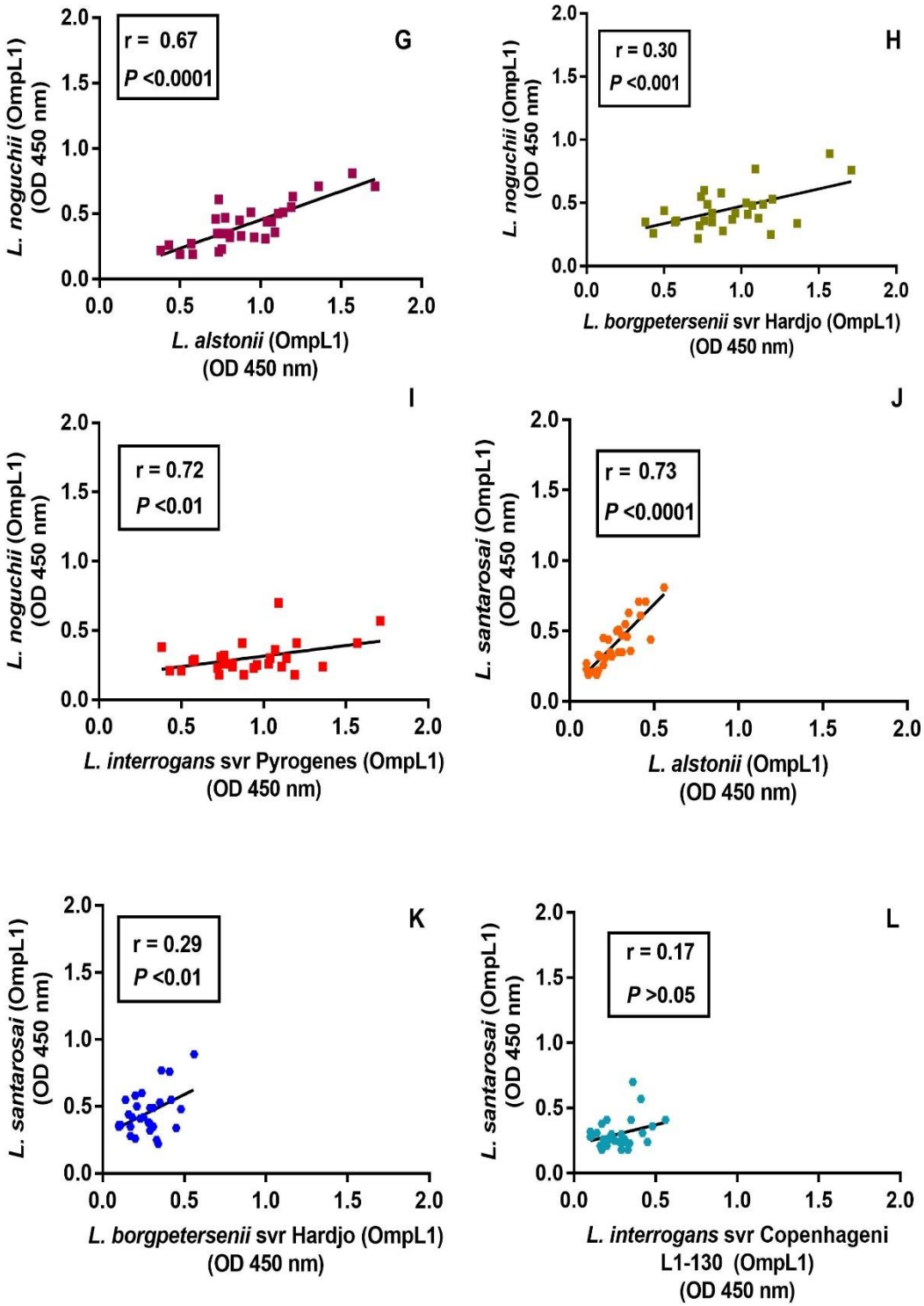


Figure 6.16 (A-D): Cattle sera antibody responses to all Lsa49 variants across selected Leptospira genomospecies represented in scatter dot-plot graphs.

Data represent the mean absorbance of secondary antibody concentration (mouse anti-bovine IgG1 and IgG2) at 450 nm ± the standard error of the mean (SEM) of two independent experiments. Both IgG1 and IgG2 titres were compared between positive and negative samples by Mann-Whitney U test with a P-value of < 0.05 was considered significant.


A
r = 0.78
P <0.0001
L. noguchii (OmpL1) (OD 450 nm)
L. interrogans svr Pyrogenes (OmpL1) (OD 450 nm)
B
r = 0.56
P <0.0001
L. santarosai (OmpL1) (OD 450 nm)
L. interrogans svr Pyrogenes (OmpL1) (OD 450 nm)
C
r = 0.70
P<0.0001
L. alstonii (OmpL1) (OD 450 nm)
L. interrogans svr Pyrogenes (OmpL1) (OD 450 nm)
D
r = 0.18
P <0.05
L. borgpetersenii svr Hardjo (OmpL1) (OD 450 nm)
L. interrogans svr Pyrogenes (OmpL1) (OD 450 nm)
E
r = 0.11
P >0.05
L. interrogans svr Copenhageni L1-130 (OmpL1) (OD 450 nm)
L. interrogans svr Pyrogenes (OmpL1) (OD 450 nm)
F
r = 0.50
P <0.0001
L. noguchii (OmpL1) (OD 450 nm)
L. santarosai (OmpL1) (OD 450 nm)


G
r = 0.67
P <0.0001
L. noguchii (OmpL1) (OD 450 nm)
L. alstonii (OmpL1) (OD 450 nm)
H
r = 0.30
P <0.001
L. noguchii (OmpL1) (OD 450 nm)
L. borgpetersenii svr Hardjo (OmpL1) (OD 450 nm)
I
r = 0.72
P <0.01
L. noguchii (OmpL1) (OD 450 nm)
L. interrogans svr Pyrogenes (OmpL1) (OD 450 nm)
J
r = 0.73
P <0.0001
L. santarosai (OmpL1) (OD 450 nm)
L. alstonii (OmpL1) (OD 450 nm)
K
r = 0.29
P <0.01
L. santarosai (OmpL1) (OD 450 nm)
L. borgpetersenii svr Hardjo (OmpL1) (OD 450 nm)
L
r = 0.17
P >0.05
L. santarosai (OmpL1) (OD 450 nm)
L. interrogans svr Copenhageni L1-130 (OmpL1) (OD 450 nm)

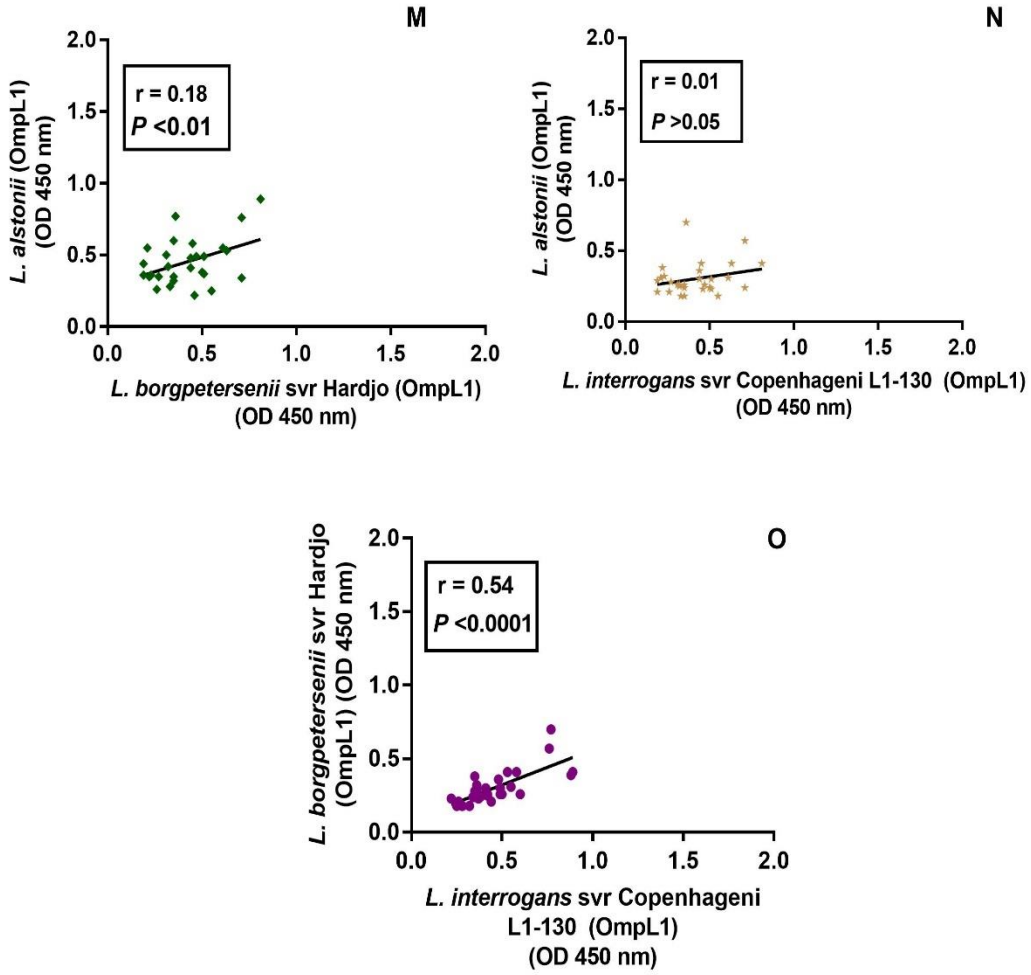


Figure 6.17 (A-O): The association between cattle sera IgG1 antibody titres to different recombinant OmpL1 variants across selected Leptospira genomospecies represented in scatter plot linear regression analysis.

The trend line represents the correlation coefficient, denoted as (r), which estimates the relationship between the OD of both results. The r values are ranging from -1.0 to +1.0 and the closer r is to +1 or -1, the more closely the two variables are related (+ integer; positively correlated, -integer; negatively correlated). P-values determine the significant level of association between two assays of which P-value of at least <0.05 was considered significant.

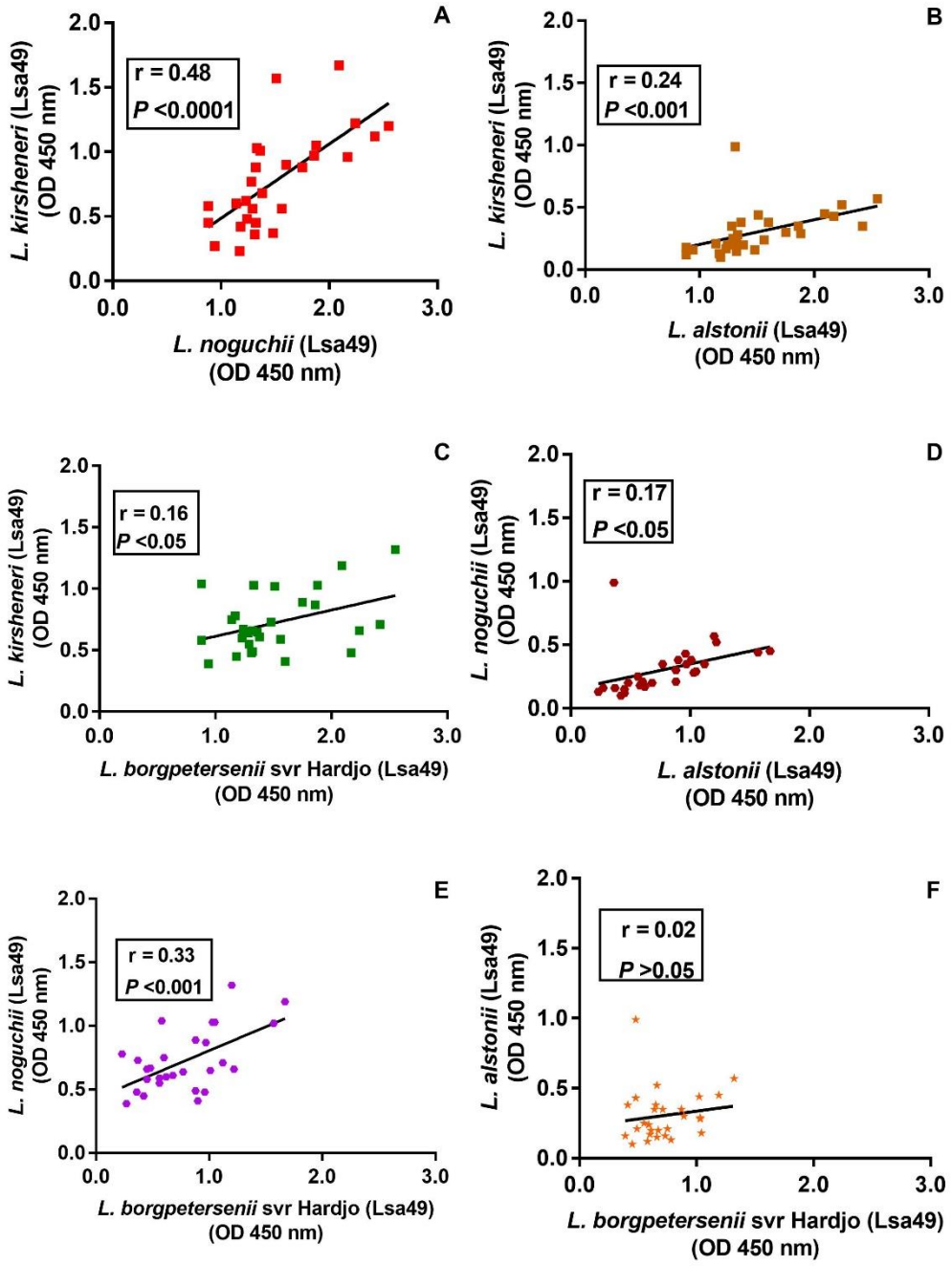


Figure 6.18: (A-F): The association between cattle sera IgG1 antibody titres to different recombinant Lsa49 variants across selected Leptospira genomospecies represented in scatter plot linear regression analysis.

The trend line represents the correlation coefficient, denoted as (r), which estimates the relationship between the OD of both results. The r values are ranging from -1.0 to +1.0 and the closer r is to +1 or -1, the more closely the two variables are related (+ integer; positively correlated, -integer; negatively correlated). P-values determine the significant level of association between two assays of which P-value of at least <0.05 was considered significant.

Table 6.5: The summary matrix of r values of IgG1 of OmpL1 against one another with their corresponding P values and comparison of amino acid identity (%).

Species	*L. interrogans* svr Pyrogenes	*L. noguchii*	*L. santarosai*	*L. alstonii*	*L. borgpetersenii* serovar Hardjobovis L550	*L. interrogans* serovar Copenhageni L1-130
***L. interrogans* serovar Pyrogenes**		**r = 0.78 (*P* <0.0001) ID: 90%**	**r = 0.56 (*P* <0.0001) ID: 84%**	**r = 0.70 (*P* <0.0001) ID: 84%**	**r = 0.18 (*P* <0.05) ID: 84%**	r = 0.11 NS ID: 84%
L. noguchii			**r = 0.58 (*P* <0.0001) ID: 86%**	**r = 0.67 (*P* <0.0001) ID: 89%**	**r = 0.30 (P <0.001) ID: 86%**	**r = 0.17 (*P* <0.01) ID: 95%**
L. santarosai				**r = 0.73 (*P* <0.0001) ID: 85%**	**r = 0.29 (*P* <0.01) ID: 94%**	r = 0.17 NS ID: 87%
L. alstonii					**r = 0.18 (*P* <0.05) ID: 85%**	r = 0.01 NS ID: 88%
***L. borgpetersenii* svr Hardjobovis L550**						**r = 0.54 (*P* <0.0001) ID: 86%**
***L. interrogans* serovar Copenhageni L1-130**						

Abbreviations: NS: Not significant (P-values >0.05), ID: Amino acid identity between two genomospecies

Table 6.6: The summary matrix of r values of IgG1 of Lsa49 variants against one another with their corresponding P values and comparison of amino acid identity (%).

Species	*L. kirschneri*	*L. noguchii*	*L. alstonii*	*L. borgpetersenii* serovar Hardjobovis L550
L. kirschneri		**r = 0.48 (*P* <0.0001) ID: 73%**	**r = 0.24 (*P* <0.001) ID: 71%**	**r = 0.16 (*P* <0.05) ID: 65%**
L. noguchii			**r = 0.17 (*P* <0.05) ID: 73%**	**r = 0.33 (*P* <0.001) ID: 66%**
L. alstonii				r = 0.02 NS ID: 69%
***L. borgpetersenii* serovar Hardjobovis L550**				

Abbreviations: NS: Not significant (P-values >0.05), ID: Amino acid identity between two genomospecies

6.4 Discussion

6.4.1 Phylogenetic analysis of OMP variants

Leptospiral OMPs have been widely studied due to their location within the membrane and their potential antigenic interaction with the host. However, information on functional and immunological diversities based on OMP molecular evolution is extremely limited, although it is known that the leptospiral genomes are greatly influenced by recombination events (Zuerner *et al.,* 1993). For example, comparative sequence analysis of several characterised leptospiral surface proteins; OmpL1, LipL41 and LipL32 revealed that OmpL1 genes and amino acids are more variable than the others, and these variations are thought to be due to positive selection or genetic reassortment including acquisition of a segment of sequence from an unknown allele source (Haake *et al.*, 2004). A rather similar observation was seen in *Treponema pallidum* subspecies *pallidum* OMP, where selection pressure has resulted in OMP diversity, and genetic recombination in OMP loci, which was hypothesised could potentially alter the protein's function, driving treponemal evolution (Kumar *et al.*, 2018).

In this study, we have selected several OMP variants based on the phylogenic analysis of OmpL1 and Lsa49 across *Leptospira* pathogenic genomospecies. The leptospiral OmpL1, the porin protein found only in pathogenic species, was previously characterised through its

binding properties and immunological evaluations (Dong *et al.*, 2008; Fernandes *et al.*, 2012), however, functional diversity between OmpL1 across several *Leptospira* species has never been studied before. Additionally, the newly-discovered adhesin (Lsa49) is another OMP of interest that belongs to the family protein containing the domain of unknown function (DUF1566/FP07603) that are found in *L. interrogans* (Atzingen *et al.*, 2012). This novel protein was discovered through the application of a reverse vaccinology approach in this thesis (Chapter 3) and was successfully expressed and characterised as a novel leptospiral adhesin (Lsa49) (Chapter 4 and 5), and again diversity of function across genomospecies had not been studied .

6.4.2 Expression, purification and determination of structural integrity of OMP variants

Majority of OMP alleles were successfully expressed as recombinant proteins via modular cloning, with exceptional of Lsa49 of *L. santarosai*. Protein analysis on SDS-PAGE confirmed recombinant OmpL1 band migration at 31 kDa (denatured) and 25 kDa by Shang *et al.* (1995). Similarly, SDS-PAGE analysis of recombinant Lsa49 proteins across *Leptospira* species showed a migratory band at ~49 kDa again indicative of β-barrel. The secondary structure of all OMP variants by CD analysis showed predominantly β-sheets, and these findings are consistent with OmpL1 and Lsa49 of *L. borgpetersenii* serovar Hardjobovis L550 produced in Chapter 4.

