Leptospirosis in UK vet visiting dogs, wild rodents and the pathogenomics of *Leptospira* species

By

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This thesis is submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

Memorandum

Apart from the help and advice acknowledged, this thesis represents the unaided work of the author:

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Abstract

Canine infection from pathogenic *Leptospira* serovars remains an issue within the UK, despite the availability of a canine vaccine. Canine leptospirosis cases are non-reportable and data regarding current levels for both suspected and confirmed cases is limited. A questionnaire based survey was undertaken to determine the number of canine leptospirosis cases within UK practices over a 12 month period. Average canine vaccination coverage across responding practices was determined as 60%, with 1669 vaccines administered per practice on average within the responding practices. No significant difference was witnessed between doses administered in either mixed or dedicated small animal practices (1692.40 and 1653.38 respectively), demonstrating that vaccination habits vary between individual clinicians rather than practice type. Diagnosing leptospirosis remains an issue, particularly relating to vague clinical signs during early infection. Survey results emphasised the priority that clinicians base on initially vague signs, with leptospirosis being typically considered once icteric signs present, where mortality rates are greater and treatment is less effective.

Despite well documented associations linking leptospirosis and rodents, a current uncertainty remains regarding serovars maintained within the UK. In an attempt to clarify the situation, 283 wild rodents were sampled from rural (n=7) and urban sites (n=8). Infection was identified within 23 (8.13%) samples belonging to wood mice (n=16/152), bank voles (n=5/47) and field voles (n=2/10). Initial *Leptospira* identification using direct sequencing of PCR amplicons showed a single infecting pathogenic species (*Leptospira interrogans*). Serology data was obtained for 71 rodents using the microscopic agglutination test (MAT). Positive samples from pooled antigen testing (n=7/71; 9.86%) were further tested using four individual antigens. Data further confirmed a single infecting species (*L. interrogans*) and serogroup (Australis). Interestingly, we did not detect *Leptospira* within the portion of 67 rat kidney samples investigated. Stained kidney sections (n=11) showed limited association between inflammation and leptospire presence, indicating that rodents may shed the bacteria asymptomatically.

Multi-locus sequence typing (MLST) schemes have been successfully applied to identify the sequence types (STs) of pathogenic *Leptospira* strains. The PCR positive rodent samples were tested using MLST (n=23). All samples with a full profile (n=11) were shown to belong within ST-24, with five partial profiles also likely to belong to the same ST. To date, three serovars are within ST-24 (Jalna, Bratislava and Muenchen), that belong to the Australis serogroup. This was the first study to utilise DNA extracted directly from kidney tissue to perform *Leptospira* MLST analysis. MLST data further emphasises a single infecting species and presents evidence for a single infecting serogroup.

Further work involved full genome sequencing of ten strains not previously investigated, covering pathogenic, intermediate and saprophytic species. Sequence data for each strain was obtained using the MiSeq platform. The 'core' genome was identified across 17 strains (n=1,095; 28.76%), with pathogenic strains being more conserved with a greater shared core genome (n=2,859; 69.30%). Single nucleotide polymorphism (SNP) data was generated for strains (n=6), with an average of 35,346 SNPs per strain (range=686 to 55,303). *L. interrogans* serovar Icterohaemorrhagiae had the lowest SNP count (686) and was the only strain with a greater number of non-synonymous SNPs compared to synonymous (1.8:1); indicating close relatedness between serovars Icterohaemorrhagiae and Copenhageni. Coding sequences were identified within genome regions that may relate to antigenic differences (high SNP variation) or relate to key cellular processes (low SNP variation). Due to poor gene characterisation, hypothetical proteins make up a high proportion of coding sequences within such regions. Further work to characterise identified coding sequences may identify future therapeutic or diagnostic targets.

This project aimed to investigate the current situation concerning canine and rodent *Leptospira* research within the UK. Results presented within this thesis demonstrate several wild rodent species within England are capable of maintaining and potentially shedding pathogenic strains known to infect both humans and dogs. Serogroup Australis (found infecting rodents) is now protected within a tetravalent canine vaccine; however annual booster vaccinations are required for optimal immunity. Extended urban sampling would be of great benefit considering the absence of positive urban samples. Suspected and confirmed canine cases are still witnessed within UK practices despite the reported majority having current vaccinations. Continued monitoring of serogroups would benefit vaccination strategies, and an emphasis on early detection within infected dogs would allow for the greatest chance of survival.

List of abbreviations

5-FU	5-fluorouracil
ABADH	4-aminobutyraldehyde dehydrogenase
AHVLA	Animal Health and Veterinary Laboratories Agency
BLAST	Basic Local Alignment Search Tool
CAAT	Cross-agglutination absorption test
CC	Clonal complex
CDC	Centre for Disease Control
CGR	Centre for Genomic Research
CMG	Comparative microbial genomics tools
CSF	Cerebral spinal fluid
DOI	Duration of immunity
ECM	Extra-cellular matrix
ELISA	Enzyme linked immunosorbent assay
EMJH	Ellinghausen-McCullough-Johnson-Harris
FOM	Phosphomycin
GABA	Gamma-aminobutyrate
GATK	Genome analysis toolkit
H&E	Haematoxylin and eosin
HPA	Health Protection Agency
IFAT	Immunofluorescence absorption test
IS	Insertion sequence
IUB	International Union of Biochemistry
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAT	Microscopic agglutination test
MDCK	Madin-Darby canine kidney
MLST	Multi-locus sequence typing
MLVA	Multi-locus variable number tandem repeat analysis
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
NCBI	National Center for Biotechnology Information
OMP	Outer membrane protein
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
PSS	Practice Standards Scheme
RAST	Rapid Annotation using Subsystem Technology
RCVS	Royal College of Veterinary Surgeons
SBS	Sequencing by synthesis
SNP	Single nucleotide polymorphism
SPDS	Spermidine synthase33
SPHS	Severe pulmonary haemorrhagic syndrome
ST	Sequence type
T2SS	Type two secretion systems
TLR4	Toll-like receptor 4
TTP	Thrombotic thrombocytopenic purpura
VNTR	Variable number tandem repeat
WHO	World Health Organisation

Chapter One

Introduction

1. Introduction

1.1 Overview

Pathogenic *Leptospira* spp. are the causative agents for leptospirosis, regarded as the most widespread zoonotic disease in the world ((WHO), 1999), with estimated human global infection rates at 5 cases per 100,000 (or 1 in 20,000) ((LERG), 2010). The bacterium itself is Gram negative and responsible for both Weil's disease in humans and canine leptospirosis within the UK. Weil's disease is caused by serovar Icterohaemorrhagiae and can lead to renal and hepatic failure if left untreated. Humans and dogs are incidental hosts, with small rodents being the typical reservoir animal for pathogenic variants. Human cases are self-limited, with only a minority of cases progressing into late stage clinical signs with no treatment intervention. Early stage clinical signs can be vague and indicate a range of diseases, and it is believed to be under-diagnosed within all end hosts as a result (Prescott et al., 1991; Sarkar et al., 2012).

1.2 Discovery & naming of the *Leptospira* genus

The disease now known as leptospirosis was first described in 1886 by Adolf Weil, although its presence can be traced back from infectious disease outbreaks throughout history (Faine S., 1999). Stimson was the first to describe the morphology of the bacteria within kidney tissue, specifically the renal tubules. From this, the nomenclature *Spirochaeta interrogans* was derived (Stimson, 1907). Wolbach and Binger attempted to culture the bacteria during 1914 on various media, including sterile water, with many failed attempts (Wolbach and Binger, 1914). A year later, Inada and Ido successfully identified the bacteria within a guinea pig injected with blood serum from a patient suffering with Weil's disease, naming it *Spirochaeta icterohaemorrhagiae* (Inada et al., 1916). It wasn't until 1918 that Noguchi described the name *Leptospira* based on morphological characteristics when compared to other members of the *Spirochaeta* family (Noguchi, 1918).

1.2.1 *Leptospira* species and serovars

Up until 1987, only three *Leptospira* species were identified; pathogenic (*L. interrogans*), saprophytic (*L. biflexa*) and intermediate (*L. parva* (Hovind-Hougen et al., 1981)). Following DNA hybridization studies a further four pathogenic, two intermediate and two saprophytic species were nominated for inclusion in the genus (Yasuda et al., 1987). Now the genus *Leptospira* contains 19 known species.

Within each species, strains are further classified based on antigen recognition to give a serovar and serogroup. Serovars were first identified using the cross-agglutination absorption test (CAAT) (Faine S., 1999). CAAT classifies strains based on their expressed surface exposed epitopes within the lipopolysaccharide (LPS), specifically the sugar composition and protein orientation within the membrane (Adler and de la Pena Moctezuma, 2009). A single serovar can exist within multiple species, including both pathogenic and saprophytic species, due to the serogrouping system (Zakeri et al., 2010a). For example the serogroup Canicola contains 11 serovars in *Leptospira interrogans* and 3 serovars in *Leptospira kirschneri* (Cerqueira and Picardeau, 2009).

The variability in LPS constituents gives rise to the multitude of serovars. DegT (amino transferase) proteins are involved with the synthesis of LPS O side-chains. For example, the serovar Copenhageni genome contains 9 *degT* genes, whereas serovar Lai only has 7 (Nascimento et al., 2004). Variations of this nature may contribute to host specificity seen with distinct serovars.

Recombination arising from horizontal gene transfer has meant that serovars are now represented across multiple species (Brenner et al., 1999; Haake et al., 2004). One example can be seen with serovar Grippotyphosa now represented within both *L. interrogans* and *L. kirschneri* (Table 1.1). This is a result of the same O antigen that characterises the serovar becoming transferred between strains (Levett, 2004). However to date, no evidence has been presented for mobile elements surrounding such antigenic genes.

Serogroup	Species	Serovar	Strain
Hebdomadis	*L. kirshneri*	Kambale	Kabura
	L. borgpetersenii	Nona	Nona
	Genomospecies 2	Manzhuan	A 23
Icterohaemorrhagiae	*L. interrogans*	Canicola	Hond Utrecht IV
	L. interrogans	Icterohaemorrhagiae	RGA
	L. borgpetersenii	Tonkini	LT 96-98
Grippotyphosa	*L. interrogans*	Grippotyphosa	Andaman
	L. kirschneri	Grippotyphosa	Moskva V

Table 1.1. Examples of how a single serovar and serogroup can be represented within multiple pathogenic species.

The catalogue of known serovars is ever increasing, and as such there is a range of total serovar numbers amongst published work, with between 240-260 pathogenic serovars reported (Adler and de la Pena Moctezuma, 2009; Galloway and Levett, 2010; Jimenez-Coello et al., 2010). In 2010, a novel serovar belonging to *L. weilii* was discovered within infected grey kangaroos in Eastern Australia (Roberts et al., 2010). Further novel serovars have since been discovered in both rodents and humans (Paiva-Cardoso et al., 2013; Valverde Mde et al., 2013).

1.2.2 Emergence of genomospecies

The emergence of recent genomospecies demonstrates that the serovar database is still growing and changing as typing and identification methods evolve. In 2007, the subcommittee on the Taxonomy of *Leptospiraceae* recognised five genomospecies presented by Brenner and colleagues (1999) as newly defined species (Brenner et al., 1999; Levett and Smythe, 2008). *L. alexanderi* was given to genomospecies 2, with the remaining four characterised with species names in 2012 (Smythe et al., 2013).

The full genome composition for each newly discovered genomospecies have not yet been investigated. Comparison between themselves and other closely related species would provide insight into how genetically related they really are to the serovars they were previously classified with.

Given the high number of serovars, and difficulties encountered for using them in epidemiology studies, identification methods are now favouring genomospecies. As a result, difficulties have emerged with aligning results for genomospecies with the traditional antigenic methodology.

1.2.3 Cellular structure

All members of the genus possess the same spiral shape morphology (Fig 1.1). The bacterium is composed of a double membrane structure with a selectively permeable outer membrane for specific binding and uptake of molecules. The outer membrane contains phospholipids, outer membrane proteins (OMPs) and lipopolysaccharide (LPS) (Cullen et al., 2004). Porins (e.g. OmpL1) and type two secretion systems (T2SS) are also located within the outer membrane and have been shown to have antigenic properties (Adler and de la Pena Moctezuma, 2009).

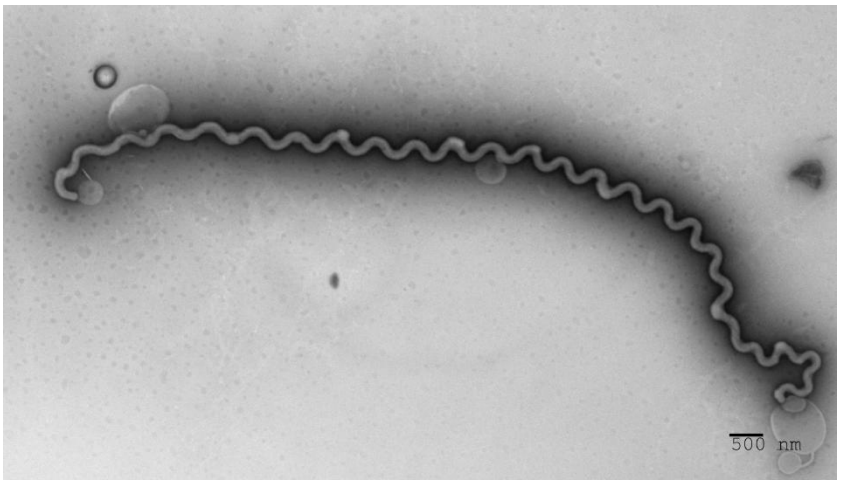

Figure 1.1. Electron microscope image of *Leptospira spp* (2400x magnification). Courtesy of Lee Smith (AHVLA).

Pathogenic strains adhere to host cells with one or two termini. The adhering positions are located within the terminal knobs at the flagella basal bodies. The bottom of the flagella consists of the MS ring (formed by both the M and S rings) and a cytoplasmic C ring (Liao et al., 2009). Adhesion to the extra-cellular matrix (ECM) is seen as essential in the initial stage of infection (Oliveira et al., 2010).

Two periplasmic flagella with polar insertions into the periplasmic space at each end are responsible for motility. They comprise of both a flagellar sheath (made up from FlaA proteins) and a core (FlaB proteins). A mutant deficient in *flaB* loses endoflagella and becomes non-motile (Picardeau et al., 2008). Phosphorylation of CheY leads to an interaction with the flagella to change the motor rotation bias (Li et al., 2006).

Within the leptospiral genome, 1,496 proteins (41% of total) have been found to contain at least one transmembrane segment. Within those, 346 (9.3% of total) have been shown as having four or more. Previous studies have documented that between 40-60% of proteins that include at least four transmembrane segments are related to transport (Meidanis et al., 2002; Paulsen et al., 2000). Of the 346, one protein contains 18 transmembrane segments, and at least 111 have not yet been identified as having a specific function (Nascimento et al., 2004).

LPS facilitates innate immune recognition and clearance by the host, therefore expression once within a host may be disadvantageous towards the survival of the organism (Haake and Matsunaga, 2010). However, the human innate immune system does not detect the LPS of *Leptospira* via human Toll-like receptor 4 (TLR4) (Werts et al., 2001). As a result, the human immune response is instead activated by TLR2. Further evidence is shown with TLR2/4 double

knock-out mice, normally asymptomatic hosts, that become highly susceptible to a leptospiral challenge (Chassin et al., 2009).

Once shed into the environment, pathogenic strains can adapt their membrane constituents for survival. This is in part down to the range of stimulus response systems within the large genome e.g. *L. interrogans* have 107 signal transduction proteins (Haake and Matsunaga, 2010). One example being with the regulation of Lig production (an OMP), that has been shown to be dependent on osmolarity changes (Haake and Matsunaga, 2010). An osmolarity increase in the local environment activates an up-regulation of the production of both LigA and LigB. This situation is similar to when the bacteria encounter host cells, and both Lig proteins can mediate the osmolarity for enhanced adherence to fibronectin (Choy et al., 2007).

1.2.4 Growth conditions

Leptospira are an obligate aerobe with an optimum growth temperature of 28-30^{o}C (Adler and de la Pena Moctezuma, 2009; Palmer and Zochowski, 2000). However, some saprophytic strains have demonstrated their potential to grow at lower temperatures, albeit with a lower efficiency.

When observed from a sample of stagnant water in 1914, culture was attempted using sterile water, synthetic media and broth with no success (Wolbach and Binger, 1914). Ito and Matsuzaki first described the isolation of *Spirochaeta Icterohaemorrhagiae (Inada)* on both solid and semi-solid blood media, particularly with greater growth seen on blood gelatine instead of blood agar (Ito and Matsuzaki, 1916). Following the first isolation, advances have been made using supplemented media, with semi-solid media (such as Fletcher's or Vervoort's) becoming commonly used for isolation (Czekalowski et al., 1953; Lawrence, 1951).

Now, *Leptospira* are commonly grown on Ellinghausen-McCullough-Johnson-Harris (EMJH) media. The nutrient rich media contains supplements of long chain fatty acids, ammonium salts and vitamins B1 and B12, of which all are essential for successful growth. EMJH is a modified version of the original developed by Ellinghausen and McCullough in 1965 that contains tween alongside the original components (Johnson and Harris, 1967). As cultures are extremely fastidious and slow growing, rabbit serum is typically added to further supplement growth. Cultures can take up to 6 months before a definitive negative result can be ascertained. Components such as bovine serum albumin (BSA) also need to be fresh to give the greatest chance of a positive culture. Due to the nutrient rich media, contamination is a problem; precautions are required to limit the likelihood of this occurring.

When growing multiple strains, cross-contamination must be prevented as saprophytic strains can outgrow pathogenic strains in a mixed culture (Ganoza et al., 2006; Wilson and Fujioka, 1995). This presents issues when isolating pathogenic species from environmental sources, such as standing water or damp soil, as it has been shown that saprophytic species are in the majority under such conditions (Benacer et al., 2013b). It is recommended that cultures are checked regularly (at least once a week) using a dark field microscope for evidence of growth or potential contamination (Fig 1.2).

Another media currently in use for isolating *Leptospira* is Korthof's media, again supplemented with rabbit serum (Babudieri, 1961). Saprophytic strains have been found to reach stationery phase within 5 days of inoculation with Korthof's compared to 30 days on EMJH (Saito et al., 2013).

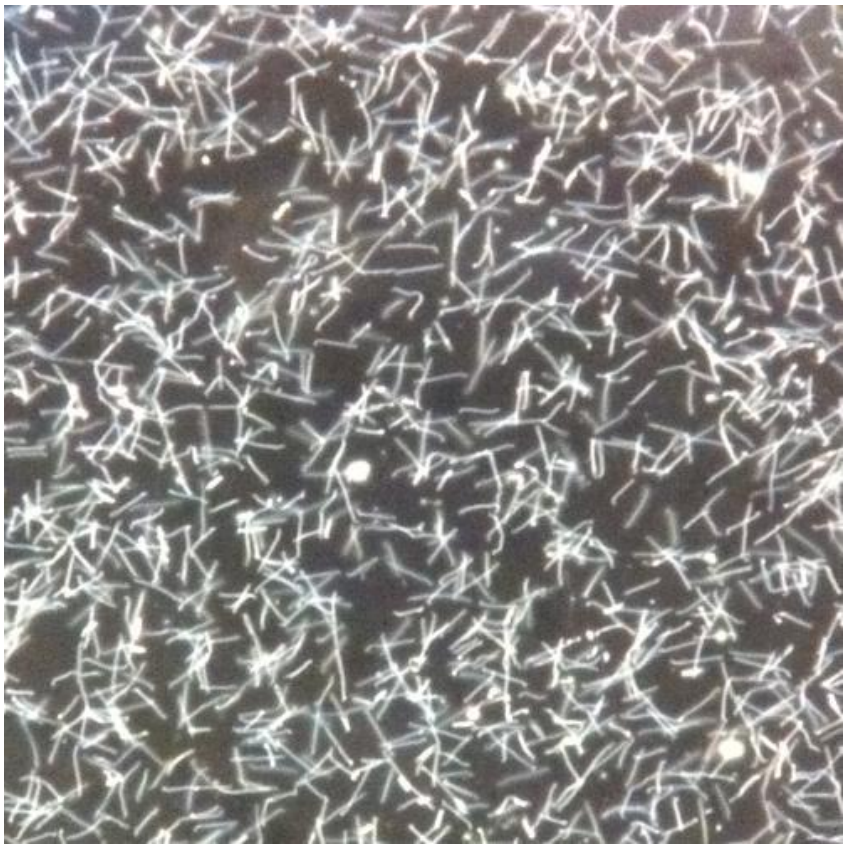

Figure 1.2. Growth of *L. interrogans* as seen using dark field microscopy (x400 magnification). Image courtesy of Lee Smith, AHVLA.

Antimicrobials are often included within *Leptospira* growth media, particularly during early sub-culturing from clinical material or environmental sources. Several antimicrobials have been suggested for inclusion into EMJH that do not impair growth of leptospires, such as 5-fluorouracil (5-FU). The pyrimidine analogue of 5-FU shows no impairment of *Leptospira*

growth up to concentrations of 200µg/ml, whereas it has demonstrated a bacteriostatic effect on other bacteria species (Heidelberger et al., 1957). Phosphomycin (FOM) also shows no inhibition of *Leptospira,* even at greater concentrations of 1,600µl/ml (Oie et al., 1986). Once inside a bacteria, FOM prevents the production of new cellular wall structures by inhibiting the biosynthesis at an early stage (Forsgren and Walder, 1983).

ATP is generated by an F_0F_1-type ATPase, as seen in other bacterial species such as *Mycoplasma pneumonia* (Nascimento et al., 2004). Fatty acids are utilised as a sole carbon source and metabolised by beta-oxidation, whereas ammonium salts are metabolised for nitrogen (Adler and de la Pena Moctezuma, 2009; Palmer and Zochowski, 2000), and both are key components of any commercial *Leptospira* media.

Environmental survival can be sustained for prolonged periods by certain species such as *L. interrogans*; however saprophytic species are better adapted for such conditions. The genomes for saprophytic strains contain the metabolic pathways necessary to thrive in environmental niches instead of those contributing to survival within a host.

1.2.5 Survival & acquisition of metabolites within a host and the surrounding external environment

Following the full genome sequencing of *Leptospira* strains, it became possible to identify the genes and pathways involved in acquiring nutrients and metabolites, both within a host and the external environment.

The mechanism for glycerol metabolism and further glycerol enzymes indicated towards glycerol and fatty acids being acquired through phospholipids degradation (Nascimento et al., 2004). All genes necessary for a complete glucose utilisation pathway are present but only one uptake system is utilised, which is dependent on a sodium gradient across the membrane. Difficulties in utilising glucose for energy are a result of insufficient uptake rather than a lack of ability to generate glucose-6-phosphate (Nascimento et al., 2004).

During infection, the host immune response can reduce the rate of iron absorption and increase production of transferrin, thus limiting the amount of free iron available to the bacteria. The low iron environment has been suggested as one explanation for the signs of jaundice and haemorrhagic lesions seen in patients, as the bacteria lyse host cells to acquire the iron they require (Sridhar et al., 2008). A number of proteins have been linked with degrading membranes including 5 sphingomyelinase C-type haemolysins, one phospholipase D and orthologs for other haemolysins (Nascimento et al., 2004).

Genes relating to capsular polysaccharides and secreted exopolysaccharides are present, despite no experimental evidence of leptospiral biofilm formation existing (Faine S., 1999). One possible explanation may relate to colonisation mechanisms in renal tubules and methods for survival outside of a host (Nascimento et al., 2004).

Mutant clones of *metW, metX* and *metY* demonstrated the presence of two distinct pathways for methionine biosynthesis within two pathogenic species of *Leptospira* (Picardeau et al., 2003). These include both transsulphuration and sulphhydration pathways, similar to those seen in other bacteria such as *E. coli*. The importance of additional sources for methionine may be emphasised from the range of hosts that pathogenic species can infect, and so require the genes for nutrient acquisition within a number of differing environments. EMJH contains high levels of sulphate (Johnson and Harris, 1967) which may be utilised during the biosynthesis of methionine when grown on media.

1.3 Pathogenic *Leptospira* Species

Pathogenic strains are able to infect and cause disease in humans and a wide range of animals (Albatanony and El-Shafie, 2011; Gamage et al., 2012; Mayer-Scholl et al., 2013; Miraglia et al., 2012; Runge et al., 2013; Suwancharoen et al., 2013). In particular, serovars known to infect companion animals (Arbour et al., 2012; Arent et al., 2012; Markovich et al., 2012) have been reported in the UK. Disease manifestations can differ between serovars to a certain extent; however the specificity to a host is of greater importance to the pathogenic outcome. For example, rats act as the reservoir for serovar Icterohaemorrhagiae, whereas dogs are maintenance hosts for serovar Canicola as they shed the serovar into the local environment (Klaasen et al., 2003).

1.3.1 Canine and human infection

Canine leptospirosis is a widespread zoonotic issue, with a large range of pathogenic serovars able to infect and cause disease. Even within countries with vaccine availability, infection is still an issue due to a lapse in duration of immunity (DOI), differences with infecting serovars or a lack of vaccination entirely. Following colonisation, antibodies can be detected within 10-15 days (Andre-Fontaine, 2013). Clinical signs are typically vague during the early stages of infection (such as diarrhoea, vomiting, fever and malaise) and can be associated with a range of diseases. If left untreated, the disease can develop into late stage leptospirosis and cause renal and hepatic complications, which can be fatal. It is during these stages where the

characteristic jaundice can present. Not only is disease an issue but as dogs can shed into the local environment, humans or other unvaccinated animals in their vicinity are at risk.

Previous infection studies have demonstrated the effects that certain serovars can have following infection of a canine host. Greenlee and colleagues (2005) demonstrated L*. interrogans* serovar Pomona to be highly infectious, with 11/12 8-week old female beagles becoming infected following a challenge dose. Typical signs of lethargy and fever presented at day 7 post-inoculation, with lesions and pulmonary haemorrhage from day 10 onwards (Greenlee et al., 2005). An earlier study by Navarro and colleagues (1981) showed the presence of fever, dehydration, depression and icterus following infection with *L. interrogans* serovar Icterohaemorrhagiae (Navarro et al., 1981).

Human leptospirosis can be caused by a range of pathogenic serovars regularly maintained within animal hosts. Since 2008 in the UK, the HPA have reported infections from serovars, Icterohaemorrhagiae, Autumnalis, Australis, Grippotyphosa, Saxkoebing, Bataviae and Hardjo (HPA, 2012). Humans are incidental hosts that can also shed the bacteria for up to a year after infection (Bal et al., 1994; Chow et al., 2012; Ko et al., 2009). Human to human infection is not common, but can occur when residing in cramped conditions with poor sanitation that favour leptospire growth in the environment (Ganoza et al., 2010).

Not only does leptospirosis pose a detrimental effect to dogs and humans, pathogenic strains can also cause equine abortions (Whitwell et al., 2009). This was the first report of leptospirosis causing abortions in the UK, however this has been seen in cattle (Atxaerandio et al., 2005), sheep (Leon-Vizcaino et al., 1987) and pigs (Paz-Soldan et al., 1991; Ramos et al., 2006).

1.3.2 Mechanisms for infection for *Leptospira*

Once inside a host, pathogenic strains have the ability to evade defences, such as complement, due to expressed *Leptospira* proteins (LenA and LepA for example) (Fraga et al., 2011). In contrast, saprophytic strains (such as *L. biflexa*) lack these (or similar) proteins and are cleared rapidly from host serum by reticuloendothelial phagocytosis in a matter of minutes (Fraga et al., 2011; Merien et al., 1997). The bacteria travel through the bloodstream to rapidly disseminate into target organs and tissues (Stevenson et al., 2007), further facilitating the ability to evade host immune responses (Ko et al., 2009). Evidence for such mechanisms has been published using pathogenic strains on polarised Madin-Darby canine kidney (MDCK) cells (Barocchi et al., 2002). Plasmin activity arising from interacting with plasminogen on host cell

surfaces can help facilitate this translocation across the endothelial cell barrier (Vieira et al., 2013), a practice lacking in non-virulent strains. Proteolytic degradation of the extra-cellular matrix (ECM) may also aid invasion of host cells. Enzymes are transported to the surface as lipoproteins and then utilise type two secretion systems (T2SS) (Nascimento et al., 2004). Further to this, leptospires can target areas protected from the immune response such as the meninges or the eye.