6.4.3 Adhesion of OMP variants to host molecules

From screening binding analysis, we successfully determined the binding profile of each OMP from a range of genomospecies against various host components. In the case of OmpL1, all selected *Leptospira* species bound significantly to fibrinogen, and other host molecules binding variations were observed in three species; *L. borgpetersenii* serover Hardjobovis L550, *L. santarosai* and *L. interrogans* serovar Copenhageni L1-130, whilst no significant adhesiveness of other host molecules were seen in *L. interrogans* serovar Pyrogenes and *L. noguchii*.

Two OmpL1 from *L. santarosai and L. alstonii* showed laminin-binding similarly to the positive control *L. interrogans* serovar Copenhageni L1-130 (rLIC10973). However, only OmpL1 from *L. alstonii* demonstrated binding saturation for laminin with a K_d value of 0.54 ± 0.37. Surprisingly, both OmpL1 from *L. borgpetersenii* serovar Hardjobovis L550 and *L. santarosai* showed binding preference to many host molecules, including elastin. To our knowledge, this is the first study that demonstrates significant adherence to elastin, which contradicted with the previous finding by Fernandes *et al.* (2012). A subsequent binding analysis using human

elastin (skin and aorta) revealed that OmpL1 *L. borgpetersenii* serovar Hardjobovis L550 bound to both types of human elastin and binding saturation was achieved, thus fulfilling a specific interaction of a typical receptor-ligand interaction. Additionally, OmpL1 of *L. santarosai* can also adhere selectively to human (aorta) elastin. However, the binding saturation studies were not performed due to limited protein solution stock, which is recommended for the future studies to study the possible interaction of OmpL1 and host elastin that enables *Leptospira* transmission as previously demonstrated by other *Leptospira* transmembrane OMP, such as Omp37 (Pinne *et al.,* 2010).

In order to determine the differences in an amino acid site that may responsible for a specific host molecule binding, comparative analysis of OmpL1 and Lsa49 amino acid sequences in all species used in this study was performed. Analysis of the OmpL1 amino acid sequence on the surface-exposed loops (Figure 6.12) revealed that the OmpL1s from *L. borgpetersenii* and *L. santarosai* have the most conserved variable regions compared to OmpL1 in other species, thus suggesting both strains may originally derive from the same source and therefore retain similar binding preferentially to selected host molecules.

The comparison of the amino acid sequence of OmpL1 surface loops and transmembrane regions based on topology by Haake *et al.* (1993) in many species prompted the interesting hypothesis that sequence alignment taken together with adherence phenotype may allow for one or several amino acids responsible for binding to host molecules to be identified. In this analysis, we found that leucine on the surface loops (**SL 1**: site 88; **SL 4**: site 249) is present in all species that bind to laminin and fibronectin, respectively. It is known that pathogenic, *Leptospira* strains have an extension of genes containing Leucine-Rich Repeat (LRR) sections which are not seen in non-pathogenic strains, which may be involved in host invasion (Picardeau *et al.*, 2008). Moreover, LLR sections are also present in other spirochetes (e.g. *Treponema denticola*) protein (LrrA), which previously exhibited binding and host tissues penetration (Ikegami *et al.*, 2004). Additionally, several studies have identified the roles of LRR binding to fibronectin, which suggested a possible mechanism for host colonisation (Sharma *et al.*, 1998; Davis *et al.*, 2006).

Using the same topology map Figure 6.12 and Figure 6.13, we also identified several groups of amino acids in 13 variable regions (VR) of both surface-loops regions (**SL 1**: site 67-guanine, 68-threonine, 70-arginine, 73-alanine ; **SL 4**: 267-isoleucine, 271-serine, 272-threonine,274-alanine, 278-threonine) and multiple transmembrane segments (**TS 1**: site 2-serine ; **TS 7**: site 202-valine, 203-threonine ; **TS 8**: site 222-isoleucine) correspond to elastin-binding profiles

present in *L. borgpetersenii* and *L. santarosai* but absent in other species. This finding may possibly explain why OmpL1 of *L. borgpetersenii* L550 and *L. santarosai* can bind to elastin, but not *L. interrogans* Copenhageni L1-130. This further supports the concept that through the evolution of *Leptospira*, both species acquired an additional binding function possibly through intragenic recombination of OmpL1 DNA. However, we could not stipulate the origin of the genes that are responsible for the elastin-binding property. Moreover, it is not possible to discriminate the binding sites responsible for fibrinogen binding, assuming all sites may potentially bind to this molecule.

The differences of host molecules binding between two interrogans serovars are an interesting finding. From the homology analysis of OmpL1 DNA sequences (Figure 6.19), this revealed that the *L. interrogans* group is divided into two sub-clusters. The first sub-cluster (of which includes serovar Copenhageni) appears to be highly conserved and the second interrogans cluster further diverges into two deeper branches (one branch contains serovar Pyrogenes). From this analysis, deeper branch tends to lose one of more host-binding functions (depicted by the loss of laminin and fibronectin-binding of serovar Pyrogenes compared to serovar Copenhageni). Additionally, it may be possible that the separation of two major groups between *L. interrogans* and *L. noguchii* is probably due to limited binding functions (as depicted by *L. noguchii* binding to only fibrinogen) compared to *L. interrogans* Copenhageni.

Through the same analysis, we found that both OmpL1 of *L. borgpetersenii* and *L. santarosai* belong to a major cluster which has an additional binding function to elastin. As seen in Figure 6.19, *L. alstonii* belongs in a major group together with *L. santarosai* and *L. borpetersenii* Hardjobovis serovar L550. However, this species splits forming its own subgroup. This may explain that *L. alstonii* appears to lose elastin-binding function while retaining several binding preferences similar to both *L. borgpetersenii* and *L. santarosai* species.

We consider that the differences in binding function in different *Leptospira* species and serovar could be the key to their adaptation or survival in the hosts. For instance, serovar Copenhageni can infect a wide range of hosts including dogs, rats and man (Faria *et al.*, 2008; Zwijnenberg *et al.*, 2008; Koizumi *et al.,* 2009a) whereas serovar Pyrogenes is commonly found in cattle reservoirs (Feresu *et al.*, 2009). It should be noted that distribution of pathogenic *Leptospira* serovars maintained by an animal host is often geographically unique; such as serovar Sokoine in African cattle and serovar Sarawak in Malaysian cattle (Mgode *et al.*, 2015; Daud *et al.*, 2018). This may be possible because OmpL1 is an important leptospiral

adhesin contributing to host binding preference as they are one of the few proteins that can be only found in pathogenic species (Fouts *et al.*, 2016). Therefore, future studies are warranted to highlight the range of OmpL1 binding across various pathogenic species that are locally present in endemic leptospirosis area that may provide more information on *Leptospira* species/serovar evolution and their adaptations in a particular mammalian host.

In comparison to OmpL1, the binding functions of the other protein's studies are likely conserved in each species. All Lsa49 proteins bound to fibrinogen and the majority are bound to laminin and fibronectin, respectively. The homology analysis of Lsa49 protein sequence of each species concurs with the 16S rRNA sequences indicating that both sequences are conserved and are likely to retain similar binding function (Results not shown). Additionally, the comparative amino acid analysis of Lsa49 in all species revealed high variabiality of amino acids in both strand and loop regions (Figure 6.14) and multiple variable sites were predicted for laminin and fibronectin binding for *L. borgpetersenii* serovar Hardjobovis L550 and *L. alstonii* (Appendix C.6). Interestingly, we found that both OmpL1 and Lsa49 of *L. noguchii* was only bound to fibrinogen. It is not known whether the limited binding to host molecules may have an effect the transmission of the bacteria to other hosts (e.g. host restriction), or it could be the species is restricted to certain host's tissues (e.g. kidney tubules).

6.4.4 Immunological evaluation of OMP variants in cattle sera

The OMP variants were used as antigens to screen for specific bovine leptospiral antibodies that may be present in cattle serum samples via Ig based-ELISA. In this study, IgG1 is the predominant antibody and was detected in all samples, with lesser amounts of IgG2. This further supports that both OmpL1 and novel adhesin Lsa49 are expressed by *Leptospira*, and also detected by the host immune system and therefore these OMPs are likely to be surface-exposed.

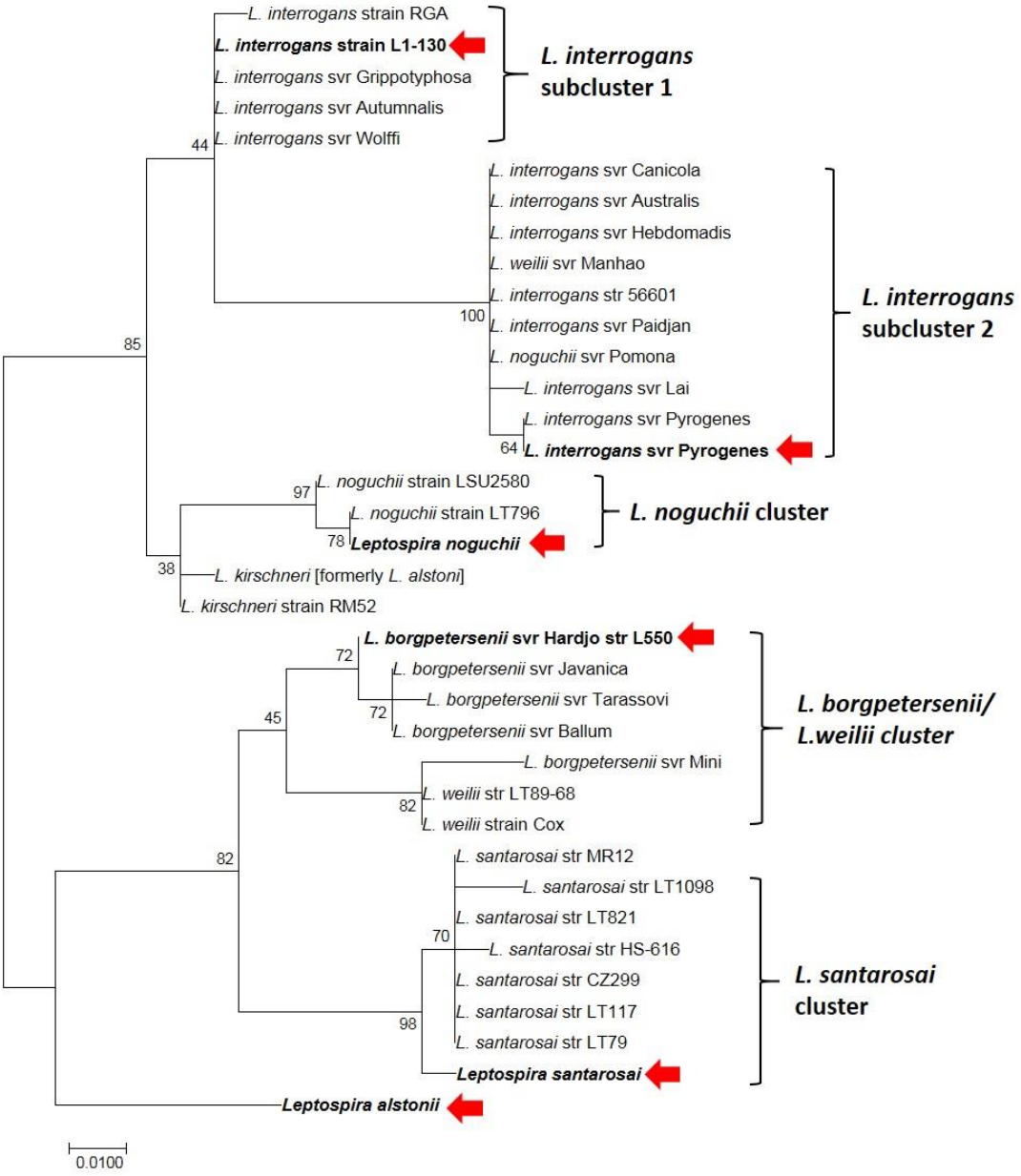


Figure 6.19: The homology tree of OmpL1 amino acid sequences of several representative pathogenic genomospecies and serovars.

L. interrogans separates from L. noguchii and forming two distinct groups, each representing at least one species/serovar in this study, indicated by arrows as shown. The difference in each group is corresponding to adherence to host components observed in this study; both L. interrogans serovar Pyrogens and L. noguchii bound to only fibrinogen, and L. interrogans L1-130 bound to laminin, fibronectin and fibrinogen. Both L. borgpetersenii and L. santarosai are grouped in the same subset, formally diverged from L. interrogans main cluster which showed binding variety towards fibrinogen, fibronectin, laminin and elastin (Table 6.4).

However, there is no discrimination between the *Leptospira* positive and negative serum samples (pre-determined by commercial leptospira kit) against all proteins and only OmpL1 (rLBL2510) from *L. borgpetersenii* serovar Hardjobovis L550 shown statistically significant differences between positive and negative sera. This result is similar to the finding in cattle milk (Chapter 5), where the OmpL1 from *L. borgpetersenii* serovar Hardjobovis L550 (bovine strain) showed a significant difference between positive and negative bulk milk samples. The test kit to determine infected and naïve animals in this chapter, utilised inactivated *L. interrogans* serovar Hardjobovis L550 as the sole crude antigen (Appendix C, Figure C.2) for the detection of leptospiral antibodies in cattle serum samples (Bercovich *et al.,* 1990). The true status of disease could be more complicated since tested cattle may be infected with other *Leptospira* spp. which could explain some differences in agreement between OMPs and diagnostic test kit ELISA.

In addition to this finding, IgG1 response towards our OMP antigens suggests that these proteins do likely to trigger host immune responses. It is thought that both IgG isotypes are present during active infection (Adler *et al.*, 1982). However, in this study, bovine IgG2 was poorly detected or detected at low levels in all samples. The reason for this is not clear; one possible explanation for this is due to the antigen proteins that selectively bind to B cell membrane IgG1 rather than IgG2, even though IgG2 is mainly present in cattle serum (Wallner *et al.,* 1987). A study of papillomatous digital dermatitis (PDD)-associated spirochetes cattle by Elliott and Alt (2009) concluded that robust bovine IgG1 responses compared to IgG2 of cattle experimentally infected with treponemes is due to the binding preference, which resulted in the lower level of antigen-specific bovine IgG2 detected in the serum. Moreover, a study by (Naiman *et al.*, 2002) demonstrated that IgG2 response in vaccinated cattle showed a two-fold increase upon *Leptospira* challenged, compared to naïve cattle. The cattle in this study were unlikely to be vaccinated, therefore, this agrees with low IgG2 titres that were measured. A limitation of this study is that the true infection status of the cattle in this study is not known, as it was only determined using a commercial test kit and lacks corresponding epidemiological data. Therefore, further longitudinal outbreak study is warranted in the future to ascertain the status of leptospirosis via a serological method to compare the immune response of infected versus uninfected herd by ELISA method. The lack of association via linear regression between antibody titres (IgG1) from the *Leptospira* test kit (PrioCHECK™ *L. hardjo* kit/whole-cell antigen) and all recombinant OMPs is noted in this study (results not shown). We suggest that this could be due to the different antigen-antibody interactions between whole-cell and the recombinant proteins. This indicates that

the antibodies detected by OMPs were not interacting with the whole-cell antigen contained in this kit. Interestingly, when comparing the OMPs, the majority of OmpL1s across the species (except *L. interrogans* serovar Copenhageni L1-130) demonstrated moderate to strong correlations of IgG1 titres to one another (r values +0.20-0.80, *P*-values <0.001, <0.0001). A similar observation is seen among the majority of Lsa49 proteins where all species (except *L. borgpetersenii* serovar Hardjobovis L550 versus *L. alstonii*) exhibited positive association (r values +0.20-0.70 *P*-values <0.001, <0.0001). These findings suggest that the immunogenicity responses towards both OMPs are conserved among species and are likely to share identical epitopes that are recognised by host immune system. Moreover, a previous study showed that different OmpL1 are able to cross-agglutinate with OmpL1 antisera indicating evidence of conserved immunogenicity (Dong *et al.,* 2008).

Furthermore, a positive correlation of antibody titres in the majority of OMPs indicates that differences in OMP binding function may not affect the immunogenicity of the proteins as antigens, despite a large degree of amino acid diversity (Table 6.5 and Table 6.6). However, it is difficult to interpret the lack of correlation of OmpL1 of *L. interrogans* serovar Copenhageni L1-130 and with the rest of species (except to *L. borgpetersenii* serovar Hardjobovis L550). The reason for this is not clear, although the OmpL1 sequence identity matrix of amino acids sequences among tested species showed all species are highly identical (>85%) to one another. A similar observation was seen in two of Lsa49 proteins of *L. borgpetersenii* serovar Hardjobovis L550 versus *L.alstonii* where the correlation was reduced to non-significant, although Lsa49 shared moderately high identity of amino acid (>65%). Whilst there are variabilities seen in amino acids of OMPs, this does not appear to agree with the changes of epitopes that antibody binds, based on there being no similarity between linear corrleation of antibody serotitres and protein simlarity scores. Further study such as epitope mapping, which has been used for other spirochete vaccine candidates (Livey *et al.*, 2011; Arnaboldi and Dattwyler, 2015) may be useful to evaluate the protein's structure to determine why amino acid variation and seroreactivity may not agree.

6.4.5 Limitation of the study

The use of a diagnostic kit to determine leptospirosis status in this study is a limitation. Tested animals in this study may have already been exposed to *Leptospira* strains previously, ideally a longitudinal serological study which includes detailed clinical signs of leptospirosis should be undertaken. Secondly, although our study is likely to suggest that these proteins are likely to be expressed on the *Leptospira* membrane which carries essential gene for host

interaction, more studies (e.g. membrane expression studies) (Grisshammer, 2006; Jensen *et al.*, 2017) are warranted to confirm that these proteins can demonstrate similar functional and immunological profile as seen in this study. Lastly, as our study only includes OMPs from several representative *Leptospira* genomospecies, more studies are needed to include a large number of alleles from other species (pathogenic/intermediate) to fully understand the functional and immunological diversity and better disect host-pathogen interactions.

6.5 Conclusion

In the present study, we have successfully expressed and purified two groups of leptospiral OMP alleles across five pathogenic genomospecies; OmpL1 and Lsa49 (previously known as DUF1566-49kDa) as recombinant proteins. Interestingly, these proteins showed binding variations towards different host components, which may be due to genetic recombination or through positive selection from different *Leptospira* pathogenic species. To our knowledge, this is the first study to demonstrate leptospiral OMP functional diversity across genomospecies. Furthermore, our findings on OmpL1 binding diversity provide insight into potential mechanisms of bacterial tropism for mammalian hosts, worthy of future investigation. Interestingly, in addition to functional diversity, the immunological assessments indicate that the immunological reaction to these proteins among *Leptospira* species is relatively conserved, despite the functional variation, suggesting these OMPs need future consideration as candidates for a universal leptospirosis vaccine. Therefore, these may be useful as recombinant vaccine candidates and as diagnostic tools for the serodiagnosis of leptospirosis. More studies are needed to; 1) study the leptospiricidal activity of these antigens and 2) implement these antigens for immunogenicity trial in cattle host as the next step towards engineering the new line of bovine leptospirosis vaccines and diagnostic purposes.

In summary, this study elucidates the molecular evolution of leptospiral OMPs across pathogenic species, and how the changes influence the functional and immunological behaviour toward the host. These findings could be useful information to further explore *Leptospira* species adaptation or survival in multiple animal species, and also may help researchers to understand their ability to bind to several host components to establish infection in the host.

Chapter 7: Survey of UK ruminants for *Leptospira*

7.1 Introduction

7.1.1 Ruminant leptospirosis

As previously noted, *Leptospira* can infect a wide range of species and ruminants are one of the most susceptible hosts. Cattle can be infected and may also serve as a maintenance host depending on the group of serovars geographically circulating within the area. Bovine leptospirosis (BL) has a global distribution (Table 1.5, Chapter 1), and the disease can cause a profound effect on cattle productivity. The majority of infection is commonly caused by serovar Hardjo from two distinct pathogenic species (*L. interrogans* and *L. borgpetersenii*), although other associated serovars (e.g. serovar Kennewicki, Sarawak) may be associated with cattle infection but are geographically restricted within particular regions worldwide (Daud *et al.*, 2018; Zarantonelli *et al.*, 2018). The global prevalence of cattle leptospirosis is unknown due to the lack of data and many unreported cases, possibly due to asymptomatic infection. Despite such data limitations, seroprevalence studies across the world have shown a high prevalence of *Leptospira* infected animals when reported. For instance, recent studies among unvaccinated cattle in northeastern Malaysia and the Republic of Ireland reported the overall prevalence of BL of 81.7% and 91.0%, respectively (Barrett *et al.*, 2018; Daud *et al.*, 2018). Other reported BL prevalence studies from across the world have been based on various screening methods accompanied by isolation of circulating strains and are indicative of an endemic presence in these tropical regions including India (Natarajaseenivasan *et al.*, 2011), Uganda (Zarantonelli *et al.*, 2018) and Brazil (Pinna *et al.*, 2018). Regardless of the increasing prevalence of BL globally, the prevalence of BL on UK cattle farms, especially in beef cattle remain elusive. Surveys on UK dairy herds estimated a farm prevalence of between 47-72% in unvaccinated farms (Williams and Winden, 2014; Velasova *et al.*, 2017). Additionally, a recent national survey on several farms across the country in 2015 recorded 55% of dairy cattle farms and 33% of beef cattle farms tested positive (MSD Animal Health, 2015). Data from the GB Cattle Disease Surveillance Dashboard from 2012-2018 confirmed 23 abortion cases and 11 cases of milk drop syndrome were reported across the country due to leptospirosis (APHA Data, 2019). However, these surveys failed to provide comprehensive detail about strain identity, as they are only reported through blood or milk ELISA for a single serovar (Hardjo), with no further characterisation through laboratory isolation or molecular

typing. It is known that BL in the UK is commonly caused by serovar Hardjo from *L. interrogans* or *L. borgpetersenii* although several other serovars (e.g. serovar Pomona, Grippotyphosa and Icterohaermorrhagiae) may also be present (Roberts, 2009; Ellis, 2015; Arent *et al.*, 2017).

Besides cattle, small ruminants such as sheep and goats can also be infected with leptospirosis. It was thought that sheep are relatively resistant to the infection as indicated by low seroprevalence and the small number of associated serovars, such as serovar Pomona, Grippotyphosa, Icterohaemorrhagiae, Australis, Sejroe and Canicola (Leon-Vizcaino *et al.,* 1987; Vermunt *et al.*, 1994; Ellis, 2015). These infections caused reproductive failure in adult animals and other related signs such as haematuria, jaundice, haemoglobinuria and death in lambs. Despite the lack of studies surrounding leptospirosis in sheep and goats, *Leptospira* spp. are present globally, and animal exposure to various pathogenic serovars is indicated by global serological surveys (Martins and Lilenbaum, 2014).

7.1.2 The gastrointestinal (GI) tract as a potential infection reservoir of *Leptospira*

In many pathogens, the transmission to the host is enhanced through colonisation of the host's specific anatomical passage where they can co-exist as normal flora within the host. For example, *Neisseria meningitidis* (meningococcus) is a Gram-negative bacterium that can cause bacterial meningitis, leading to life-threatening sepsis in human, especially in children (Rouphael and Stephens, 2012). The bacteria commonly reside in the nasopharynx tract of humans in the form of non-pathogen. It has been reported between 10-35% of healthy adults carry the bacteria on their upper respiratory tract, of which the bacteria can be easily spread through respiratory secretions and saliva (Cartwright *et al.*, 1987; Orr *et al.*, 2003). It appears that these bacteria exclusively inhabit a specific anatomical area of a host and become a carriage site to transmit the bacteria to another vulnerable host. Furthermore, it is considered that bacterial invasion is through the nasopharynx also. For this microbe, the point of invasion is also considered as the point of carriage.

Thus far, pathogenic *Leptospira* are only able to be carried in the kidneys or urogenital tract of maintenance host particularly ruminants. Infected animals tend to shed the microorganism through renal excretion or uterine discharges/aborted foetuses to contaminate the environment (Farina *et al.*, 1996; Lilenbaum *et al.*, 2008; Director *et al.*, 2014; Adugna, 2016). However, it is not known if the bacteria are able to be carried to any other anatomical site of the host, especially as there has been a dearth of molecular surveys reported. The GI tract is another potential leptospires reservoir due to its anatomical

structure, which consists of an extensive mucosal lining from the mouth to the anus that enables leptospires to penetrate and colonise the region (Inada *et al.*, 1916). Furthermore, due to the close proximity to the urinary tract, leptospires may be able to invade to the adjacent recto-anal junction and colonise and survive within the region. However, this site was potentially overlooked, although there has been a report of swine GI tract epithelia containing leptospires (Fossi *et al.*, 2005). This shows that the mid-GI tract too may potentially be reservoirs for leptospires.

One of the important *Leptospira* transmission routes is the ingestion of infected material and invasion of the mucous membranes. Several reports have described transmission via this route. For instance, the first study on GI transmission was reported by Inada *et al.* (1916) who observed exposure of pathogenic leptospires in an experimental animal model through a oral or anal route resulted in death. The oral cavity was also suggested as the main *Leptospira* entry point as several human leptospirosis outbreaks related to contaminated water consumption (Cacciapuoti *et al.*, 1987; Corwin *et al.*, 1990; Levett, 2001). There have been no studies into the oral or rectal tissues as leptospires carriage site in animals despite them considered points of invasion.

Ruminants such as cattle and sheep are continuously exposed to leptospires that may present in the slurry. Farm slurry is a mixture of faeces and urine, and therefore, is an external reservoir of *Leptospira* infection for ruminants. *Leptospira* can survive in diverse environments and may persist alive for several days outside of the host which can allow transmission of the bacteria from one susceptible animal to one another. A previous study showed that a large number of leptospires were isolated from cattle slurry, indicating slurry is a convenient transit for leptospires to survive outside the host (Jones and Matthews, 1975). Furthermore, recent studies demonstrated that the environment serves as a temporary infection reservoir, and the presence of even low pathogenic leptospire concentrations may allow the transmission of disease (Casanovas-Massana *et al.*, 2018; Schneider *et al.*, 2018). Ruminants are frequently being exposed to leptospires via slurry contamination to water and feed sources, especially in housing condition which may lead to possible oral cavity invasion.

Given *Leptospira* spp. may invade the upper and lower GI tract, this current study investigates whether there is a continued leptospires presence/carriage at these sites of pathogenic (or non-pathogenic) leptospires. The identification of such additional carriage sites could underpin future control methods.

7.2 Materials and methods

7.2.1 Animal sample collection

Samples from both dairy and beef cattle, and sheep from farms across England and Wales were obtained from previous studies carried out in this laboratory by Bell, (2017) and Sullivan *et al.*, (2015). These comprised of tissues samples from rectal-anal junctions and gingiva. The previous workers had already extracted the DNA and stored it at -80°C (Table 7.1).

Table 7.1 : Origin of samples from cattle and sheep used in this study.

Samples	Region	No of samples	Reference in this study
Sheep			
Rectum	England/Wales, UK	36	(Sullivan *et al.*, 2015)
Gingiva	England/Wales, UK	39	(Sullivan *et al.*, 2015)
Total		**75**	
Cattle			
Beef rectum	England/Wales, UK	35	(Sullivan *et al.*, 2015)
Beef gingiva	England/Wales, UK	34	(Sullivan *et al.*, 2015)
Dairy cattle rectum	England, UK	115	(Bell, 2017)
Dairy cattle gingiva	England, UK	114	(Bell, 2017)
Total		**298**	

7.2.2 PCR optimisation assay for the detection of *Leptospira* spp.

Three leptospiral PCR primer pairs that specifically detect pathogenic and/or saprophytic *Leptospira* spp. were obtained from previous studies (Table 7.2). These primers were used to detect leptospires using PCR in various biological human samples such as urine, blood and cerebrospinal fluid, including direct detection using pure culture collections.

These primers were synthesised and purchased directly from Eurofins, UK. Leptospiral PCR assays were performed using Taq polymerase according to manufacturer's instructions and PCR conditions initially revalidated using a range of annealing temperatures. Upon verifying the annealing temperature and also magnesium chloride ($MgCl_2$) concentration, the specificity of each assay was determined using relevant control material including *L. interrogans* serovar Copenhageni L1-130, *L. borgpetersenii* serovar Hardjobovis L550, *L. biflexa* serovar Patoc 1, *Treponema phagedenis* T320A and *Treponema pedis* T3552B respectively. All PCR assays were performed in triplicates.

Table 7.2: The list of diagnostic leptospiral PCR primer pairs used in this study.

Primer	Target gene	Sequence	Size (bp)	Specificity	Reference in this study
Lep F Lep R	16S rRNA	5-'GGC GGC GCG TCT TAA ACA TG- 3' 5'–TCC CCC CAT TGA GCA AGA TT- 3'	330	*Leptospira* spp. (Universal)	(Merien *et al.*, 1992)
F1 R_i	23S rRNA	5'-GAA CTG AAA CAT CTA AGT A- 3' 5'- CAG CGA ATT AGA TCT G- 3'	115	Pathogenic leptospires (e.g. *L. interrogans*)	(Woo *et al.*, 1997)
F1 R_b	23S rRNA	5'-GAA CTG AAA CAT CTA AGT A- 3' 5'- TTC GCC TTC GAG ATT C-3'	523	Non-pathogenic leptospires *(*e.g. *L. biflexa*)	(Woo *et al.*, 1997)

7.2.3 PCR assays for the detection of *Leptospira* spp.

All extracted DNA samples (*n*= 373) were subjected to the three leptospiral PCR assays that had been optimised (Table 7.3) to detect both pathogenic and/or saprophytic *Leptospira* spp. in infected animals. The PCR products were then analysed on 1% (w/v) agarose gel (Section 2.10).

7.2.4 Direct sequencing of PCR products

Positive samples from repeated PCR assays were purified (Section 2.11). The purified PCR products were then sequenced at Source Bioscience (Cambridge, UK) using the amplification primers. The outcome of the DNA sequences was analysed using Staden Package software (Cambridge, UK) (Bonfield, Smith and Staden, 1995) and compared using BLAST search tool based on the 16S and 23S database to identify the *Leptospira* species from the positive samples.

7.2.5 Phylogenetic analysis of positive *Leptospira* samples

A phylogenetic tree was constructed to determine the relationship of the PCR positive *Leptospira* detected in ruminant samples and related *Leptospira* genomospecies using MEGA7 (Tamura *et al.*, 2013) based on the maximum-likelihood statistical method. The phylogeny test was performed by 10,000 bootstrap replications and the final model for nucleotide substitution is based on Tamura-Nei model (Tamura and Nei, 1993).

7.3 Results

7.3.1 PCR validation

Using the three different *Leptospira* primer pairs as stated, the annealing temperature was verified using a range of annealing temperatures and cycling condition of each PCR assay. The gradient PCR of each assay and determination of cycling condition and concentration of $MgCl_2$ are shown in Table 7.3.

7.3.2 PCR detection of leptospires in sheep and cattle rectal and gingiva tissues

From the PCR survey (Table 7.5), several samples were detected as *Leptospira* positive. Overall, 5/36 (8.33%) and 3/39 (7.70%) of sheep rectal and gingival samples were considered positive, respectively. No positives were found in both beef cattle gingiva and rectal samples. In dairy cattle samples, only two animals (1.74%) were found positive in rectal samples and two (1.75%) in gingiva samples. Interestingly, most of the positive samples were detected using the 16S rRNA PCR assay, and none were detected using 23S rRNA *L. interrogans* assays. However, only one gingiva sample from dairy cattle was detected positive using the 23S rRNA *L. biflexa* assay.

Table 7.3: PCR validation for the detection of Leptospira spp. in this study.

Primer	Target gene	Target taxa	Annealing temp (C)	Extension time (s)	$MgCL_2$ (mM)	Annealing time (s)	Cross-reactivity with relevant bacterial DNA			
							L. interrogans **L1-130**	***L. biflexa*** **Patoc 1**	***T. pedis*** **T3552B**	***T. phagedenis*** **T320A**
LepF/LepR	16S rRNA	*Leptospira* spp.	60.4	60	1.5	30	+	+	-	-
F1/R_i	23S rRNA	Pathogenic *Leptospira* spp.	48.0	60	1.5	60	+	+	-	-
F1/R_b	23S rRNA	Commensal *Leptospira* spp.	53.0	30	1.5	60	-	+	ND	-

Primer cross-reaction against relevant bacterial DNA is denoted by the following symbols: (+) positive reaction, (-) negative reaction. ND: Not determined

Table 7.4: PCR detection results of leptospires in both sheep and cattle gastrointestinal samples.

Source	No. of samples	PCR Profile		
		L[1]	P[2]	S[3]
Sheep				
Rectum	31	-	-	-
Rectum	5	+	-	-
Gingiva	36	-	-	-
Gingiva	3	+	-	-
Total (Sheep samples)	**75**	**8**	**0**	**0**
Cattle				
Beef rectum	34	-	-	-
Beef gingiva	34	-	-	-
Dairy rectum	113	-	-	-
Dairy rectum	2	+	-	-
Dairy gingiva	112	-	-	-
Dairy gingiva	1	+	-	-
Dairy gingiva	1	-	-	+
Total (Cattle samples)	**298**	**3**	**0**	**1**

All positives samples (+) were sent for direct sequencing (Table 7.6)

[1] PCR assay detecting all Leptospira spp.

[2] PCR assay detecting pathogenic Leptospira spp.

[3] PCR assay detecting non-pathogenic Leptospira spp.

7.3.3 Direct DNA sequencing analysis

Whilst PCR results suggested *Leptospira* DNA in twelve samples in Table 7.4, sequencing results were only able to confirm that two samples contained leptospires, each from sheep rectal (3RJE) and dairy cattle gingiva samples (476) corresponded to *L. interrogans* and *L. biflexa* serovar Patoc 1. Further 16S rRNA gene phylogenetic analysis (Figure 7.1) with other *L. interrogans* representative serovars revealed that the positive sheep sample shared 100% identity with all *L. interrogans* serovar representatives, including two important serovars; Hardjo and Canicola, which are consistent with both maintenance and bystander infections in sheep (Hassanpour *et al.*, 2011; Director *et al.*, 2014).

The majority of positive samples from sheep and dairy cattle were demonstrated to be false positive results having high sequence identity with non-leptospire microorganisms (Table 7.6). As a result in this study, the true distribution is that all tested tissues were *Leptospira* negative except for a single sheep rectal tissues (2.78%) and a single dairy cattle gingiva (0.88%), respectively (Table 7.5).

Table 7.5: Leptospira PCR positive samples.

Animal	Positive gingival tissue	Positive rectal tissue	True *Leptospira* positive by gingiva DNA sequencing	True *Leptospira* positive by rectal DNA sequencing
Sheep	3/39 (7.70%)	5/36 (8.33%)	0/36 (0.00%)	**1/36 (2.78%)**
Cattle (Beef)	0/34 (0.00%)	0/35 (0.00%)	0/34 (0.00%)	0/35 (0.00%)
Cattle (Dairy)	2/114 (1.75%)	2/115 (1.74%)	**1/114 (0.88%)**	0/115 (0.00%)

Table 7.6: DNA sequencing analysis of Leptospira PCR assay products.

Samples	PCR assay[a]	Identical organism	Origin	Max score	Total score	Query cover	E-value	Percentage ID	Accession No.
Sheep									
2RJE	L	*Collinsella intestinalis* str. JCM 10643	Rectum	340	340	100%	1.00E-93	96.15%	NR_113165.1
3R	L	*Collinsella intestinalis* str. JCM 10643	Rectum	335	335	93 %	7.00E-92	92.98%	NR_113165.1
3RJE	**L**	***L. interrogans* str. RGA**	**Rectum**	**104**	**104**	**100%**	**4.00E-23**	**100.00%**	**NR_116542.1**
5R	L	*Atopobium deltae* str. HHRM1715	Rectum	222	222	95%	5.00E-58	84.93%	NR_133972.1
6JA	L	*Alkalibacterium iburiense str. JCM 12662*	Gingiva	69.4	69.4	100%	6.00E-13	100.00%	NR_112660.1
7JA	L	*Collinsella intestinalis* str. JCM 10643	Gingiva	503	503	98%	3.00E-142	95.25%	NR_113165.1
1839	L	*Collinsella aerofaciens* str. JCM 10188	Rectum	436	436	99%	2.00E-122	98.01%	NR_113316.1
S7G	L	*Collinsella aerofaciens* str. JCM 10188	Gingiva	556	556	98%	2.00E-158	97.26%	NR_113316.1
S2R	L	*Peptoniphilus coxii* str. RMA 16757	Rectal	87.9	87.9	89%	5.00E-18	93.22%	NR_117556.1
Dairy cattle									
473	L	*Atopobium fossor* str. ATCC 43386	Rectum	318	318	100%	6.00E-87	93.91%	NR_044646.1
476	**S**	***L. biflexa* svr Patoc str. 'Patoc 1 (Ames)'**	**Gingiva**	**340**	**340**	**100%**	**7.00E-90**	**100.00%**	**NR_103964.1**
489	L	*Collinsella aerofaciens* str. JCM 10188	Rectum	549	549	100%	3.00E-156	95.87%	NR_113316.1

The selection of identical organism was based on the top line of BLAST database sequence.