Pathogenic species are also resistant to the alternate complement pathway and acquire complement factor H through ligands such as leptospiral endostatins (Len proteins). Factor H binds to the outer membrane and degrades C3b and C3 convertase. This inhibits the formation of the membrane attack complex (MAC). Saprophytic strains lack the ability to bind factor H (Adler and de la Pena Moctezuma, 2009). There is also some degree of protection against the classical pathway by strains binding the C4b-binding protein alpha chain (C4BPA) to the leptospiral surface (Ko et al., 2009).

Should an infection become chronic (as typically seen in reservoir hosts), the bacteria colonise the luminal surface of the proximal renal tubules, allowing continuous shedding into the environment via urine (Haake and Matsunaga, 2010; Stevenson et al., 2007).

It has been shown *in vitro* that virulent strains of *L. interrogans* killed macrophages via apoptosis to invade Vero cells (renal fibroblast cell line) (Merien et al., 1998). However prior treatment with formaldehyde strongly inhibited the internalisation of virulent cells (Merien et al., 1997).

Four of the five genes in the BatI operon seen in *Bacteriodes fragilis* have also been found in *Leptospira* (including *batA* and *batB*). They encode for proteins containing a Von Willebrand factor type A domain (Nascimento et al., 2004) and has been inferred that the proteins are involved in the loss of haemostasis in a host during infection (Ren et al., 2003).

Mutations in genes involved with motility (LA0025, LA2417, LA2069, LA2215 & LA2592) demonstrated no effect on motility in liquid culture or inhibited growth, suggesting a degree of functional redundancy (Murray et al., 2009a). Following the addition of leptospiral antiserum to EMJH, a reduction in motility was witnessed *in vitro*. At high concentrations the bacteria intertwined initially and then slowly regained motility after two hours. It was suggested that the constituents of the LPS react to antibody binding by shifting the antigens towards the bacterial cell ends, which aids motility and the ability to break away from membranes (Guo et

al., 2013). Pathogenic strains may have evolved methods to circumvent or aid in reducing the time taken to regain motility however further investigation would be required.

Temperature is a well documented factor that can affect the growth and survival of the bacteria (Palmer and Zochowski, 2000), although the exact role on differential gene expression was not known until recently. Under conditions simulating the shift between an outside environment and that inside a host, 507 genes were witnessed to be differentially expressed. Of those, 299 were up-regulated and included signal peptidases (*lepB*), DNA repair proteins (*radC*) and ion chaperones (*copZ*) (Lo et al., 2006). Such proteins would further aid survival and replication within a host.

1.3.3 Clinical manifestations of *Leptospira* infections

Humans are incidental hosts and cannot transmit the disease to others (Ko et al., 2009), whereas dogs act as the maintenance host for certain serovars (such as Icterohaemorrhagiae). Generally, canine leptospirosis is only considered in a differential diagnosis when typical later stage (anicteric) symptoms present themselves, such as jaundice and renal failure (Segura et al., 2005). With the disease presenting with varying manifestations (potentially due to different infecting serovars), a diagnosis of leptospirosis may be neglected altogether or only considered post-mortem (Zaki et al., 1996).

Distinct serovars can manifest with differing symptoms within a host. Serovar Canicola can cause Stuttgart's disease in humans (acute renal or gastrointestinal infection) and serovar icterohaemorrhagiae can cause Weil's disease in canines (acute haemorrhagic form) (Klaasen et al., 2003) but is also known to cause acute hepatic syndrome, uraemia or haemorrhagic enteritis (Andre-Fontaine and Ganiere, 1990). Acute renal failure can also manifest as tubulo interstitial nephritis as indicated by polyuria (Atzingen et al., 2008).

Within both canines and humans, the majority of leptospirosis cases present as mild and vague signs, such as fever, diarrhoea and vomiting (Levett, 2001). However, if left untreated during this early stage, a percentage of cases can develop into late stage leptospirosis where the survival likelihood is reduced.

Between 5-10% of human patients contracting leptospirosis develop late stage complications, with an overall mortality rate reported between 1-5% (Dolhnikoff et al., 2007a). Adler and de le Pena Moctezuma (2009) described the potential for four syndromes to arise from late stage leptospirosis, of which severe pulmonary haemorrhaging syndrome is becoming increasingly

common, reported in France, Mexico and Brazil (amongst others) (Gouveia et al., 2008; Paganin et al., 2011; Zavala-Velazquez et al., 2008).

Severe pulmonary haemorrhagic syndrome (SPHS) was first identified within humans during an outbreak in Nicaragua, 1995 (Trevejo et al., 1998). A study in Brazil identified a further 47 cases of SPHS between 2003-2005 as a result of a *Leptospira* infection, with a fatality rate of 74% within them (Gouveia et al., 2008). Since the first identification of SPHS there have been growing reports from human infections (Croda et al., 2010; Dolhnikoff et al., 2007b; Duplessis et al., 2011; Trevejo et al., 1998). However no definitive link between SPHS and leptospirosis has been made in dogs to date (Klopfleisch et al., 2010). Seven pulmonary leptospirosis cases were identified using real-time PCR within endemic urban areas of Peru which presented with symptoms not traditionally associated with leptospirosis (Segura et al., 2005). Of the seven, five died with late stage pulmonary leptospirosis which further emphasises the high mortality rates previously described (Dolhnikoff et al., 2007a).

Late stage symptoms have been suggested to have associations with certain serogroups, such as serogroup Icterohaemorrhagiae linked to jaundice, elevated bilirubin and oliguria relating to renal failure (Katz et al., 2001). Serovar Icterohaemorrhagiae has been associated with severe leptospirosis in humans (Herrmann-Storck et al., 2010). However, despite this perceived association in humans, it is yet to be investigated if the same level of significance is seen between serovars and the chance of developing severe leptospirosis within canine cases.

Thrombotic thrombocytopenic purpura (TTP) is a condition that is classically characterised by fever, anemia, renal failure and thrombocytopenia. If left untreated, the condition has a mortality rate of up to 90% (Amorosi and Ultmann, 1966; Rock et al., 1991). Recently there have been human cases of TTP witnessed that have been linked to a *Leptospira* infection (Booth et al., 2011; Quinn et al., 2013). In the case presented by Quinn and colleagues (2013) the abnormal liver function results were not traditionally indicative of TTP, indicating a possible mis-diagnosis based on the vague symptoms. However, whether this extends to canine infections is yet to be determined.

Meningitis can also be caused by a small number of late stage human infections, typically from neuroleptospirosis, which is biphasic; with septic and immunological phases (Panicker et al., 2001). It is during this second stage where classical meningitis symptoms can be seen. Aseptic meningitis has been seen in up to a quarter of human leptospirosis cases (Bharti et al., 2003).

The cholinergic responses to mild leptospirosis have also been demonstrated, with both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) detected within the blood of infected rat models, particularly five days post infection. Increased levels of AchE can contribute to inflammation caused by a leptospirosis infection and may act as a marker for inflammation within an infected host (da Silva et al., 2012; Das, 2007). BChE can also act as a marker in similar fashion. Following the host response of inhibiting AChE to prevent further inflammation, BChE can compensate, and an increase of BChE activity was witnessed 30 days post infection (da Silva et al., 2012). Both enzymes may be beneficial as markers for assessing inflammation arising from infection.

It has been suggested that white-spotted kidneys of ruminants & cattle following slaughter are an indicator of an infection from *Leptospira* (Dorjee et al., 2009). With 79.2% of cattle presenting with the white spots were positive by PCR for the bacteria (Azizi et al., 2012). While it is not indicative of an infection, the presence of white-spotted kidneys should in turn raise suspicion of either an active or chronic infection. Despite the association in farm animals, no link has been identified within companion animals for such clinical signs.

1.3.4 Treatment of Leptospirosis in both humans and dogs

Due to vague anicteric clinical signs witnessed during early stage canine leptospirosis, treatment is typically prescribed with broad spectrum antibiotics. In the past, penicillin has been used to combat infections, and has been suggested for use with severe, late stage infections (Watt et al., 1988), however a situation was witnessed in humans requiring an increased dialysis following late stage penicillin use (Brett-Major and Coldren, 2012). Whether this also applies to canine treatment has not yet been investigated.

For treating human infections, penicillin is reported to be effective only within the first four days of infection (Panicker et al., 2001) but a course of treatment, coupled with dialysis to treat the renal complications, has been shown to be sufficient for treating complications arising from infection (Patil et al., 2011). Despite the advantages, due to severe Jarish-Herxheimer reactions previously witnessed from large scale endotoxin release, other antibiotics are now commonly prescribed (Hartskeerl et al., 2011). A report in 2000 backed the use of both penicillin and doxycycline for leptospirosis treatment (Guidugli et al., 2000).

In vitro studies have shown that pathogenic strains of *Leptospira* are susceptible to ampicillin, cefepime and macrolides (Ressner et al., 2008). Tilmicosin (a macrolide) was demonstrated to show greater bacteriocidal properties against four strains when compared to amoxicillin and

enrofloxacin (Kim et al., 2006). However, any clinical implications are yet unknown as extensive testing on side effects for a host is yet to be carried out in regards to leptospirosis treatment.

Alternatives treatments such as corticosteroids (Shenoy et al., 2006) that have demonstrated a reduced mortality rate during human studies may also prove useful in canines. To what extent however is currently unknown as they are yet to be investigated for such purpose.

1.3.5 Reservoir hosts and environmental survival of *Leptospira*

Rodents are an important part of the transmission cycle for leptospirosis in both canines and humans. Not only do they maintain their survival as asymptomatic carriers, but they also shed live leptospires into the local environment. Once shed, certain pathogenic species can survive within damp soil or bodies of standing water for prolonged periods before being taken up by a new host.

The most common rodent reservoir across the literature is the black rat (*Rattus rattus*) and the brown or Norwegian rat (*Rattus norvegicus*). Wood mice, bank voles, house mice and yellow necked mice are also associated with the disease (Wisseman et al., 1955). Renal carriage levels vary depending on local endemic rates, with reports of infection in 84.6% (11/13) of mice and 65.9% (54/82) from regions of high leptospirosis infection rates (Desvars et al., 2012).

Once inside a reservoir host, the bacteria rapidly disseminate from the bloodstream into target organs such as the liver or kidneys in order to avoid host defences. Once there, they colonise and establish an infection within the renal tubules. From the kidneys they are shed via the urine of the infected animal which can then infect an incidental or reservoir host.

It has been suggested that the sex of a rodent is not a definitive risk factor for the rodent to contract an infection, whereas others suggest that females have an increased risk for infection (Krojgaard et al., 2009).

Currently, reports are conflicting on the risk of urban leptospirosis. Expansion of urban populations into previously rural land has increased leptospirosis incidence rates from greater contact with favourable growth conditions, particularly in areas with poor sanitation (Reis et al., 2008). Rats living in a confined, damp space e.g. sewers show an increased prevalence of *Leptospira* infections. The environmental conditions facilitate leptospire survival and a limited movement space increases contact and the chance of transmission between rodents (Krojgaard et al., 2009). Cases within cities and towns also vary. A study of rodent populations

within Tokyo, Japan, revealed that of the total 127 rats sampled, 22 (17%) were shown to be PCR positive (Koizumi et al., 2009). As a contrast however, two studies from New Zealand identified no presence of urban *Leptospira* over two separate studies despite identifying the bacteria within rural samples (Blakelock and Allen, 1956; Brockie, 1977).

To emphasise the worldwide extent of the type of host susceptible to leptospirosis, the common tenrec (*Tenrec ecaudatus*) is a small mammal native to islands around Madagascar and Africa. Interestingly one study discovered that although the species were found with circulating antibodies in their blood (confirmed by MAT), no evidence of renal presence was confirmed by PCR (Desvars et al., 2012).

When outside a host, pathogenic strains can survive for short periods in bodies of water and damp soil. Damp, alkaline conditions favour the growth of all *Leptospira* (Smith and Self, 1955) and temperature is a limiting factor in their survival (Levett, 2001). Reports from cases in Argentina (Vanasco et al., 2008), Korea (Jung et al., 2010) and Guadeloupe (Storck et al., 2008) all show an increase in leptospirosis cases during wet and warm conditions.

1.3.6 Vaccination and prevention methods

The vaccine itself is typically a non-adjuvanted liquid vaccine containing inactivated whole cells of each serogroup that it protects against. A number of human and canine vaccines have been produced in countries with endemic leptospirosis within local reservoir populations. Vaccine effectiveness in a region primarily focuses on two areas, serovar specificity and the duration of immunity. Knowing the endemic serovars within a region can allow for vaccines to be tailored for adequate coverage and increase their effectiveness. Typically, two vaccine doses are required for full immunity against the disease when vaccinating for the first time. The same is true for including a new serovar within the annual booster, as a single dose may not be sufficient to supply adequate protection (Moore, 2013). Failing to maintain an up to date vaccination by letting the duration of immunity (DOI) lapse exposes a companion animal to infection should they be challenged with *Leptospira* exposure. Typical vaccines against leptospirosis protect for 12 months before a booster is required to maintain optimal protection (Klaasen et al., 2003).

A study in 2003 investigated the duration of immunity (DOI) that the canine vaccines available at the time of the study offered. They conferred a suitable level of protection for 13 months from the serovar Canicola with no signs of shedding. Two of six unvaccinated dogs became carriers of the serovars Icterohaemorrhagiae whereas none of those vaccinated did (Klaasen et

al., 2003). More recently a study by Minke and colleagues (2009) looked into the DOI offered by the vaccine EURICAN® L (Merial). This vaccine was shown to offer protection against both Canicola and Icterohaemorrhagiae for at least 14 months. None of the 18 vaccinated puppies developed a renal carrier state and only 2 out of the 16 adult dogs presented clinical signs (Minke et al., 2009). This was however following a challenge with doses much larger than those naturally occurring in the environment.

Several canine leptospirosis vaccines have been registered within the UK. Due to previously reported cases, most are typically bivalent to include two common serovars in the UK (*L. interrogans* serovars Canicola and serovar Icterohaemorrhagiae). The vaccines do offer limited cross-protection to other serovars within the same serogroup; however this effect can vary. It has been suggested that serovar Canicola can impart protection against Ballum and Copenhageni strains (Rosario et al., 2012).

Licensing requirements differ between countries, meaning the serovars that vaccines are registered to protect against can vary. For the US, there are four tetravalent vaccines that protect against four serogroups including Canicola, Icterohaemorrhagiae, Grippotyphosa and Pomona (Klaasen et al., 2013).

In 2013, MSD Animal Health published details of a new tetravalent canine vaccine that protects against challenge from four serogroups (Klaasen et al., 2013). Serogroups Australis and Grippotyphosa are included alongside Canicola and Icterohaemorrhagiae. The HPA have reported human infection arising from the Australis serogroup (HPA, 2013b) and previous studies in the UK have shown that serovars belonging to the Australis serogroup are present in wild rodents (Hathaway et al., 1983b).

Due to the perceived low rates of infection in the UK, the canine leptospirosis vaccine is also not currently considered a 'core' vaccine in the UK (unlike the vaccines for parvovirus, parainfluenza virus, canine distemper and infectious hepatitis). Alongside this leptospirosis was removed from the list of notifiable diseases, meaning labs are not required to report positive leptospirosis cases to the AHVLA.

Human vaccines are available alongside those for companion animals and livestock. As with canine vaccines they are typically bivalent or trivalent and contain serovars endemic to the region. However, human vaccines are typically administered for regions with high risk factors such as following serious environmental conditions like a period of heavy flooding (Adler and

de la Pena Moctezuma, 2009). In the UK, the human vaccine is not routinely administered due to the low incidence of cases (between 50-60 a year) (HPA, 2012).

Proteins residing in the outer membrane have been scrutinised for their efficacy within vaccines. Following the full sequencing of several *Leptospira* genomes, motifs within the membrane have been identified and suggested for inclusion within vaccines due to their antigenic properties. The protein LemA was shown as a potential candidate, given its conserved nature amongst pathogenic species. Initial testing of LemA indicated a reduced mortality rate from a range of vaccine production methods, including a strong level of protection using a prime-boost strategy (Hartwig et al., 2013). However the protection that is conferred amongst each endemic serogroup would need further investigation to determine its true potential.

Hap1 is also known as *lipL32*. Previous reports describe *hap1* as encoding for a haemolysin, located downstream from *sphH* (Lee et al., 2000). It is highly conserved and located within pathogenic strains *L. interrogans* serovar Lai and *L. kirschneri* serovar Grippotyphosa (Branger et al., 2005). Studies demonstrate the potential of *hap1* as a vaccine candidate, as the gene conferred a level of protection within gerbil models (Branger et al., 2001). However, studies should be compared to those of *lipL32*.

Leptospirosis can also be avoided by preventing an infection within a host. By preventing colonisation, the level of bacterial shedding into the environment is also restricted. By creating a physical barrier between a host and the bacteria, the potential for host invasion is restricted. The threat of such has been highlighted within clinicians examining infected rodents and canines (Whitney et al., 2009).

Alongside vaccination, removal or prevention of reservoir hosts can help prevent incidental canine or human infections. During 2011, in an attempt to curb endemic human infections, the Indonesian government culled the local rat population following an over-average increase in leptospirosis cases. In one month before the culling, fifteen cases were reported that led to four deaths in humans (ProMED-mail, 2011).

While many strains cannot be eradicated from areas due to the large range of animal reservoirs such as rodents, there is one exception. Serovar Hardjo is maintained within cattle and sheep reservoirs with no other known hosts (Hartskeerl et al., 2011). Given these characteristics, it may well be possible to eradicate the serovar from herd populations through extensive vaccination programmes.

1.4 Saprophytic *Leptospira* species

To date there are six saprophytic species of *Leptospira*. Within the six, there are much fewer serovars documented compared to the pathogenic species which emphasises the greater ratio of pathogenic strains currently in circulation throughout the world.

Historically, all saprophytic strains were grouped within one species name, *L. biflexa* until the discovery of several genome divergences which culminated in the discovery of new species in 1987. Yasuda et al., (1987) proposed the names of two new saprophytic strains *L. meyeri* and *L. wolbachii*. Since then a further two were discovered, including a genomospecies, to bring the total to six species within the saprophytic sub-group.

Isolating the bacteria from environmental sources has proven to be difficult due to the nature of sampling, the presence of other contaminants, and a slow growth process (Saito et al., 2013). The issue with contaminants can be prevented to an extent with the addition of multiple antimicrobial agents which do not affect leptospires (Chakraborty et al., 2011). The combination of five antimicrobial agents were able to isolate a potentially new saprophytic species from water obtained in Japan within close proximity to a swimming pool (Saito et al., 2013). The same study also suggested that saprophytic strains may not grow as efficiently in EMJH compared to pathogenic species. This finding is conflicting with previous reports of how the saprophytic strains can outgrow pathogenic if in the same EMJH inoculation (Ganoza et al., 2006).

While saprophytic strains have no clinical relevance in terms of causing disease, the genetics of the strains can still remain useful in identifying pathways and survival mechanisms utilised that could also be part of pathogenic serovars. To date, saprophytic strains have never been isolated from a human host (Merien et al., 1997).

The inability of saprophytic strains to cause disease, lack of clinical importance and the difficulty of isolating new strains from environmental sources means that new pathogenic serovars have a greater chance of discovery. This may be one reason for explaining the greater number of pathogenic serovars currently identified.

1.5 Diagnosing a *Leptospira* infection

Diagnosis of leptospirosis infections are currently carried out with serological or molecular protocols, although successfully culturing any bacteria recovered from a host is highly beneficial to research into the organism. Diagnosis via PCR is more effective, compared to the

MAT, during the early phase of infection as antibody levels are low or even non-existent (de Souza, 2006). In the immunological phase, while antibodies can be detected in the cerebral spinal fluid (CSF), it is not possible to isolate the bacteria (Bharti et al., 2003).

DNA for molecular detection can be extracted from a range of tissues for PCR including the spleen, liver, lungs and kidneys (Lourdault et al., 2009). Studies have demonstrated a greater number of positive samples from tissue extracts compared to urine or blood (Azizi et al., 2012), presumably due to the bacterial load present. However this approach presents issues with material availability. Typically either blood or urine samples are submitted for testing, meaning this approach may not be feasible under such circumstances.

In 2007, a range of serological techniques were tested against the MAT to compare their specificity and sensitivity. Methods investigated include the IgM ELISA, dip stick tests, ELISA EIE-IgM-Leptospirose and the Dri-Dot test. The IgM ELISA demonstrated the greatest sensitivity during the acute (<7 days) and the convalescent (8-10 days) phases of infection. Although with a reported sensitivity during the acute phase lower than 67%, a second result may be required for a definite diagnosis (McBride et al., 2007).

When required, kidney tissue can be stored in formalin, embedded in paraffin and then cut into sections for histology, such as the Warthin-Starry stain (Athanazio et al., 2008). The technique is not useful for clinical application, only for post-mortem confirmation of infection. However, it is important for research due to having the greatest chance for visualising internalised bacteria within a host. The haematoxylin and eosin (H&E) stain can infer sites of inflammation as a result of an infection when applied to the same section of tissue.

1.5.1 Serological identification of *Leptospira* serogroups

Serological testing still remains the most widespread method to confirm a potential leptospirosis case, with the microscopic agglutination test (MAT) established as the 'gold standard'. Laboratories across the world utilise the MAT for both diagnosing clinical cases and identifying the serogroup of infections for research purposes (Faine S., 1999).

Fundamentally, the MAT relies on agglutination of live *Leptospira* antigens to host antibodies, meaning the antigen needs to be a specific serogroup in order to detect an infection. Sample serum is diluted down a titre gradient and mixed with live culture of *Leptospira* and saline on a 96 well plate. The samples are then incubated for two hours to allow for agglutination to take place. Following this, each sample is observed under a dark field microscope for the presence of agglutination against a black background (Fig 1.3). More than 50% agglutination is

considered as a positive titre. Each sample is read down the titre gradient to determine the final reading.

Countries differ in their cut-off point for positive titre values. In the UK, a titre of 1:100 is usually required for a positive result, whereas in Australia and New Zealand, a higher titre of 1:400 is required. This value can differ for countries where the disease is more prevalent and values of 1:200-1:320 are regarded as the minimum diagnostic criteria (Katz et al., 2001; Sekhar et al., 2000). A second sample taken a few days later demonstrating a rising titre value is a definitive diagnosis of a *Leptospira* infection. Relying on a single sample is not ideal as it may be skewed by a recent vaccination or a previous challenge that has since been cleared.

The MAT offers a useful diagnostic tool for clinicians. Diagnostic labs that are maintaining a full panel of serovars can run the test in a matter of hours. If ran on multiple pooled samples, the test can give a positive or negative result, whereas further testing with individual serovars will go some way to identifying the serogroup of the infecting strain. Laboratories responsible for testing samples using MAT are regularly monitored by the Leptospirosis Reference Centre (Royal Tropical Institute, Amsterdam), who administer blind tests to ensure a high degree of competency. As MAT results are subjective, this testing allows for a confidence in the results generated between labs.

Epidemiologically, the MAT has been utilised to detect circulating antibodies in a range of susceptible animals. Studies demonstrate that the level of seropositive results can vary to a wide degree, depending on the sampling location and host investigated. One study in Brazil identifying 39.4% of sows from a slaughterhouse was seropositive (Miraglia et al., 2008), whereas a separate study in the same country found that 68.1% of domestic brown rats were seropositive (de Faria et al., 2008). Further studies have shown that seropositive rates in rodents can also vary depending on the region, particularly within countries were leptospirosis is considered endemic (Agudelo-Florez et al., 2009; Benacer et al., 2013a; Rahelinirina et al., 2010; Scialfa et al., 2010).

A main disadvantage to conducting the MAT is with serovar identification and specificity. While the test is able to determine serogroups, it is unable to definitively distinguish between serovars within a serogroup due to the large amount of cross-reaction that can occur (Cerqueira and Picardeau, 2009). This cross-reaction can be more severe within some serogroups compared to others (Levett, 2003) and can restrict the potential for epidemiological studies investigating the circulating strains within reservoir populations.

Figure 1.3. Agglutination of antibodies to *Leptospira* antigens as typically seen as an outcome of a microscopic agglutination test (MAT). Image courtesy of Lee Smith, AHVLA.

The need for a second sample for a definitive diagnosis is also a major drawback with MAT. A four-fold rising titre result on the second sample indicates an active infection. In contrast, a second sample showing the same titre result (especially a low titre) is indicative of a previous infection, or even a recent vaccination. Alongside this, the technique is labour intensive, requires trained expertise to perform and is time consuming (Yitzhaki et al., 2004).

An alternative serological test to the MAT that is used in the UK is the immunofluorescence absorption test (IFAT). Antigens from live *Leptospira* cultures are fixed to slides. Sample serum is then applied and incubated to allow interaction between the antigen and any present antibodies. Following this, fluorescent conjugated anti-human IgG is added to the slides and incubated further for attachment. Once excess IgG has been removed, the slides are observed under a fluorescent microscope for the presence or absence of fixed fluorescent tags (Zakeri et al., 2010b). Just like the MAT, the IFAT is only able to give definitive sample identification to the serogroup level and is unable to identify the serovar. The test offers a high-throughput alternative to the MAT, as a full 96 well plate can be used instead of needing a titre gradient.

The enzyme-linked immunosorbent assay (ELISA) has been utilised both commercially and for researching *Leptospira*. Anti-guinea pig IgM or IgG is conjugated to horseradish peroxidase to detect cross-reactive antibodies, absorbance results are then analysed (Lourdault et al., 2009).

For commercial tests the non-pathogenic *L. biflexa* serovar Patoc is used as it can detect cross-reactive antibodies in a broad selection of serovars. However it has been suggested that results gained from commercial test kits should only be used as an initial diagnosis and further confirmation from another test e.g. MAT/PCR may be needed (Levett and Branch, 2002).

There is also a commercially available IgM-dipstick test that can detect *Leptospira*. This method may be better suited for smaller sample sizes, compared to large sample numbers which are more suited for ELISA. The test itself uses minimum equipment and takes around two hours, making it possible for an initial diagnosis in areas without the facilities for the full ELISA test, or when out in the field (Levett et al., 2001). However the downside is a low sensitivity for both acute and convalescent phases (32.9% and 80.0% respectively) (McBride et al., 2007)

1.5.2 Molecular identification of *Leptospira* serovars

A number of genes have been proposed as targets for serovar identification using molecular methods, the most common being the 16S rRNA gene. Not only can it be used for differentiating *Leptospira* from other spirochetes (Paster et al., 1991), the infecting species can also be determined. A PCR assay targeting the 16S rDNA gene was first developed in 1992 (Hookey, 1992) and has now been incorporated into a range of conventional, nested and real-time PCR assays (Djadid et al., 2009; Merien et al., 1992; Patarakul and Lertpocasombat, 2004; Slack et al., 2006; Tansuphasiri et al., 2006; Woo et al., 1998).

Published data for utilising the DNA gyrase gene, *gyrB*, demonstrates the potential for species identification from direct amplicon sequencing (Slack et al., 2006). The gene encodes for a DNA gyrase subunit and has a 100% agreement with direct 16S rDNA amplicon sequencing, with the added benefit of a greater BLAST score and lower E value. The *gyrB* gene has been shown in other bacteria to have a much higher rate of base substitution when compared to 16s rDNA, a rate of 0.7-0.8% per 1 million years compared to 1% every 50 million years (Yamamoto and Harayama, 1996). This level of genetic variation suggests that *gyrB* could be the better candidate for phylogenetic analysis.

Several outer membrane proteins (OMPs), such as OmpL1 and the LipL family, have also been investigated for their use in distinguishing between serovars. As they contain antigenic properties, as recognised for the MAT, then the potential exists for OMPs to convey the ability to distinguish on a serovar level.