[a] The PCR assay that corresponded with the positive detection of the tested tissue sample. L: Leptospira spp. specific, P: commensal Leptospira specific

Max score; indicates the maximum score of single best aligned sequence, Total score; indicates the sum of scores of all aligned sequences, Query cover; indicates the percentage a query sequence aligned to a sequence in Genbank, E-value; indicates the expect value that the number of match hits which can be expected when searching a database of a particular size, Percentage ID: indicates the percent of identical match of a query sequence to closest to related organism. Samples with positive Leptospira PCR result are in bold

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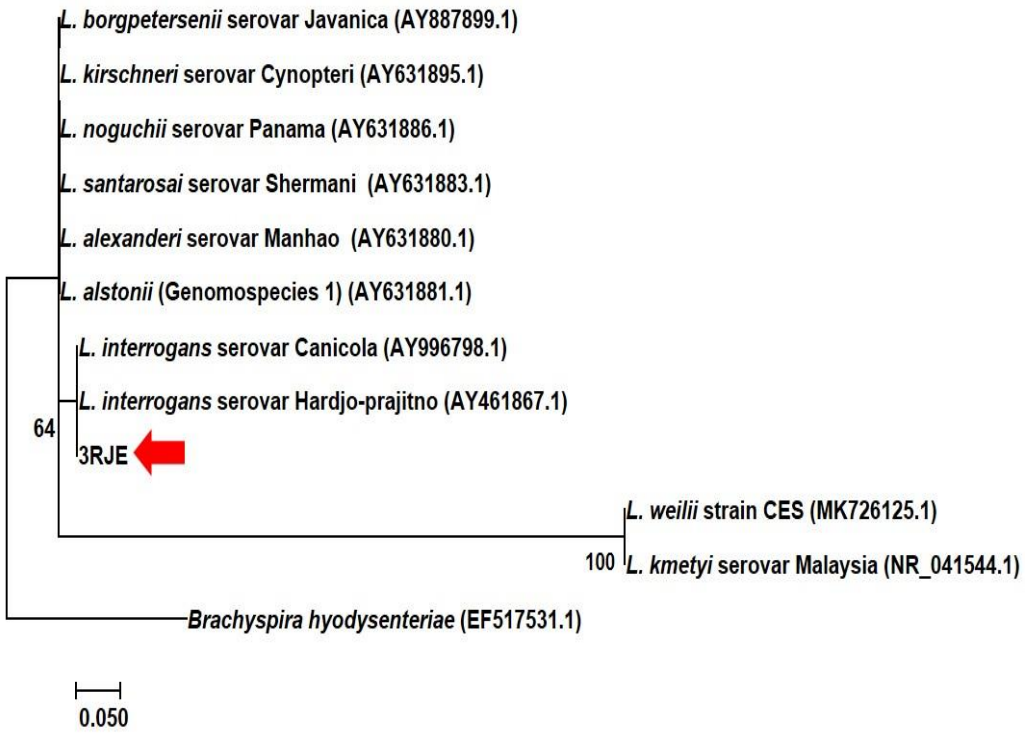


Figure 7.1: The phylogenetic analysis of 16S rRNA gene sequence comparisons of all representative pathogenic Leptospira species using the maximum-likelihood method by 10,000 times bootstraps.

The tree shows the relationship between a positive sample (indicated by an arrow) from sheep rectal tissue sample and associated Leptospira species.

7.4 Discussion

To our knowledge, this is the first study targeting the presence of leptospires in the ruminant gastrointestinal tract using molecular methods. The mucosal surface of the intestinal cavities may act as potential leptospire reservoir/carriage site with microbes eventually passed in faecal material to the environment potentially enabling a previously unreported faecal-oral transmission route. Furthermore, slurry provides a favourable environment for the survival of spirochetes and may potentially be an important reservoir on the farm (Tilahun, Reta and Simenew, 2013). For example, previous studies identified that for another spirochete, the digital dermatitis treponeme, that there is presence in gastrointestinal tract of ruminants (Evans *et al.*, 2012; Sullivan *et al.*, 2015) indicating the gastrointestinal tract is likely to be an important additional reservoir for transmission of digital dermatitis in ruminants apart from the foot. However, there is a lack of studies investigating the link between *Leptospira* transmission through the ruminant gastrointestinal tract, and therefore the present study is focusing on the involvement of digestive tract as a possible reservoir of ruminant leptospirosis.

From the PCR results obtained in this study, the percentage of positive samples using three different previously described PCR assays (reference the 3 assays) for both cattle and sheep samples were relatively low. Although several DNA samples are labelled as 'positive' *Leptospira* in 16S rRNA PCR and one from 23S rRNA (*L. interrogans*) PCR, only two samples were confirmed as true positive from sequencing data, identified as *L. interrogans* (3RJE) and *L. biflexa* (476) respectively. The results show that the 16S rRNA gene *Leptospira* genus-specific PCR assay lacks specificity as it demonstrates cross-reactivity with contaminating enteric bacteria, which resulted in false positives, whilst the 23S rRNA *L. biflexa and L. interrogans* specific PCR assays appears specific. It has been previously reported that there can frequently be non-specific amplification from other DNA in stool samples using 16S rRNA PCR assays due to the presence of polymicrobial flora in the gut that may result in PCR cross-reactivity and therefore decreasing the PCR specificity (McLain *et al.*, 2009).

The low detection rates of *Leptospira* in cattle and sheep gastrointestinal tracts suggests a minor or brief interaction between these bacteria and the ruminant host's tissues surveyed. In man and dogs, acute leptospirosis is reported to cause gastrointestinal bleeding and intestinal motility disorder (Schweighauser *et al.*, 2009; Legris *et al.*, 2014). These complications are thought to be due to disrupted homeostasis or intestinal inflammation induced by leptospirosis infection that may alter intestinal motility and flexibility. However, in the ruminant host, bleeding and gastrointestinal signs caused by leptospirosis are rarely observed, except in young animals. Additionally, it is unlikely that the *Leptospira* can survive in the ruminant digestive tract. A study Asoh *et al.* (2014) by the roles of saliva as a natural defence against leptospires cells, although this study does not verify the effect of high pH on the bacteria. Ruminant saliva has high pH at approximately 8.2-9.0, which is not favourable for *Leptospira* survival. Moreover, the microenvironment condition in rumen and reticulum also is not conducive for *Leptospira* growth or survival despite having a relatively good pH range between pH 5.7-7.2. The anaerobic environment is only beneficial to commensal rumen microbes (anaerobic bacteria, protozoa and fungi) whose growth may potentially suppress the ability of *Leptospira* to replicate and survive, and extremely low pH in the abomasum (stomach) will further destroy the bacteria.

In contrast to the gastrointestinal tract, the ruminant kidney is a favourable environment for *Leptospira* survival and multiplication in the renal tubules and is excreted via the urinary passage. As such, the optimal pH range for *Leptospira* growth is slightly alkaline (7.2-7.6) (Cameron, 2015), which is similar to a ruminant kidney condition. Additionally, *Leptospira*

localisation in the proximal renal tubules (Prescott, Miller and Nicholson, 1987; Skilbeck, Forsyth and Dohnt, 1988) and passing of microorganism via urine is the primary mode of transmission and may further contaminate the farm slurry. As previously noted, *Leptospira* may be able to survive in the moist environment for months (Khairani-Bejo *et al.*, 2004), and therefore slurry could be an important *Leptospira* reservoir in ruminant farms, rather than the gastrointestinal tract.

Although a small percentage of DNA samples were found to be true positives for *Leptospira* spp., (Table 7.5) we believe that these are chance findings. One gingival sample from dairy cattle was positive to *L. biflexa*, and we suspect the animal acquired the bacteria directly from the environmental sources such as from slurry/pasture or water. *L. biflexa* is one of the commensal, non-pathogenic leptospires that is commonly found in water and soil (Henry *et al.*, 1971; Henry and Johnson, 1978). However, it is not easy to understand the presence of *L. interrogans* found in one of the sheep rectal tissues in this study. It may be possible that this animal could be actively being infected/invaded or there is maybe occasional carriage for the leptospires. Studies by several authors had previously isolated several *Leptospira* strains from the genital tract of sheep that indicates the persistent and potential venereal transmission of *Leptospira* (Lilenbaum *et al.*, 2008; Arent *et al.*, 2013; Director *et al.*, 2014). Close anatomical distance between urinary tract and anus may enable possible spread of the bacteria from the urethra to the anus via urinary dropping, which is likely to contaminate around the perineal area. Future investigations are recommended to investigate whether leptospires are actively invading and damaging rectal tissues using immunohistopathology studies. Moreover, the use of an advanced genotyping technique such as Multi locus sequence typing (MLST) can be applied to further verify if the same isolate can be found in different tissues within the same animal to determine whether it is within host spread of the same microbe (Bell, 2017).

In terms of study limitations, the specific leptospirosis status based on serology of each individual animal tested, and leptospire PCR detection across a period of time was not known. Therefore, it was not clear if these animals may have just acquired the disease or previously become *Leptospira* carriers before sampling. Secondly, *Leptospira* isolation using slurry, rectal and gingival tissues samples were not performed in this study. Therefore, it is not possible to give additional evidence and characterisation for the presence of leptospires in the ruminant gastrointestinal tract. Although the *Leptospira* specific PCR had previously shown higher sensitivity and specificity with various clinical samples in the previous studies,

this PCR is deemed here as not ideal to be used for *Leptospira* detection in gastrointestinal samples due to higher cross-reactivity with other GI bacteria.

From the results shown in this study, more investigations are needed to rule out the possibility of disease transmission and infection reservoirs via the ruminant gastrointestinal tract and farm slurry. Further improvements could include; 1) *Leptospira* isolation from gingiva and rectal tissues, including the slurry samples from positive farms, 2) use of more sensitive PCR assay such as nested-PCR and/or quantitative PCR that were previously demonstrated in both human and cattle leptospirosis studies (Stoddard, 2013; Hernández-Rodríguez *et al.*, 2014; Chen *et al.*, 2015) would improve the *Leptospira* detection sensitivity and specificity using gastrointestinal samples as well as quantify the actual leptospire burden within the infected animals. Lastly, 3) a longitudinal ruminant outbreak study including both serological analyses could be used to determine the relationship between disease burden and potential carriages sites to better dissect transmission routes of this important endemic infectious disease.

7.5 Conclusion

The low detection rates for *Leptospira* presence within the gastrointestinal tract of ruminants in this study does not suggest a possible carriage site/infection reservoir of *Leptospira* for these locations. Future studies should either concentrate on detection methods of increase sensitivity or investigate alternative infection reservoirs such as detection and survival of leptospires in contaminated pasture/slurry.

Chapter 8: General Discussion

8.1 Leptospirosis- An insight into a complex pathogenesis

It has been nearly one hundred years since leptospirosis was discovered and identified as a significant global threat to both humans and animals. Subsequently, a substantial foundation of research was undertaken in order to understand the pathogenesis of the disease in the host. Before the arrival of the genomic era, the knowledge of disease progression in mammalian hosts was mainly based on observed disease aspects, and as a result, the disease is well-described in a clinical manner (Turner, 1967; Bruce *et al.*, 2005; Gouveia *et al.*, 2008). However, details on *Leptospira*-host interactions, including specific mechanisms of virulence and host defence that leads to a disease outcome, is somewhat limited. Hence, the pathogenesis of leptospirosis at the cellular level is poorly understood. Lack of knowledge in this area has hampered efforts to identify important virulence factors that could be utilised as disease markers for diagnostic tools and could underpin vaccination.

Recent advances in genomic tools provide an insight into the evolution of *Leptospira* and the pathogenesis of leptospirosis at both the cellular and molecular level. Despite the availability of *Leptospira* whole genome sequences and development of mutagenesis studies, there are still many virulence factors in *Leptospira* which are understudied when compared with other bacterial pathogens. This is supported by a large number of hypothetical function genes within *L. interrogans*, where 78% out of 655 unique *L. interrogans* and 58% out of 308 unique *L. borgpetersenii* genes have no assigned function (Adler *et al.*, 2011). Mutagenesis studies have begun to enable the discovery of numerous *Leptospira* virulence factors as reviewed by Murray, (2015). However, these studies were mostly centred on *L. interrogans* associated with human leptospirosis, and there is still a huge knowledge gap for other pathogenic *Leptospira* species, which includes essential characterisation of pathogenic and host-adaptation mechanisms worthy of investigation.

The leptospiral surface-exposed proteins (e.g. OMP) are one of the major virulence factors that play a major role in bacterial interaction to the host enabling attachment and colonisation and also serve crucial biological purposes in maintaining the cell's life such as providing structural integrity, nutrient transports and resistance against antibiotic attack. In recent years, several leptospiral OMPs have been successfully discovered and functionally

identified. However, as previously mentioned, the majority of these OMPs were identified in a single strain of *Leptospira interrogans* (Table 1.6, Chapter 1).

One *Leptospira* species of interest, *L. borgpetersenii* is an important pathogenic species maintained by cattle and rats and is potentially zoonotic to humans (Benacer *et al.*, 2013; Gamage *et al.*, 2014; Chideroli *et al.*, 2016). From a previous study, a total of 263 predicted surface-exposed proteins identified in *L. borgpetersenii* Hardjobovis L550 were identified through bioinformatic analysis and 238 proteins were successfully expressed as recombinants and immunologically evaluated, resulted in high immune response (>70%) in tested animals (Murray *et al.*, 2013). Although the study provided a useful immunological reference for future vaccine perspective, it did not confer information about the specific individual leptospiral protein interaction mechanisms within the host, and therefore it was not clarified how these proteins were able to underpin invasion and colonisation. Thus, the present study was prompted to investigate the extent of *L. borgpetersenii* surface-exposed OMPs interaction with the host.

8.2 Reverse vaccinology - a rational direction towards leptospirosis vaccine design

The RV approach has proven to be an advantageous method for prediction and identification of surface-exposed OMP. The method was previously applied in many bacterial studies, including in some leptospiral studies (Maneewatch *et al.*, 2007; Pinne and Haake, 2009). While this study only utilised the *in silico* approach, it is clear that the method is practical for screening for target genes from the entire genome using various prediction algorithms, and one can quickly evaluate the several gene functions through expression and *in vitro* characterisation at once, saving time and resources. Using a similar approach focusing more on OMPs, we had successfully identified and characterised several novel OMPs in a bovine *Leptospira* species (*L. borgpetersenii* serovar Hardjobovis L550) via functional and immunological evaluation investigations.

To date, the use of RV has not been extensively applied in leptospirosis field, although previous studies had successfully identified a broad list of potential vaccine candidates using either near complete RV methodology or by using the bioinformatics approach alone (Dellagostin *et al.*, 2017). Moreover, the increasing availability of genomes and proteomics data provides the opportunity to explore more potential vaccine candidates/virulence factor in other pathogenic *Leptospira* species using the RV application.

8.3 *Leptospira borgpetersenii* Hardjobovis L550 OMP functional and immunological characterisation

From the results obtained in this study, five genes encoding transmembrane surface proteins were successfully cloned and expressed as recombinant OMPs (Chapter 4). Through host-ligand binding studies, we were able to identify that three of these *L. borgpetersenii* OMPs (assigned as Lsa37 and Lsa49) including OmpL1 of the same species, exhibited strong adherence to multiple host ligands, with an exception to the rLBL0375 OMP, which may tentatively have other functional roles not identified in the present study. Interestingly, the binding variations seen in this study suggest the involvement of functional redundancy between these OMPs (Chapter 5). Functional redundancy has been widely described for many bacterial pathogens (Antonara *et al.,* 2011; Ghosh and O'Connor, 2017), and the phenomenon is also considered normal in *Leptospira* spp. (Adler *et al.*, 2011). The reason for this is thought to be due to a process of genomic expansion among pathogenic species (e.g. *L. interrogans*) through gene duplication (Bulach *et al.*, 2006). Surprisingly, our results revealed that even though *L. borgpetersenii* species underwent genome reduction which resulted in restricted host transmission (Bulach *et al.*, 2006), this species is able to retain some degree of functional redundancy which exhibited by the ability of Lsa37, Lsa49 and OmpL1 to bind selectively to similar host molecules such as laminin, fibrinogen, fibronectin and elastin seen in this study. This further suggests that leptospiral OMPs possess a high level of functional redundancy both within and between species. This is further supported by the fact that pathogenic leptospires have more paralogs compared to saprophytic species which explain the occurrence of gene and functional redundancy are more common pathogenic species (Picardeau *et al.*, 2008).

However, despite functional redundancy seen within these bovine OMPs, there are slight functional variations observed. For example, Lsa49 was able to bind to laminin, whereas Lsa37, rLBL0375 and OmpL1 (*L. borgpetersenii* serovar Hardjobovis L550) do not possess this function. Interestingly, a study by Fernandes *et al.* (2012) demonstrated that OmpL1 (from *L. interrogans*) bound significantly to laminin, which suggested OmpL1 of *L. interrogans* differ in function compared to OmpL1 of *L. borgpetersenii*. It is not certain if the loss of binding function may have an effect on the pathogenicity of *L. borgpetersenii* species, however, we consider that this could be a contributor to host-restriction, other than the already reported genome reduction (Bulach *et al.*, 2006). This is supported by a recent study describing the loss of function due to mutations in unique virulence factors identified in *Salmonella enterica*

subsp. *enterica* serovar Typhimurium which is likely to affect the bacteria ability to infect a wide host range and promote systemic infection (Larock, Chaudhary and Miller, 2015).

Functional overlap can be limited to proteins of similar structure. For instance, a group of paralogue leptospiral protein such as Ligs (A and B) and Lens (ABCDEF) proteins were reportedly able to interact with similar range of host molecules as demonstrated *in vivo* studies (Barbosa *et al.*, 2006; Choy *et al.*, 2007; Stevenson *et al.*, 2007). On the other hand, functional redundancy may also observe between proteins belong to different domains. For example, the majority of leptospiral proteins such as LipL32, LipL53, Lsa21, Lsa30, MFn6 are reported to bind to fibronectin (Atzingen *et al.*, 2008; Hauk *et al.*, 2008; Pinne, Matsunaga and Haake, 2012; Souza *et al.*, 2012; Vieira *et al.*, 2014), including several novel OMPs discovered in this study (Chapter 5). The redundancy feature, as seen in these proteins is difficult to understand as these proteins may present and operate differently in certain disease stage, different tissues or may operate simultaneously.

What is lacking in the present study is further confirmation that these proteins do indeed demonstrate *in vivo* what we have identified they bind to *in vitro*. To do this, an animal disease model would need to be used, and measures put in place to try and reduce issues around functional redundancy need to be taken into account to enable something useful to be measured. Mutagenesis studies would be beneficial to determine if these proteins are required for virulence, which can be determined via mutagenesis and *in vivo* investigations to determine whether molecular Koch's Postulates are fulfilled (Falkow, 2004). One classic example is a study of prominent surface-exposed lipoprotein with OMP-A domain (Loa22) by Ristow *et al.*, (2007) who successfully proved that Loa22 is essential for leptospiral ability to cause disease and pathological lesions in experimental animal models. In contrast to this, another study using a major surface protein LipL32 revealed that despite being abundant on the cell-surface of pathogenic *Leptospira* spp., the mutagenesis study revealed that this protein is not essential during infection, although may be a key during leptospiral transmission cycle (Haake, 2001; Murray *et al.*, 2009).

Despite this many leptospiral adhesins or other virulence factors (mainly from *L. interrogans*) have been identified and expressed over the years, although surprisingly, there is lack of mutagenesis studies to support their functional roles which would be advantageous. Therefore, mutagenesis studies may provide useful information about OMP virulence as to eliminate the possibility that the *in vitro* binding assay characteristics for these OMPs are

artefacts and to further identify that the molecules do come into contact with the host and are responsible for pathogenesis.

The immunological interaction of cattle milk with the novel bovine OMPs identified in this study revealed interesting outcomes. While all the tested OMPs can be considered to have previously been detected by the host immune response (depicted by high IgG1 titres), only OmpL1 and rLBL0375 titres agreed with the commercial test kit designation of leptospire infected or naïve. OmpL1 has previously been demonstrated as a promising antigen as it is recognised by the immune cell in the blood of infected patients during both acute and immune phase leptospirosis (Guerreiro *et al.*, 2001; Fernandes *et al.*, 2012), but has never been tested for in milk. Interestingly, an OMP of unknown function (rLBL0375) exhibited a significant titre corresponding with the test kit's disease designations. This shows that although this OMP has no defined function, it may be useful as an antigen to assign disease status in bovine leptospirosis. Interestingly, a strong linear regression correlation between OMP's antibody titres in the study (Lsa37 vs Lsa49, Lsa37 vs rLBL0375, and Lsa49 vs rLBL0375) indicates that several of these OMPs (Chapter 5) may share identical epitopes and be antigenically related to being recognised by the immune system. Proteins (antigen) sharing similar specific epitopes are likely to be recognised by host antibody, thus forming antigen antibody complex. It would be interesting to identify the binding site of an antibody to individual OMPs (antigen) through epitope mapping that would further confirm if these OMPs are sharing identical binding site corresponding to positive correlation of antibody titres in this study.

Additionally, while the majority of OMPs did not agree with the commercial *Leptospira* kit, OmpL1 (from the bovine species) exhibited significant antibody titres between positive and negative leptospirosis cattle milk samples. Although this appears promising, we cannot disregard the potential roles of other OMPs in this study as the true infection status of tested cattle/herd is not known and it was only determined by a commercial ELISA kit which may lack some specificity and is not the gold standard method (MAT) recommended by OIE (OIE, 2008). It may be possible that these OMPs were expressed in certain stages of disease (e.g. acute, chronic, convalescent/immune), which are not potentially detected in cattle milk. Furthermore, the titre differences between OmpL1s and other bovine OMPs could be evidence of reciprocal expression. The mechanism, although not yet reported in *Leptospira* spp., is described in other Gram-negative bacteria such as *Borrelia burgdorferi* (Schwan *et al.*, 1995; Yang *et al.*, 2004) and *Pseudomonas aeruginosa* (Ventre *et al.*, 2006) which is essential for bacterial survival within the host.

8.4 The functional diversity of leptospiral OMP variants across genomospecies - A key to species survival and host adaptation

The *Leptospira* genus is considered one of the most diverse, with such diversity enabling adaptation within mammalian hosts and enhancing survival in various ecological niches. The ability to sustain in disparate conditions is thought to be due to substantial genome and diversification enabling several new functions to be acquired through gene transfer associated with adaptation of *Leptospira* species to new hosts (Fouts *et al.*, 2016; Xu *et al.*, 2016; Vincent *et al.*, 2019). The leptospiral outer membrane is known to have antigenic diversity which was presumably related to the adaptation of leptospiral genomospecies and/or serovars to specific mammalian host due to high genetic variations seen in LPS (Haake and Matsunaga, 2005), which also account for serovar diversity (Zuerner *et al.*, 2000). Surprisingly, leptospiral OMPs (e.g. LipL32) also display antigenic variations and are genetically conserved among various serogroups. Despite conservation of OMPs among *Leptospira* spp., the gene sequence variation among species may be highly variable, which suggests multiple evolutionary mechanisms acting on leptospiral genes encoding OMPs (Haake *et al.*, 2004). The gene sequence variation mostly occurs on the surface loops of OMPs, which have direct interaction with the host. This diversification of amino acid sequences on the surface loops may tentatively affect the ability of the OMPs interaction with the host.

Two leptospiral OMPs, OmpL1 that was previously identified and described in multiple studies (Haake *et al.*, 1993; Shang *et al.*, 1995; Natarajaseenivasan *et al.*, 2005) and Lsa49 (identified in this study-Chapter 6) were selected to study the functional diversity that may have an effect on the binding ability to host molecules. Genes encoding OmpL1 and Lsa49 were selected from five different genomospecies, and the binding of these OMPs to several host molecules was measured. Our results showed that OmpL1 has much greater detected binding variation across species, compared to Lsa49. Furthermore, the comparative amino acid sequence indicates both surface loops and transmembrane region of OmpL1 across pathogenic genomospecies have either one or several amino acid sites conserved among species that correspond to each host molecule specificity. Surprisingly, in the comparative analysis of OmpL1 primary structure of *L. borgptersenii* and *L. santarosai* that belong to similar sister groups showed an additional host binding preference to elastin. Additionally, a deeper branch of *L. interrogans* subcluster tended to lose several binding functions compared to main *L. interrogans* groups. These findings showed that the molecular evolution of OmpL1 affects host-binding interactions, which likely has implications for host adaptation

and survival. Although Lsa49 variants have more variable regions on their amino acid sequences, their binding functions appear more conserved as the majority of Lsa49 proteins across genomospecies demonstrated similar binding preferences to several host molecules.

The immunological evaluation of OMP variants (OmpL1 and Lsa49) across pathogenic genomospecies suggests these OMPs have conserved immunogenicity (demonstrated by a strong correlation of anti-OmpL1 vs anti-OmpL1 and anti-Lsa49 vs anti-Lsa49 titres), although they could still exhibit some antigenic diversity. This conservation would be a major advantage to overcome the problem with serovar-specificity as seen in bacterin (whole-cell) *Leptospira* vaccines, as they contain identical epitopes to allow antibody binding which is likely able to induce cross-reactions among species. The ability of the immune system to cross-react would confer broad protection against multiple species/serovars that would be ideal as a vaccine component. Additionally, antibody responses against leptospiral OMPs seen in this study highly suggested that these OMPs are indeed expressed as surface proteins and recognised by cattle immune system. However, it is not clear such responses were triggered either by humoral or cell-mediated immunity of cattle host. Previous studies by Naiman *et al.* (2001, 2002) demonstrated that immune response in induced by vaccinated cattle (with bacterin vaccine) upon challenged was strongly stimulated by Th1 response, which suggested that cattle may develop cell-mediated immunity that normally associated with intracellular microbial infections. Interestingly, humoral-mediated immunity which largely derived against LPS is equivalently important to confer protection against leptospirosis in humans, dogs and hamster, but failed to protect cattle (Bolin *et al.*, 1989; Fraga, Barbosa and Isaac, 2011). This indicates that different types of antigen contribute to different types of immune response stimulated by the host. We would consider to further characterise the type of immune response in cattle in future studies via vaccination using these OMPs as testing antigens.

The high genetic diversity observed within OmpL1 genes and encoded amino acid diversity of the encoded receptor, especially in regions encoding extracellular loops, suggests that *Leptospira* may also undergo positive selection. Positive selection is quantified by analysing nucleotide changes acting on a protein-coding region and is measured by comparing the rate of nonsynonymous substitutions (denoted as d/N) with synonymous substitutions (denoted as d/S) and is also referred to as the dN/dS ratio. This ratio measures the strength and mode of natural selection, where neutral selection is achieved when the ratio is equivocal/ at unity (dN/dS = 1), which indicates that a protein-coding region is unlikely to experience selection. On the other hand, if more nucleotide substitutions result in amino acid change than

substitutions lead to no change then the term is described as 'positive selection' when (dS/dN) ratio of a particular gene exceeds unity (>1), whereas (dS/dN) ratio less than unity (<1) implies that a gene undergone 'negative (purifying) selection' which prevents change of a particular protein-coding region, resulting in gene stabilisation and frequently occurs due to conservation of a required function (Kryazhimskiy and Plotkin, 2008; Jeffares *et al.*, 2014).

The mechanism of positive selection occurs within a wide range of genes of variable functions in a particular genome, including genes encoded for cell-surface proteins (e.g. OMPs) as they usually become a target for host immunity (Petersen *et al.*, 2007). Antigenic variation may occur in which the pathogen has the ability to transform the surface protein that is detected by host immune recognition, thus producing a subpopulation with surface-protein diversities which may evade the host immune system, thus preventing eradication and allowing re-infection to the host (Kotwal, 2006; Deitsch *et al.,* 2009). For such a strategy to work, the pathogen must possess a tightly controlled antigenic profile and produce antigenic variants at regular intervals. Theoretically, antigenic variation has several mechanisms that are largely divided into two categories; random variation and programmed variation. Random (or unprogrammed) variation occurs as a result of DNA/RNA alteration produced by nucleotides recombination, errors in DNA/RNA replication or repair, and reassortment of gene segment potentially cause the transfer of stretches of DNA/RNA and entire genes to other region of genomes to generate variations. These mechanisms which are commonly seen in viruses (e.g lentivirus and influenza), may alter the level of expression of the amino acid sequence of its products and therefore may result in antigenic site change (shift) or formation of a new virus subtype (drift) (Folks, 1994; Chen and Deng, 2009).

In contrast, the programmed variation (also referred to as phase variation/antigenic variation) may affect the expression of a gene encoding protein without changing its primary nucleotide sequence. Phase variation regulates the ON-OFF expression of a particular antigen (van der Wouder and Baumler, 2004). The strategy is widely applied to a family of paralogous genes encoding proteins with similar functions and ability to express one protein at a time, and generally irreversible or temporarily. One common phase variation mechanism is slipped-strand mispairing of repetitive DNA segments in homopolymeric tract consisting of a single nucleotide, or several multimetric classes of short sequences repeats (SSR). Phase variation may occur by altering the SSR region that is positioned either upstream of a gene (affecting transcription), or within the gene (altering translational reading frame) (Chandler and Fayet, 1993; van Belkum *et al.*, 1998). The outcome from both events will result in 'phase ON or OFF' expression either by blocking of transcription promoter or producing premature

stop codon. For example, transcriptional control seen in surface-proteins of pathogenic bacteria such as *Neisseria meningitidis* and *N. gonorrhoea* (Opa proteins) and translational controls seen in *E. coli* (fimbriae) are essential for mediating bacterial attachment to host cell surface (Meyer, Gibbs and Haas, 1990; Dixon *et al.*, 2007).

In this study, intragenic recombination of OmpL1 amino acids sequence on the surface-exposed loops across several *Leptospira* species/strains is highly suggestive for evidence of antigenic variation as previously described by Haake *et al.* (2004). However, it was noted that the positive selection was only observed on two amino acid sites on a single surface-loop, and other additional variable loops (denoted by several nonsynonymous substitutions in four variable regions encoding surface loops), indicates that such amino acid sequence variability on these regions is unlikely due to the immunological pressure. The outcome of gene recombinant events which involve the movement of entire genes, or a small portion of the gene producing a chimeric DNA may result in non-expression into pathogen expression site, which is commonly adapted by many bacteria to regulate antigenic variation to circumvent immune host. This is further supported by the fact that leptospiral colonisation in renal tubules in the reservoir hosts could be driven by adaptations to the specific hosts that leads to increase the variability of amino acid sequence to tolerate within the host environment. However, changes of the amino acids on OmpL1 surface-exposed loops may evidently result from recombinant DNA of antigenic variation, although the actual effect from this variation may not be significant in terms of disease outcome, apart from binding preference to host molecules observed in our study.

Interestingly, the effect of recombinant DNA, causing antigenic variation was previously studied in other pathogenic spirochetes such as *Borrelia burgdorferi* and *Borrelia hermsii* which are causative agents for Lyme disease and relapsing fever, respectively. Hosts infected with either one spirochete (e.g. *B. burgdorferi*) can last from several months to years due to the ability of *Borrelia* to escape from host immune invasion, and may eventually lead to persistent infection (Steere *et al.,* 2004). The key of evasion strategy adapted by *B. burgdorferi* is the gene recombination at the *vls* (Vmp-like sequence) locus which composed of an expression site (*vlsE*) encoding for surface lipoprotein VlsE and 15 silent cassette (unexpressed) located on the 28-kb linear plasmid in the organism (lp28-1) (Zhang *et al.*, 1997; Norris, 2006). During infection, changes at the *vls* locus may occur through recombination events and resulting in gene conversion between expression cassette and silent cassette. Silent cassette segment serves as a source of non-reciprocal recombinant events with the expression cassette which eventually express as distinct VlsE variants during

infection. This resulted in alteration of the surface epitope of VlsE, thus avoiding host immunity (Bankhead and Chaconas, 2007).

The *Leptospira* genome possesses an extensive heterogeneity across species which suggest for large gene rearrangements due to the various types of recombinants events (Zuerner *et al.,* 1993). It is possible that antigenic variation is likely to occur, although this has never been well-described compared to other spirochete species, possibly due to the difficulty to manipulate leptospiral genome (Picardeau, 2015a). We hypothesise that given the high antigenic diversity observed in OMPs from pathogenic *Leptospira* spp., it could be expected that they already also have specific mechanisms that would affect gene expression that would be key for *Leptospira*-host interaction and evasion from host immune response. The evolutionary divergence observed for OmpL1 concurs with other leptospiral proteins such as LipL32 and LipL41, and the Lig proteins, albeit those protein sequences, are more conserved (Haake *et al.*, 2004; McBride *et al.*, 2009; Vedhagiri *et al.*, 2009). However, neither is required during infection, although all are largely recognised by host humoral immune response and therefore their roles during infection remain unexplained. However, the genetic variability observed in Lig proteins are thought to be a key factor contributing towards species adaptation to mammalian host for serovar-host specificity (McBride *et al.*, 2009).

Additionally, changes in amino acids acting on the surface-exposed loops of OmpL1 across pathogenic *Leptospira* species are driven by a strong positive selection (Haake *et al.*, 2004), of which contributed to the significant binding preference shown in our study. Moreover, the evidence of positive selection was proven to be essential for pathogenic *Leptospira* spp. survival and adaptation in the new host during the early stage of infection in the recent study (Kurilung *et al.*, 2019). However, we could not stipulate the same conclusion in the case of Lsa49. It is not clear if this gene could also develop similar selection pressure, which would be an interest. Therefore, future investigation is warranted to compare genomic data of Lsa49 genes to identifying the possible site(s) affected by selection pressure and to investigate if changes of genes may contribute to the adaptation of *Leptospira* in the host, or to avoid evasion of host immunity.

Combining of both functional and immunological data of OMP variants in Chapter 6, we can assume that the genetic exchanges between *Leptospira* species are translated with additional gaining or losing of binding function to various host components without affecting their immunogenicity which would be ideal for vaccine development. This information is crucial to determine if the variation seen in a particular OMP may; 1) be involved in *Leptospira*

adaptation and survival in particular hosts such as cattle or human, 2) affect *Leptospira* virulence (self-limiting versus acute or chronic infection or ability to induce cross-protection) 3) relate to geographical distribution (e.g. Africa versus Asia). A further recommendation is to study leptospiral OMP (e.g. OmpL1) in all pathogenic and intermediate genomospecies species through functional binding diversities and immunological evaluations to determine the extent of antigenic variations among species, which would reveal insight into physiology and pathogenesis of pathogenic *Leptospira* spp. Moreover, it is expected that such findings will help researchers to understand the mechanism(s) that possibly are involved in lieu of selection pressure with antigenic or phase variations as seen in other pathogenic bacteria.

8.5 OmpL1- The future of vaccine development

Among of all OMPs studied, we found that OmpL1 is undoubtedly an ideal candidate to be developed as a vaccine candidate and diagnostic tool which concurred with the previously published findings (Haake *et al.*, 1999; Maneewatch *et al.*, 2007; Dong *et al.*, 2008; Subathra *et al.,* 2013). Previously, there was a lack of further studies for this OMP, possibly due to the difficulty to express toxic OmpL1 gene as recombinant proteins resulting in low protein yield. Here, our recombinant protein expression protocol has proven suitable to express the recombinant OmpL1 gene and provide a substantial yield. Therefore this protocol should be further optimised for future OmpL1 studies the which including; 1) to study the OmpL1 structure via crystallography approaches, 2) to produce mutant OmpL1 alleles across pathogenic species incapable of binding host ligands to verify if key amino acids responsible for adhesive properties to host cells, 3) to develop a complement-mediated serum leptospirocidal assay using OmpL1 to investigate whether they may interact with specific site(s) of host complement regulators for immune interference. Collectively, such studies may be useful for future therapeutic approaches and further understanding of the immuno-pathogenesis of *Leptospira.*

8.6 *Leptospira* reservoir in the ruminant gastrointestinal tract

Our study provides important information regards the possibility of *Leptospira* transmission via the ruminant GI tract. From the literature, it was known that *Leptospira* could be transmitted directly through the oral route. However, whether the oral or rectal tissues act as a carriage site/infection reservoir also had never been investigated in ruminant leptospirosis studies. Although our findings did not support the identification of new carriage

sites/infection reservoirs (Chapter 7), more studies are needed to investigate how long leptospires may survive in farm slurry and to determine the risk of further infections from any GI presence. Such studies include; 1) Epidemiological/outbreak investigation of the ruminant herd to determine the disease association between the presence of positive leptospires detected in GI tract with leptospirosis status of the animal, 2) Identification and characterisation of leptospires in GI tissues using reliable and more sensitive detection methods (e.g. qPCR). Furthermore, dissecting whether the occasional positives we observed were active disease through immunohistopathology or evidence of occasional contamination from the kidney tubule urine route would seem most valid.

One risk factor of accidental leptospirosis infection in ruminants is mixed farm practice; including co-grazing and direct contact of infected sheep/cattle/pigs/deer (Lilenbaum and Souza, 2003; Schoonman and Swai, 2010; Subharat *et al.*, 2012). While urine is a known source of infection when presented as slurry, the combined urine and faecal droppings may result in differences in leptospire survival. Future work should study whether slurry may act as a potent leptospire reservoir and take into account differences in survival in this material at different temperatures in the future. Such studies may help to limit disease transmission into the farm while maintaining a good husbandry practice.

8.7 Conclusion

With this worldwide human affliction staggering at nearly 60,000 deaths and more than one million cases reported every year (Torgerson *et al.*, 2015), leptospirosis is a global public threat, and an urgent intervention is needed to tackle the spread of infection. Addressing domestic animals as the main source of direct transmission of *Leptospira* to humans, vaccination is deemed necessary to prevent infection in both human and animals. While human vaccination has yet to be approved, major drawbacks in leptospirosis vaccination as observed in cattle and dogs are lack of cross-protection, inability to protect against renal colonisation and limited duration of protection (Dellagostin *et al.*, 2017). These disadvantages call for the new lines of modern *Leptospira* vaccines that are preferably able to confer universal protection, long-lasting duration, and have zero side effect using the leptospiral OMPs as the next potential antigens. The availability of *Leptospira* genomes and the development of recombinant DNA technology have enabled for the identification of potential OMPs that would be ideal for recombinant vaccine development.

Using a whole genome of *L. borgpetersenii* serovar Hardjobovis L550 (bovine leptospiral strain), here we identified six novels transmembrane OMPs encoding genes with a porin protein (OmpL1) using RV approach of which four genes were successfully expressed as recombinant OMP. Three of these recombinant OMPs (including OmpL1) showed the significant adhesive property to various host molecules which reflect their ability to adhere to the host. The resulting two novels OMPs were each re-assigned as Leptospiral Adhesin (Lsa) protein followed by their respective molecular sizes; Lsa37 and Lsa49. Immunological evaluation of these OMPs highly suggestive for that they are likely expressed, exposed and recognised by host immunity, which in turn would be considered as diagnostic tools. Our study on OmpL1 across pathogenic genomospecies showed a significant immunogenic property which supports an extensive body of literature considering this protein as a perfect recombinant vaccine candidate and improvement diagnostic method. Additionally, our research verified that OmpL1 appears to be a thermostable antigen, which may enable the production of a heat-stable vaccine, which would be a major advantage especially in the majority of leptospirosis endemic tropical countries where the cold chain access is hardly accessible.