The *lipL32* gene (also known as *Hap1)* is only present in pathogenic strains and codes for the OMP LipL32. To date, the gene has been targeted for several published PCR assays and direct sequencing of amplified amplicons can also determine the species of a strain (Foronda et al., 2011; Haake et al., 2004; Tansuphasiri et al., 2006).

Multi-locus sequence typing (MLST) is a highly discriminative molecular technique for bacterial identification and typing, which relies on sequencing multiple loci within the genome. The first scheme was developed in 1998 to strain type isolates of *Neisseria meningitides* (Maiden et al., 1998). Since then, the technique has been successfully applied to a wide range of bacterial taxa, including spirochetes (Margos et al., 2008; Rasback et al., 2007). The first MLST scheme for *Leptospira* was published by Ahmed and colleagues (2005) which targets six genes, including genes contained within previously published PCR assays. Thaipadungpanit and colleagues (2007) published an alternate scheme in 2007 which amplifies seven loci, based on housekeeping genes (Thaipadungpanit et al., 2007).

The first scheme put forward by Thaipadungpanit and colleagues (2007) was only able to discriminate between two pathogenic species (*L. interrogans* & *L. kirschneri*) and is inconclusive for typing other species. Cerquiera and colleagues (2010) observed that when two additional loci (*lipL41* & *rpoB*) were included with the original scheme it was possible to distinguish between a further four pathogenic species (Cerqueira et al., 2010).

The online MLST database (http://leptospira.mlst.net) currently contains allele data for 338 isolates belonging to 201 sequence types (STs). Recently, the scheme was updated to incorporate a total of seven pathogenic species (Boonsilp et al., 2013). By switching the *fadD* locus for *caiB* and introducing degenerate bases into the primers, it is now possible to identify an additional five species to improve the accuracy and potential for the technique. The database holds information for alleles based on both the original scheme and the updated primers.

Multi-locus variable number tandem repeat analysis (MLVA) is a technique similar to MLST that has been described as having the discriminative potential to distinguish the serovars of three pathogenic species. Similar to MLST, the technique is based on running the products from amplified loci on a sequencer to identify the size and total number of repeats. However, the nucleotide sequence is not examined with MLVA, only the size of the amplicons. Several loci where identified as potential targets for a VNTR scheme in 2005 (Majed et al., 2005). Following the study, a full MLVA scheme was subsequently first published in 2006 (Salaun et al., 2006) based on five loci. Each locus is selected based on tandem repeat regions within the

genome. Different serovars contain differing replicate numbers for each repeating region. It was found that by combining the number of repeats in up to five loci, it was possible to determine the serovar of three pathogenic species (*L. interrogans, L. borgpetersenii* and *L. kirschneri*). However MLVA has its own limitations, with some serovars sharing the same number of tandem repeats, highlighting a reduced discriminatory ability to distinguish genetically close serovars. Within *L. interrogans*, serovars Copenhageni and Icterohaemorrhagiae have the same profile, as do serovars Sejroe and Istrica from *L. borgpetersenii* (Salaun et al., 2006).

1.6 The *Leptospira* genome

The size of the bacteria's genome differs from species to species. This is to be expected as pathogenic and saprophytic strains require different pathways for survival. However, there is also a differentiation within individual pathogenic species. The *L. interrogans* genome is roughly 4.7Mb with the *L. borgpetersenii* genome having a smaller genome with roughly 4.1Mb (Table 1.2). However, most pathogenic genomes are generally conserved, with serovars Lai and Copenhageni sharing 95% homology (Nascimento et al., 2004). All tRNA and rRNA genes are found on Chromosome 1 (Ren et al., 2003).

The first full genome sequencing of *Leptospira* was carried out in 2003, when Ren and colleagues (2003) sequenced the pathogenic *L. interrogans* serovar Lai (Ren et al., 2003); using the same sequencing method applied to other spirochete species (Fraser et al., 1997). In the same year, the first mutant strain was produced in *Leptospira meyeri*, using *trpE,* the gene responsible for coding the alpha subunit for the tryptophan biosynthetic pathway (Bauby et al., 2003). The ability to produce mutants for *Leptospira* presented the potential for virulence trait investigations in knock out models.

Following the success from whole genome sequencing, the first virulence factor was genetically defined in 2007 by Ristow and colleagues (2007). They discovered that the lipoprotein Loa22 was essential for virulence when inserted into guinea pig and hamster models. Despite being essential for virulence, its exact role is yet unknown. A transposon was inserted into the *loa22* gene and attenuating the resulting strain, which failed to express the protein *in vivo* (Ristow et al., 2007).

Five years following the first *Leptospira* genome sequencing, the whole genome for a saprophytic strain of *Leptospira biflexa* was published (Picardeau et al., 2008). In total, the genome size of *L. biflexa* was more similar to *L. borgpetersenii* rather than *L. interrogans* (Table

1.2). In addition to this, the saprophytic strain contained a replicative plasmid (p74) along with the two chromosomes found in all other *Leptospira* species.

Within all strains sequenced to date, a 'core' genome has been reported that consists of 2,052 (61%) genes. This core includes essential genes associated with DNA repair, cellular structure, metabolism amongst others (Picardeau et al., 2008).

A total 31 open reading frames code for the enzymes involved with the O-antigen of LPS (Werts et al., 2001). This would go some length to explain the large variation of epitope expression, and the large range of serovars.

Strain	Chromosome Size (Mb)		Number of Genes		Number of Proteins	
	C I	*C II*	*C I*	*C II*	*C I*	*C II*
L. interrogans Copenhageni str. Fiocruz L1-130	4.28	0.35	3,486	276	3,399	268
L. interrogans Lai str. 56601	4.34	0.36	3,457	293	3,409	293
L. interrogans Lai str. IPAV	4.35	0.36	3,469	293	3,421	293
L. borgpetersenii Hardjo-bovis str. JB197	3.58	0.3	2,980	262	2,645	235
L. borgpetersenii Hardjo-bovis str. L550	3.61	0.32	3,003	270	2,703	242
L. biflexa Patoc str. Patoc 1 (Ames)[1]	3.6	0.28	3,351	267	3,277	266
L. biflexa Patoc str. Patoc 1 (Paris)[1]	3.6	0.28	3,440	276	3,391	276

Table 1.2. Genome sizes, gene numbers and protein numbers for seven species that have been sequenced to date. [1] Contains an additional plasmid (p74)

Differences in genome size and composition of strains within the same species have previously been highlighted (Bulach et al., 2006). Pathogenic strains may become adapted to infect specific hosts, which can lead to differences in the receptors utilised for cell attachment within alternative hosts. This can be demonstrated within *L. interrogans* due to its widespread presence in a range of animal species whereas *L. borgpetersenii* is considered to be maintained mainly within cattle herds. As a result, the genome of *L. borgpetersenii* has undergone a restriction, with a lower total number of functional genes (Bulach et al., 2006).

Following recombination of genome arrangements via insertion sequences (IS), the organism can acquire genes that are considered non-essential. To increase efficiency, production of

enzymes and proteins involved with active pathways for survival and growth are either up or down regulated when within a host in response to the change in environment. Despite the evidence for horizontal gene transfer within intermediate species, there is a distinct lack of IS elements amongst both the intermediate and saprophytic species (Ricaldi et al., 2012).

One example of non-essential gene deletion can be seen in all pathogenic strains that contain the cobalamin riboswitch, which is concerned with the regulation of genes involved with synthesising and transporting vitamin B_{12}. Vitamin B_{12} is essential for growth and included as a supplement in all commercial growth media. However it is not present in the saprophyte *L. biflexa,* indicating a lack of a response to local environmental cobalamin levels from saprophytic species (Ricaldi et al., 2012). This potentially indicates the presence of an alternative process of acquiring B_{12}, given its importance for growth.

Mutant construction has been carried out on saprophytic strains since 2003, when a *trpE* double cross over mutant was produced in *L. meyeri* (Bauby et al., 2003), however the first pathogenic mutant was only produced in 2008 with an Spc^R cassette inserted into a portion of the *ligB* open reading frame (Croda et al., 2008).

1.6.1 Comparison between serovars within the same species: *L. interrogans*

Comparisons of serovars within the same serogroup (Icterohaemorrhagiae) can be analysed following the full sequencing of *L. interrogans* serovars Copenhageni & Lai. The average identity between the two genomes was 95% (Nascimento et al., 2004). While chromosome II in both are collinear, a large inversion exists in chromosome I, with evidence suggesting that the inversion took place in Lai.

The rDNA genes do not exist in operons, but are instead scattered across chromosome I, with the only difference being an extra 23S *rrl* gene in Copenhageni. A key part to note is that many of the genes unique to Lai are based in a 54-kb insertion containing 81 genes, not present in Copenhageni (Nascimento et al., 2004).

Feature	Copenhageni		Lai	
	CI	*CII*	*CI*	*CII*
Size (bp)	4,277,185	350,181	4,332,241	358,943
GC Content (%)	35.1	35	36	36.1
Protein Coding Genes				
With assigned function	1,811	161	1,901	159
Conserved & Hypothetical	1,643	113	2,459	208
Total	3,454	274	4,360	367
Transfer RNA	37	0	37	0
Ribosomal RNA genes				
23S	2	0	1	0
16S	2	0	2	0
5S	1	0	1	0
Insertion Sequences	26	0	48	9
Transfer-Messenger RNA	0	0	0	0

Table 1.3. Comparison of genome constituents between *L. interrogans* serovars Copenhageni and Lai

1.6.2 Comparison between two pathogenic species, *L. interrogans* & *L. borgpetersenii*

The normal route of transmission for pathogenic leptospires is through urine from a carrier host. This can be either through direct urine contact or via contact of contaminated water sources following from urine passage. However, it was suggested that the pathogenic *L. borgpetersenii* may have adopted a different mode of transmission (when compared to *L. interrogans*) that requires more direct contact. This may have contributed to a reduction in overall genome size (compared to *L. interrogans*). Around 22% of the *L. borgpetersenii* genome now comprises of pseudogenes, transposases or insertion sequences (Adler et al., 2011). This resulted in the total size being roughly 700Kb smaller than *L. interrogans* (Bulach et al., 2006).

The gene products absent from *L. borgpetersenii* are involved with adaptation to diverse environments (e.g. host immune defence avoidance, metabolic functions and solute transportation), resulting in a limited range of nutrients utilised by the bacteria in both host and natural environments. Two genes which are absent from *L. borgpetersenii,* but present in *L. interrogans* carry out a process of preparing the bacteria for long term survival in water and closely resemble *devR* and *devS* in *Myxococcus Xanthus* (Bulach et al., 2006; Thony-Meyer and Kaiser, 1993). Studies identified *L. interrogans* outbreaks to usually result from contaminated

water (Cacciapuoti et al., 1987; Stern et al., 2010) implying an ability to survive compared to *L. borgpetersenii*.

Despite the genome reduction, the outer membrane protein (OMP) *lipL32* is still retained in the *L. borgpetersenii* genome, with suggestions of a further role for the protein. Murray and colleagues (2009) described an up-regulation of genes involved with the haem and vitamin B_{12} pathways within a *lipl32* mutant, suggesting an association with the uptake and/or metabolism of these co-factors (Murray et al., 2009c).

Upon investigation, *L. interrogans* contains more genes responsible for signal transduction, transcription factors and solute transport functions when compared to *L. borgpetersenii* (Sridhar et al., 2008), which may be in part a result of the overall genome reduction.

1.6.3 Comparison between genomes of pathogenic and saprophytic species

Genes shared by pathogenic and saprophytic strains are mostly responsible for essential functions, such as metabolism, protein formation and maintaining homeostasis and structural integrity. One third of the genes within the saprophytic *L. biflexa* genome are not present in pathogenic species. These relate to processes developed to aid survival under more diverse, potentially extreme, environmental conditions such as a greater number of nutrient acquisition and sensing mechanisms (Adler et al., 2011).

While present in pathogenic species, it has been suggested that the lack of sphingomyelinases within saprophytic strains propose a role in pathogenesis over nutrient acquisition (Adler et al., 2011). However, in this instance, nutrient acquisition itself may be part of pathogenesis. Further investigation is required before an alternate role for the proteins can be definitively stated.

Genes encoding for a haem-oxygenase are present in both pathogenic and saprophytic strains. As it is used for iron acquisition in a host, its presence in *L. biflexa* is unusual since iron would be acquired from other sources outside a host.

Many target genes that have been utilised for PCR are only present in pathogenic strains. As saprophytes cannot cause infection within a host, such genes lend themselves well to molecular studies of leptospirosis infections. A strict presence within pathogenic strains has led to many being suggested or investigated as potential virulence factors.

1.6.4 Virulence Factors

Identifying virulence factors within the *Leptospira* genome has become a major focus for research in recent times. In theory, identifying key virulence factors may have a direct result on future vaccine production. By knowing the role of genes involved with virulence, vaccines can target the proteins or genes to prevent infection, shedding and disease.

It was only in 2005, following the full genome sequencing of *L. interrogans,* that the *Himar1 mariner* transposon was identified to allow for random mutagenesis (Bourhy et al., 2005). The high A+T content of *Leptospira* allow for suitable mutants to be produced by this system as all insertions occur at a TA dinucleotide (Murray et al., 2009a).

The first genetically defined virulence factor of *Leptospira* was published in 2007 (Ristow et al., 2007). An antigenic protein known as Loa22 had previously been shown to be expressed only in pathogenic strains and while it is located within the outer membrane, a part of the protein is left exposed on the cell surface (Koizumi and Watanabe, 2003). Loa22 has previously been described as having a strong correlation with *Leptospira* virulence (Koizumi and Watanabe, 2003) and several studies attempting to identify candidates for vaccine production have highlighted *loa22* to have potential as a virulence factor (Gamberini et al., 2005; Yang et al., 2006). As there is no replicative plasmid present in pathogenic *Leptospira* strains, a spectinomycin-resistant mutant was utilised. A *loa22* transposon was inserted to disrupt the gene. When the animals were challenged with the *loa22*$^{-}$ strain in an infective dose of 4e10^{8}, an amount shown to be 100% lethal in guinea pigs, all eight of the subjects remained alive and demonstrated no clinical signs within the 21 day follow-up investigation. Bacteria were recovered from the blood of the animals 3 days post-infection (4/4), and from the kidneys (5/7) for culture with none shown to have reverted back to the wild-type. Complementation restored the wild type phenotype (Ristow et al., 2007). This evidence complies with the Molecular Koch's postulates for virulence (Falkow, 1988).

It has been shown that the haem oxygenase gene, *hemO*, has a major role in iron acquisition by *Leptospira* spp. HemO degrades the haem tetrapyrrole ring (tetrapyrroles are the precursor molecule to haem) and releases ferrous iron for uptake (Frankenberg-Dinkel, 2004). Using this, the bacteria can survive solely with haem or haemoglobin as it's only iron source (Murray et al., 2009b). A knock-out mutation of *hemO* showed reduced virulence in a hamster model suggesting towards a significant role for *in vivo* survival (Murray et al., 2009b). The production of *hemO* is up-regulated in low iron conditions, increased osmolarity and in the presence of serum (Adler et al., 2011).

One set of proteins that have been targeted as potential factors are a group of OMPs that are anchored into the outer membrane. As they play a role in either attachment to host cells or translocation through the membranes, then it is feasible to hypothesise that they may also play a key role in virulence.

Being the most abundant OMP with 38,000 copies per cell (Haake and Matsunaga, 2010), one study looked into the virulence traits of *lipL32*. LipL32 also demonstrated the highest immunogenic response, with over 95% of patients during one study producing antibodies towards the protein (Guerreiro et al., 2001). Despite this, it lacks sensitivity towards polymixin B (toxic towards Gram negative bacteria) (Werts et al., 2001), with no other antibiotics further studied. Recently the protein has been re-evaluated and suggestions have been made that due to its abundant nature, the actual location of the protein within the cell may have been mis-calculated (Pinne and Haake, 2013). It was demonstrated that the protein is not in fact surface exposed and this previous mis-conception may have been from cellular degradation exposing LipL32 on the surface.

Following gene knock-out studies, LipL32 was suggested to play a role in virulence, however with a degree of redundancy. Within knock-out models, production of other genes was up-regulated to compensate for the absence of *lipL32*. As the bacteria retained its pathogenic status despite losing the gene, the study concluded that *lipL32* was not a key factor for the virulence of *Leptospira* (Murray et al., 2009c) and has been shown as not necessary for acute infection or kidney colonisation (Adler and de la Pena Moctezuma, 2009).

Another OMP with potential as a virulence factor is *lipL53*, an adhesin that can bind to several components of the extra-cellular matrix. As with *lipL32*, the gene is only present in virulent strains and has been demonstrated to be up-regulated by temperature and osmolarity (Oliveira et al., 2010), conditions that may be similar to those within a host. It was suggested as a potential vaccine target, however only conferred a limited protection response from a challenge dose of *Leptospira*.

There are three high-molecular weight leptospiral immunoglobulin-like repeated (Lig) proteins, *ligA*, *ligB* and the pseudogene *ligC*. Both LigA and LigB mediate host cell attachments during infection and invasion and are present on the surface of pathogenic strains (Matsunaga et al., 2003). They contain Ig-like domains with 90 amino acid tandem repeats (Lin et al., 2008). LigA and LigB have structural and virulence traits relating to microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), the bacterial proteins involved with colonisation of host tissue (Choy et al., 2007). One study demonstrated that when both *ligA*

and *ligB* were inserted into the replicative plasmid in *L. biflexa* they conferred a virulent phenotype and allowed for binding to eukaryotic cells and fibronectin when tested *in vitro* (Figueira et al., 2011). Despite this however, *ligB* is not necessary for virulence. While it plays a role in infection, including up-regulation with physiological osmolarity (Matsunaga et al., 2005) and a high affinity between human sera and the 2nd to 6th Big domains of LigB (Croda et al., 2007), it has been shown that a *ligB* mutant did not demonstrate a loss of virulence (Croda et al., 2008). As a result, it has been suggested that single gene inactivation is not a suitable method for determining *Leptospira* virulence factors (Adler and de la Pena Moctezuma, 2009).

Leptospira have demonstrated chemotactic movement towards haemoglobin (Yuri et al., 1993) and the genome contains more than double the amount of methyl-accepting chemotaxis proteins (MCP) than other spirochetes (11 in Copenhageni and 12 in Lai) (Nascimento et al., 2004). It was demonstrated that heme oxygenase may contribute significantly to the virulence of *L. interrogans* since the bacteria utilise haem as their only source of iron (Murray et al., 2009b).

A complete set of flagella genes are present in the genome of *Leptospira* including 4 flagella motor switch proteins; FliG, FliM, FliN and FliY, which play a critical role in motor direction. FliY contains a carboxy-terminal domain of 60 amino acid residues which is homologous with *Yersinia pestis* flagella proteins that are involved in Type 3 secretion systems. Mutant *fliY* strains demonstrated a reduced pathogenicity, however it was not clear if *fliY* is a confirmed virulence gene, or was a result of down-stream effects following insertion (Liao et al., 2009).

Lsa21 is a surface adhesin 21kDa in length. When grown on EMJH containing a salt supplementation the expression of *lsa21* is enhanced; demonstrating that up-regulation is dependent on osmolarity (Atzingen et al., 2008). In a similar fashion to Lig proteins, it binds to fibronectin along with laminin (basal membrane proteins) and collagen IV. During infection, damaged host cell walls may present adhesive glycoproteins for attachment by Lsa proteins (Ljungh et al., 1996).

OmpL1 is an outer membrane protein with a molecular mass of approximately 31 kDa and in line with other proposed virulence factors, is only present in pathogenic strains. It is considered to be a primary target of the host immune response (Haake et al., 1993). However no work to date has focused on the feasibility of targeting OmpL1 for either therapeutics or vaccination purposes.

Lipopolysaccharide (LPS) is a major component of the leptospiral outer membrane, as with most gram negative bacteria. Its role as a virulence factor was hypothesised and proven by Murray and colleagues (2010) following transposon mutations in two separate mutant strains of *L. interrogans* serovar Manilae. This result makes LPS the fourth *Leptospira* virulence factor to be identified (Murray et al., 2010). The mutants failed to cause acute infection or establish chronic infection in the kidneys of hamsters.

The role of LPS as a virulence factor is unique, as most pathogenic spirochetes (including *Treponema* & *Borrelia*) do not have LPS, opting for a majority of lipoproteins instead (Murray et al., 2010). In 2005, two spontaneous mutations in *L. interrogans* serovar Pomona were isolated. These mutants had an altered LPS size and the presence of an insertion sequence designated IS*1501*, suggesting that this insertion sequence may be capable of gene activation (Zuerner and Trueba, 2005).

Microarray studies investigated the effects of variables such as temperature, physiological osmolarity, serum presence and iron levels in the medium. From these, 14 genes were differentially expressed in at least three experiments and six were absent from *L. biflexa* (Adler et al., 2011). Any of these fourteen genes could potentially encode for virulence factors and further work would be required as to their role in pathogenesis.

1.7 Leptospirosis in the UK

Human infections are perceived as incidental in the UK, with humans being a dead-end host. Human to human transmission is not common due to an absence of direct contact between the urine of a shedding individual and another. Infection is typically likely to arise following contact with water sources contaminated with the urine of infected reservoir animals, or following direct contact with shedding animals.

Unfortunately, due to issues relating to both the diagnosing and reporting of clinical canine leptospirosis, there is currently very limited data on the present situation in the UK. Current information on suspected cases and the serovars causing infection would aid vaccination production and ultimately, protection for dogs. However, the same serovars can infect both humans and dogs and cause disease (potentially transmitted by dogs), so current human data may go some way to describe circulating serovars.

Reports for human cases in recent years have linked to both occupational and recreational infections with hazards of this nature. In 2009 the Health Protection Agency (HPA) reported 52 confirmed cases in England & Wales, with 38 being indigenous and 14 acquired abroad (HPA,

2013a). This figure was reduced in 2010, with 39 confirmed cases of which 17 were acquired abroad (HPA, 2011). Of the indigenous infections, only one case was presented in a female; however previous reports have identified no link between infection and gender (Alton et al., 2009). Studies showing a higher prevalence in males typically link an infection to occupational risks (Cowie and Bell, 2012). Infection rates remained at the same level in 2011, with 52 cases in the UK, with sixteen cases linked to recreational exposure and fourteen linked to occupational risks. The infecting serovar was only identified in 21 cases (HPA, 2013b).

Occupational risks are mostly linked to water exposure, with 36% infected from water exposure in 2010 (HPA, 2011). A further five cases were a result of direct handling of rodents, or material that rodents could have contaminated. Both routes of transmission are also important with regards to canine infections. Veterinary surgeons are at risk when examining potentially infected companion animals and physical barriers such as gloves are highly recommended. A greater emphasis on a patient's occupation and history when clinical signs related to early stage leptospirosis are presented would improve the accuracy and efficiency for a diagnosis.

Water sports can be a key risk factor for contracting the disease. A recent high profile case in the UK was seen when Andy Holmes, a canoeist, became infected and subsequently died in October 2011. Since then, another outbreak was reported when five individuals contracted the disease on the River Itchen, Southampton. Five cases were suspected with one lab confirmed by the HPA.

Climate plays a role in *Leptospira* infection rates, with temperate climates not having extreme weather situations that may contribute to infection rates. According to the Köppen climate classification, the UK has a rating of Cfb, meaning cooler summers but also milder winters. The classification reflects the milder climate changes between seasons which reduce the likelihood of leptospirosis.

1.7.1 Epidemiology of leptospirosis outside the UK

Humans and canines within a large proportion of countries (including the UK) are most commonly infected with serovars Canicola and Icterohaemorrhagiae (HPA, 2012; van den Broek et al., 1991). However infection arising from other serovars is dependent on serovars in circulation within a region. An example being that infections from *L. interrogans* serovars Pyrogenes and Tarassovi, two serovars not commonly witnessed have been identified in dogs in Chiapas, Southern Mexico (Jimenez-Coello et al., 2010).

Several outbreaks that have been linked to canines include North Dakota 1950, Texas 1971, Portland and St Louis 1972, Barbados 1988 (Levett, 2001) and Nicaragua 1995 (Trevejo et al., 1998). Recent vaccinations, improved sanitation and public health awareness have meant that canine-related outbreaks have decreased over the past 20 years. However one conflicting study presented an increasing trend of cases in Ontario, Canada, between 1998-2006 (Alton et al., 2009).

The United States and Canada showed a decline in human cases between 1970 and 1982, however the following 15 years between 1983 and 1998 presented an increase (Ward et al., 2002). A follow up study concluded that the greater level of annual rainfall had a positive effect on the number of cases (Ward, 2002).

There are 50-60 annual cases of human leptospirosis in England and Wales (HPA), giving an average incidence rate of 0.1 per 100,000. Incidence rates were also relatively low for the rest of Europe between 2000 and 2008. Croatia had the greatest, averaging 1.56 (cases per 100,000) with the lowest in Romania at 1.47. Latvia witnessed a high level in the early 1990's at 5.45 but has declined each year to 0.13 in 2008 (WHO).

A seasonal pattern is seen in Eastern Europe with most infections occurring between July and November (Roczek et al., 2008). A study in Germany from 1962-2003 showed the median number of cases to be 59 in a year, however cases were on the decline over the 41 year period (Jansen et al., 2005).

Leptospirosis is more commonly associated with a recreational risk within countries where higher *Leptospira* infection rates are not endemic. Recent recreational outbreaks include an endurance length swamp race in Florida 2005 (Stern et al., 2010), white water rafters in Costa Rica 1996 ((CDC), 1997) and triathlons in Wisconsin and Illinois 1997 ((CDC), 1998).

Occupational risks are also evident outside of the UK. A survey of both non-veterinary and veterinary students within Trinidad and Tobago showed a higher percentage of vet students were positive for antibodies towards *Leptospira* (James et al., 2013). Whilst a serosurvey in the USA outlined the degree of danger direct canine contact poses to vets (Whitney et al., 2009). A recent case report showed an incidental case of human leptospirosis from *L. interrogans* in a fish market worker in Tokyo. Infection was contracted as a result from cleaning out the sewage system whilst having an exposed cut on his hand. Following a patient interview it was revealed that the sewers were host to many rats which were the presumed source (Kokudo et al., 2009), the serovars present where not investigated.

Travel associated infections are also common, with tourists not knowing the risks associated with the disease. Two cases in Venice were reported following Australian tourists submerging themselves in contaminated waters. Rising MAT titres were reported for serogroup Icterohaemorrhagiae, along with a positive urine PCR result, demonstrating shedding of the bacteria via urine (Lagi et al., 2013).

Reservoir hosts within a region can also determine which serovars are maintained. Some serovars are relatively specific, whereas others are known to infect several different hosts. This can be demonstrated in Spain, where a common serovar (Icterohaemorrhagiae) has known to infect a range of hosts from feral cats to the mongoose (Millan et al., 2009).

Two communities that still practice traditional survival and hunting techniques in Canada were investigated for the presence of *Leptospira* antibodies. Within the 250 patients examined, 23% were positive for leptospiral antibodies, the highest out of 10 zoonotic bacteria investigated (Campagna et al., 2011). Of the 58 positive cases, only 4 had documented any symptoms commonly associated with the disease.

Heavy flooding is commonly associated with outbreaks of leptospirosis, particularly in climates with extreme seasonal changes. One example was shown during a large outbreak in Nicaragua during October-November 1995 following a period of heavy flooding. It was assumed that infection was with the serovar Canicola as 60% of the dogs tested were confirmed to be infected (despite the small sample size); however there was no associated jaundice (Trevejo et al., 1998). In 2010, 33 cases were reported in the 8 weeks leading up to the end of September. Outbreaks such as this are not uncommon in the region during the rainy season (ProMED-mail, 2010).