The functional diversity of leptospiral OMPs in this study revealed an insight into the molecular evolution of the leptospiral genome. Through a comparative analysis of the amino acid sequences of both OmpL1 and Lsa49 across multiple genomospecies, we successfully demonstrated diversity in binding profiles assessed when expressing these OMPs as recombinants. This subsequently enabled identification of several binding sites corresponding specific host-ligand binding. Additionally, the immunological evaluation using these OMP variants (OmpL1 vs OmpL1 and Lsa49 vs Lsa49) revealed that the majority showed positive association which reflect that these OMPs are equally recognised by the host immune system. A high degree of variation seen in a particular short amino acid stretch or single amino acid on the surface-loop regions of both OMP variants suggest evidence of molecular evolution possibly through selection pressure causing antigenic variation and has eventually resulted in functional variation across genomospecies.

The survey of ruminants for the presence of leptospires in the GI tract eliminates the possibility of GI as potential leptospires reservoir and carriage site that allow disease transmission. This highly suggests that GI tract microenvironment may not be ideal for *Leptospira* survival in the ruminant. Although slurry contains urine, this continues to act as an infection reservoir which needs further characterisation as to how it contributes to disease transmission among healthy herd and re-infection in the farm.

Using the outcomes obtained from this study, we hope to contribute and improve our understanding of *Leptospira*-host interactions at both the cellular and molecular levels and therefore, narrow the knowledge gap on the complex leptospirosis pathogenesis as well as further clarify leptospire transmission pathways of leptospires on ruminant farms. The information might serve as a valuable reference for future development in the field of therapeutic and prophylactic approaches in leptospirosis, which may help to eradicate the disease in both animals and humans worldwide.

Appendix A

Supplementary material for Chapter 3

List of L550 genes with predicted signal peptide cleavage sites and desirable OMP criteria

>LBL_0012	>LBL_1344	>LBL_2896
>LBL_0024	>LBL_1351	>LBL_2925
>LBL_0072	>LBL_1355	>LBL_2998
>LBL_0145	>LBL_1532	>LBL_3015
>LBL_0150	>LBL_1552	
>LBL_0164	>LBL_1570	
>LBL_0198	>LBL_1590	
>LBL_0216	>LBL_1592	
>LBL_0238	>LBL_1695	
>LBL_0240	>LBL_1719	
>LBL_0247	>LBL_1751	
>LBL_0252	>LBL_1771	
>LBL_0303	>LBL_1773	
>LBL_0307	>LBL_1779	
>LBL_0319	>LBL_1846	
>LBL_0353	>LBL_1866	
>LBL_0375	>LBL_1874	
>LBL_0396	>LBL_1884	
>LBL_0511	>LBL_1892	
>LBL_0514	>LBL_1893	
>LBL_0543	>LBL_1898	
>LBL_0553	>LBL_1930	
>LBL_0585	>LBL_1934	
>LBL_0636	>LBL_1949	
>LBL_0707	>LBL_1981	
>LBL_0708	>LBL_1994	
>LBL_0719	>LBL_2026	
>LBL_0720	>LBL_2063	
>LBL_0726	>LBL_2133	
>LBL_0727	>LBL_2149	
>LBL_0732	>LBL_2155	
>LBL_0733	>LBL_2239	
>LBL_0765	>LBL_2273	
>LBL_0803	>LBL_2366	
>LBL_0823	>LBL_2376	
>LBL_0858	>LBL_2510	
>LBL_0885	>LBL_2559	
>LBL_0972	>LBL_2618	
>LBL_0976	>LBL_2650	
>LBL_0977	>LBL_2683	
>LBL_0997	>LBL_2697	
>LBL_1010	>LBL_2706	
>LBL_1040	>LBL_2718	
>LBL_1054	>LBL_2732	
>LBL_1104	>LBL_2757	
>LBL_1138	>LBL_2785	
>LBL_1284	>LBL_2800	
>LBL_1327	>LBL_2804	
>LBL_1330	>LBL_2831	
>LBL_1341	>LBL_2857	

Prediction of protein localisation sites		Domain ID	Prediction of transmembrane β-barrel proteins				
PSORTb	CELLO	PFAM[a]	TMHMM[b]		MCMBB	BOMP	PRED TMBB
LBL_0069	LBL_0145	LBL_0072	LBL_0012	LBL_1592	LBL_1054	LBL_1930	LBL_0072
LBL_0121	LBL_0198	LBL_0511	LBL_0024	LBL_1695	LBL_0765	LBL_0238	LBL_0145
LBL_0154	LBL_0216	LBL_0396	LBL_0072	LBL_1719	LBL_1341	LBL_1054	LBL_0511
LBL_0198	LBL_0238	LBL_0319	LBL_0145	LBL_1751	LBL_2618	LBL_0072	LBL_0727
LBL_0225	LBL_0240	LBL_0303	LBL_0150	LBL_1771	LBL_1930	LBL_0765	LBL_0765
LBL_0279	LBL_0252	LBL_0252	LBL_0164	LBL_1773		LBL_1341	LBL_0823
LBL_0366	LBL_0303	LBL_0216	LBL_0198	LBL_1779		LBL_0976	LBL_0885
LBL_0398	LBL_0307	LBL_0636	LBL_0216	LBL_1866		LBL_0353	LBL _0976
LBL_0515	LBL_0319	LBL_0707	LBL_0240	LBL_1874			LBL_1010
LBL_0690	LBL_0511	LBL_0708	LBL_0247	LBL_1884			LBL_1054
LBL_0692	LBL_0720	LBL_0240	LBL_0303	LBL_1893			LBL_1284
LBL_0693	LBL_0726	LBL_0198	LBL_0307	LBL_1898			LBL_1327
LBL_0764	LBL_0765	LBL_0727	LBL_0319	LBL_1930			LBL_1341
LBL_0765	LBL_0885	LBL_0733	LBL_0353	LBL_1934			LBL_1570
LBL_0809	LBL_0976	LBL_0765	LBL_0375	LBL_1949			LBL_1930
LBL_0862	LBL_0977	LBL_0858	LBL_0396	LBL_1981			LBL_1934
LBL_0935	LBL_0997	LBL_0976	LBL_0511	LBL_1994			LBL_1949
LBL_0936	LBL_1054	LBL_0997	LBL_0514	LBL_2026			LBL_2239
LBL_1002	LBL_1327	LBL_1010	LBL_0543	LBL_2063			LBL_2366
LBL_1054	LBL_1341	LBL_1104	LBL_0553	LBL_2133			LBL_2618
LBL_1337	LBL_1570	LBL_1138	LBL_0585	LBL_2155			LBL_2683
LBL_1341	LBL_1695	LBL_1327	LBL_0636	LBL_2239			LBL_2718
LBL_1432	LBL_1719	LBL_1330	LBL_0707	LBL_2273			LBL_2785
LBL_1447	LBL_1771	LBL_1351	LBL_0708	LBL_2366			LBL_2857
LBL_1453	LBL_1874	LBL_1590	LBL_0719	LBL_2376			LBL_2998
LBL_1530	LBL_1930	LBL_1695	LBL_0720	LBL_2510			LBL_0150
LBL_1575	LBL_2155	LBL_1773	LBL_0726	LBL_2559			LBL_0164
LBL_1645	LBL_2366	LBL_1846	LBL_0727	LBL_2618			LBL_0198
LBL_1647	LBL_2683	LBL_1866	LBL_0732	LBL_2650			LBL_0238
LBL_1718	LBL_2697	LBL_1874	LBL_0733	LBL_2683			LBL_0247
LBL_1773	LBL_2718	LBL_0972	LBL_0765	LBL_2697			LBL_0252
LBL_1821	LBL_2785	LBL_1930	LBL_0803	LBL_2718			LBL_0307
LBL_1847	LBL_2998	LBL_1949	LBL_0858	LBL_2732			LBL_0319
LBL_1882		LBL_2063	LBL_0885	LBL_2757			LBL_0514
LBL_1907		LBL_2155	LBL_0972	LBL_2785			LBL_0636
LBL_1948		LBL_2239	LBL_0976	LBL_2800			LBL_0726
LBL_2023		LBL_2366	LBL_0977	LBL_2804			LBL_1344
LBL_2080		LBL_1898	LBL_0997	LBL_2831			LBL_1335
LBL_2139		LBL_2706	LBL_1040	LBL_2857			LBL_1552
LBL_2242		LBL_2697	LBL_1054	LBL_2925			LBL_1592
LBL_2418		LBL_2618	LBL_1104	LBL_2998			LBL_1771
LBL_2425		LBL_2376	LBL_1138	LBL_3015			LBL_1884
LBL_2518		LBL_2510	LBL_1284				LBL_2155
LBL_2697		LBL_2925	LBL_1327				LBL_2376
LBL_2791		LBL_2998	LBL_1330				LBL_2804
LBL_2806		LBL_3015	LBL_1341				
LBL_2818		LBL_2831	LBL_1344				
LBL_2998		LBL_2857	LBL_1351				
		LBL_2785	LBL_1355				
		LBL_0150	LBL_1532				
		LBL_0145	LBL_1552				
			LBL_1590				

Abbreviations:
PSORTb: List of genes with beta-barrel prediction
CELLO: List of genes WITH most-likely as outer membrane
PFAM: List of genes with domains
TMHMM: List of genes which are NOT α-helices
MCMBB: List of genes with predicted beta-barrel with hidden markov chain model
BOMP: List of genes with predicted beta-barrel integral outer membrane proteins
PRED-TMBB: List of genes with predicted transmembrane beta-strands

Appendix B

Supplementary material for Chapter 4

On the next few pages are the cloning vectors used in Chapter 4, and the summary for both gene cloning, recombinant protein expression, refolding and purification.

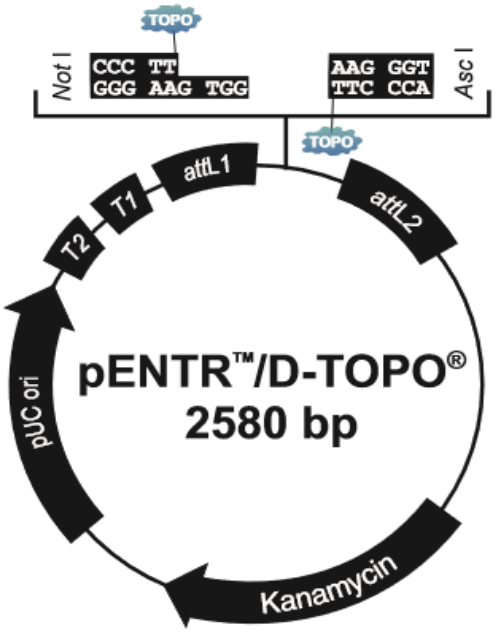


Figure B.1: The map of pENTR™/D-TOPO® vector and its features.

The following features are described as follow. rrnB T1 (bases 268-295) and T2 (bases 427-470) transcription termination sequences; preventing basal expression of PCR products that is toxic to E. coli host. M13 forward priming site (bases 537-552); to allow sequence of the insert. attL1 (bases 569-668 containing TOPO® recognition site 1: bases 680-684, overhang bases, GTCC 685-688) and attL2 (bases 705-804 containing TOPO® recognition site 2: bases 689-693); Recombination sequences derived from bacteriophage γ-derived to allow recombination cloning of DNA insert in the entry construct with Gateway® destination vector (Landy, 1989). *T7 promoter (bases 821-840); allows in vitro transcription and sequencing of the insert. M13 reverse primer priming site (bases 845-861); to allow sequence of the insert. Kanamycin resistance gene (bases 974-1783; allows selection of the plasmid E. coli. pUC origin of replication (ori) (bases 1904-2577); allows high copy replication and maintenance in E. coli competent host. Figure and description were adapted from clone manager software and pENTR™ Directional TOPO® Cloning Kits user guide (2012). Figure and descriptions were adapted from E. coli Expression System with Gateway® Technology User Guide (Life Technologies, Carlsberg, USA 2012).*

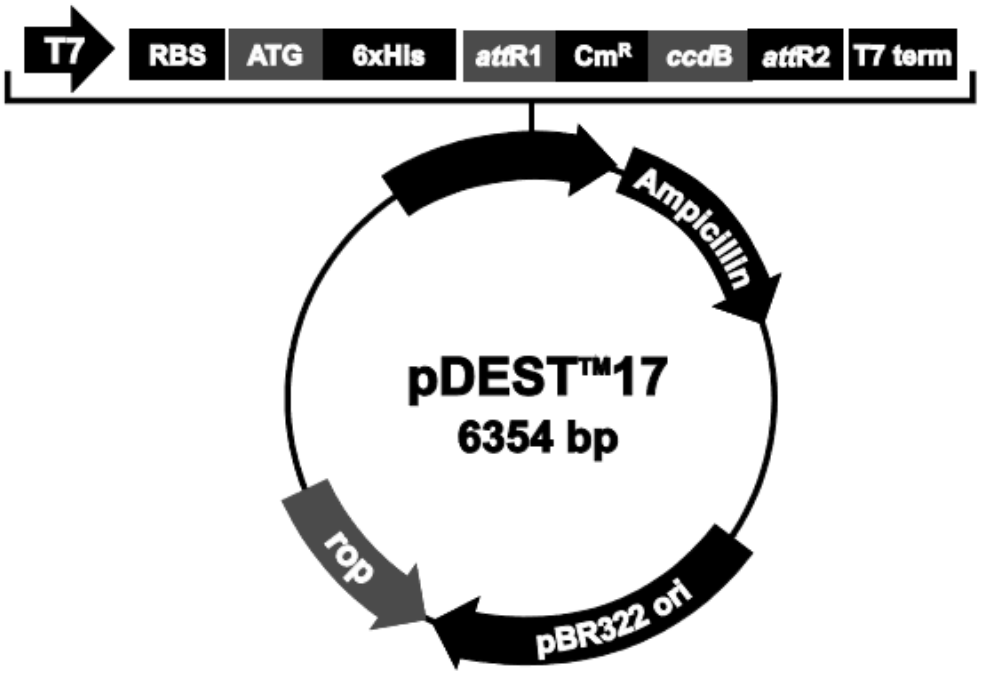


Figure B.2: The map of pDEST17™ and its features.

The following features are described as follow. T7 promoter (bases 21-40) allows high level expression of recombinant in bacterial (E. coli) expression strains expressing T7 RNA polymerase. Ribosome binding site (RBS) (bases 86-92) an optimum space from the initiation ATG in the N-terminal tag for efficient translation of DNA. N-terminal 6XHis-tag allows affinity purification fusion protein using a metal-chelating resin. AttR1 (bases 71-195) and attR2 (bases 1651-1775) sites; Bacteriophage λ-derived DNA recombination sequences that allows recombination cloning of the gene of interest from Gateway® entry cloning. Chloramphenicol resistance gene (Cm^R) (bases 304-963); permits counterselection of the plasmid. T7 transcription termination region (bases 2466-2594); Sequence from bacteriophage T7 for transcription termination. Ampicillin (bla) resistance gene (base 3181-4041); permits expression of the ampicillin resistance gene. The pBR322 ori (bases 4186-4859); allows replication and maintenance in E. coli. Repression of primer, open reading frame (Rop, ORF) (bases: 5230-5421); Interacts with the pBR322 origin to facilitate low-copy replication in E. coli. Figure and descriptions were adapted from E. coli Expression System with Gateway® Technology User Guide (Life Technologies, Carlsberg, USA 2012).

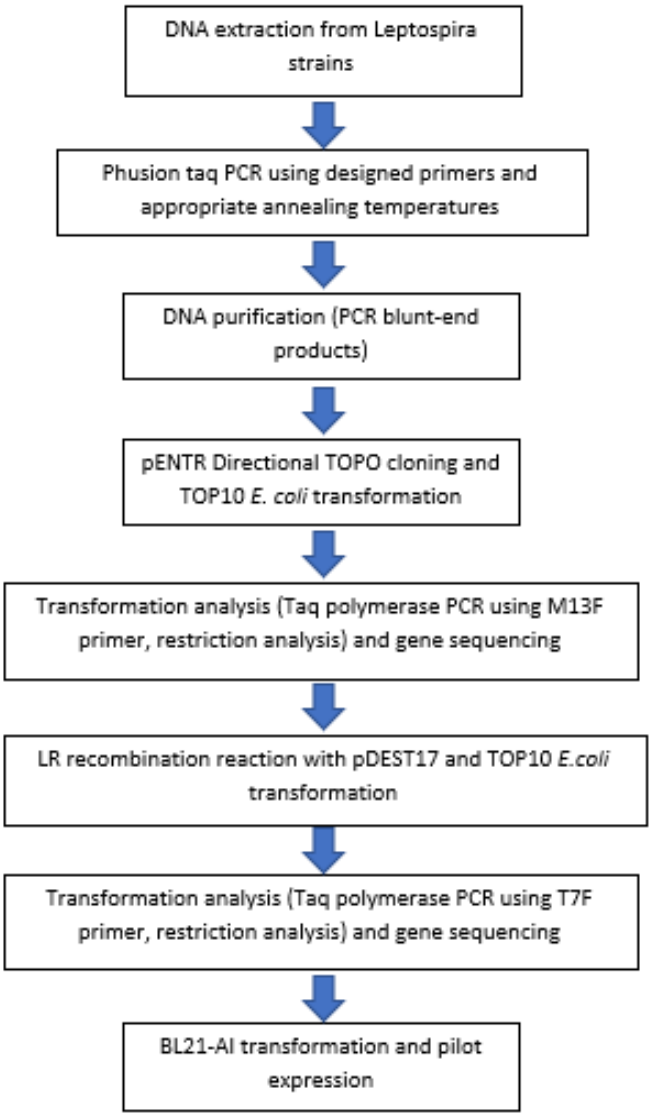


Figure B.3: The summary of gene cloning and transformation.