Co-infections with other endemic bacteria are not common, however they can occur, and to what extent either facilitates infection from the other is not known. India had an outbreak of leptospirosis and dengue fever occurring within the same area in 2002. Neither leptospirosis nor dengue occurred in the same host but malaria was also witnessed in a small number of cases alongside either disease (Leptospirosis = 3.33% and Dengue = 3.33%) (Karande et al., 2005). A further outbreak occurred in 2010, with 77 dengue cases and 22 leptospirosis cases. However, there were only two cases where both bacteria infected the same individual (Zaki and Shanbag, 2010). While this is not evidence to either prove or disprove any methods of co-infection it certainly suggests that dengue and leptospirosis are not likely to facilitate each other's infection. Additional evidence identified a co-infection in three further cases following clinical signs being compared to those seen in 18 previous patients with the same infection

(Gurjar et al., 2011). A conflicting study in 2010 investigated the presence of *Leptospira* in children submitted to hospital in Mumbai during an outbreak of both leptospirosis and dengue fever. No cross-over or co-infection was subsequently reported on a significant scale in this instance (Zaki and Shanbag, 2010).

A historical review looked into an epidemic within Native Americans in 1616-1619. Symptoms and witness reports from the time suggest a possible link towards leptospirosis, due to jaundice being a key symptom. Previous work investigating the same outbreak suggests alternate diseases such as the bubonic plague, smallpox and influenza which have all been discounted, with the exception of influenza. Living conditions and surroundings are ideal for *Leptospira* survival, such as black rat presence, close living and working contact to soil and opportunistic routes of entry into the body (Marr and Cathey, 2010).

Crowded areas close to bodies of water are at a greater risk from zoonotic infections, particularly those prone to flooding (Agudelo-Florez et al., 2009). Developing countries living in poor sanitation and housing also have higher zoonotic risks from an increased exposure to rodents and other host reservoirs. As a contrast, it has been suggested that regions experiencing a drought or dry season could also encounter a rise in cases as bodies of water reduce, potentially concentrating any bacteria within them. This decrease can also increase the chance of coming into contact with the bacteria as rodents, animals and humans may take from the same source (Gubler et al., 2001).

With regards to animal incidental hosts that aren't companion or farm animals, Korea witnessed *Leptospira* infections in horses and ponies bred for racing (Jung et al., 2010). Due to limited or no interaction with other horses, environmental exposure to reservoir hosts such as rodents have been hypothesised as the main risk factor. This is not an isolated situation, with horse infections also being prominent in Poland, with 620/1588 (39%) showing a positive MAT titre. Serogroups Grippotyphosa and Sejroe where found as the most common causative serovars (Arent and Kedzierska-Mieszkowska, 2013), which are also known to cause disease in both canines and humans.

1.8 Aims of this thesis

The overall aim of this thesis was to investigate the presence and diversity of *Leptospira* currently circulating within the UK. Further to this, expansions on molecular methods for detection were explored for an accurate identification method. Potential virulence traits were also considered from full genome sequencing of a panel of strains across multiple species. In more detail, the major aims of the project were as follows:

- Assess the prevalence of *Leptospira* species in the UK vet-visiting dog population
- Assess the environmental prevalence of *Leptospira* species pathogenic to dogs and humans.
- Development of a sensitive and specific assay for the detection of pathogenic *Leptospira* species from environmental and canine samples
- Assess molecular markers for virulence by full genome analysis of representative *Leptospira* species

Chapter Two

Materials and Methods

2. Materials & Methods

This chapter detailing general materials and methods is divided into four sections. The first outlines the process for practice selection and design during the questionnaire study. The second outlines the general methodology for molecular studies, including DNA extraction from both field samples and cultures, and subsequent PCR assays. Section three describes serological testing and histopathology staining. The final section describes the process and analysis for full genome sequencing of *Leptospira* strains. Further detailed protocols are available within relevant chapters.

2.1 Questionnaire design and protocol

A mail shot questionnaire was designed and sent to 419 vet practices within the UK. The UK was stratified into 23 regions and practices were randomly selected from each region. One member of staff at each practice was used for correspondence in attempt to increase the likelihood of return. An information sheet outlining the study and providing information regarding the purpose was included also (Appendix One). Participants were informed of their right to withdraw from the study at any point should they wish. Due to the nature of the study, the questionnaire itself was kept relatively short to increase compliance.

Initially, questions were asked about the practice itself and covered practice size and vaccination habits (Appendix One). The second part enquired for details regarding the last date a case of either suspected or confirmed leptospirosis was seen by the practice. If a case had presented to the practice within the last 12 months we then asked for further details. This included the age and breed of the dog, vaccination status and the outcome.

2.2 Molecular identification of infecting *Leptospira* strains

2.2.1 Maintenance and DNA extraction for cultured strains

Two control strains (*L. interrogans* serovar Australis & *L. kirschneri* serovar Grippotyphosa) were maintained on commercial EMJH media provided by MSD. Direct DNA extractions on low passage cultured strains was carried out using the DNeasy® Blood and Tissue (Qiagen, Crawley, UK) kit according to manufacturer's instructions. The success of each extraction was subsequently confirmed using the duplex PCR assay as described below.

Extraction of genomic DNA from ten strains (*L. interrogans* serovar Icterohaemorrhagiae, *L. interrogans* serovar Bratislava, *L. interrogans* serovar Pomona, *L. interrogans* serovar Prajitno, *Leptospira alexanderi* serovar Manzhuang, *L. borgpetersenii* serovar Hardjo-bovis,

Leptospira kirschneri serovar Grippotyphosa, *Leptospira weilii* serovar Mengding, *Leptospira fainei* serovar Hurstbridge and *L. biflexa* serovar Andamana) for full genome sequencing was carried out with the Wizard Genomic DNA Kit (Promega, Southampton, UK) according to manufacturer's instructions. A total of 5ml of live culture were centrifuged at 16,000g for five minutes. The resulting pellet was then used for the DNA extraction. Ethanol precipitation ensured no salt contamination in the final extraction. Quantification of the DNA yield was determined via the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Paisley, UK) according to manufacturer's instructions.

2.2.2 DNA Extraction from rodent kidney tissue

From obtained field samples, roughly 1g of kidney tissue was used for each extraction. The DNeasy® Blood and Tissue (Qiagen, Crawley, UK) kit was used according to manufacturer's instructions. Incubated protease digestion was extended depending on size of the tissue used. Each extraction yielded a total of 60µl in elution buffer. Following elution, DNA was split into 10µl aliquots and stored at -80^{o}C until required. This removed potential DNA degradation following repeated freeze-thawing.

2.2.3 Duplex PCR assay for detecting *Leptospira*

Two sets of primers from previously published studies were utilised in a duplex conventional PCR assay throughout the whole of this project. This assay targeted two gene candidates to detect the presence of pathogenic *Leptospira* and allowed for direct sequencing of amplicons, identifying the species of infecting strains.

Gene Target	Primer	5' to 3' Sequence	Size (bp)	Reference
rrs	*rrs*F *rrs*R	GGAACTGAGACACGGTCCAT GCCTCAGCGTCAGTTTTAGG	430	Tansuphasiri et al., 2006
gyrB	2For 504Rev	TGAGCCAAGAAGAAACAAGCTACA MATGGTTCCRCTTTCCGAAGA	502	Slack et al., 2006

Table 2.1. Primers used for the duplex PCR assay to detect the presence of *Leptospira*

A total reaction volume of 25µl including ReddyMix PCR Master Mix (Thermo Scientific, Asheville, United States) containing 1.3 units of ThermoPrime *Taq* DNA polymerase, 150mM Tris-HCl, 40mM $(NH_4)_2SO_4$, 3mM $MgCl_2$, 0.01% (v/v) of Tween® 20 and 0.4mM each of dATP,

dCTP, dGTP and dTTP with 20pmol of each primer (Eurofins MWG Operon, Acton, UK) and 1µl of sample DNA was used per reaction.

Cycle conditions for the assay were as follows; 94°C for 15 seconds, followed by 35 cycles of 94°C for 10 seconds, 50°C for 20 seconds, 72°C for 40 seconds and a final extension step of 72°C for 5 minutes. Strains belonging to pathogenic species demonstrated an amplified band for both primers, whereas saprophytic species only amplify the *rrs* gene.

2.2.4 *Leptospira* MLST PCR assay

Following the success of applying MLST to *Leptospira* (Boonsilp et al., 2013; Thaipadungpanit et al., 2007), the scheme was applied to field samples obtained in this study. Separate PCR assays were performed for each locus in a 50µl reaction volume using ReddyMix PCR Master Mix (as described in 2.2.3). Due to the larger reaction volume, 2µl of sample DNA was included.

Primers included with the updated scheme were as described in table 2.2. The MLST PCR was carried out under the following cycle conditions: Initial denaturing at 95°C for 2 minutes, followed by 30 cycles of denaturing at 95°C for 10 seconds, annealing at 46°C for 15 seconds and extension at 72°C for 30 seconds with a final extension of 72°C for 7 minutes.

Gene	Primer	Sequence (5' - 3')	Amplicon Size (bp)
pntA	Forward	TAGGAAARATGAAACCRGGAAC	621
	Reverse	AAGAAGCAAGATCCACAAYTAC	
sucA	Forward	TCATTCCACTTYTAGATACGAT	640
	Reverse	TCTTTTTTGAATTTTTGACG	
pfkB	Forward	CGGAGAGTTTTATAARAAGGACAT	588
	Reverse	AGAACACCCGCCGCAAAACAAT	
tpiA	Forward	TTGCAGGAAACTGGAAAATGAAT	639
	Reverse	GTTTTACRGAACCHCCGTAGAGAAT	
mreA	Forward	GGCTCGCTCTYGACGGAAA	719
	Reverse	TCCRTAACTCATAAAMGACAAAGG	
glmU	Forward	AGGATAAGGTCGCTGTGGTA	650
	Reverse	AGTTTTTTTCCGGAGTTTCT	
caiB	Forward	CAACTTGCGGAYATAGGAGGAG	650
	Reverse	ATTATGTTCCCCGTGAYTCG	

Table 2.2. Primer pairs utilised for the seven locus scheme, with expected amplicon sizes (Boonsilp et al., 2013).

2.2.5 Variable number tandem repeat (VNTR) PCR assay

A recently published VNTR protocol was investigated for its use in determining the serovars infecting wild rodents for this project (Salaun et al., 2006). Two positive controls were initially included to test the viability of the scheme (*L. interrogans* serovar Australis and *L. kirschneri* serovar Grippotyphosa).

Each locus was assayed individually using primers described in table 2.3. As described above in 2.2.4, reactions were carried out in a 50µl volume. Cycle conditions for all loci were as follows: 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and a final elongation step at 72°C for 10 minutes.

Amplified products were purified and sent for direct sequencing as described below to identify associated alleles.

Primer	5' to 3' Sequence	Size (bp)		
		Leptospira interrogans	*Leptospira kirschneri*	*Leptospira borgpetersenii*
VNTR4-F	AAGTAAAAGCGCTCCCAAGA	425+34n	425+34n	425+34n
VNTR4-R	ATAAAGGAAGCTCGGCGTTT			
VNTR7-F	GATGATCCCAGAGAGTACCG	299+46n	299+46n	No Product
VNTR7-R	TCCCTCCACAGGTTGTCTTG			
VNTR10-F	GAGTTCAGAAGAGACAAAAGC	420+46n	347+46n	333+46n
VNTR10-R	ACGTATCTTCATATTCTTTGCG			
VNTR-Lb4 F	AAGAAGATGATGGTAGAGACG	No Product	No Product	573+60n
VNTR-Lb4 R	ATTGCGAAACCAGATTTCCAC			
VNTR-Lb5 F	AGCGAGTTCGCCTACTTGC	668+39n	668+39n	722+36n
VNTR-Lb5 R	ATAAGACGATCAAGGAAACG			

Table 2.3. Primers used for the VNTR analysis to identify the serovar from infecting strains (Salaun et al., 2006).

2.2.6 Agarose gel electrophoresis

All PCR assay products were separated and analysed on a 1.5% agarose gel (Alpha Laboratories, Hampshire, UK) containing ethidium bromide, or RedSafe® (iNtRON Biotechnology) when available, and 1x Tris-acetate buffer (Alpha Laboratories). Given the size of expected amplicons, ΦX174 DNA/BsuRI (HaeIII) (Thermo Scientific) was utilised as the DNA

marker. During the project, agarose gels were run at 120V, with time dependant on the gel size.

2.2.7 Purification of PCR amplicons

All PCR products were purified using the commercial QIAquick® PCR purification kit (Qiagen) according to manufacturer's instructions. The purified DNA was quantified on a Nano-drop 1000 spectrophotometer (Thermo Scientific) for a concentration estimate.

2.2.8 Sequencing of PCR products

Following quantification, products were diluted to 100µg/µl/100bp. Sanger sequencing was carried out commercially (Source Bioscience Ltd, Nottingham, UK). Both forward and reverse reads were sequenced to allow for construction of a consensus sequence of each reaction. Primers initially used for each assay were also used for all amplicon sequencing.

2.2.9 Downstream analysis of sequenced amplicons

Following commercial sequencing of amplicons (Source Bioscience), signal qualities of reads were analysed using ChromasPRO v1.7.3 (http://technelysium.com.au). The presence of background interference was also checked for, with poor quality sequences re-sequenced to ensure high quality output.

Consensus alignments between sequences for the same gene were carried out using MEGA5 were possible (Tamura et al., 2011). Both forward and reverse sequences were aligned for each sample to ensure high compliance consensus sequences to reduce any error potentials. For ambiguous base calls, degenerate IUB codes were included. Maximum likelihood analysis to infer evolutionary trees were constructed using default settings with 1000 bootstrapping re-sampling in MEGA5.

BLAST analysis was conducted on all sequencing results deemed of a high enough quality. Briefly, sequence data was trimmed to remove primer sites and compared against the National Center for Biotechnology Information (NCBI) database. This allowed for species identification from the *rrs* and *gyrB* assay products.

MEGA5 was also use to aid in the analysis of MLST sequence reads. Alignment to reference alleles for each locus allowed for trimming to the correct size. Allele data for each query locus was then obtained from the online MLST database.

2.3 Serological testing and histopathology

Serology testing of samples was personally carried out at the AHVLA (Woking, Surrey), using their facilities and stock antigen panels. Histopathology staining was done by the histopathology department at the University of Liverpool.

2.3.1 Multiple pooled serovar antigens

Serum samples obtained from rodents were tested using the microscopic agglutination test (MAT). The MAT is a previously well documented method for detecting circulating leptospiral antibodies and is widely regarded as the gold standard for comparing diagnostic methodology (Bajani et al., 2003). For pooled antigens, 5ml of live culture for each strain were mixed. 25µl of each serum sample were diluted in saline to obtain 100µl total serum. In a 96-well plate, 25µl of pooled antigen was added to each well. On top of that, 25µl of sample was added individually followed by a further 25µl of saline. Plates were incubated at 30^{o}C for two hours and analysed under dark field microscopy at 400x magnification. Each sample was observed for the presence of 50% agglutination against a black background.

2.3.2 Single serovar titrations

To identify single serovar titres, each antigen from a positive pooled result was tested for on individual plates. 25µl of saline was added to lanes 2-11 on a 96-well plate and 25µl of the 1:50 sample dilution was added into lanes 1 and 2. From lane 2, 25µl was taken and serial diluted down to lane 10 to give a final titration of 1/12800. Lane 11 was left as a negative control with no antigen or sample. Antigen was added (25µl) to lanes 1-10 and each plate was incubated for two hours at 30^{o}C. Plates were read on a dark field microscope starting at 1/25 and working across the titration gradient if agglutination was witnessed until a negative result was seen. The lowest titration that showed more than 50% agglutination against a black background was confirmed as the titre result for that sample.

2.3.3 Histopathology testing of formalin fixed rodent kidney tissue

During dissection, one kidney from each rodent was stored in 1% formalin. Sections from each sample were sent for staining at the histopathology department at the University of Liverpool. Two stains were used to analyse each kidney section during this study. The hematoxylin and eosin (H&E) stain was applied to identify the presence of inflammation and any morphological changes resulting from infection. A second stain that has previously been used to visualise

leptospires in tissue, the Warthin-Starry silver stain, was also applied for a visualisation of the bacteria.

2.4 Full genome sequencing of *Leptospira* strains

The final aim of the project involved the sequencing and analysis of *Leptospira*, with a view to identify potential virulence factors. Following extraction and quantification, next generation sequencing was carried out on ten strains using the high-throughput MiSeq platform (Illumina, UK) by colleagues at the Centre for Genomic Research (CGR) (Liverpool, UK).

2.4.1 Analysis of sequenced genomes

After successful sequencing of each strain, paired end reads from each strain were constructed into contig predictions. Further *de novo* pipeline work combined the files into a draft genome. The Prokaryotic Genome Annotation System (Prokka) (http://vicbioinformatics.com/) was used to annotate the genomes. BLAST (Basic Local Alignment Search Tool) analysis to confirm annotations and to derive potential matches for hypothetical proteins was used. GATK (Genome Analysis Toolkit) detected single nucleotide variations (SNPs) between sequenced strains and closely related reference strains, allowing for potential virulence genes to be identified. Genbank files were uploaded for annotation by RAST (Rapid Annotation using Subsystem Technology) to compare functional differences within each strain. CMG-tools (Comparative microbial genomics tools) allowed for the calculation of both the total number of unique genes between all sequenced strains and the total number of shared genes to derive core genomes (Vesth et al., 2013).

Chapter Three

Investigating suspected or confirmed *Leptospira* cases in the UK vet visiting dog population

3.1 Introduction

Canine leptospirosis is believed to be under-diagnosed due to non-specific symptoms (Sarkar et al., 2012), including within the UK (Forbes et al., 2012). Leptospirosis infections in canines can be classified as anicteric (before jaundice) or icteric (jaundice). Primary clinical signs observed during the anicteric stage are common within other diseases; including vomiting, diarrhoea, malaise and fever. The majority of cases will present anicteric symptoms only, with the minority (5-10%) of cases continuing into the icteric, jaundice stage where uraemia from renal failure and hepatic haemorrhaging also occur. Haemorrhagic gastroenteritidis (Stuttgart disease) may also develop following fever and has been linked to infection by the serovar Canicola (Andre-Fontaine and Ganiere, 1990).

Survival rates in dogs contracting the disease have been previously reported at around 78% (Adin and Cowgill, 2000; Goldstein et al., 2006), however no figures on survival are currently available for the UK. Pathogenic serovars associated with canine leptospirosis include Copenhageni, Icterohaemorrhagiae and Canicola (Millan et al., 2009; Roach et al., 2010; Suepaul et al., 2010). However this is dependent on the endemic serovars within a country, as pathogenic serovars are capable of causing disease in other animal species given the opportunity.

There are no current reports of canine leptospirosis case numbers in the UK. However, the HPA reports an annual incidence in humans of 50-60 cases per year, equating to an incidence rate of 0.1 per 100,000 (HPA, 2012).

In the UK, diagnosis of canine leptospirosis is routinely carried out using either the microscopic agglutination test (MAT) (Faine S., 1999) or the immunofluorescent antibody (IFA) test (Appassakij et al., 1995; Naigowit et al., 2000; Torten et al., 1966). In most diagnostic labs, a MAT titre is regarded as positive at dilutions above 1:100. A second blood serum sample is recommended to be taken 3-5 days following the initial sample. A four-fold titre increase in the second sample confirms a positive MAT titre.

There are a number of canine vaccines currently licensed in the UK which protect against two serogroups, Canicola and Icterohaemorrhagiae (and specifically serovars Canicola and Icterohaemorrhagiae respectively); however it is believed to provide cross-protection to other serovars within these serogroups. A level of protection between antigenically similar serogroups has been previously documented (Plesko and Lataste-Dorolle, 1970; Sonrier et al., 2000) however currently a vaccine can only claim protection against the serogroups

administered. Details for a tetravalent vaccine in Europe were recently published to protect against challenge from additional serogroups Australis and Grippotyphosa (Klaasen et al., 2013). The extent of vaccination coverage in UK dogs also remains unexplored, including whether booster vaccinations are administered annually to maintain an up-to-date vaccination and therefore sufficient immunity from strain challenges.

The vaccine itself targets the lipopolysaccharide (LPS) which determines the variability of serovars and causes the very limited level of cross-protection amongst different serovars. Prevention remains one of the greatest defences against infection, and creating a physical barrier when in contact with potential shedders of the bacteria is highly recommended. Previous cases have been observed in clinicians following handling of animals actively shedding leptospires without wearing gloves (Baer et al., 2010).

According to the World Small Animal Veterinary Association (WSAVA) guidelines, leptospirosis is not considered a 'core' vaccine (unlike the vaccines for parvovirus, parainfluenza virus, canine distemper and infectious hepatitis) (VGG, 2010). It is however, recommended to be administered annually to maintain protection against this disease. It has been determined that the duration of immunity for the bivalent vaccine covered between 12-14 months, following that the level of protection declined (Klaasen et al., 2003; Minke et al., 2009).

Human leptospirosis is primarily a recreational and occupational disease in the UK. From the 22 human laboratory confirmed cases in 2010 that were acquired domestically, eighteen were from direct contact with animals or water (HPA, 2011). The same threat is present to canines. Dogs living in close vicinity, or with more regular contact with, water or woodland are at a higher risk of contracting leptospirosis (Meeyam et al., 2006; Raghavan et al., 2012; Ward et al., 2004), a similar situation as seen in humans (Tangkanakul et al., 2000). Including this information alongside clinical signs can aid in a more rapid and accurate diagnosis. Vaccination history should also be taken into consideration with all potential cases. A recent vaccination can affect a MAT titre result if testing for the same serogroups within the vaccine and give a false-positive result (Midence et al., 2012).

The aim of this work was to investigate the perceived and confirmed number of canine leptospirosis cases within UK vet visiting dogs, by surveying small animal vets. The level of up-to-date (within 12 months) vaccinations within their practice was also investigated alongside the clinical signs that veterinarians associate with the disease for a diagnosis. Information on both suspected and confirmed cases was collected from targeted practices over the previous 12 months.

3.2 Materials and Methods

3.2.1 Practice selection

A questionnaire survey was conducted across the full length of the UK. To ensure full coverage, practices in the UK were stratified into 23 regions and 15 practices were randomly chosen from each region. The only inclusion criteria included the practice residing within a UK postcode. Practices both with and without Royal College of Veterinary Surgeons (RCVS) Practice Standards Scheme (PSS) accreditation were included for the study in accordance with the randomised selection. This selection process allowed for an unbiased approach for full coverage of all areas within the UK.

In total, 472 practices were targeted. This enabled good coverage of UK practices across different environment types (rural, semi-rural and urban).

3.2.2 Questionnaire design

A two page questionnaire was designed as a mail shot approach to a large number of practices. In order to keep compliance as high as possible, the questionnaire was kept to only the necessary questions to obtain the information needed. An additional information sheet was included outlining the purpose of the study and also the overall aim of what was hoped to be achieved (Appendix One).

To improve compliance and enable practices to reply as easily as possible, methods employed for returning questionnaires included freepost envelopes, email and a fax number. Alongside this, an article was published in the Veterinary Record prior to the first questionnaires being sent out (Ball et al., 2011).

Practices were asked to return the questionnaire even if they had not seen a case either in the last 12 months or at all. Full consent for the study was granted by the University of Liverpool ethics committee.

3.2.3 Study protocol

Initially 239 practices were targeted. However due to the low compliance rates, an additional number of practices were contacted, bringing the total to 472.

To determine the size of the practice and how much time was dedicated to small animal practice, participants were asked questions covering their practice type and the number of small animal vets employed in their practice, including part time staff. Information was also

asked to determine the number of dogs seen in the practice per day. This information could then be used for a comparison between the practice size and number of small animal vets. This information can be used to test two hypotheses. The first being that the more vets employed at a practice (and so the larger the practice is), the more dogs will be seen per day. Following on from this, the second hypothesis is that the more dogs a practice receives per day, the greater the likelihood is of reporting a leptospirosis infection.

Vaccination status within the vet visiting dog population was determined. Firstly, participants were asked the total number of vaccination doses administered over the previous 12 months. Secondly, they were also asked to estimate the percentage of dogs that were up to date (within the last 12 months) with a leptospirosis vaccine. Using this data we can test the hypothesis that as dogs seen per day increase in a practice, the amount of vaccine doses administered also increases.

This study also aimed to determine the number of suspected or confirmed leptospirosis cases that were witnessed within UK practices. Participants were asked when their practice last saw a case of leptospirosis, either suspected or laboratory confirmed, and also information pertaining to cases seen within the last 12 months. If no cases had been seen over 12 months this then provides information detailing the point at which the last suspected or confirmed leptospirosis case was observed at a practice. If a practice had seen a case within the last 12 months, clinicians were then asked for further details regarding the age, breed, vaccination status, clinical outcome and if the case was laboratory confirmed.

The final section asked participants what clinical signs they looked for when making a diagnosis of leptospirosis. As the anicteric symptoms are vague in most cases presenting to a vet, determining what signs they look for would give an indication of the level of awareness when making a differential diagnosis for a case. It would also indicate if vets look more for the symptoms related during the icteric stage – where treatment intervention may have a limited effect due to the late stage of the disease.

As a follow up, practices that reported lab confirmed case details within the last 12 months were contacted for further details. Practices that had opted out on the initial response were not contacted. Information was then collected concerning the diagnostic test carried out and which serogroup/serovar, if any, was reported.

3.2.4 Response inclusion criteria

All responses received either by mail, email or fax either during or after the 12 week study were included if they fell within the inclusion criteria. Responses that contained ≤3 blank fields (not including case details) would be included, however responses containing >3 blank fields would be excluded.

3.2.5 Correlation analysis of responding practices

SPSS Statistics 20 (IBM) was utilised for analysing correlation of variables obtained through the questionnaire responses. R^2 values were obtained for comparisons of dogs seen per day against practice size and vaccine doses administered.

3.3 Results

3.3.1 Total Questionnaire Responses

From the combined total of 472 questionnaires sent out, a final total of 89 were returned completed over the 12 weeks that the study ran for, with a compliance rate of 18.86% for the study. In total three questionnaires were returned due to the practice no longer being at the address. These were excluded from any further part in the study.

All responses were divided into 3 distinct groups based on the presence or absence of a leptospirosis case at the practice. Group 1 = Leptospirosis case in the last 12 months (n=13), Group 2 = Leptospirosis case > 12 Months (n=29), Group 3 = No reported leptospirosis cases at all (n=45).

Out of the 89 replies in the study, 13 (14.61%) reported either a suspected or laboratory confirmed *Leptospira* case within the last 12 months (Table 3.1). However, only five of the practices had their suspected cases confirmed by a laboratory, with the remaining eight either not sending a sample (single or paired) or having the result back as negative. Only one practice reported a confirmed case of leptospirosis in a canine with an up to date vaccination.

Practice Reference	Lab Confirmed		Up to date Vaccination		Age (Years)	Breed	Outcome
	Yes	*No*	*Yes*	*No*			
1004	+			+	N/A[1]	Yorkie Cross-breed	Died
1007	+			+	5	Australian Cattle Dog	Cured
1009	+		+		2	Springer Spaniel	Cured
1014		+		+	5	Cross-breed	Cured
1018		+		+	N/A[1]	Working Labrador	Cured
1027		+		+	4	Border Collie	Died
1029		+		+	5	Springer Spaniel	Died
1129	+			+	3	Old English Sheep Dog	Cured
1315		+		+	8	Staffordshire Bull Terrier	Died
2021		+		+	6	Huntaway	Died
2146		+		+	7	Boxer	Died
2166	+			+	0.4	Labrador	Died
2171		+		+	10	Jack Russell	Died

Table 3.1 Case details for the 13 practices reporting a case in the last 12 months. [1]N/A = Information not given.

A further 29 practices reported having seen a case within the last 15 years. No practice in the study reported seeing two or more suspected (or confirmed) cases in the 12 previous months.

From the study, no apparent age or breed of dog leading to greater susceptibility to contracting the disease was determined.

The final outcome of each case was asked for. The majority of the cases (n=8/13) ended with the dog either dying or being put to sleep.

Information concerning the practice itself was also collected (Table 3.2). The majority of the practices (n=8/13) specialised in small animal practice. The remaining five were mixed. The mean number of small animal vets at the practices worked out to be 4.125, slightly higher than the amount employed at the mixed practices ($n \geq 4$).