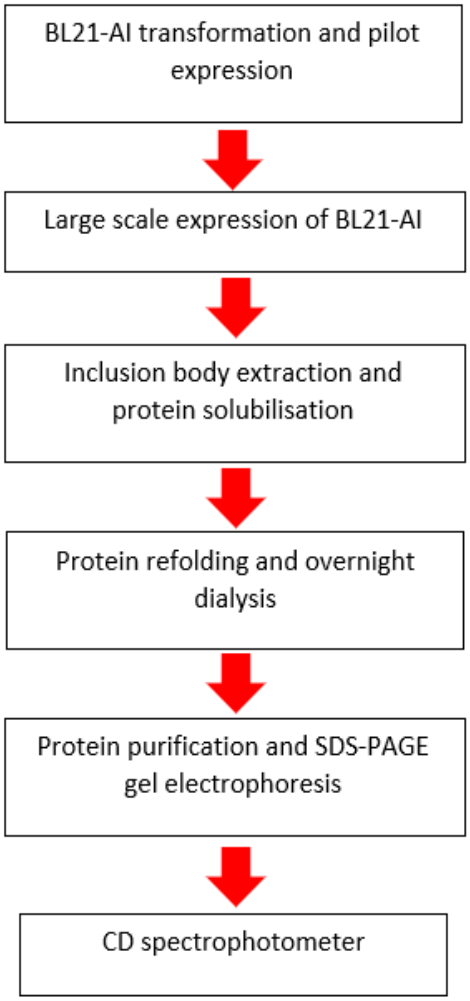
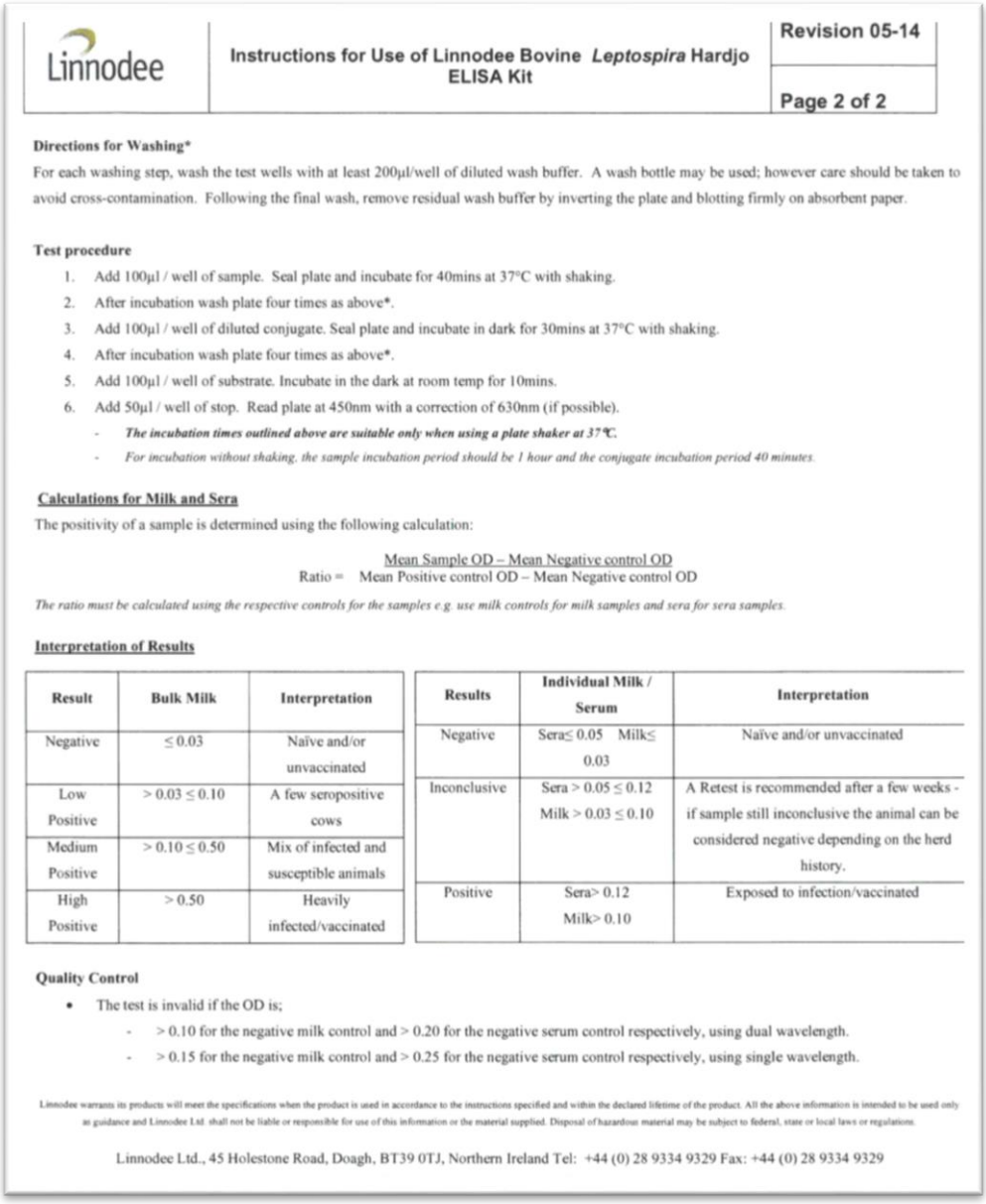


Figure B.4: The summary of protein overexpression, refolding and purifications.

Appendix C

Supplementary material for Chapter 5 and 6

On the next few pages are the commercial *Leptospira* test kits used as reference, their results as described in Chapter 5 and 6, including CD spectra analysis and the predicted sites of conserved amino acids of Lsa4 corresponding to host molecules binding.



Linnodee

Instructions for Use of Linnodee Bovine *Leptospira* Hardjo ELISA Kit

Revision 05-14

Page 2 of 2

Directions for Washing*

For each washing step, wash the test wells with at least 200µl/well of diluted wash buffer. A wash bottle may be used; however care should be taken to avoid cross-contamination. Following the final wash, remove residual wash buffer by inverting the plate and blotting firmly on absorbent paper.

Test procedure

1. Add 100µl / well of sample. Seal plate and incubate for 40mins at 37°C with shaking.
2. After incubation wash plate four times as above*.
3. Add 100µl / well of diluted conjugate. Seal plate and incubate in dark for 30mins at 37°C with shaking.
4. After incubation wash plate four times as above*.
5. Add 100µl / well of substrate. Incubate in the dark at room temp for 10mins.
6. Add 50µl / well of stop. Read plate at 450nm with a correction of 630nm (if possible).
   - ***The incubation times outlined above are suitable only when using a plate shaker at 37°C.***
   - *For incubation without shaking, the sample incubation period should be 1 hour and the conjugate incubation period 40 minutes.*

Calculations for Milk and Sera

The positivity of a sample is determined using the following calculation:

$$\text{Ratio} = \frac{\text{Mean Sample OD} - \text{Mean Negative control OD}}{\text{Mean Positive control OD} - \text{Mean Negative control OD}}$$

The ratio must be calculated using the respective controls for the samples e.g. use milk controls for milk samples and sera for sera samples.

Interpretation of Results

Result	Bulk Milk	Interpretation
Negative	≤ 0.03	Naïve and/or unvaccinated
Low Positive	> 0.03 ≤ 0.10	A few seropositive cows
Medium Positive	> 0.10 ≤ 0.50	Mix of infected and susceptible animals
High Positive	> 0.50	Heavily infected/vaccinated

Results	Individual Milk / Serum	Interpretation
Negative	Sera≤ 0.05 Milk≤ 0.03	Naïve and/or unvaccinated
Inconclusive	Sera > 0.05 ≤ 0.12 Milk > 0.03 ≤ 0.10	A Retest is recommended after a few weeks - if sample still inconclusive the animal can be considered negative depending on the herd history.
Positive	Sera> 0.12 Milk> 0.10	Exposed to infection/vaccinated

Quality Control

- The test is invalid if the OD is;
  - > 0.10 for the negative milk control and > 0.20 for the negative serum control respectively, using dual wavelength.
  - > 0.15 for the negative milk control and > 0.25 for the negative serum control respectively, using single wavelength.

Linnodee warrants its products will meet the specifications when the product is used in accordance to the instructions specified and within the declared lifetime of the product. All the above information is intended to be used only as guidance and Linnodee Ltd shall not be liable or responsible for use of this information or the material supplied. Disposal of hazardous material may be subject to federal, state or local laws or regulations.

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Figure C.1: Manual instructions for use of Linnodee Bovine L. Hardjo ELISA test kit®.

The commercial test kit used to determine the Leptospira bulk cattle milk status in Chapter 5, performed by Cattle Information System (CIS, UK).

PrioCHECK® L.hardjo Ab

ELISA for *in vitro* detection of antibodies directed against Leptospira interrogans serovar hardjo in serum and milk of cattle

5 plate kit for 440 samples
©Prionics AG
Version 1.0_e

Package Insert

For *in-vitro* veterinary diagnostic use only
Store at 5±3°C
Product No.: 7442080

Introduction

Leptospirosis is a contagious disease of animals and humans caused by infection with the spirochete Leptospira. The genus Leptospira is at present divided into two species L.interrogans (parasitic) and L.biflexa (saprophytic)*. Many serovars appear to have a certain animal species as a natural host, but animals and humans can be infected with a wide variety of serovars. Leptospirosis occurs worldwide. In many European countries, Leptospira interrogans serovar hardjo (L.hardjo) is the most common cause of bovine Leptospirosis. The indirect ELISA described by Bercovich et al has been modified into a sensitive, specific and robust ELISA that is suitable for large scale screening. The PrioCHECK® L.hardjo Ab can be used for:
L.hardjo eradication programs; Monitoring of the L.hardjo free status of cattle herds; Monitoring of L.hardjo antibody level in cattle after vaccination; Individual and herd diagnosis.

Test Principle

The PrioCHECK® L.hardjo Ab is an indirect ELISA and detects antibodies (Ab) against Leptospira interrogans serovar hardjo (L.hardjo) in cattle. A microtiterplate is coated with inactivated antigen. Serum- and/or milk samples are dispensed in the coated wells of a Test Plate. Antibodies directed against L.hardjo that are present in the test sample will bind to the antigen during incubation. The bound antibodies are detected using an anti-bovine monoclonal antibody conjugated to the enzyme horseradish-peroxidase. Subsequently, the bound Conjugate is visualized by incubation with the Chromogen (TMB) Substrate. Finally, color development is stopped and measured at a wavelength of 450 nm.

Kit Components

Store kit at 5±3°C until expiry date. See kit label for actual expiry date. The shelf life of diluted, opened or reconstituted components is noted below, when appropriate. Chemical hazard data are available in section "Safety Regulations and R&S Statements" (Appendix II).

Component 1
Test Plate
Five Test Plates are delivered in vacuum bags which contain a desiccant sachet.

Component 2
Conjugate (30x)
(30x concentrated, dilute before use)
One vial contains 2.5 ml Conjugate.
Diluted conjugate is not stable, prepare just before use.

Component 3
Dilution Buffer (5x)
(5x concentrated, dilute before use)
One vial contains 60 ml Dilution Buffer.
Shelf life of dilution buffer working solution: 4 hours at 22±3°C.

Component 4
Horse Serum (lyophilized)
One vial contains 3.5 ml lyophilized Horse Serum.
Shelf life of reconstituted horse serum: until expiry date at -20°C.

Component 5
Demineralized Water
One vial contains 10 ml Demineralized Water.

Component 6
Washing Fluid (200x)
(200x concentrated, dilute before use) One vial contains 60 ml Washing Fluid.
Shelf life of washing solution: 1 week at 22±3°C.

Component 7
Reference Serum 1 (lyophilized)
One vial contains 0.5 ml Reference Serum 1 (positive control).
Shelf life of reconstituted serum: until expiry date at -20°C.

Component 8
Reference Serum 2 (lyophilized)
One vial contains 0.5 ml Reference Serum 2 (negative control).
Shelf life of reconstituted serum: until expiry date at -20°C.

Component 9
Reference Serum 3 (lyophilized)
One vial contains 0.5 ml Reference Serum 3 (weak positive control).
Shelf life of reconstituted serum: until expiry date at -20°C.

Component 10
Chromogen (TMB) Substrate (Ready-to-use)
One vial contains 60 ml Chromogen (TMB) Substrate.

Component 11
Stop Solution (Ready-to-use)
One vial contains 60 ml Stop Solution.

Additional Kit Contents:
- Package Insert
- 10 plate sealers
- Certificate of Analysis

Additional Material Required

Dummy plates
Dummy plates to make pre-dilutions of the milk and serum samples. We advice U-bottom shaped plates (Greiner, art. nr. 6501101). However, also other non binding plates or tubes can be used.

General:
Laboratory equipment according to national safety regulations.

Analysis of Results:
Plate Reader e.g. Multiscan EX or equivalent.
The reader has to have an appropriate filter set to read the plates at 450 nm.

Optional:
Plate washer e.g. Tecan EIA Tray Washer or equivalent.

Test Procedure

Precautions
National guidelines for working with animal samples must be strictly followed. The PrioCHECK® L.hardjo Ab must be performed in laboratories suited for this purpose.
Samples should be considered as potentially infectious and all items which contact the samples as potentially contaminated.
Chemical hazard data are available in section "Safety Regulations and R&S Statements" (Appendix II).

Notes
To achieve optimal results with the PrioCHECK® L.hardjo Ab, the following aspects must be considered:

- **The Test Procedure protocol must be strictly followed.**
- All reagents of the kit must be equilibrated to room temperature (22±3°C) before use.
- Pipette tips have to be changed for every pipetting step.
- Separate solution reservoirs must be used for each reagent.
- Kit components must not be used after their expiry date or if changes in their appearance are observed.
- Kit components of different kit lot numbers must not be used together.
- Demineralized or water of equal quality must be used for the test.

SOLUTIONS TO BE MADE IN ADVANCE

Dilution buffer working solution
The Dilution Buffer (5x) (Component 3) must be diluted 1/5 in demineralized water (1 part Dilution Buffer + 4 parts demineralized water).

Horse serum
Equilibrate the vial to 22±3°C and reconstitute[1] the Horse Serum (Component 4) with 3.5 ml Demineralized Water (Component 5). Can be stored at -20°C until expiry date.

ELISA buffer
Dilute reconstituted horse serum 1/100 in dilution buffer working solution; e.g. for 1 plate prepare 40 ml (add 400 µl horse serum to 39.6 ml dilution buffer working solution). The ELISA buffer can be stored up to 4 hours at 22±3°C.

Conjugate dilution
Dilute the Conjugate (30x) (Component 2) 1/30 in dilution buffer working solution; e.g. for 1 plate prepare 12 ml (add 400 µl Conjugate to 11.6 ml dilution buffer working solution).
Note: The working dilution must be prepared just before use.

Reference sera
Reconstitute the sera (Component 7 – 9) with 0.5 ml Demineralized Water (Component 5). Reconstituted reference sera are preferably aliquoted and can be stored at –20°C until expiry date. Mix sera gently after thawing and do not refreeze.

Washing solution
The Washing Fluid (200x) (Component 6) must be diluted 1/200 in demineralized water and is sufficient for a final volume of 12 liters washing solution.
Stability of washing solution: 1 week stored at 22±3°C.

Note: see Appendix IV for sample preparation procedure and storage.

PRE-TREATMENT OF THE TEST PLATES

Note: Pre-treatment is only necessary when milk samples are tested and not for serum samples. However, sera may be tested on a pre-treated plate (test results are not affected).

1.1 Dispense 100 µl ELISA buffer to all wells of the Test Plate (Component 1).
1.2 Seal or cover the Test Plate and incubate for 60±2 minutes at 37±1°C.

[1] Reconstitution of lyophilized reagents should be performed as follows:
- Equilibrate the vials to 22±3°C.
- With the vial in an upright position, tap the vial gently against the worktop to ensure that the content is on the bottom of the vial.
- Open the vial.
- Add the required amount of Demineralized Water (see label on vial).
- Replace the stopper on the vial and gently rotate the vial so that any remaining dry material will be dissolved.
- Allow the lyophilized material to stand for 15 minutes at 22±3°C.
- Occasionally gently invert the vial (formation of foam should be avoided).

PrioCHECK® L.hardjo Ab

1.3 Discard the ELISA buffer and wash the Test Plate 6 times with washing solution.

PRE-DILUTION OF REFERENCE SERA AND TEST SERA

2.1 Make a 1:20 dilution of Reference Serum 1, 2, 3 and of the test sera in a dummy plate by mixing 10 µl serum with 190 µl ELISA buffer.

INCUBATION OF TEST SAMPLES

3.1 In case of testing serum samples, unpack the required number of Test Plates. In case of testing milk samples use the plates as described under 1.1 to 1.3.
3.2 Dispense 100 µl of ELISA buffer to wells A1 and B1 of the Test Plate (=blanks).
3.3 Dispense 90 µl of ELISA buffer to wells C1 to H1.
3.4 Dispense 10 µl of 1:20 diluted reference serum 1 (=positive control) to wells C1 and D1. Final serum dilution 1:200.
3.5 Dispense 10 µl of 1:20 diluted reference serum 2 (=negative control) to wells E1 and F1. Final serum dilution 1:200.
3.6 Dispense 10 µl of 1:20 diluted reference serum 3 to (=weak positive control) wells G1 and H1. Final serum dilution 1:200.
3.7 When testing **serum samples**, dispense 90 µl ELISA buffer into the remaining wells. Dispense 10 µl of 1:20 diluted test sera in each of these wells. Final serum dilution 1:200.
Serum samples can be titrated by making two-fold serial dilutions in dilution buffer.
3.8 When testing **individual milk samples**, dispense 75 µl ELISA buffer into the remaining wells. Dispense 25 µl of milk sample in each of these wells of the plate. Final milk dilution 1:4. Take defatted milk sample from below the creamy layer.
3.9 When testing **bulk milk** samples, dispense 100 µl of milk sample into the remaining wells. Take defatted milk sample from below the creamy layer.
3.10 Seal and shake the test plate gently and incubate for 60±5 minutes at 37±1°C.

INCUBATION WITH CONJUGATE AND CHROMOGEN (TMB) SUBSTRATE

4.1 Wash the Test Plate 6 times with washing solution.
4.2 Dispense 100 µl of diluted conjugate to all wells.
4.3 Cover the Test Plate and incubate for 60±5 minutes at 37±1°C.
4.4 Wash the Test Plate 6 times with washing solution.
4.5 Dispense 100 µl of the Chromogen (TMB) Substrate (Component 10) to all wells.
4.6 Incubate the Test Plate for 15 minutes at 22±3°C.
4.7 Add 100 µl Stop Solution (Component 11).
4.8 Agitate the Test Plate to mix the content of the wells prior to measuring.

Note: Start the addition of Stop Solution 15 minutes after the first well was filled with Chromogen (TMB) Substrate. Add the Stop Solution in the same order and at the same pace as the Chromogen (TMB) Substrate was dispensed.

READING OF THE TEST AND CALCULATING THE RESULTS

5.1 Measure the optical density (OD) of the wells at 450 nm within 15 minutes of stopping the color development.
5.2 Calculate the mean OD_{450} value of the blanks (wells A1 and B1).
5.3 Calculate the corrected OD_{450} value of all samples by subtracting the mean OD_{450} of the blanks.
5.4 Calculate the percentage positivity (PP) of the reference samples 2, 3 and the test samples according to the formula given below.

The corrected OD_{450} values of all samples are expressed as percentage positivity (PP) relative to the corrected mean OD_{450} value of Reference Serum 1 in wells C1 and D1.

$$PP = \left[\frac{\text{corrected } OD_{450} \text{ test sample}}{\text{corrected } OD_{450} \text{ Reference Serum 1}} \right] \times 100$$

RESULT INTERPRETATION

Validation criteria
6.1 The mean OD_{450} of the blanks (wells A1 and B1) must be <0.150.
6.2 The corrected OD_{450} of Reference Serum 1 (wells C1 and D1) must be ≥1.000.
6.3 The mean PP of Reference Serum 2 must be <20.
6.4 The mean PP of Reference Serum 3 must be between 20 and 60.
6.5 The above mentioned criteria have to be met in order to validate the results of test samples.

Note: If the corrected mean OD_{450} of Reference Serum 1 is below 1.000 possibly the Chromogen (TMB) Substrate solution is too cold. In that case pre-warm the solution to 22±3°C or incubate up to 30 minutes.

Interpretation of the percentage positivity

Serum samples
PP = <20%: Negative for L.hardjo specific antibodies.
PP = 20% - 45%: Inconclusive (antibodies may be present)
PP = >45%: Positive for L.hardjo specific antibodies.