The amount of dogs seen per day does not significantly correlate to the number of small animal vets at a practice ($R^2 = 0.282$). Despite that however, there is a degree of positive correlation between the two variables. The figures for dogs per day per vet varied to a great degree. Overall, the average was calculated to be 7.1 dogs per day per vet.

Comparing the number of dogs seen per day to the reporting of a case can determine whether or not bigger and busier practices are more likely to see a leptospirosis case. In this study, practices that reported seeing no cases saw a higher number of dogs per day compared to practices that did (40.22 compared to 30.21).

The number of annual vaccine doses administered differed to a large degree between all practices, ranging from 473 to 3600. The mean number of vaccine doses administered between practices witnessing a leptospirosis case was 1668.38. Mixed practices reported a higher number of doses with an average of 1692.4 compared to 1653.38 in dedicated small animal practices. Although there is a positive trend between the variables, no significant correlation was witnessed between vaccine doses administered and dogs seen per day at a practice ($R^2 = 0.195$).

The mean reported level of vaccinated dogs at practices observing a case was lower than the overall average (60.37%) at 58.77%. It was also lower than the practices that hadn't seen a case in the last 12 months which averaged out at 60.67%.

Practice Reference	Practice Type	Small Animal Vets[1]	Dogs seen per Day	Vaccine Doses administered over past 12 months	Percentage of dogs registered at the practice with an up to date vaccination (%)	Last *Leptospira* Case - Suspected or Lab Confirmed
1004	Small Animal	2	30	1200	30	Confirmed
1007	Small Animal	5	40	500	70	Confirmed
1009	Small Animal	N/A[2]	6	3136	55	Confirmed
1014	Small Animal	4	40	960	56	Suspected
1018	Mixed	9	40	3000	50	Suspected
1027	Mixed	4	35	900	65	Suspected
1029	Mixed	4	8	775	60	Suspected
1129	Small Animal	2.5	12	884	80	Confirmed
1315	Small Animal	3	50	3600	30	Suspected
2021	Mixed	6	65	2800	53	Suspected
2146	Small Animal	3	30	2474	60	Suspected
2166	Small Animal	1	10	473	85	Confirmed
2171	Mixed	6	15	987	70	Suspected

Table 3.2 Practice details for the 13 practices reporting a case in the last 12 months. [1]Includes part time vets at 0.5. [2]Information not given

Practices reporting a case within the last 12 months were analysed based on their location in the UK to explore any potential hot-spots or environmental conditions were leptospirosis is more common in dogs. Due to the low number of respondents, no definite area of the UK was identified as having a higher risk for the disease. There was also no apparent prominent environment type based on the practice locations. Six of the practices were from urban areas, whereas the other seven came from rural or semi-rural practices.

3.3.2 Practices reporting a *Leptospira* case later than 12 months

From the 89 responses, 29 (32.58%) reported their practice having seen a *Leptospira* case (Table 3.4) at a date later than the previous 12 months. The total number of practices reporting seeing either a suspected or confirmed leptospirosis case was 42 (47.19%) (Table 3.3).

Time before Study	Suspected	Lab Confirmed	Total
≤ 12 Months	8	5	13
> 12 Months	18	11	29
Total	26	16	42

Table 3.3 Total number of suspected and lab confirmed cases for all practices reporting a *Leptospira* case

Practice Reference	Practice Type	Small Animal Vets[1]	Dogs seen per Day	Vaccine Doses administered over past 12 months	Up to Date Vaccination (%)	Last *Leptospira* Case - Suspected or Lab Confirmed
1005	Mixed	2	30	N/A[2]	60	Suspected
1010	Small Animal	4	30	843	50	Suspected
1011	Small Animal	3	25	1000	20	Suspected
1015	Small Animal	4	25	600	45	Suspected
1016	Small Animal	4	20	1200	70	Confirmed
1017	Small Animal	6	34	900	76	Confirmed
1019	Small Animal	6	60	5000	73	Suspected
1022	Mixed	2.5	20	1800	0	Confirmed
1024	Mixed	6	15	1138	90	Confirmed
1028	Small Animal	2	25	846	75	Suspected
1108	Mixed	6	60	1940	80	Suspected
1135	Small Animal	2	15	480	75	Suspected
1170	Mixed	4	25	2400	30	Suspected
1171	Small Animal	4	27	1931	51	Suspected
1214	Mixed	5	25	1	90	Suspected
1299	Small Animal	2	20	250	70	Confirmed
1310	Small Animal	8	80	3600	80	Suspected
2003	Small Animal	11	10	605	45	Suspected
2047	Small Animal	3	20	800	70	Suspected
2055	Small Animal	1	15	835	N/A[2]	Confirmed
2080	Mixed	3	30	1350	80	Confirmed
2084	Small Animal	3	30	240	75	Suspected
2122	Mixed	7	45	2245	75	Confirmed
2150	Small Animal	3	20	1600	85	Suspected
2151	Small Animal	8	60	3000	50	Suspected
2175	Small Animal	5	50	2886	20	Confirmed
2187	Small Animal	5.5	25	3000	50	Confirmed
2196	Small Animal	4	35	1500	50	Confirmed
2215	Small Animal	1	12	300	90	Suspected

Table 3.4 Practices reporting a leptospirosis case > 12 Months before the study. [1]Includes part time vets at 0.5. [2]Information not given

The majority (n=22) of the 29 practices reported a case within 5 years of the study, with the earliest reported suspected case in 1985.

On average, there were 30.6 dogs seen per day per practice. This figure is roughly compliant with those reporting a case ≤ 12 Months (29.3).

The vaccine doses administered were on average, lower than the overall responses with 1510.36 compared to 1669.41. This is also lower than the doses administered to the ≤ 12 Months group (1668.38).

3.3.3 Comparison of vaccination status between the three defined groups

In total, from the 89 participants who responded, 42 (47.19%) reported seeing a case at their practice. The average percentage of dogs vaccinated was the same level for practices reporting a case and those that had seen none (60.71% compared to 60.55% respectively). However despite this, the amount of vaccine doses administered was lower for the practices reporting a case (1560.46 compared to 1708.67 per year) (Table 3.5). The group that reported a case ≤ 12 months before the study also reported the lowest average number of dogs with an up to date vaccination.

Group	Mean Vaccine Doses administered over past 12 months	Mean Up to Date Vaccination (%)
≤ 12 Months	1668.38	58.77
>12 Months	1510.36	61.61
No Cases	1708.67	60.55
Overall	1669.41	60.31

Table 3.5 Comparison of vaccination doses and up to date vaccinations between the three groups and the overall figures.

3.3.4 Clinical signs identified for a leptospirosis case by vet surgeons

All vets completing the questionnaire were asked to list the clinical signs they look for when diagnosing a suspected leptospirosis case. Of the 89 that responded, 70 listed at least one clinical sign (78.66%).

The clinical signs most frequently reported where usually characteristic of the disease. Jaundice was the sign cited most, with 64.04% of clinicians reporting it (Figure 3.1). The other two main reported signs were fever and vomiting (52.8 and 41.57% respectively), both common in the anicteric stage.

Identifying contact with rodents or water was only reported as a risk factor from seven practices. This amount is lower than expected considering the importance that contaminated urine or water plays in the transmission of *Leptospira*. A current vaccination against the

disease was only determined as a risk factor when making a diagnosis in 17% of practices, whereas symptoms such as lethargy, polydipsia/polyuria and renal failure were more common.

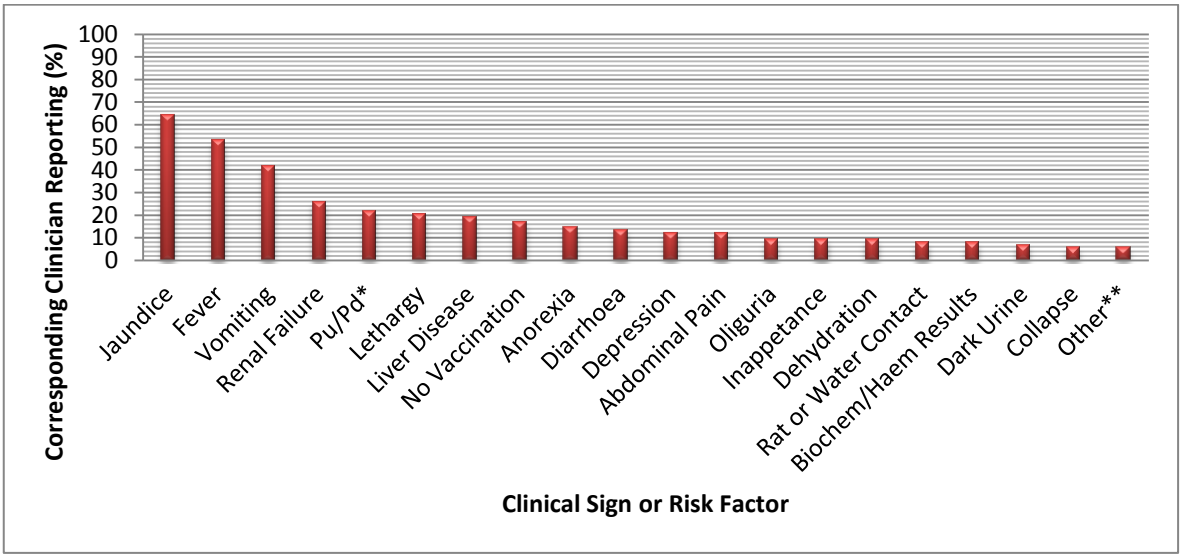

Fig 3.1 Percentage of corresponding vets reporting clinical signs and risk factors. *Pu/Pd = Polyuria & Polydipsia. **Other = Clinical signs reported in <5% of practices including toxaemia, coughing, age, breed & shock.

3.4 Discussion

The aim of this study was to gain information on the occurrence of suspected or confirmed *Leptospira* cases seen in the UK vet visiting dog population. To date, the level of practices witnessing a suspected case of leptospirosis in the UK over a given time period has not been investigated. Neither has the level of vaccination coverage against the disease. Knowledge of current data regarding case numbers and vaccination levels will help vaccination programs to become more effective and efficient. For the study, a questionnaire based approach was employed to gain a better insight into how practices perceive and deal with the disease.

In theory, a bigger and busier practice with more dogs seen per day will have an increased likelihood of witnessing a leptospirosis case. This hypothesis was investigated during this study by comparing the means between those that have ever seen a case and those that haven't. On average, practices that haven't seen a case reported 40.23 dogs per day, whereas those that have seen a case only reported 30.21 per day. This contradicts the original hypothesis as the 42 practices that had seen an infection received roughly 10 dogs less per day on average. A larger sample size available would go further to improve the accuracy of this finding and ascertain if this report is true for a greater population.

The only breeds to be represented more than once during the study were Labradors and Springer spaniels (n=2 for both). This may be a result of their popularity in the UK, with the breeds coming 1st and 3rd on the Kennel Club's registration statistics in 2007 (KC, 2008). This finding echoes the reports that have been seen in countries across the world (Alton et al., 2009; Oliveira Lavinsky et al., 2012; Raghavan et al., 2011). One report indicated that dog breeds used as farm working dogs were more susceptible to the disease (Harland et al., 2013). This however is more likely to be linked to the environmental exposure rather than the breed itself. Only two clinicians claimed to include a dog's breed or age in a differential diagnosis, indicating that vets place a low priority on these factors when making a diagnosis for leptospirosis.

A further interesting point to focus on is the lack of clinicians that consider environmental factors in their diagnosis. As *Leptospira* environmental survival tends towards certain conditions (neutral pH and damp soil, room temperature water sources etc.) (Parker and Walker, 2011; Smith and Turner, 1961) this restricts the sources of infection for dogs. Canine exposure to this habitat on a regular basis can increase the likelihood for contracting the disease. *Leptospira interrogans* have the ability to survive for weeks in these conditions (Smith and Turner, 1961) meaning the likelihood of an infection from serovars Canicola and

Icterohaemorrhagiae increases with exposure to those risk factors. As the bivalent vaccine protects against these serovars, maintaining an up to date vaccination will limit the potential for an infection. Contact with rodents either directly or indirectly (urine contact) will also have a large impact on the risk of contracting the disease.

While there is currently no data for dogs in the UK, there are reports of human cases linked to water or rodent contact through either occupational or recreational activities (Forbes et al., 2012). As the same serovars can infect humans and dogs, these risk factors can't be ignored. The dog's exposure to both damp conditions and rodents, and a lack of vaccination should be considered when taking a suspected leptospirosis case into account. This study indentified no apparent locations or environments that presented an increased risk for contracting leptospirosis. While this contradicts the studies previously mentioned, it suggests infection to be linked more to the level of exposure and lifestyles of the dogs in the UK, as these will vary to a degree within each geographical area. However as this study used only a small demographic, such associations may be missed that would be more apparent if a larger sample size was obtained.

Interestingly, the practices that reported a lower level of both vaccine dose administered and overall percentage of vaccinated dogs were also those reporting cases within the last 12 months. This reduced level of vaccine coverage within dogs, or a lower rate of annual vaccinations to maintain protection, may be contributing to the increased risk for contracting leptospirosis. Despite this observation, a greater sample size available would be required to draw any significant conclusions from the data.

For 12 of the cases reported by practices within the last 12 months, no up to date vaccination was maintained in the individual animals. This further emphasises the need for regular, maintained *Leptospira* vaccinations in dogs. The only case that did have an up to date vaccination was confirmed by a laboratory and the dog survived. It was not possible to obtain information regarding the infecting serogroup due to confirmation by PCR. One possible explanation may be the infection being caused by a serovar not protected by the vaccine. As the vaccines are serogroup specific (with a varying degree of cross-protection within serogroups) then a host will not be protected from a challenge by a different strain.

In recent years a number of different serovars have been reported to cause human disease in the UK, including serogroups Australis and Autumnalis. These serogroups weren't covered by the bivalent vaccine and also demonstrated the potential to infect and cause disease in dogs (Collings, 1984; Iwamoto et al., 2009; Mastrorilli et al., 2007; Weekes et al., 1997). Further

investigation would be required to determine if infection was due to a serogroup not covered by the bivalent vaccine. Details regarding a novel tetravalent vaccine that incorporates an additional two serogroups (Grippotyphosa and Australis) were recently published (Klaasen et al., 2013). The emergence of these serovars reinforces the need to have current data available regarding strains in circulation. Not only does this improve the accuracy of serological testing but also ensures vaccines are kept relevant and efficient.

Despite the low case numbers, the amount of cases resulting in death were relatively high (n=8/13; 61.54%) when compared to the 78% survival rate previously reported (Adin and Cowgill, 2000; Goldstein et al., 2006). This can possibly relate back to the diagnostic issue to an extent. If the diagnosis of leptospirosis is delayed to allow late stage signs to manifest, then the chances of recovering are reduced. This study collected no information on when the dog presented at the vet. The earlier an infection is detected, the more effective treatment is. For the cases reported, no dog was older than 10 years old and given the expected life spans for the breeds reported, it is reasonable to link leptospirosis as the cause of death.

A key limitation for the study was the level of dependence on vet practices having to recall previous figures over the last 12 months and longer. While certain questions would be easier to gather figures for (vaccine doses administered), the questions relating to previous cases may be affected by issues such as recall bias or a change of practice personnel. One issue that can be identified with practices recalling suspected cases from longer than 12 months ago is the degree of recall bias. When presented with the question, they would be more likely to associate a previous suspected case to the disease. This potential bias could affect the reports for this question, however to what extent is unknown. As this study was carried out using a mail shot approach a number of factors were taken into consideration in an attempt to maintain a good compliance rate, however rates seen were relatively low (18.86%). Despite this, 89 responses were received from all practices targeted. As there have been no similar studies of this nature carried out in UK practices regarding *Leptospira*, it is not possible to compare compliance rates.

The returning number of practices reporting a case (n=13) may be underestimating the true number of cases if dogs showing only mild clinical signs (such as vomiting or diarrhoea) are not presented to vets or a differential diagnosis is not made. Doxycycline can help to rapidly clear a *Leptospira* infection in its anicteric stage from the dog within a matter of days by preventing the growth of any bacteria within a host (Truccolo et al., 2002). This would hamper the possibility of any future samples giving a definitive positive result. While this is good practice

for the health of the animal, it removes the possibility of a definite leptospirosis case from a fourfold increase in serological test results.

The vague anicteric symptoms are one of the main problems with diagnosing an early infection. As highlighted in this study, of the three main clinical signs, two presenting before jaundice can be applied to a range of other diseases. Jaundice is characteristic of the disease and is only seen in the minority of cases that progress to the more aggressive state. Dogs can become carriers and shed the bacteria should they become infected (Harkin et al., 2003; Rojas et al., 2010). Due to this, the zoonotic potential hence remains high even in cases only presenting with mild clinical signs. Vaccination not only protects against the disease, but can also prevent asymptomatic colonisation and shedding. During a differential diagnosis, more prevalent and reported diseases in the UK will take precedence. If treatment for other diseases overlaps with leptospirosis (such as prescribing doxycycline) then it's possible a *Leptospira* infection can be treated without ever knowing it was there. A lack of information to the general public and published information to veterinarians will mean that leptospirosis will remain to be perceived as an infrequent disease in dogs.

Current diagnostic testing in the UK focuses around the MAT and the IFAT test. One major drawback for both is the requirement for a paired second sample to see a 4-fold titre increase. This increase in the second sample is indicative of a current infection and indicates a definite positive result, as a recent vaccination (or previous recent exposure) can affect results by circulating antibodies causing agglutination resulting in a false-positive result. A second sample taken 5-7 days following the first negates the chance of this occurring. Another issue is the limited serovar and serogroup specificity of the MAT test. When testing a suspected sample, a vet must request which serogroups or serovars to test for. With the incorrect information about which serovars are in circulation within UK reservoirs, this reduces the effectiveness for the test to return the correct result. The development of an accurate, rapid and reliable diagnostic test will benefit the identification of future suspected cases and improve monitoring in the UK.

In summary, this study has shown that *Leptospira* infections are still an issue in the UK despite the availability of a vaccine. As there was a case seen in a previously vaccinated dog, the possibility of serovars different to those in the vaccine in circulation was also presented. Increasing awareness of the disease, the early symptoms and the risk factors involved will improve diagnostic testing. Identifying an infection early will also benefit treatment and increase the likelihood of the dog surviving. The current vaccine still remains relevant and key

to protecting a dog against a challenge from either serovars Canicola or Icterohaemorrhagiae, however with the emergence of new serovars in the UK; there is a need to keep vaccines up to date. Monitoring of these new serovars passing through practices will benefit vaccination programs and ultimately keep dogs protected from leptospirosis.

Chapter Four

Investigating and identifying the presence of pathogenic *Leptospira* in wild rodents in North West England

4.1 Introduction

Pathogenic *Leptospira* are maintained and transmitted within asymptomatic reservoir hosts with a wide range of animal species capable of maintaining and transmitting the bacteria (Cacciapuoti et al., 1987; Timoney et al., 2011; Tulsiani et al., 2011a). Once infected, the bacteria can colonise various internal organs including the kidneys and liver. Following an established infection in the kidneys, leptospires are then shed into the environment via urine (Rojas et al., 2010). The large majority of reservoir animals are asymptomatic and can shed the bacteria for their entire lifespan without ever showing external signs of infection (Rojas et al., 2010). Small rodents have the ability to shed *Leptospira* into the local environments of a wide range of animals and humans.

Whilst in the environment the bacteria can survive for weeks, dependant on environmental conditions, and certain species (such as *Leptospira interrogans*) have been proven to survive for longer periods outside a host than others. *L. interrogans* has demonstrated a greater tolerance towards external conditions compared to *L. borgpetersenii*, which has become much more host dependant and can only last for a matter of days outside the host. This is likely due to either acquisition, through horizontal gene transfer, or loss of genes essential to pathways for environmental survival (Bulach et al., 2006; Picardeau et al., 2008). Saprophytic species are more fastidious in the environment as their genome contains the pathways required for utilising alternative methods for acquiring metabolites (Picardeau et al., 2008). To date, saprophytic strains have been isolated from water sources but never from a host (Merien et al., 1997).

Traditionally, certain serovars have been suggested to be reservoir host specific. Serovar Canicola is maintained in dogs, Icterohaemorrhagiae in rats (Klaasen et al., 2003), Pomona in pigs (Chappel et al., 1998) and Hardjo in cattle (Ryan et al., 2012). This serovar-host specificity has an effect on transmission potential to other animal species depending on the living and housing conditions. Infections of Hardjo and Pomona are more likely to be contained and maintained within domestic animal herds (Boqvist et al., 2002; Ryan et al., 2012) whereas Canicola and Icterohaemorrhagiae may have a greater potential to infect other animals, including humans. However, an increasing number of reported infections are involving serovars not traditionally associated with that particular animal species (Felt et al., 2011; Oliveira Lavinsky et al., 2012). Serovars can be only host specific but also region specific with a variety of animal hosts more likely to be infected with certain serovars due to their geographical location than others.

Due in part to their ubiquitous nature, small rodents play an extremely important role in the transmission of the bacteria to a wide range of incidental hosts across the UK. It has been suggested that larger herd sizes and rodent presence on farmland increases potential transmission routes between animals within a herd, or even animals grazing in adjacent fields, due to their mobility (Schoonman and Swai, 2010). Presence of infected rodents in urban communities increases transmission risk amongst companion animals and humans. However, human populations within urban areas are less likely to have direct contact with wild rodents and so the potential for contracting an infection from wildlife is decreased. Incidents of flooding can cause infected sources (such as urine) to spread and potentially lead to outbreaks of leptospirosis. *Leptospira* has been reported to cause infection within 17.1% of outbreaks following periods of flooding or heavy rain (Cann et al., 2012).

Human infections in the UK are usually either occupational or recreational, and are usually consistent with either water or rodent contact (Forbes et al., 2012). UK figures from 2010 indicate that ten out of the 12 human cases arising from recreational exposure had contact with either rodents or water. This included keeping pet rats, a canoeist, a mouse bite, fishing and clearing out property infested with rats (HPA, 2011).

In the UK, traditionally serovars Canicola and Icterohaemorrhagiae have been the cause of infection in both humans and dogs (HPA, 2011). Either serovar can be maintained within small rodent populations around the world (Doungchawee et al., 2005; Scialfa et al., 2010); however the extent of this in the UK is currently unknown. Earlier studies carried out in the UK showed both bank voles and wood mice to shed serovars Saxkoebing and Muenchen (Hathaway et al., 1983b; Little et al., 1987). However, more recently human infections have been reported in the UK arising from emerging serovars not normally observed in UK infections (HPA, 2013). There is a lack of current data regarding the serovars maintained within rodent reservoirs in the UK, with little data also available across Europe. Serogroups Australis and Grippotyphosa were recently detected in bank voles and yellow necked mice during a study in Croatia (Tadin et al., 2012). Further to this, serogroups Icterohaemorrhagiae and Sejroe have been identified in wild rodents, including rats, at five areas within France (Aviat et al., 2009). Serovars from these serogroups have demonstrated the potential to cause infection in humans within the UK (HPA, 2011).

Outside of Europe, studies into reservoir animals are more prominent. Field mice and rats were both shown as PCR positive for *Leptospira* presence during a study in China that also identified a positive canine case from the same areas (Yalin et al., 2011).

Identification of the presence of *Leptospira* from companion animals is normally carried out using the microscopic agglutination test (MAT) that can also be applied to other animal species (Aviat et al., 2009; Mohamed-Hassan et al., 2010; Rahelinirina et al., 2010). This method however requires the user to have the competence to successfully determine the correct titre and results can vary from lab to lab. Another drawback of the MAT test is it does not give serovar identification due to cross-reactions between serovars within the same serogroup.

The MAT relies on the production of antibodies in a host specific for the bacteria; these react with live antigens added across titrated dilutions to give a resulting titre reaction result. In the early stages of infection, the IgM antibodies produce a non-specific reaction to live antigens. This can result in a certain degree of cross-reaction between serovars within the same serogroup. As a host's antibody reaction develops, IgG antibodies are more specific for individual serovars. A second test on the same animal will result in at least a four-fold titre increase for the infecting serovar due to the accumulation of serovar-specific IgG antibodies.

Furthermore, MAT has limited usefulness for determining an infection in a reservoir host. As chronic infections in the kidneys potentially evade the immune system (Monahan et al., 2009), then circulating antibodies towards *Leptospira* can be absent or below the determined level to give a positive MAT result.

Molecular methods, such as PCR assays, allow labs to rapidly test samples from urine, blood and even tissue. There has been a shift towards utilising PCR for identification due to its accuracy, ease of use and allowing for comparable results between labs. Direct sequencing of the 16S gene has demonstrated the ability to identify the species of an infecting *Leptospira* (Morey et al., 2006). As an alternative, *gyrB* has also demonstrated this ability, having 100% compliance with 16S sequencing results (Slack et al., 2006).

Leptospira are Gram negative; however they are difficult to stain using conventional methodology and individual leptospires are difficult to visualise. Warthin-Starry silver staining allows better visualisation of *Leptospira* within infected tissue (Fornazari et al., 2012; Leon et al., 2006). Under these conditions it is possible to see aggregates as well as individual bacteria within infected tissue. Analysing the same section with the hematoxylin and eosin (H&E) stain allows for comparison between the two stains to investigate if inflammation is present, along with morphological changes (De Brito et al., 1996).

Culture of leptospires from urine, serum or tissue samples, while beneficial for diagnostic and identification capabilities, is problematic. There are numerous technical issues with

establishing a grown culture in liquid EMJH media, in particular contamination. Given the long growth period, and nutrient rich media, careful consideration is required to prevent contaminated cultures. Dark field microscopy is used to help visualise and confirm any positive cultures.

The aim of this study was to investigate the presence of pathogenic *Leptospira* strains in a range of wild rodent species across multiple sites in the North West of England. Alongside this, molecular techniques were used to identify the species, serogroup and serovar of any infections discovered. Histopathology and serology testing was also carried out on the same samples to allow direct comparison of results between each test.

4.2 Materials and Methods

4.2.1 Trapping protocol and site selection

Trapping locations were chosen primarily based on their location, along with factors such as closeness to bodies of water, previous rodent sightings and if dog walking had been witnessed previously in that area (Table 4.1 and Figure 4.1). Suitable sites in Liverpool were chosen based on recommendations by the environmental health as potential hotspots. Open public areas were not included to prevent any theft or tampering with the traps. Differing locations were chosen to represent different habitats and to allow different rodent species to be investigated.

Location	Habitat Type	Rural/Semi-Rural/Urban
Livestock Farm	Open Farmland	Semi-Rural
Equine Livery	Managed field	Semi-Rural
Ruthin Forest	Managed woodland	Rural
Forest of Bowland	Managed woodland	Rural
Beef Farm	Open Farmland	Semi-Rural
Public Gardens	Field/Woodland	Semi-Rural
Frodsham Marsh	Field	Semi-Rural
Liverpool Port	Urban	Urban
Allotment 1	Urban	Urban
Allotment 2	Urban	Urban
Allotment 3	Urban	Urban
Allotment 4	Urban	Urban
Allotment 5	Urban	Urban
Allotment 6	Urban	Urban
University of Liverpool Campus	Urban	Urban

Table 4.1. Locations and habitats of trapping sites for both rats and small rodents. All allotment sites were within a single Liverpool postcode.

Rats were live trapped using standard rat traps and Longworth traps were used for small rodents (such as mice and voles). Grain, bedding and small pieces of fruit or vegetable were included in each trap. The traps where checked every morning over a four night period (Monday to Friday) each time they were placed. Trapping was carried out multiple times at different points in the year at the Livestock Farm and Equine Livery. Full ethical approval was obtained from the University of Liverpool ethics committee and land owner's permission was sought prior to work starting.

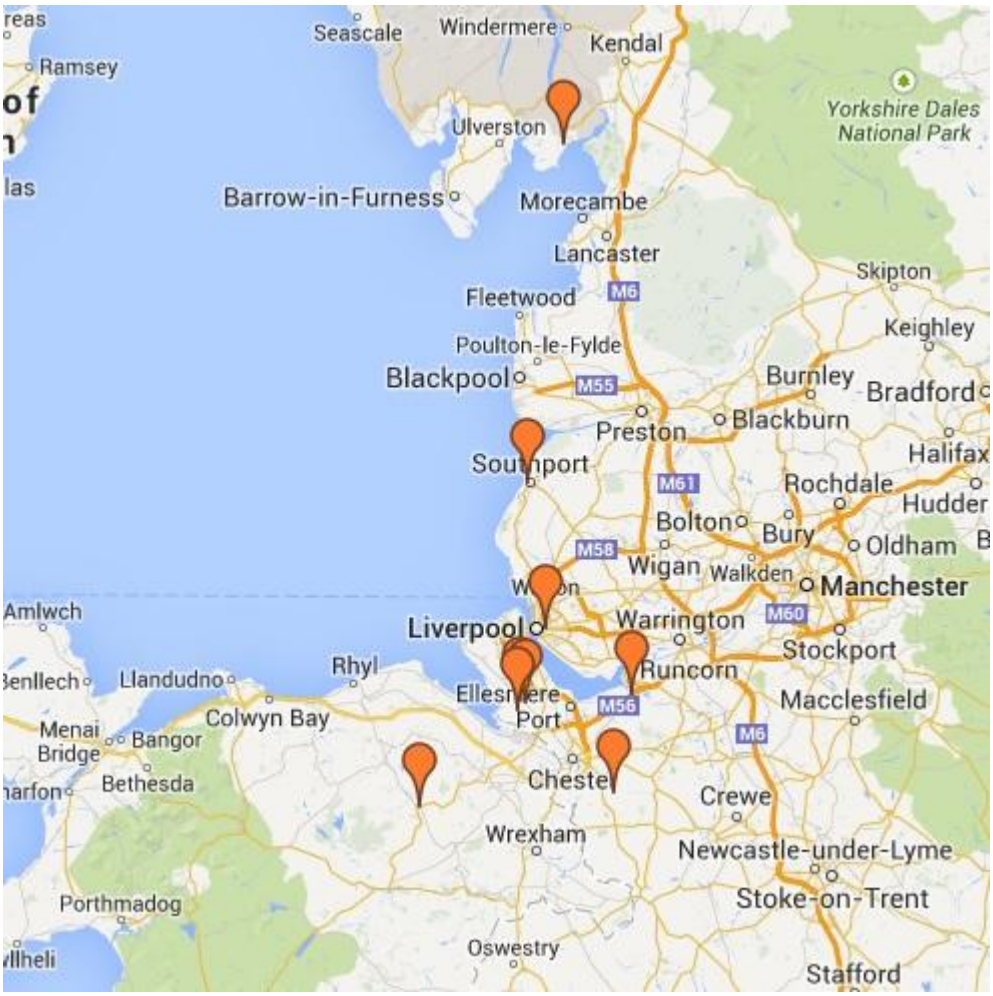

Figure 4.1. Location of rat and small rodent sampling sites during the study. Sites within Liverpool are grouped.

Rodent euthanasia was carried out according to Schedule 1 methods as described in the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (2000). Gender and size were determined for each rodent post mortem. Dissection was carried out and both kidneys were removed. To reduce any contamination risk, each rodent was sprayed with 70% ethanol. One kidney was stored at -80^{o}C for DNA extraction while the other was stored in formalin for histopathology testing. Blood was taken via a heart puncture immediately following euthanasia and stored at -80^{o}C for MAT testing at a later date.

4.2.2 DNA extraction from kidney tissue

Roughly 1g of kidney tissue was taken from each sample for a DNA extraction. The tissue was cut into small pieces to allow for more efficient extraction. The DNeasy® Blood and Tissue

(Qiagen) kit was used according to manufacturer's instructions. This method gave a total of 60µl of extracted DNA in elution buffer. The DNA was then aliquoted into 10µl tubes and stored at -80^{o}C.

4.2.3 Rodent species identification using PCR

Each DNA extract was subjected to a standard PCR reaction and subsequent sequencing analysis to confirm the species of rodent. This allowed for confirmation of species that can be difficult to distinguish on gross examination.

The PCR reaction targeted the *cytB* gene (Schlegel et al., 2012) and following sequencing, allows for BLAST comparison against other known species sequences. PCR was carried out in a 25µl reaction volume using ReddyMix PCR Master Mix (Thermo Scientific) which 0.625 units of ThermoPrime *Taq* DNA polymerase, 75mM Tris-HCl, 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01% (v/v) of Tween® 20 and 0.2mM each of dATP, dCTP, dGTP and dTTP with 10pmol of each primer and 1µl of sample DNA.

Cycle conditions were as follows: 94^{o}C for 3 minutes, followed by 40 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 47^{o}C for 30 seconds, and extension at 72^{o}C for 1 minute, with a final extension at 72^{o}C for 10 minutes. The 946bp product was run on a 1.5% agarose gel against a ΦX174 ladder with controls. The amplicon was cleaned up and sent for sequencing at Source Bioscience. The resulting sequence was then compared to the existing database using BLAST.

4.2.4 Establishing a screening PCR for presence of pathogenic *Leptospira*

To identify any *Leptospira* DNA in acquired samples, a screening PCR was established for use with kidney tissue DNA extracts that utilises two primer sets; one to identify any leptospiral DNA and a second to identify only pathogenic serovars. Two targets were primarily identified for this, the 16S gene *rrs* and the gene for the outer membrane protein *lipL32*.

A series of primers were selected from the literature to test for both gene targets (Table 4.2). These where then tested on two control strains *L. interrogans* serovars Canicola and Icterohaemorrhagiae to identify the set that were able to work in duplex and had the highest binding efficiency.

Gene	Primer	Sequence	Reference
lipL32[1]	Forward 1	ATCTCCGTTGCACTCTTTGC	Ahmed et al., 2006
	Reverse 1	ACCATCATCATCATCGTCCA	
	Forward 2	AAGAATGTCGGCGATTATGC	Tansuphasiri et al., 2006
	Reverse 2	CCAACAGATGCAACGAAAGA	
	Forward 3	TATAAGCTTTGTGGTGCTTTCGGTGGTCT	Hoke et al., 2008
	Reverse 3	TTAACCTAGATCTTTGTTTAAACAG	
	Forward 4	CGCTTGTGGTGCTTTCGGTGGT	Fernandes et al., 2008
	Reverse 4	CTCACCGATTTCGCCTGTTGGG	
	Forward 5	GTCGACATGAAAAAACTTTCGATTTTG	Cheemaa et al., 2007
	Reverse 5	CTGCAGTTACTTAGTCGCGTCAGAAGC	
rrs[2]	Forward 1	GGAACTGAGACACGGTCCAT	Tansuphasiri et al., 2006
	Reverse 1	GCCTCAGCGTCAGTTTTAGG	

Table 4.2. Primers sets used in preliminary duplex PCR testing. [1]*lipL32* is an outer membrane protein found in pathogenic strains. [2]*rrs* is a 16S RNA gene present in all strains.

From all the different primers tested, the two primers *rrs* (Tansuphasiri et al., 2006) and *lipL32* (Cheemaa et al., 2007) were initially selected to be used in duplex for the screening PCR. Cycle conditions were as follows; 94°C for 10 minutes, followed by 40 cycles of 94°C for 1 minute, 55°C 1 minute, 72°C for 1 minute, and a final extension of 72°C for 10 minutes.

One problem experienced when applying the PCR to DNA extracts was that the *lipL32* primers bound inefficiently to the control strains. Due to this, a different target for pathogenic strains was identified from a list of potential candidates (Table 4.3), all of which amplify pathogenic serovars only. All cycle conditions were kept as published.

Gene Target	Primer	5' to 3' Sequence	Size (bp)	Reference
ligB[1]	PSBF	ACWRVHVHRGYWDCCTGGTCYTCTTC	380	Cerqueira et al., 2009
	PSBR	TARRHDGCYBTAATATYCGRWYYTCCTAA		
gyrB[2]	2For	TGAGCCAAGAAGAAACAAGCTACA	502	Slack et al., 2006
	504Rev	MATGGTTCCRCTTTCCGAAGA		
flaB[3]	flaB Forward	TCTCACCGTTCTCTAAAGTTCAAC	793	Krishna et al., 2008
	flaB Reverse	CTGAATTCGGTTTCATATTTGCC		

Table 4.3. Additional potential targets for differentiation of pathogenic serovars by PCR. [1]*ligB* plays a role in cell adhesion. [2]*gyrB* is involved with DNA production. [3]*flaB* is a component of the rotational flagella.

From the potential candidate genes, *gyrB* was chosen for its efficient binding when compared to either the *ligB* or *flab* primers. The final duplex screening PCR was carried out according to the protocol in chapter two. Figure 4.2 demonstrates the expected band sizes for both *rrs* and *gyrB* (502bp).

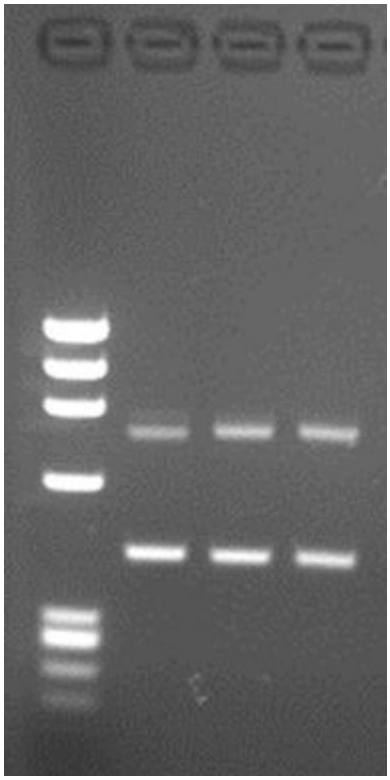

Figure 4.2 Example of amplified bands for both the *rrs* (430bp) and *gyrB* (502bp) loci used in the duplex PCR assay, against a ΦX174 ladder. *L. interrogans* serovar *Australis* was utilised as the positive control.

All samples were then re-tested using the updated duplex PCR reaction. Samples were tested in triplicate to ensure none were identified as a false-negative. Any samples with faint bands or smearing on the agarose gel were re-tested for confirmation. Positive PCR samples were then purified and sent for commercial Sanger sequencing of both the *rrs* and *gyrB* gene amplicons.

4.2.5 Clean up of PCR reactions and sequencing of amplicons

Amplicons gained from positive PCR assays were purified for sequencing. This was carried out using the QIAquick® PCR purification kit (Qiagen) according to manufacturer's instructions. The subsequent solution was tested by Nano-drop to estimate the concentration for sequencing and diluted to 100µg/µl/100bp.

Commercial Sanger sequencing of the purified amplicons was carried out at Source Bioscience and MWG Eurofins. Both forward and reverse reads were obtained for each sequence. This allowed for alignment of consensus sequences to prevent potential base variation errors.

Sequences were analysed in ChromasPRO v1.7.3 (http://technelysium.com.au), for read quality. Primer locations were identified and the sequences were trimmed appropriately. Alignments and comparisons between sample sequences were carried out in MEGA5 (Tamura et al., 2011). Phylogenetic analysis and BLAST searches were also carried out using MEGA5.

4.2.6 Microscopic agglutination testing of rodent serum samples

Blood samples were taken via cardiac puncture post mortem and serum was stored at -80^{o}C. All serum samples were tested at the Animal Health and Veterinary Laboratories Agency (AHVLA) against their stock panels of antigens.

All serum samples were centrifuged at 2,500rpm for six minutes to separate the serum from any clotted blood. Where possible, 80µl of serum was taken from each and diluted down by a factor of 12.5 in saline to obtain a total of 1ml, however up to a minimum of 15µl was used to dilute giving a minimum total of 187.5µl.

If the serum was contaminated with serum proteins, lipids etc. then it was necessary to filter the sample prior to testing through a 0.2µM filter to remove impurities which could potentially give false-positive results.

4.2.7 Multiple serovar pooled samples

Serum samples were initially tested with pooled antigens as described in Chapter Two. Previous serovar identification from direct sequencing of PCR assays were included, along with those typically seen with UK infections. Serovars included in the pool were *L. interrogans* serovar Canicola, Icterohaemorrhagiae, Australis, Bratislava, Muenchen and Jalna.

4.2.8 Single serovar titrations

Samples indicating an infection from pooled antigen testing were tested further with single serovar plates as described in Chapter Two. Each plate contained a serum sample serial diluted and incubated with an individual antigen. Two hours of incubation was then followed by observation under dark field microscopy as with the pooled antigens. The serogroup that demonstrated agglutination at the lowest tire was deemed the infecting serogroup.

4.2.9 Histopathology of kidney tissue sections

To attempt to visualise the bacteria within rodent tissue, any samples found positive by the screening PCR were subjected to histopathology staining by the Histopathology department at the University of Liverpool. Chapter Two describes the two stains used for this project and the reasoning behind them. Key areas looked for with the H&E stain included capsular indentations and lymphoplasmacytic infiltration in the interstitial tissue, cortex and medulla.

The Warthin-Starry silver stain allowed for potential visualisation of either individual or aggregates of leptospires within tissue. Using both stains in tandem allowed identification of any association between sites of inflammation and the sites that the bacteria are located within.

4.2.10 Variable number tandem repeat (VNTR) analysis of positive samples

A VNTR method has been developed for *Leptospira* which has the potential to identify individual serovars (Salaun et al., 2006). In this study, the primers for the VNTR analysis were primarily tested on positive controls. If shown to be effective they would then be applied to all positive samples. Published cycle conditions were kept the same, as were the primers (Table 4.4).

Primer	**5' to 3' Sequence**	**Size (bp)**		
		L. interrogans	*L. kirschneri*	*L. borgpetersenii*
VNTR4-F	AAGTAAAAGCGCTCCCAAGA	425+34n	425+34n	425+34n
VNTR4-R	ATAAAGGAAGCTCGGCGTTT			
VNTR7-F	GATGATCCCAGAGAGTACCG	299+46n	299+46n	No Product
VNTR7-R	TCCCTCCACAGGTTGTCTTG			
VNTR10-F	GAGTTCAGAAGAGACAAAAGC	420+46n	347+46n	333+46n
VNTR10-R	ACGTATCTTCATATTCTTTGCG			
VNTR-Lb4 F	AAGAAGATGATGGTAGAGACG	No Product	No Product	573+60n
VNTR-Lb4 R	ATTGCGAAACCAGATTTCCAC			
VNTR-Lb5 F	AGCGAGTTCGCCTACTTGC	668+39n	668+39n	722+36n
VNTR-Lb5 R	ATAAGACGATCAAGGAAACG			

Table 4.4. Primers used for the VNTR analysis to identify the serovar from infecting strains (Salaun et al., 2006).

4.2.11 Isolation of *Leptospira* strains from caught wild rodents

As rodent reservoirs are more likely to have a higher bacterial carriage in the kidney tissue, culture was attempted on single kidneys from a subset of samples. The PCR assay on the second kidney would be carried out alongside to give early confirmation of an infection. Positive controls were *L. interrogans* serovar Canicola and serovar Icteroheamorrhagiae.

Ellinghausen-McCullough-Johnson-Harris (EMJH) media was obtained from MSD Animal Health. Media containing 1% rabbit serum and 5-fluorouracil was used for the primary culture. To reduce any contamination, both the rodent and the kidney were sprayed with 70% ethanol prior to inoculation.

Roughly 1g of kidney tissue and 9ml of liquid culture were placed into a Stomacher tissue homogeniser for 5 minutes. From this, 1ml was taken and inoculated into 9ml of semi-solid EMJH media. This was mixed for 10 seconds and one 1ml was transferred into 9ml of liquid media to give a 10^{-3} dilution. For the final culture, 100µl of the 10^{-3} dilution was inoculated into 6ml of EMJH media and incubated at 29^{o}C. The final inoculation was carried out in triplicate to ensure maximum likelihood of a successful culture.

All cultures were checked on a weekly basis for growth using a dark-field microscope. Additionally, DNA extracts were carried out on the media to detect growth via PCR assays. Only after six months were negative cultures identified.

4.3 Results

In total, 283 rodents were caught over the seven locations; samples from multiple sites around Liverpool were pooled together as one location (Table 4.5). All samples from Liverpool were provided by Kieran Pounder from the University of Liverpool. Species caught were identified and confirmed using the *cytB* PCR assay and sequencing. These included wood mice (*Apodemus sylvaticus*), house mice (*Mus musculus)*, bank voles (*Myodes glareolus)*, field voles (*Microtus agrestis),* and brown rats (*Rattus norvegicus)*.

Trapping was carried out at different times of the year, at different locations. Live trapping was carried out between May 2010 and July 2012. Some locations were visited on more than one occasion at different points throughout the year.

The total number of small rodents caught and tested over the study was 283. This broke down into wood mice (n=152), bank voles (n=47), field voles (n=10), house mice (n=7) and brown rats (n=67). The only species that was sampled at all locations were wood mice, with bank voles present at all locations apart from Ruthin Forest and sites within Liverpool.

As it was, a high majority of brown rats were obtained in the urban locations at Liverpool; two were captured on semi-rural sites, but none were captured from rural sites. No voles were caught at any of the urban sites in Liverpool.

Location[1]	**Time of Sampling**	**Rodent Species**					**Total**
		A. sylvaticus	*M. glareolus*	*M. agrestis*	*R. norvegicus*	*M. musculus*	
Livestock Farm	06/2010 – 03/2012	22	21	2	0	0	45
Equine Livery	09/2010 – 07/2011	43	6	1	0	0	50
Forest of Bowland	08/2010	1	1	2	0	0	4
Ruthin Forest	10/2010	8	5	0	0	0	13
Beef Farm	08/2011	6	2	3	0	0	11
Liverpool	06/2010 – 08/2011	37	0	0	67	7	111
Public Gardens	11/2011 – 12/2011	35	12	2	0	0	49
Total		152	47	10	67	7	283

Table 4.5. Numbers and species of rodents caught at each trapping location. [1]Locations from table 4.1 from which no samples were obtained are not included.

4.3.1 Wild rodent samples tested via the screening PCR for pathogenic *Leptospira* presence

All 283 rodent samples were tested using the PCR assay described in Chapter Two, the results of which are in table 4.6. Amongst the five rodent species between the seven sites, 23 samples were PCR positive for the presence of pathogenic *Leptospira.* The remaining 260 tested negative for the presence of any leptospires.

From the 283 samples, infections were identified within the kidneys of 23 small rodents (8.13% of total sample size) using PCR. This comprised of 15 wood mice (9.87% of wood mice sampled at seven locations), six bank voles (12.77% of bank voles sampled at six locations) and two field voles (28.57% of field voles sampled at five locations).

Location	Total number tested	Total Number Positive (%)
Livestock Farm		
Apodemus sylvaticus	22	2 (9.09)
Myodes glareolus	21	2 (9.52)
Microtus agrestis	2	0
Equine Livery		
Apodemus sylvaticus	43	3 (6.98)
Myodes glareolus	6	0
Microtus agrestis	1	0
Forest of Bowland		
Apodemus sylvaticus	1	1 (100)
Myodes glareolus	1	1 (100)
Microtus agrestis	2	1 (50)
Ruthin Forest		
Apodemus sylvaticus	8	4 (50)
Myodes glareolus	5	0
Beef Farm		
Apodemus sylvaticus	6	0
Myodes glareolus	2	1 (50)
Microtus agrestis	3	1 (33.33)
Liverpool		
Apodemus sylvaticus	37	0
Rattus norvegicus	67	0
Mus musculus	7	0
Public Gardens		
Apodemus sylvaticus	35	5 (14.29)
Myodes glareolus	12	2 (16.67)
Microtus agrestis	2	0

Table 4.6. PCR results by location and species investigated in this study

All but one of the locations sampled presented evidence of pathogenic *Leptospira* being present in rodents (Table 4.6). Three sites that were tested multiple times presented evidence for the same species being maintained (Livestock Farm, Equine Livery and the Public Gardens). Sites at Liverpool all tested negative (the majority of which were rat samples), despite numerous repeats in triplicate and a second DNA extraction on additional tissue.

Species of both mice and voles (specifically wood mice, bank voles and field voles) demonstrated their potential to be reservoir hosts of the bacteria. The brown rat and house mouse were all negative; however samples were only obtained from sites in Liverpool for both species which was the only location testing completely negative.

BLAST analysis of the trimmed *rrs* and *gyrB* consensus sequences confirmed identification as *L. interrogans* for 22 samples, with one being *L. borgpetersenii,* identified within a field vole.

Both wood mice and bank voles were shown to be host to the same *Leptospira* species, whereas the positive field vole sample was infected with a different species. Both the *rrs* and *gyrB* consensus sequences were compared for each sample and showed 100% homology when identifying the species of each infecting leptospire. It is not possible to determine the serovar or serogroup using this method.

4.3.2 Microscopic agglutination test on rodent serum samples to determine the infecting serogroup

Serum samples taken from 71 wild rodents were tested using the standardised MAT protocol at the AHVLA. Samples were initially tested against pooled samples that contained a range of serovars from different serogroups that corresponded to the findings of the initial BLAST results (Table 4.7).

From the pooled testing, seven samples resulted in a positive titre (>50% agglutination against a black background) with antigen pools containing strains belonging to the Australis serogroup. These samples where then tested against the individual antigens to determine the serovar producing the highest titre result. Trace results (<50% agglutination on a black background) were not included as a positive result.

Single antigen testing can determine to an extent the infecting serovar based on which shows the highest titre response. From the seven samples, *L. interrogans* serovar Bratislava produced the strongest titre response in four of the samples (WM72, WM80, WM93 and BV50). All three wood mouse samples were from the same trapping location. Serovar Lora (of

the same species) had the highest serovar for one wood mouse sample (WM80). Three samples failed to produce a response when tested against the four serovars (Table 4.7).

Three samples produced a reaction in all of the serovars tested for, demonstrating the potential cross-reactivity that can occur between serovars within the same serogroup.

All samples identified to have a positive MAT titre had previously been shown as PCR positive using the screening PCR (Table 4.4). Three samples identified as PCR positive were shown to be negative for an MAT titre.

Sample[1]	Pooled Antigen Result	Serovar Antigen			
		Australis	*Bratislava*	*Lora*	*Jalna*
BV3	+	-	-	-	-
BV13	-	-	-	-	-
BV14	-	-	-	-	-
BV15	-	-	-	-	-
BV16	-	-	-	-	-
BV17	-	-	-	-	-
BV18	-	-	-	-	-
BV19	-	-	-	-	-
BV20	-	-	-	-	-
BV21	-	-	-	-	-
BV22	-	-	-	-	-
BV26	-	-	-	-	-
BV36	-	-	-	-	-
BV38	-	-	-	-	-
BV39	-	-	-	-	-
BV40	-	-	-	-	-
BV41	-	-	-	-	-
BV43	-	-	-	-	-
BV44	-	-	-	-	-
BV45	-	-	-	-	-
BV46	-	-	-	-	-
BV47	-	-	-	-	-
BV48	-	-	-	-	-
BV50	+	-	*1:25*	-	-
FV3	-	-	-	-	-
FV6	-	-	-	-	-
FV8	-	-	-	-	-
FV9	-	-	-	-	-
FV10	-	-	-	-	-
WM25	-	-	-	-	-
WM26	-	-	-	-	-
WM27	-	-	-	-	-
WM28	-	-	-	-	-
WM42	-	-	-	-	-
WM43	-	-	-	-	-
WM44	-	-	-	-	-
WM58	-	-	-	-	-
WM59	-	-	-	-	-
WM67	-	-	-	-	-
WM68	-	-	-	-	-
WM69	-	-	-	-	-
WM70	-	-	-	-	-

Sample[1]	Pooled Antigen Result	Serovar Antigen			
		Australis	*Bratislava*	*Lora*	*Jalna*
WM72	+	1:100	*1:800*	1:200	1:100
WM72	-	-	-	-	-
WM73	+	-	-	-	-
WM73	-	-	-	-	-
WM75	-	-	-	-	-
WM76	-	-	-	-	-
WM77	-	-	-	-	-
WM78	-	-	-	-	-
WM79	-	-	-	-	-
WM80	+	1:50	1:100	*1:200*	1:100
WM80	-	-	-	-	-
WM81	-	-	-	-	-
WM83	-	-	-	-	-
WM84	-	-	-	-	-
WM85	-	-	-	-	-
WM86	-	-	-	-	-
WM87	-	-	-	-	-
WM88	-	-	-	-	-
WM89	-	-	-	-	-
WM90	-	-	-	-	-
WM91	-	-	-	-	-
WM92	-	-	-	-	-
WM93	+	1:100	*1:400*	1:200	1:100
WM93	-	-	-	-	-
WM94	+	-	-	-	-
WM94	-	-	-	-	-
WM96	-	-	-	-	-
WM97	-	-	-	-	-
WM98	-	-	-	-	-
WM99	-	-	-	-	-
WM100	-	-	-	-	-
WM101	-	-	-	-	-

Table 4.7. Titre results for the all 71 samples examined using the MAT. Italics denote suggested serovar identification. [1] Bank vole (BV), field vole (FV) and wood mouse (WM).

4.3.3 Visualising a *Leptospira* infection using conventional silver staining histopathology

Eleven formalin fixed samples that had previously been shown as PCR positive were tested using both the Warthin-Starry and the H&E stain. From two samples inflammation and leptospires were identified (Fig 4.3A and 4.3B). Locations of leptospires were not directly linked to the presence of inflammation. Inflammation alone was witnessed in a further six.

Capsular multifocal indentations were witnessed with several samples (n=4), including samples where no *Leptospira* were visualised. Multiple membrane indentations at several locations within the kidney tissue indicate an inflammation response by a host to an infection. Multifocal lymphoplasmacytic infiltration in the interstitial tissue was seen in six of the samples tested (Fig 4.3C and 4.3D). The presence of lymphocytes and plasma cells within the tissue indicate either an active or chronic infection. In this instance it is more likely to be a chronic infection maintained within the rodent. Inflammation was not directly linked to the same sites where bacteria were visualised.

Three samples showed no signs of either an inflammatory response or the presence of any *Leptospira*.