Milk (individual and bulk) samples
PP= < 40%: Negative for L.hardjo specific antibodies
PP = 40% – 60%: Inconclusive* (antibodies may be present)
PP = >60%: Positive for L.hardjo antibodies

* Bulk milk samples with a doubtful test result can be retested in the PrioCHECK® L.hardjo Ab.
When an inconclusive result is confirmed it may be followed by collecting blood samples of the (possibly) infected herd.

Note: Cut off values may need to be refined for the local situation in order to obtain acceptable percentages of false-positive and false-negative results. Testing of individual serum samples (1:200 diluted) has preference over testing of individual milk samples (1:4 diluted), because of non-specific reactions that incidentally may occur when testing individual milk samples.

Appendix I

Notice
This manual is believed to be complete and accurate at the time of publication. In no event shall Prionics AG be liable for incidental or consequential damage in connection with or arising from the use of this manual.

Liability
Prionics AG warrants its products will meet their applicable published specification when used in accordance with their applicable instructions and within the declared products life time. Prionics AG makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose. The warranty provided herein and the data, specifications and descriptions of Prionics AG products appearing in Prionics AG published catalogues and product literature may not be altered except by express written agreement signed by an officer of Prionics AG. Representation, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.
In the event of a breach of the foregoing warranty, Prionics AG's sole obligation shall be to repair or replace, at its option, the applicable product or part thereof, provided the customer notifies Prionics AG promptly of any such breach. If after exercising reasonable efforts, Prionics AG is unable to repair or replace the product or part, then Prionics AG shall refund to the customer all monies paid for such applicable product or part.

Prionics AG shall not be liable for consequential, incidental, special or any other indirect damages resulting from economic loss or property damage sustained by any customer from the use of its products.

Prionics AG and Prionics Lelystad B.V. are ISO 9001:2000 certified companies.

Appendix II

Safety Regulations and R&S Statements

National Safety Regulations must be strictly followed.

Component 1
Test Plate
Hazard Code: This product is not classified according to EU regulations.

Component 2
Conjugate (30x)
Hazard Code: This product is not classified according to EU regulations.

Component 3
Dilution Buffer (5x)
Hazard Code: This product is not classified according to EU regulations.

Component 4
Horse Serum (lyophilized)
Hazard Code: This product is not classified according to EU regulations.

Component 5
Demineralized Water
Hazard Code: This product is not classified according to EU regulations.

Component 6
Washing Fluid (200x)
Hazard Code: This product is not classified according to EU regulations.

Component 7
Reference Serum 1 (lyophilized)
Hazard Code: This product is not classified according to EU regulations.

Component 8
Reference Serum 2 (lyophilized)
Hazard Code: This product is not classified according to EU regulations.

Component 9
Reference Serum 3 (lyophilized)
Hazard Code: This product is not classified according to EU regulations.

Component 10
Chromogen (TMB) Substrate (Ready-to-use)
Hazard Code: This product is not classified according to EU regulations.

Component 11
Stop Solution (Ready-to-use)
Hazard Code: R35: Causes severe burns.
S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S36/37/39: Wear suitable protective clothing, gloves and eye/face protection.
S45: In case of accident or if you feel unwell, seek medical advice immediately (show label on vial).

Appendix III

References
(1) International Leptospirosis Society, First meeting, Nantes, France 9-12 September 1996.
(2) OIE Manual of Standards for Diagnostic Tests and Vaccines, Third Edition, 1996
(3) Bercovich, Z, Taaljke, R and Bokhout, BA. Vet. Microbiol. 21, 255-262, 1990

Appendix IV

Sample preparation procedure and storage

Serum
- Test sera can be stored at –20°C prior to testing.
- Test sera have to be prediluted in ELISA buffer.

Milk
- Milk samples can be stored at 5±3°C prior to testing. If milk samples are not tested within 3 days of collection add 0.02% sodium azide as a preservative.
- The fluid of the milk sample to be tested should be collected from underneath the creamy layer.
- Milk samples (bulk) undiluted.
- Individual milk samples 1:4 diluted in ELISA buffer.

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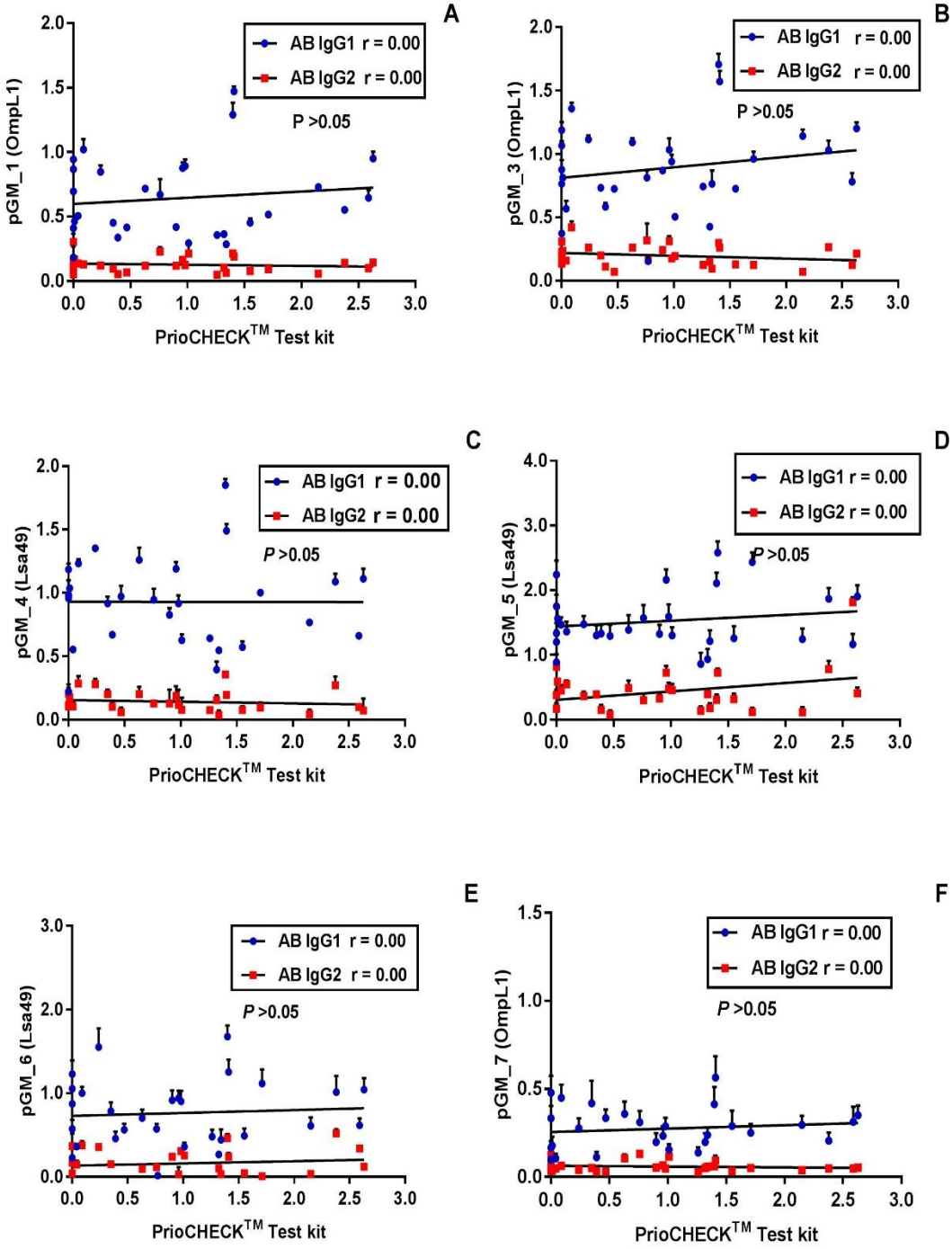
Figure C.2: Manual instructions for use of PrioCHECK® L. Hardjo ELISA test kit.

The commercial test kit used to determine the Leptospira cattle sera status in Chapter 6.


A
AB IgG1 r = 0.00
AB IgG2 r = 0.00
P >0.05
pGM_1 (OmpL1)
PrioCHECK™ Test kit
B
AB IgG1 r = 0.00
AB IgG2 r = 0.00
P >0.05
pGM_3 (OmpL1)
PrioCHECK™ Test kit
C
AB IgG1 r = 0.00
AB IgG2 r = 0.00
P >0.05
pGM_4 (Lsa49)
PrioCHECK™ Test kit
D
AB IgG1 r = 0.00
AB IgG2 r = 0.00
P >0.05
pGM_5 (Lsa49)
PrioCHECK™ Test kit
E
AB IgG1 r = 0.00
AB IgG2 r = 0.00
P >0.05
pGM_6 (Lsa49)
PrioCHECK™ Test kit
F
AB IgG1 r = 0.00
AB IgG2 r = 0.00
P >0.05
pGM_7 (OmpL1)
PrioCHECK™ Test kit

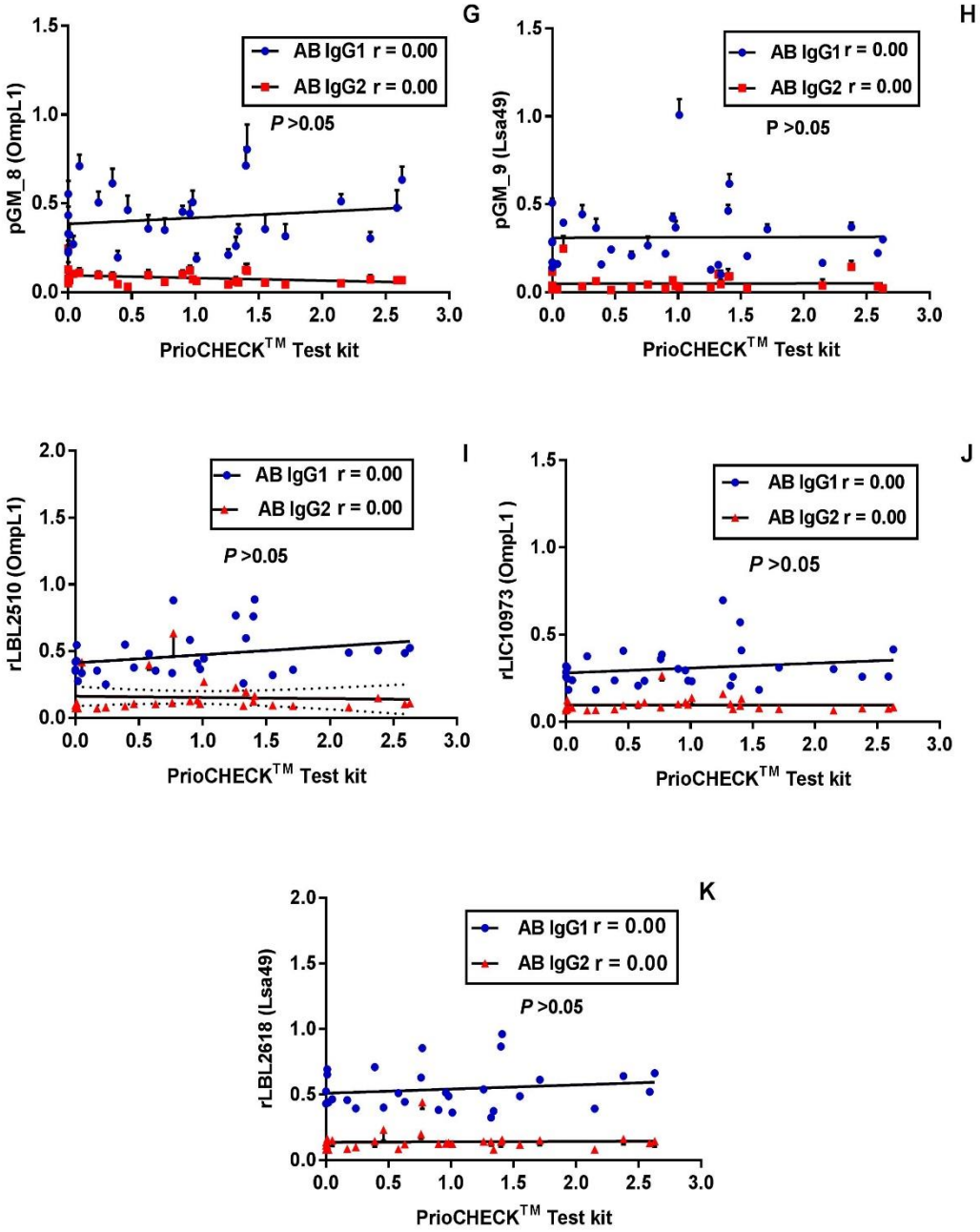


Figure C.3 (A-K): The association between PrioCHECK™ test kit results and antibodies (IgG1 and IgG2) binding to leptospiral OMP variants tested in cattle sera (Chapter 6).

The trend line represents the correlation coefficient, denoted as (r values), which estimates the relationship between the OD (450 nm) of both results. P-values determine the significant level of association between two assays of which P-value of at least <0.05 is considered significant. Note that no association found in all OMPs versus kit in this study.

Table C.1: Analysis of the CD spectra using two online servers; BestSel and CAPITO show components of OmpL1 proteins by their secondary structures.

Species	Helix	Anti-parallel	Parallel	Turn	Others	Helix 1 (Regular)	Helix 2 (distorted)	Anti 1 (Left-twisted)	Anti 2 (relaxed)	Anti 3 (Right twisted)	Conc (mg/ml)	Molar conc (µM)	Predominant spectra by BestSel	Predominant spectra by CAPITO
L. interrogans svr Pyrogenes	3.50	45.4	0.00	12.5	38.7	3.50	0.00	6.30	20.2	18.8	1.20	38.7	β-sheet	β-sheet
L. noguchii	8.40	35.8	0.00	12.8	42.9	3.60	4.9	6.3	18.2	11.3	1.70	55.0	β-sheet	β-sheet
L. santarosai	5.10	28.3	7.40	15.0	44.2	5.10	0.00	4.00	9.80	14.5	0.70	23.0	β-sheet	β-sheet
L. alstonii	3.70	22.2	8.30	14.2	51.6	2.70	0.90	2.00	9.00	11.3	0.40	12.90	β-sheet	β-sheet
L. borgpetersenii svr Hardjobovis L550	0.0	44.6	0.0	13.6	41.8	0.0	0.0	3.8	21.2	16.6	2.3	72.73	β-sheet	β-sheet
L. interrognas svr Copenhageni	0.0	44.8	0.0	13.8	41.4	0.0	0.0	4.1	21.3	19.4	1.3	41.94	β-sheet	β-sheet

The overall score of α-helix and β-sheet are defined as 'Helix' and 'Anti-parallel', respectively. The α-helix scores are based on a regular part of the helix (regular); The middle part of α-helices and the Helix 2 (distorted ends); 2-2 residues at the ends of α-helices. The β-sheet scores are based on antiparallel β-sheets which are divided into three subclasses: Anti 1; Left-handed twisted, Anti 2; relaxed (slightly right-handed twisted) and Anti 3; right-hand twisted. The definition of 'Turn' is the turn and bend segment longer than one residue. 'Others' are described any additional features present within a protein such as 33,,-helix, π-helix, β-bridge, bend, loop/irregular and invisible region of the structure. The secondary structure determination of most recombinant OMPs is in agreement in this study (Chapter 4).

Table C.2: Analysis of the CD spectra using two online servers; BestSel and CAPITO show components of Lsa49 proteins by their secondary structures.

Species	**Helix**	**Anti-parallel**	**Parallel**	**Turn**	**Others**	**Helix 1 (Regular)**	**Helix 2 (distorted)**	**Anti 1 (Left-twisted)**	**Anti 2 (relaxed)**	**Anti 3 (Right twisted)**	**Conc (mg/ml)**	**Molar conc (µM)**	**Predominant spectra by BestSel**	**Predominant spectra by CAPITO**
L. kirschneri	8.60	25.1	9.60	12.1	44.7	6.50	2.20	1.10	6.40	16.60	0.50	10.2	β-sheet	β-sheet
L. noguchii	22.3	45.3	0.00	9.50	22.9	22.3	0.00	0.00	17.8	27.6	0.40	8.16	β-sheet	β-sheet
L. alstonii	4.20	11.3	13.5	13.9	57.1	4.10	0.20	0.00	0.00	11.3	0.40	8.16	β-sheet	β-sheet
L. borgpetersenii svr Hardjobovis L550	0.0	43.8	0.0	13.7	42.5	0.0	0.0	4.6	19.5	19.6	0.3	6.12	β-sheet	β-sheet

The overall score of α-helix and β-sheet are defined as 'Helix' and 'Anti-parallel', respectively. The α-helix scores are based on a regular part of the helix (regular); The middle part of α-helices and the Helix 2 (distorted ends); 2-2 residues at the ends of α-helices. The β-sheet scores are based on antiparallel β-sheets which are divided into three subclasses: Anti 1; Left-handed twisted, Anti 2; relaxed (slightly right-handed twisted) and Anti 3; right-hand twisted. The definition of 'Turn' is the turn and bend segment longer than one residue. 'Others' are described any additional features present within a protein such as 33,,-helix, π-helix, β-bridge, bend, loop/irregular and invisible region of the structure. The secondary structure determination of most recombinant OMPs is in agreement in this study (Chapter 4).

Table C.3: The predicted site of conserved amino acids corresponding to host molecules binding that is present in Lsa49 variable regions of both transmembrane segments and surface-loop regions of selected Leptospira species in this study (Figure 6.14).

Host molecules	***Leptospira* species bind to corresponding host molecules**	**The conserved amino acid in Lsa49 predicted strand regions**	**The conserved amino acid in Lsa49 predicted loop regions**
Fibronectin and laminin	*L. borgpetersenii* svr Hardjobovis L550 *L. alstonii*	24L, 62I, 68T, 69V, 70Q, 85S, 103V, 121V, 142N, 145F, 162V, 165A, 171V, 182I, 198V, 216T, 250H, 254S, 278S, 317R, 319F, 359S, 376G, 404S, 405T, 419T, 426S, 428I,	55T, 64G, 268G, 281T, 425A, 449V

Abbreviations: A: alanine, F: Phenylalanine G: guanine, H: histidine I: isoleucine, N: Asparagine, Q: Glutamine, R: arginine, S: serine, T: threonine, V: valine

List of knowledge exchange contributions relating to this thesis

Poster presentation

Kamaruzaman, I. N. A., Staton, G. J., Ainsworth, S. R., Carter, S. D. and Evans, N. J. (2017). Characterisation Studies of Outer Membrane Proteins from Pathogenic Bovine *Leptospira* spp. In *Institute of Infection and Global Health, University of Liverpool*, *UK.*

Kamaruzaman, I. N. A., Staton, G. J., Ainsworth, S. R., Carter, S. D. and Evans, N. J. (2018). Characterisation Studies of Outer Membrane Proteins from Pathogenic Bovine *Leptospira* spp. In *Veterinary Vaccinology Network, University of Stirling, UK.*

Oral presentation

Kamaruzaman, I. N. A., Staton, G. J., Ainsworth, S. R., Evans, N. J. and Carter, S. D. (2018). Characterisation Studies of Outer Membrane Proteins from Pathogenic Bovine *Leptospira* spp. In *3rd European Scientific Meeting on Leptospirosis and Other Rodent Haemorrhagic Fevers*, Sardinia, Italy.

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