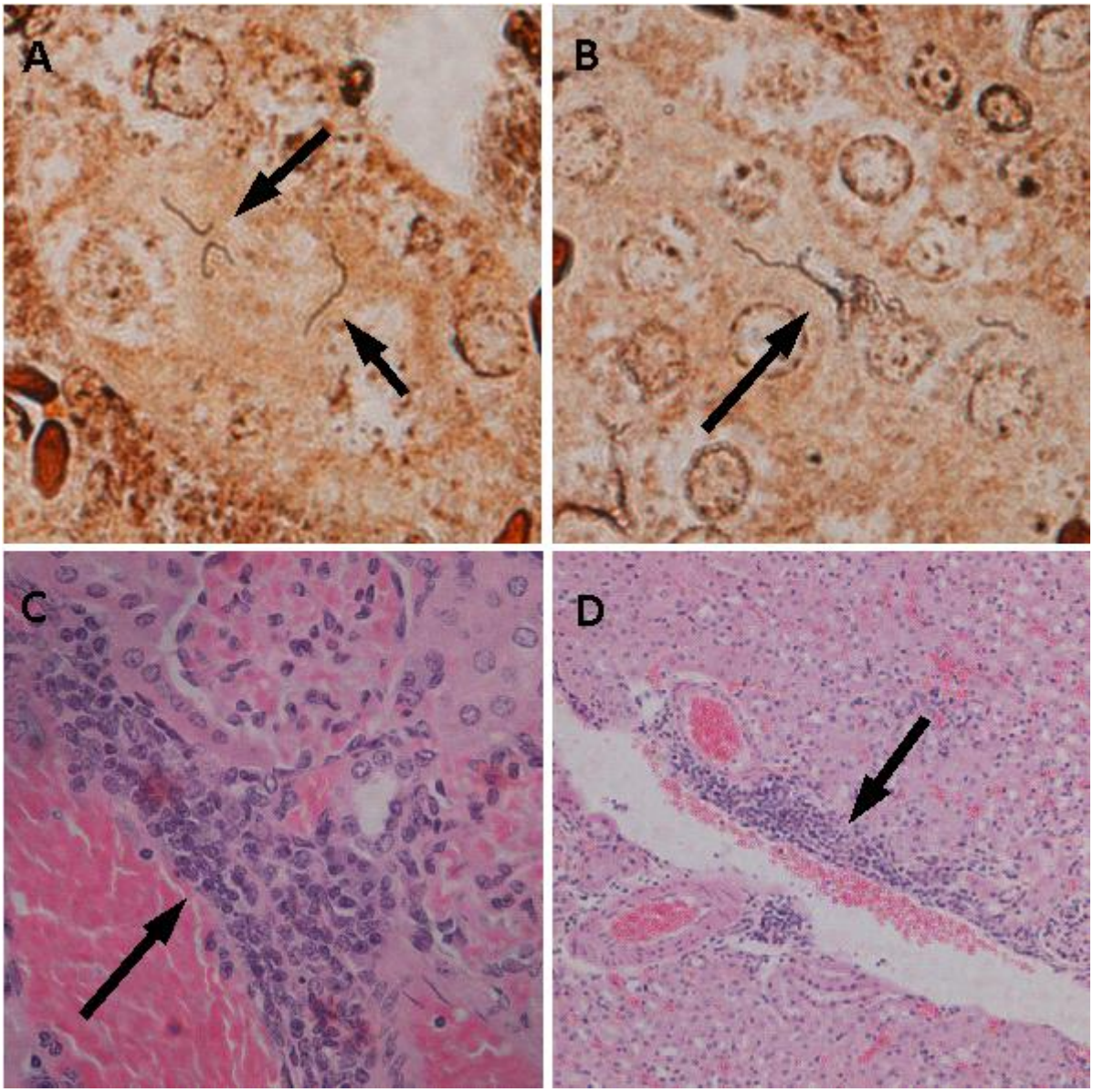

Figure 4.3. Histopathology **s**taining images of a PCR positive wood mouse sample. A: Warthin-Starry silver stain showing individual leptospires are observed. B: Warthin-Starry stain showing clumping of multiple bacteria within renal tubules. C: H & E stain showing interstitial infiltrate within kidney tissue. D: H & E stain showing chronic interstitial infiltrate of lymphocytes and plasma cells. All imaging carried out at 400x magnification.

4.3.4 VNTR analysis to identify *Leptospira* serovars

All samples positive for *Leptospira* by the screening PCR assay were examined using the VNTR primers as described in section 4.2. The primers were tested on two controls, *L. interrogans* serovar Australis and *L. kirschneri* serovar Grippotyphosa for their ability to identify serovars before their use on rodent samples.

Testing on the positive control strains proved inconsistent and unreliable. It wasn't possible to obtain bands for all loci with the controls despite altering the PCR conditions such as lowering the annealing temperature or increasing primer and sample concentration. This was the same for the field samples. As primers were unable to bind, it was not possible to produce amplicons that could be sequenced.

4.3.5 Isolating *Leptospira* from wild rodent kidney tissue

Culturing was attempted from seven samples from the Public Gardens, a site which had previously demonstrated positive rodent samples (Table 4.6).

The same samples were also tested via PCR and three of the seven samples (BV49, WM105 and WM106) were positive by PCR for the presence of pathogenic *Leptospira*. All seven cultures were diluted and maintained in liquid EMJH media obtained from MSD Animal Health at 30^{o}C.

All cultures were checked on a weekly basis for signs of growth over a six month period. This included a visible turbidity within the media and also signs of the bacteria under the dark field microscope. However over the six months, there were no signs of growth either on the microscope or visually in the media itself. All dilutions were kept for reference for up to six months as advised. Both *L. interrogans* serovar Canicola and serovar Icterohaemorrhagiae also demonstrated no growth over the time period.

4.4 Discussion

The aim of this study was to investigate pathogenic *Leptospira* serovars currently maintained within UK wild rodent populations. Although the focus of the study was a molecular testing approach, serological and histopathology results were also examined. Being able to identify pathogenic serovars maintained over a range of environments benefits both vaccination development and treatment. The presence of the bacteria within these locations would help to determine if *Leptospira* should be perceived as a viable zoonotic threat to both dogs and humans.

Rodent trapping sites were determined based on their environment type, proximity to water sources and/or the potential for canine infection through close proximity of dog walking. This could potentially allow for the selection of diverse conditions and for possible comparisons to be drawn from positive results. However final results of this study demonstrated that positive sample numbers were not high enough for definitive conclusions based on location alone.

Locations included for the study were rural woodland (n=2), semi-rural farmland, rural fields or public gardens (n=4) and urban locations within central Liverpool (n=8). The main differences between sites hampering comparisons would be the native rodent species to that area and the small number of samples obtained. The absence of voles caught within urban locations indicates their typical habitat to be more rural. Wood mice were the only rodent species obtained from all locations sampled, suggesting their widespread presence as a risk for human and canine leptospirosis.

Screening PCR assays targeted a number of genes using published primers and conditions on two control strains (*L. interrogans* serovar Canicola and serovar Icterohaemorrhagiae). Preliminary testing of each primer set allowed for a consistent and reliable PCR protocol that could detect both saprophytic and pathogenic *Leptospira* DNA in samples by targeting the *rrs* and *gyrB* genes. Exploring the 16S/23S spacer region was also suggested as it has been targeted in other spirochetes, however as the 16S and 23S regions aren't linked in *Leptospira* this is not feasible (Woo et al., 1996).

As previously mentioned in section 4.3, brown rats were not obtained from rural areas. Repeated attempts were made to sample rats on farm land and at public gardens, however despite anecdotal reports of sightings and several different bait (including grain and cooked/cured meats), it was not possible to capture and sample any rural rats. This was one major limitation of the study. Having samples from rural and semi-rural rats would have been

of benefit given their previously well documented role in the transmission of the disease (Foronda et al., 2011; Jansen and Schneider, 2011; Koma et al., 2012; Socolovschi et al., 2011).

Of the five species sampled, the brown rat and the house mouse were the only rodent species absent of any *Leptospira* infection. One reason may be due to the low sample size of the house mice as it was only possible to obtain seven in total. A greater sample size of house mice would enable definite conclusions to be drawn regarding its status as a reservoir for *Leptospira.*

Previous work demonstrated that *gyrB* sequencing analysis shares a 100% homology with full 16S sequencing in regards to determining the species of *Leptospira* strains (Slack et al., 2006). The results from this study provided further evidence to this, as both the 16S and *gyrB* sequencing identified individual samples as being the same species.

Phylogenetic analysis of *rrs* and *gyrB* sequencing showed the nucleotide sequences of all samples confirmed to be *L. interrogans* as highly conserved. Typically wood mice and bank voles occupy the same habitat (Zhang and Usher, 1991), as evidence presented here further details, thus the two species becoming infected by the same serovar is entirely feasible. The results may indicate that all the *L. interrogans* infections are in fact from the same serovar. However, as the sequences of these genes are so highly conserved, it is only possible to identify at a species level and cannot definitively state the serovar. Further work is required to investigate whether molecular methods are able to distinguish between serovars, such as multi-locus sequence typing (MLST).

To date there have been only three previous studies carried out in the UK investigating the serovars present in wild rodent hosts, the most recent of which investigated brown rats in 1995 (Webster et al., 1995). Webster and colleagues (1995) carried out the study on UK farms investigating the presence in brown rats to see if infection levels were as high as previously perceived. They discovered that 14% were currently infected, compared to the 50-70% previously thought to act as a reservoir. Our study found no presence of *Leptospira* in the urban brown rat samples, however as it was not possible to obtain samples from farmland, despite repeated attempts, it is not possible to draw a comparison at the present time. Hathaway and colleagues (1983) discovered during a study of free-living and domestic animals in the UK that *L. interrogans* serovar Muenchen was present in wood mice, bank voles and field voles from two areas of Southern England (Hathaway et al., 1983b). *L. interrogans s*erovar Muenchen belongs to the serogroup Australis. The findings of this current study have shown that serogroup Australis is still being maintained and in circulation amongst a range of

native rodent species, however the work presented in this thesis suggests a different serovar, *L. interrogans* serovar Bratislava, infecting rodents.

The results of this study indicate that the species of rodent plays a larger role in determining infection risk than the location (urban or rural). This may be due to the habitat in which the rodents occupy, with voles preferring the rural and semi-rural locations compared to urban sites. If urban rodents (primarily brown rats) live in conditions that have low levels of standing water or no contact with other infected rodents then the chances of them in turn becoming reservoir shedders are low. Bank voles and field voles are likely to habitat the same areas however interspecies transmission of bacteria is not common (Begon et al., 1999)

Cases have been reported from the AHVLA in dogs over recent years from Bratislava and Australis infections (L Smith 2012, personal communication). This study identified both mice and voles as viable candidates to maintain these serovars, potentially for prolonged periods as positive samples were identified from two sites on more than one occasion. Due to their habitat the potential for transmitting the infection to other animals, particularly on farmland, remains a possibility.

Typically the MAT is considered the standard test for serological studies and is traditionally used to describe the serogroup of an infecting strain (Koizumi et al., 2013). As this study focused more on molecular methodology for detecting and determining the serovar, the MAT was conducted post-PCR for a comparison between results obtained. The four serovars Australis, Bratislava, Jalna and Lora were included based on their initial reaction to the Australis serogroup and previous results from direct PCR sequencing. From the four, the resulting highest titre was deemed the infecting serovar (Faine S., 1999).

The potential for MAT cross-reactivity is more prominent early on in infection when non-specific IgM antibodies are in circulation. The MAT results from this study demonstrated to an extent the possibility of cross-reactivity between serovars within the same serogroup. This reaction outlines one reason why a definitive serovar cannot be stated from a single serum sample. Clinically, a four-fold rising titre from a paired-sample is required to reliably state the infecting serogroup (van de Maele et al., 2008). As a second serum sample was not feasible for each rodent in this study, it was only possible to serologically determine the infecting serogroup based on single serum samples.

Knowing the serovars in current circulation not only aids vaccination development and efficiency, but it can also have a positive impact on the accuracy of diagnostic testing,

particularly those based on serology. In the UK, the AHVLA run the MAT test on serum samples submitted from vet practices, however the vets request which pooled antigens to run the tests on. Canicola and Icterohaemorrhagiae belong in the same pool, whereas the Australis serovars are in a different pool altogether. By not knowing which serovars pose a risk to dogs, then the correct antigens may not be requested and a potential positive could be missed. There is a degree of cross-reaction particularly within the acute phase of infection (Levett, 2004; Meites et al., 2004), however the effect of such a reaction would be minimised with chronic rodent kidney infection as witnessed during the study.

Results in this study demonstrate that PCR assays on kidney extracts have greater potential to identify a *Leptospira* infection in small rodent reservoirs when compared to serological testing. Given the greater bacterial load in the kidneys of infected reservoir hosts, and the low antibody responses, this is somewhat expected.

Histopathology has a low specificity in regards to identifying *Leptospira* in infected tissue and is not routinely used for diagnostic purposes. Keeping with its fastidious nature, it can be hard to stain conventionally; however it is Gram negative. Silver staining has previously demonstrated the ability to visualise individual bacteria in tissue sections (De Brito et al., 1996; Saravanan et al., 2000), characterised by their helical shape combined with the hooked ends.

In this study, a total of eleven samples were tested using both the Warthin-Starry and H&E stain. The stains allowed for visualisation of indicators for inflammation within reservoir hosts. Despite there being no direct link, in the absence of other potential signs of infection then it is feasible to assume that *Leptospira* are the causative agent of the inflammation. The absence of inflammation in sections where no bacteria were seen further suggests this to be the case. All eleven samples were previously shown to be PCR positive, emphasising that molecular testing by PCR is faster, more cost-effective and has a greater sensitivity for detecting the presence of an infection. Another diagnostic advantage to using PCR over histopathology is that direct sequencing of PCR amplicons can also identify the infecting *Leptospira* species whereas staining can only determine presence at best. Relying on staining alone is not recommended, although it is useful post-diagnosis, particularly for research, to obtain a visual confirmation without the need to culture.

Any study of this nature that investigates the presence of an infection will look to isolate the bacteria in question. This is of particular interest with *Leptospira* as the discovery of new pathogenic and saprophytic serovars and strains from a wide range of sources is ongoing (Saito et al., 2013; Valverde Mde et al., 2013). Isolation brings its own difficulties, particularly when

attempting to isolate from urine or tissue (Faine S., 1999). Due to the size of a rodent, using the whole kidney presents the greater chance of a successful culture. Urine contains inhibitory properties that can hamper leptospire growth after only a couple of hours, meaning a mid-stream sample is typically taken and inoculated into growth media as soon as possible (Rajeev et al., 2010). This study attempted to isolate bacteria from rodents obtained at a site previously identified as having *Leptospira* positive rodent reservoirs present.

Potential reasons for the failure to isolate any bacteria may relate to the original bacterial load in the kidney tissue. If the concentration of colonised bacteria was low, then homogenisation and dilution steps may have affected their inoculation into the media. Alongside this, if the level of viable cells where low during the initial inoculation, then this would also reduce the chances of growth when diluted out into the liquid media. However, as all dilutions were kept and monitored, still with no growth, then it may have been an issue regarding the growth media itself or its constituents, such as bovine serum albumin (BSA).

The canine vaccines currently available in the UK predominantly cover two serogroups, Canicola and Icterohaemorrhagiae, which are traditionally perceived as the most common infecting serovars in humans and dogs (Blum Dominguez Sdel et al., 2013; Miraglia et al., 2012). However in recent times the picture has shifted to differing pathogenic serovars (Koizumi et al., 2013). A novel tetravalent vaccine is now available in Europe that includes the additional *Leptospira* serogroups Australis and Grippotyphosa (Klaasen et al., 2013).

The inclusion of such serogroups within the vaccine is based on diagnosis from cases observed in practices; this study provides evidence that serovars belonging to serogroup Australis are being maintained within rodent reservoirs. The 16S and *gyrB* sequencing could only prove this to the species level; however the MAT also demonstrated that the samples belonged to the Australis serogroup, of which both Canicola and Icterohaemorrhagiae do not belong. Including strains from the Australis serogroup has aided to keep the vaccine relevant to current needs.

The main aim of this study was to establish and identify pathogenic serovars that are being maintained within small rodent reservoirs. These rodents pose a large transmission risk to dogs, as well as carrying the zoonotic potential to humans. Urine carriage of pathogenic leptospires can contaminate small bodies of standing water or cause infection through direct contact. This study successfully identified the presence of pathogenic serovars within multiple sites in the UK and across multiple rodent species, particularly wood mice. It was also possible to determine that the Australis serogroup is prominent within these animals and serovars

belonging to this serogroup have been shown to cause infection in humans and a wide range of animals including dogs in the UK.

Chapter Five

A molecular approach to identifying serovars of pathogenic *Leptospira* from direct tissue DNA extracts of wild rodents in North West England

5.1 Introduction

Leptospira are notoriously fastidious and difficult to culture. When cultures have been successfully established from bacteria isolated from the environment or clinical samples, their identification was traditionally relied on the cross agglutination absorption test (CAAT) (Faine S., 1999). More recently, monoclonal antibodies have been introduced for determining the serovar of *Leptospira* strains (Adler and Faine, 1983; Masuzawa et al., 1988) and are now used routinely for identifying the species of cultured leptospires (KIT, 2013). Obtaining cultures of any *Leptospira* species encountered is ideal in regards to research.

For diagnostic purposes, culturing the bacteria is not routine, and given their slow growth, not recommended. In the UK the widely used microscopic agglutination test (MAT) is routinely carried out on serological clinical samples (Forbes et al., 2012).

The MAT can also be used for epidemiological studies, for example to determine the natural transmission pathways of *Leptospira* (Aviat et al., 2009; Mohamed-Hassan et al., 2010). Results from the MAT are open to interpretation however due to the criteria used for determining a positive result; judgement of the required 50% agglutination against a black background can be somewhat subjective. This subjectivity potentially undermines inter-laboratory comparison of results, although the availability of reference sera and independent quality control procedures contributes to countering these concerns.

The introduction of molecular methods for the direct detection of leptospiral DNA has provided a practical alternative to serology for diagnosis and for epidemiological studies. Serology can detect a recent *Leptospira* exposure whereas PCR has the discriminative ability to detect both a recent challenge and a chronic infection. The nomenclature for *Leptospira* is serological based and as a result there is currently a limited congruence between molecular and serological identification of serovars and serogroups. The availability of a discriminative molecular technique to specify individual strains would be of great value.

Multi-locus sequence typing (MLST) has now been applied to explore the population structure and diversity of many bacterial taxa (Dingle et al., 2001; Enright et al., 2000; Iredell et al., 2003). This approach involves comparative sequence analysis of six or seven different genetic loci. These loci are typically within housekeeping genes, under neutral selection and well conserved within a bacterial species.

To date two MLST schemes have been described for *Leptospira* species. The first was developed by Ahmed and colleagues in 2006 who proposed a scheme involving loci in *rrs2,*

secY, icdA, adk, lipL32 and *lipL41*. The first four of these are housekeeping genes but the latter two encode outer membrane proteins (Levett et al., 2005; Lin et al., 2009). This study revealed that *rrs2* was highly conserved amongst serovars, whereas *secY, icdA, lipL32, lipL41* and *adk* were not. Limitations of this scheme include a lack of an existing and easily accessible online database for analysis of MLST allele profiles discovered in samples from around the world and therefore the inability to directly link obtained samples to serovars.

A second MLST scheme was published in 2007 based on seven loci, all within housekeeping genes, none of which were included in the scheme by Ahmed and colleagues (2006) (Thaipadungpanit et al., 2007). This study delineated 12 sequence types (STs) from 101 human isolates within Thailand, with one dominating in 76% of isolates (ST-34; corresponding to serovar Autumnalis). It was suggested that possible selective advantages for the survival (and host infection) of ST-34 contributed to the low ST diversity within the human isolates. Eight isolates were obtained from bandicoot rats, of which seven were associated with ST-34, indicating its role as a maintenance host for this ST within the area of North East Thailand.

Since the publication of the original scheme, a further amendment has been made to allow the detection of sequence types across six pathogenic species (*Leptospira interrogans, Leptospira borgpetersenii, Leptospira kirschneri, Leptospira weilii, Leptospira noguchii* and *Leptospira santarosai*). The *fadD* locus was excluded from the scheme and in its place *caiB* was included (Boonsilp et al., 2013). To date there are a total of 201 STs and 338 isolates in the database. All allele numbers and STs are currently published and maintained in an online database at http://leptospira.mlst.net.

Ahmed and colleagues (2011) subsequently compared the two MLST schemes. They identified slightly higher nucleotide diversity (p-distance) in the seven locus scheme of 3.60% compared to 2.30% of the six locus scheme. The inclusion of *lipL32* and *rrs2* within the six locus scheme resulted in the 7L scheme providing a better resolution of STs between the two species analysed, *Leptospira kirschneri* and *L. interrogans*. As the seven loci scheme has the online database established and maintained it was proposed by Ahmed and colleagues, that it should be the scheme to be broadly adopted for use in MLST analyses of isolates (Ahmed et al., 2011). For these reasons it was chosen for use in this study.

There have now been several MLST-based surveys of *Leptospira* species (Agampodi et al., 2013; Caimi et al., 2012; Perez and Goarant, 2010; Romero et al., 2011), including one focused on isolates obtained from rodents (Li et al., 2012). However all of the studies utilise cultured isolates or blood samples for analysis. MLST schemes have been applied to infected material

(rather than isolates) for identifying other bacterial species (Arvand et al., 2010; Henriksen et al., 2009), including from paraffin fixed tissue (Arunmozhi Balajee et al., 2013). Given the fastidious nature of *Leptospira*, developing a similar approach would be useful for both diagnostic and epidemiological purposes. This will be the first study to attempt to sequence type infecting serovars from direct DNA extracts of wild rodent kidney tissue.

This study aimed to use MLST to determine the identities and diversity of leptospiral strains infecting wild rodents in the North West of England.

5.2 Materials and Methods

Wild rodent samples for this study were obtained from seven locations as described in Chapter Two. A total of 60µl of genomic DNA was directly extracted from roughly 1g of kidney tissue from each rodent using the DNeasy® Blood and Tissue (Qiagen) kit according to manufacturer's instructions, and aliquoted into 10µl amounts and stored at -80^{o}C until required.

A total of 283 samples were screened for the presence of pathogenic *Leptospira* DNA, using the PCR assay to target the *rrs* and *gyrB* genes as described in Chapter Two, table 2.1. A total of 23 samples yielded a PCR product. The identity of the strains from which these amplicons were derived was determined by amplification then sequencing of both gene amplicons.

All 23 positive samples were then used for the MLST study to determine the infecting sequence type and which serovars and serogroups they were related to. Reference strains from two different species covered by the scheme (*L. interrogans* serovar Bratislava & *L. kirschneri* serovar Grippotyphosa) were included as positive controls to ensure the primers amplified correctly and the sequencing results were accurate.

5.2.1 Primers used in the seven loci MLST scheme

The seven loci explored in this study were the same as those described previously (Thaipadungpanit et al., 2007). Following the publication of the updated scheme, primer sequences were amended and the *caiB* locus was included to comply with the current database (Boonsilp et al., 2013).

The loci in the scheme encode for the following: *glmU* (UDP-N-acetylglucosamine pyrophosphorylase), *pntA* (NAD(P) transhydrogenase subunit alpha), *sucA* (2-oxoglutarate dehydrogenase decarboxylase component), *tpiA* (Triosephosphate isomerase), *pfkB* (Ribokinase), *mreA* (Rod shape-determining protein rodA), *fadD* (putative long-chain-fatty-acid-CoA ligase) and *caiB* (carnitine dehydrataseA).

Separate PCR assays were performed for each locus in a 50µl reaction volume using ReddyMix PCR Master Mix (Thermo Scientific) which contains 1.3 units of ThermoPrime *Taq* DNA polymerase, 150mM Tris-HCl, 40mM $(NH_4)_2SO_4$, 3mM $MgCl_2$, 0.01% (v/v) of Tween® 20 and 0.4mM each of dATP, dCTP, dGTP and dTTP with 20pmol of each primer (Eurofins MWG Operon) and 2µl of sample DNA.

The MLST PCR was carried out under the following cycle conditions: Initial denaturing at 95^{o}C for 2 minutes, followed by 30 cycles of denaturing at 95^{o}C for 10 seconds, annealing at 46^{o}C for

15 seconds and extension at 72°C for 30 seconds with a final extension of 72°C for 7 minutes. Figure 5.1 demonstrates expected amplicon sizes for all seven loci used by Boonsilp and colleagues (2013).

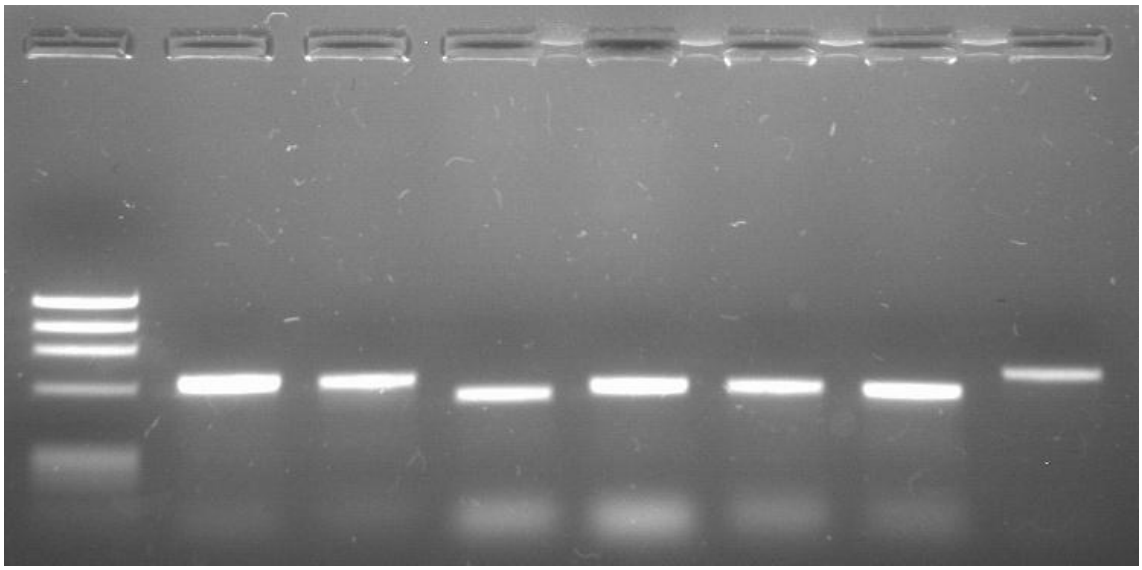

Figure 5.1. Expected amplicon sizes for each *Leptospira* MLST locus, as amplified from *L. kirschneri* serovar Grippotyphosa. Lane 1: ΦX174 ladder, Lanes 2-8: *pntA, sucA, pfkB, tpiA, mreA, glmU* and *caiB*.

Reaction products were separated and analysed on a 1% agarose gel containing ethidium bromide and Tris-acetate buffer with ΦX174 DNA marker to confirm presence of the correct sized amplicon prior to clean-up and sequencing.

Gene	Primer	Sequence (5' - 3')	Amplicon Size	Number of Alleles to date[1]
pntA	Forward	TAGGAAARATGAAACCRGGAAC	621	66
	Reverse	AAGAAGCAAGATCCACAAYTAC		
sucA	Forward	TCATTCCACTTYTAGATACGAT	640	62
	Reverse	TCTTTTTTGAATTTTTGACG		
pfkB	Forward	CGGAGAGTTTTATAARAAGGACAT	588	72
	Reverse	AGAACACCCGCCGCAAAACAAT		
tpiA	Forward	TTGCAGGAAACTGGAAAATGAAT	639	57
	Reverse	GTTTTACRGAACCHCCGTAGAGAAT		
mreA	Forward	GGCTCGCTCTYGACGGAAA	719	55
	Reverse	TCCRTAACTCATAAAMGACAAAGG		
glmU	Forward	AGGATAAGGTCGCTGTGGTA	650	52
	Reverse	AGTTTTTTTCCGGAGTTTCT		
fadD[2]	Forward	AGTATGGCGTATCTTCCTCCTT	576	20
	Reverse	TTCCCACTGTAATTTCTCCTAA		
caiB[2]	Forward	CAACTTGCGGAYATAGGAGGAG	650	51
	Reverse	ATTATGTTCCCCGTGAYTCG		

Table 5.1. Primers utilised for the seven locus scheme. [1] As of 15/12/2013. [2]*fadD* was replaced with *caiB* in the updated scheme.

5.2.2 Analysis of locus sequences and determining the ST

All PCR products were purified using the QIAquick® PCR purification kit (Qiagen) according to manufacturer's instructions. The purified DNA was quantified on a Nano-drop 1000 spectrophotometer (Thermo Scientific), and diluted to 100µg/µl/100bp. Sanger sequencing of both strands of each amplicon was carried out commercially (Source Bioscience Ltd, Nottingham, UK).

Amplification signal strength of each sequence was checked using ChromasPRO v1.7.3 (http://technelysium.com.au), as well as the presence of ambiguous background signals. Poor quality reads were re-sequenced to ensure a high quality of data. Alignments to reference alleles and sequence trimming was carried out using MEGA5 (Tamura et al., 2011). Maximum likelihood analysis to infer evolutionary trees of concatenated locus sequences were constructed using MEGA5 using the default settings with 1000 bootstrapping re-sampling. Variation within all alleles of the same locus were analysed in MEGA5 using the Kimura two parameter nucleotide substitution model.

The trimmed sequence for each locus was then entered into the allele database to identify the allele number. Allelic data for all loci were combined to yield an allelic profile that was assigned to a sequence type by comparison with profiles on the MLST database.

Genetic diversity between reference sequence types and collected data where analysed using UPGMA cluster analysis with START2 (Jolley et al., 2001) to demonstrate similarities between the taxa. eBURST analysis (Feil et al., 2004) was also carried out on the entire *Leptospira* database to determine which clonal complex the STs from this study were associated with.

Sequences for reference strains were obtained from http://leptospira.mlst.net/ and the NCBI Nucleotide database. Allele data for the previous scheme was obtained from http://leptospira.mlst.net/previous_scheme.asp for comparative analysis.

5.3 Results

From the 23 samples included in this study (Table 5.2), STs were obtained from 11. Partial allelic profiles were obtained for a further five samples. All samples for which a complete allelic profile was obtained were found to belong to ST24 (Table 5.3).

Sample ID	Rodent Species	Rural/Semi-Rural/Urban
WM1	*Apodemus sylvaticus*	Semi-Rural Open Farmland
WM12	*Apodemus sylvaticus*	Semi-Rural Managed Field
WM16	*Apodemus sylvaticus*	Rural Managed Woodland
WM18	*Apodemus sylvaticus*	Rural Managed Woodland
WM22	*Apodemus sylvaticus*	Rural Managed Woodland
WM24	*Apodemus sylvaticus*	Rural Managed Woodland
WM25	*Apodemus sylvaticus*	Semi-Rural Managed Field
WM31	*Apodemus sylvaticus*	Semi-Rural Managed Field
WM32	*Apodemus sylvaticus*	Semi-Rural Managed Field
WM64	*Apodemus sylvaticus*	Semi-Rural Open Farmland
WM74	*Apodemus sylvaticus*	Semi-Rural Field & Woodland
WM82	*Apodemus sylvaticus*	Semi-Rural Field & Woodland
WM95	*Apodemus sylvaticus*	Semi-Rural Field & Woodland
WM96	*Apodemus sylvaticus*	Semi-Rural Field & Woodland
WM105	*Apodemus sylvaticus*	Semi-Rural Field & Woodland
WM106	*Apodemus sylvaticus*	Semi-Rural Field & Woodland
BV4	*Myodes glareolus*	Rural Managed Woodland
BV18	*Myodes glareolus*	Semi-Rural Open Farmland
BV37	*Myodes glareolus*	Semi-Rural Open Farmland
BV42	*Myodes glareolus*	Semi-Rural Field & Woodland
BV49	*Myodes glareolus*	Semi-Rural Field & Woodland
FV2	*Microtus agrestis*	Rural Managed Woodland
FV6	*Microtus agrestis*	Semi-Rural Open Farmland

Table 5.2. Habitat and wild rodent species for each PCR positive sample obtained.

Following eBURST analysis it is demonstrated that ST-24 is a member of clonal complex (CC) 21. Clonal complexes define groups of sequence types based on their similarity to a central allelic profile. Within CC21 there are currently only four isolates belonging to two STs (ST-24 &

ST-25), with only one SNP differentiating the two. However to date, neither ST has been predicted as the founder of the CC. This is due to limited to low reported isolate numbers.

The 11 samples with full allelic profiles, along with the *L. interrogans* serovar Australis and *L. kirschneri* serovar Grippotyphosa positive controls, were compared to five serovars within *L. interrogans* using UPGMA cluster analysis. It was clear that there are multiple allele variations within serovars belonging to the same species and particularly within serovars of the same serogroup (Figure 5.2).

Sample	Allele								ST[1]
	glmU	*pntA*	*sucA*	*fadD*[2]	*tpiA*	*pfkB*	*mreA*	*caiB*[2]	
WM12	1	4	2	2	-	5	-	4	24
WM22	1	4	2	2	1	5	3	4	24
WM25	1	4	-	2	-	5	-	4	24
WM31	1	4	2	2	1	5	3	4	24
WM32	1	4	2	2	1	5	3	-	24
WM64	1	4	2	2	1	5	3	4	24
WM74	1	4	2	2	1	5	3	4	24
WM82	1	4	2	2	1	5	3	4	24
WM95	1	4	2	2	1	5	3	4	24
WM96	1	4	2	-	1	5	3	-	24
WM105	1	4	2	2	1	5	3	4	24
WM106	1	4	2	2	1	5	3	4	24
BV4	1	4	2	-	1	5	3	-	24
BV18	1	4	2	-	-	5	3	-	24
BV49	1	4	2	2	1	5	3	4	24
FV2	-	-	-	-	-	5	-	-	24
FV6	24	4	2	-	-	5	-	-	24
Australis	1	4	2	2	1	5	3	4	24
Grippotyphosa	19	20	13	-	22	31	18	23	110

Table 5.3. Allele numbers and sequence type (ST) results for the 23 samples found to be PCR positive for pathogenic *Leptospira*. [1] ST determined from at least 4 loci. [2] *fadD* was replaced by *caiB* for the updated scheme; both were included where possible for analysis.

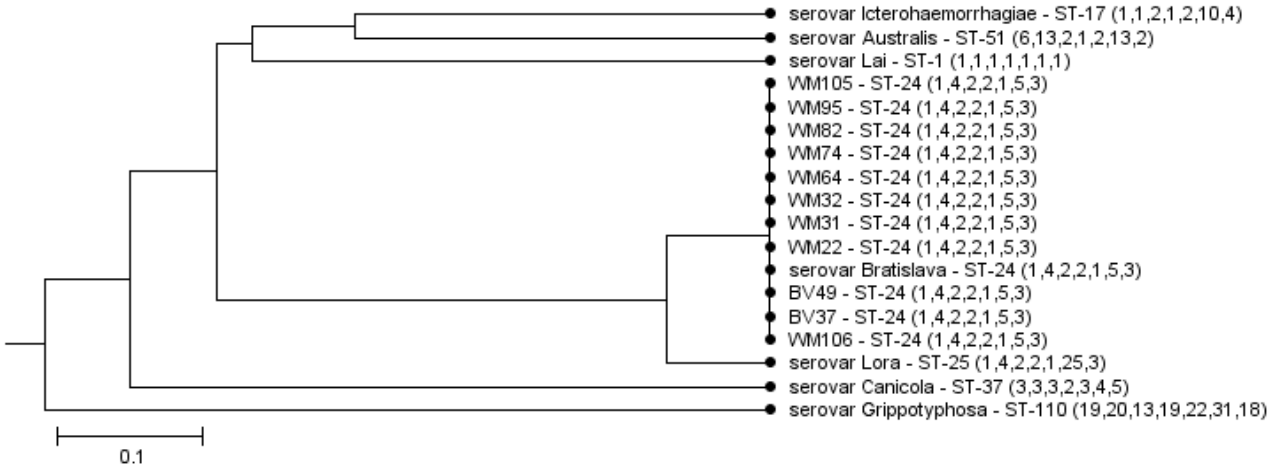

Figure 5.2. Dendrogram of the 11 samples with full allelic profiles and positive controls compared with a selection of serovars within *L. interrogans* and *L. kirschneri* from the NCBI database. Serovars Bratislava and Lora are allocated as ST-24 and ST-25respectively, and are within the Australis serogroup. Constructed using UPGMA cluster analysis.

In an attempt to obtain allele numbers for the *fadD* locus the alternate primers designed by Caimi and colleagues were utilised (Fnew: 5'-ACGTGATCTCCCTTATGCCAAGCA-3', Rnew: 5'-ATCCAACCGACAGAAGTATGGCGT-3'). The alternate primers were applied to the samples without an allele number for *fadD* from the original scheme. Despite this, I was not able to obtain any additional allele numbers using the primers.

For sample FV6 I obtained sequences for four loci. Interestingly, sequence data from the *glmU* locus shared, at best, only 87% similarity with the *glmU* sequences in the original MLST database. The overall average variation within the *glmU* locus, based on the Kimura two parameter nucleotide substitution model, was 13.8% across all 52 alleles. When compared to the updated database including seven pathogenic species, the *glmU* locus was identified as allele 24.

On the basis of its 16S rDNA sequence (Chapter 2), FV6 was initially designated as a potential *L. borgpetersenii* strain. As there are currently two full genomes published for *L. borgpetersenii* I was able to compare the sample allele sequences I obtained to genes within the existing sequences from the NCBI database. The *glmU* locus aligned perfectly with the *L. borgpetersenii* strains, however the other three sequenced loci (*pntA, sucA* and *pfkB*) aligned perfectly with alleles from *L. interrogans*.

A previous report has suggested that comparative sequence analysis of concatenated *pntA* and *glmU* data allows *L. borgpetersenii* and *L. interrogans* strains to be distinguished from one another (Perez and Goarant, 2010). Comparison of these loci from FV6 with those from representative strains of both species (Figure 5.3) indicated that FV6 was most phylogenetically similar to *L. borgpetersenii* serovar Hardjo-bovis strain JB197; however there was no distinct clustering between the two *L. borgpetersenii* reference strains included and FV6. Five strains with full published genomes were chosen as representative strains for the comparison.

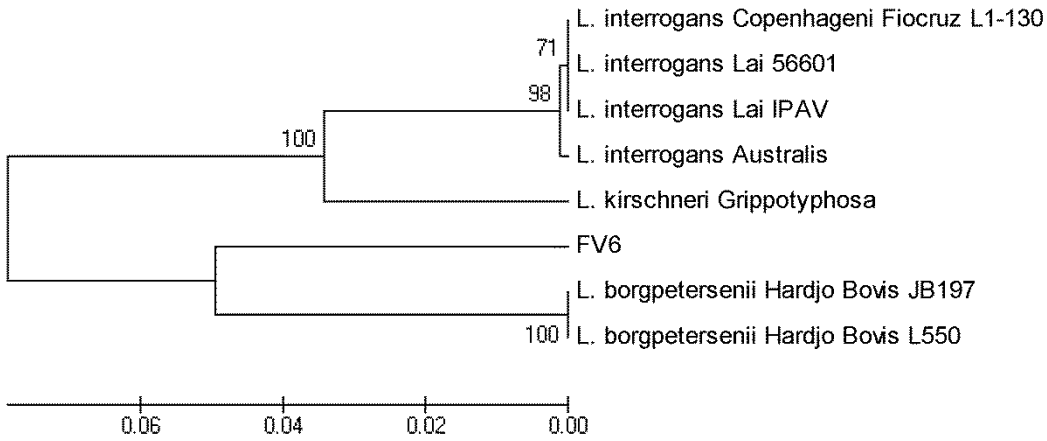

Figure 5.3. Maximum likelihood analysis of concatenated *pntA* and *glmU* sequences (969bp) for FV6 against five reference strains from the NCBI database (accession numbers: NC_004342, NC_005823, NC_008510, NC_008508, NC_017551) and two positive controls used in the study (*L. interrogans* Australis & *L. kirschneri* Grippotyphosa).

5.4 Discussion

The aim of this study was to investigate the use of a discriminative molecular typing scheme for the identification of serovars being maintained within wild rodents in the North West of England. In previous chapter four it was only been able to identify *Leptospira* within infected samples to the species level. The MLST scheme used in this study has previously been recommended as a universal scheme for adoption (Ahmed et al., 2011).

Of the 23 samples tested, it was only possible to obtain interpretable data for 16. Eleven of which were found to be the same sequence type (ST-24), with the remaining five suggested as ST-24 from the loci I was able to obtain an allele number. The data obtained suggests that ST-24 was the only infecting ST within the wild rodents at the locations sampled. The sixteen samples with interpretable data were obtained during a two year period across six locations. Two locations within a 500m radius (semi-rural open farmland and managed fields) were sampled at different time points over the two years, indicating a degree of persistence within the populations present. There are three serovars which are associated with ST-24, Bratislava, Muenchen and Jalna, all of which belong to the serogroup Australis. The closely related ST-25 (containing just serovar Lora) differs by just one SNP in the *pfkB* locus highlighting the discriminative ability of the scheme to determine the sequence type (and potentially serovar) of an infecting strain.

Not only was ST-24 identified as infecting rodents on a broad spatial and temporal scale within the North West, but was also identified within different species of wild rodents. Wood mice made up the majority of the samples identified (n=12) with bank voles contributing the rest (n=4). Unfortunately it was not possible to definitively type any of the field vole samples due to the lack of reliable amplification and sequence data for enough loci. Previously the sequence type has been isolated in Germany and the Czech Republic from a hedgehog, a yellow-neck mouse and in one case, a human. This is the first report of this sequence type being identified in the UK, and specifically within wild rodents; however the results presented here back up previously reported results on the database showing the presence of ST-24 within small wild rodents. This leads to suggest a widespread presence of this ST within Europe, although other STs have been isolated from European rodents (ST-58, 110, 115, 146 & 149) according to the online database.

Strains with an ST-24 genotype have been shown to belong to three different serovars that could be the cause of the infection (serovars Bratislava, Muenchen and Jalna). All belong to the same serogroup, are pathogenic and have the ability to cause disease in humans and dogs.

The only other previous report of this sequence type was from the Czech Republic (n=2; Jalna & Bratislava) and Germany (n=1; Muenchen) with none reported from outside of Europe. The closely related ST-25 has only been reported in Italy. The serogroup Australis is currently represented in the database with 13 serovars amongst 10 different sequence types. This highlights a potential limitation of the database, as samples will only be reported from countries that utilise the method and submit data. Given the complexities surrounding *Leptospira* identification, particularly from molecular techniques, there is a limit to what can be concluded from a limited dataset. Due to this there is a clear need for increased surveying and utilisation of MLST to generate greater allele data. From this the database can be expanded for increased effectiveness during future studies.

For one field vole sample (FV6), sequencing data for four loci were obtained (*glmU, pntA, sucA* and *pfkB*), three of which showed 100% similarity with alleles belonging to sequence types associated with *L. interrogans* serovars. However, the *glmU* locus shared only 87% similarity with the alleles within the original database. Following comparison with the current database that incorporates additional species, the sequence was assigned allele number 24. Allele 24 for *glmU* is only present in sequence types associated with *L. borgpetersenii*. Despite this similarity, the allele numbers for *pntA, sucA* and *pfkB* are still associated with *L. interrogans*. Due to this, and the absence of a full profile, it is still not feasible to assign a definitive ST to FV6.

The representation of two species within the allelic profile may indicate a potential infection from two individual serovars. The presence of multiple serovars within a single host has been previously documented (Ayanegui-Alcerreca et al., 2007), although it is not frequently witnessed. From the location where the sample was obtained (semi-rural open farmland), only one other positive sample was present that was shown to belong to ST-24. No infection from *L. borgpetersenii* alone was present at the site. As it was a beef farm, then it is possible that the species may be maintained within the herd, as it is a well documented pathogen for cattle (Bulach et al., 2006; Murray et al., 2013).

Another potential reason for the apparent incongruence of the sequence data from different loci in sample FV6 may be that the infecting strain belongs to a *Leptospira* species not currently included in the MLST database. Currently the database is restricted to seven pathogenic species and does not include *Leptospira kmetyi* and *Leptospira* genomospecies 1, along with the five intermediate species. One limitation of adapting the scheme to include other species may be the absence of loci used in the scheme from other species. For example,

pfkB, which codes for a ribokinase, is not present on either of the full *L. borgpetersenii* genomes (JB197 and L550) that have been sequenced to date.

Following concatenated analysis of *glmU* and *pntA*, it is possible to suggest that FV6 is most closely associated with *L. borgpetersenii*. Perez and Goarant included only one *L. borgpetersenii* (strain JB197) with their initial report which may have resulted in the apparent diversity, however when L550 was included along with further reference strains then it was apparent that *L. borgpetersenii* did form a separate cluster. Despite this the two concatenated loci showed limited potential beyond identifying the species. WM22 was shown to be associated with serovar Bratislava (Figure 5.1) however when based on just the two loci, it was demonstrated to have a 100% homology with serovars Australis and Copenhageni. This emphasises the limitation of specifying a serovar from just *pntA* and *glmU*. Due to the conserved nature of the locus within MLST, it is not suitable to identify a species based on this concatenation and utilising a different PCR assay is more feasible for a molecular identification simply to the species level (Slack et al., 2006).

It was not possible to amplify all loci for every PCR positive sample obtained. This issue has been demonstrated within other *Leptospira* MLST studies (Agampodi et al., 2013; Caimi et al., 2012) which presents one limitation to using direct DNA extracts from tissue. The technique itself relies on DNA being recovered at a sufficient concentration within the portion of tissue used for the extraction. This becomes more apparent within larger animal species as a biopsy would sample a smaller percentage of the overall kidney tissue when compared to a sample taken from a small rodent. On top of this there is the risk of losing DNA material during the extraction and clean-up, although precautions were taken in an attempt to maximise the total DNA output. Nested PCR assays have previously shown an improved sensitivity compared to conventional PCR for other bacterial species (Lee et al., 1998). Introducing a nested assay for locus amplification may further improve amplicon identification and also signal data produced from sequencing.

An alternative would be to use urine instead of kidney tissue. This would potentially allow for multiple attempts for isolation and/or extraction from the same animal that is actively shedding the bacteria. This approach would be particularly useful within small rodents where kidney material is limited and benefit diagnosis of canines. Extracted DNA from urine carries its own problems however. Samples are typically taken from mid-stream (Rajeev et al., 2010) and must be processed immediately to prevent the breakdown and inhibition of any bacteria that may be in there. Another issue amongst incidental hosts is that a host may only shed

pathogenic leptospires intermittently and thus hamper diagnostic capabilities, particularly when a sample is only taken from one time point (Tulsiani et al., 2011b). The difference in sensitivity and viability for DNA extracts on urine and kidney tissue has not yet been determined for *Leptospira* however hypothetically the initial bacterial load would be a factor in the concentration output.

Another limitation discovered with this study was the limited availability of sample material generated for use. The DNA extraction produced 60µl of DNA in total. Due to the size of rodent kidneys I was limited to only two extractions per sample which gave a total of 120µl per sample. As each sample required 14µl per MLST PCR for all seven loci, this gave limited material for repeated reactions. Unfortunately due to this, for some samples (WM1, WM16 and WM18) all available DNA was exhausted before obtaining sufficient sequence data for MLST analysis. This demonstrates the usefulness of obtaining cultures from any samples obtained. With a steady culture being maintained, a larger availability of material would be generated to allow for a greater number of repeats and increase the chance of obtaining full allele profiles for each sample.

During this study, the *pfkB* primers showed to be the most reliable in terms of amplicon production following PCR and signal strength from sequencing. I was able to generate good quality sequence data for 18 of the samples and it was the only locus amplified for FV2. It was also the only locus at which ST-24 and ST-25 are different (guanine to adenine at position 319). Allele 5 is currently only found in ST-24 to date, however as the database continues to expand with increased use of the scheme, it is not possible to definitively state that the sample does belong to ST-24.

In contrast, the *fadD* primers were found to be the least reliable in terms of amplification and sequencing. During the study it was only possible to obtain allele numbers for 13 samples. A similar situation has been previously reported, with alternative primers suggested for use (Caimi et al., 2012). However, even with testing the alternative *fadD* primers for this study, sequence data for the samples without the *fadD* allele could not be obtained despite multiple attempts. For three samples, data could be generated for all alleles apart from *fadD*, with that 14 full allele profiles would have been established out of the 23 samples.

The study presented here is the first to apply MLST to DNA extracted directly from infected kidney tissue to characterise the infecting leptospiral strains within wild rodents. All previous studies have used the technique on cultures that have been isolated primarily from urine, blood and tissue. One study in 2012 demonstrated the ability to apply the same *Leptospira*

MLST to DNA extracted from human blood samples during an outbreak in Sri Lanka (Agampodi et al., 2013). They were only able to produce allele numbers for 12 out of a possible 58 qPCR-positive samples (20.69%), whereas good quality sequence data was obtained for 16 of 23 PCR positive samples (69.57%). This did include repeated sequencing for some loci due to problems arising with either inefficient primer binding or low signal quality, indicating a low DNA concentration within the PCR reaction.

The benefit of having the ability to applying the technique to direct tissue extracts will allow for a rapid and accurate method for identifying the serovar of an infected human or animal. Culturing of isolates requires precise media constituents and a potential larger period of time. Even after a successful culture, further work is required to identify the serovar. MLST on direct tissue extracts presents an accurate and highly discriminative methodology, which can produce results to be easily shared between labs utilising the scheme. In this study the technique was applied to kidney tissue, however as *Leptospira* also has the potential to manifest as a chronic infection in the liver and lungs, then it is feasible that the technique could also be applied to other organs.

Other potential molecular methods for obtaining serovar identifications have previously been investigated and where considered for this study. Two main areas are macro-restriction pulsed-field gel electrophoresis (PFGE) (Galloway and Levett, 2010) and multiple locus variable number tandem repeat analysis (MLVA) (Pavan et al., 2011; Salaun et al., 2006). Both offer alternative protocols to identify the serovar of a strain; however PFGE require maintained cultures which I was not able to successfully produce in this study.

PFGE was first described to have the ability to identify infecting *Leptospira* strains in 1992 (Herrmann et al., 1992). Since then, a reference library has been developed over several studies to include over 200 strains (Galloway and Levett, 2008, 2010; Naigowit et al., 2007; Romero et al., 2009). For this study, due to a lack of viable cultures from the samples it wasn't a feasible option to carry out PFGE and instead MLST was the focus for the study.

MLVA has been demonstrated to give an accurate serovar ID based on the amplicon size from five different loci (Salaun et al., 2006). However, as demonstrated in an earlier chapter it was not possible to produce good quality sequence reads from my positive controls. Due to this the protocol was not applied to the samples obtained. In contrast to MLST, MLVA can also identify serovars belonging to the *L. borgpetersenii* species however due care must be taken when interpreting the amplicon for the correct repeat numbers.

For this study, MLST was investigated for its ability to identify the serovars of the strains found to be infecting wild rodents in the North West of England. Further to this, the potential of direct DNA extracts from kidney tissue was demonstrated with the scheme as before now, it had not been explored. An increase in the viability of MLST for tissue in comparison to previously reported blood samples was also presented in this study. Identification of a unique sequence type (ST-24) that has been infecting a range of rodent species (wood mice, bank voles and potentially field voles) across multiple sites including farmland and woodland was carried out successfully. This sequence type includes serovars Bratislava, Jalna and Muenchen which are pathogenic and have the ability to infect and cause disease in humans, dogs and other animal species.

Chapter Six

Genetic diversity within ten *Leptospira* strains of differing virulence traits

6.1 Introduction

Next generation sequencing (NGS) has revolutionised the field of genomic bacterial research and allowed the full genomes for a range of pathogenic spirochetes to be sequenced (Bellgard et al., 2009; Fraser et al., 1997; Fraser et al., 1998). Following the first full genome sequencing of a free-living organism, *Haemophilus influenza* in 1995 (Fleischmann et al., 1995), technological advancements in the field have allowed cheaper and a faster turn-around of sequence data for bacterial species. This has aided the identification of metabolite pathways, infection mechanisms and even the identification of individual virulence factors (Ricaldi et al., 2012).

Prior to the advent of NGS, the genome sizes of *Leptospira* species were estimated using pulse-field gel electrophoresis (PFGE). Using this approach, the genome of (pathogenic) *L. interrogans* was estimated to be 3.1Mb whereas the genome of (saprophytic) *L. biflexa* was estimated to be 3.5Mb (Taylor et al., 1991). Others concurrently estimated *L. interrogans* to possess a circular chromosome and a plasmid with a combined size of 4.75Mb using contour-clamped homogenous electric field gel electrophoresis (Zuerner, 1991). Subsequently, following comparison of two *L. interrogans* serovars (Icterohaemorrhagiae and Pomona) it was reported that both replicons were suggested to act as chromosomes (Zuerner et al., 1993).

To date, the whole genomes of seven *Leptospira* strains have been successfully sequenced and published. These include five strains belonging to two pathogenic species (*L. interrogans* and *L. borgpetersenii*) and two members of the saprophytic species *L. biflexa* (Bulach et al., 2006; Nascimento et al., 2004; Picardeau et al., 2008; Ren et al., 2003).

All closed *Leptospira* genomes consist of two chromosomes, with an additional plasmid (plasmid 74) in *L. biflexa* (Picardeau et al., 2008; Ren et al., 2003), and a total size of approximately 4.3Mb (range 3.88-4.71Mb). These studies suggest that, typically, chromosome I is 4Mb, chromosome II is 300kb and plasmid p74 is 30kb in size.

The GC skew demonstrates the bias for bases Guanine (G) and Cytosine (C) in either the leading or lagging DNA strand and can also be used to indicate the point of DNA replication origin. The total GC skew across both chromosomes for all *Leptospira* strains is between 35% and 40.2%. Interestingly, *L. borgpetersenii* serovar Hardjo-bovis strain JB197 has the smallest genome, but the greatest GC skew, suggesting a possible bias for choosing guanine over cytosine in the third codon position (Tillier and Collins, 2000).

Sequencing of *L. biflexa* allowed Picardeau and colleagues (2008) to suggest a 'core' leptospiral genome of 2,052 genes, comprising of essential housekeeping functions. Further to this, due to the high gene orthologue count between each genome, the authors hypothesised that lateral gene transfer had little effect on the overall gene composition.

As well as there being considerable difference in genome size and composition between pathogenic serovars, marked variation between attenuated and wild-type strains of the same serovar has also been observed. Comparison of the attenuated *L. interrogans* serovar Lai strain IPAV with the wild-type strain 56601 revealed 387 single nucleotide polymorphisms (SNPs) affecting 101 genes (Zhong et al., 2011). Within the 101 genes, only 44 were identified as encoding for a functional protein, potentially highlighting the abundance of hypothetical proteins (and variations within them) that are yet to be categorised. Furthermore, transcriptomic studies demonstrated a difference in up-regulated genes was observed when grown in EMJH. A total of 149 proteins were up-regulated in IPAV, compared to 187 in 56601. Those up-regulated in strain 56601 were typically related to DNA replication/repair, whereas those up-regulated in IPAV were involved with energy production/conversion and lipid metabolism. The variations witnessed offer an insight into how strain attenuation can affect functional genes within pathogenic serovars. With 44 functional proteins having nucleotide variations following attenuation, the study further suggests a number of genes that potentially play a role within pathogenesis.

The variation in the antigenic properties of outer membrane constituents is used as the basis for the serological nomenclature that is widely applied to leptospires. Different serovars also demonstrate an affinity for infecting particular reservoir hosts (e.g. Icterohaemorrhagiae within rodents), which may be a result of differential antigenic expression between serovars. Alternatively, as strains adapt to survive within local environments (or hosts), they diverge and antigenic differences emerge as a result. Identifying the underlying genetic variations within antigenic differences may go some way to linking the serological classification with genetic identification.

Comparison of available genomes (Bulach et al., 2006; Picardeau et al., 2008) has revealed marked variation. One striking difference between the two pathogenic species is the level of constriction in genome size within *L. borgpetersenii*. This reduction possibly presents evidence for evolution into an exclusively parasitic organism, as metabolic pathways associated with environmental survival may become obsolete (Bulach et al., 2006). As a result it is feasible to consider a similar situation within the other pathogenic species not investigated to date.

L. borgpetersenii has long been considered an exclusive pathogen for cattle (Bomfim et al., 2008) and sequencing of its genome would help identify the extent to which this species has adapted to a parasitic lifestyle, exploiting cattle as a reservoir host.

Genetic diversity within the *Leptospira* genus has previously been explored on an extensive panel of strains using a wide variety of molecular techniques. Conventional PCR assays have been developed to target a range of genes (Ahmed et al., 2012; Gravekamp et al., 1993; Slack et al., 2006), while multi-locus sequence typing (MLST), PFGE and variable number tandem repeat (VNTR) analysis have also been used (Galloway and Levett, 2010; Koizumi et al., 2013; Pavan et al., 2011; Zuerner and Alt, 2009).

One complication experienced to date is correlating molecular results based on nucleotide sequences with antigen recognition from serological tests. As traditional nomenclature is based on antibody reaction to surface antigens, there have been difficulties in identifying a molecular scheme to discriminate between every identified serovar to date. While MLST & VNTR have come close, there are instances with both approaches where serovars have not been distinguished. For example, L. *interrogans* serovars Icterohaemorrhagiae and Copenhageni share the same repeat numbers for VNTR, and within a number of MLST sequence types (STs) there are multiple strains sharing the same allelic profile. By having the full genome of multiple *Leptospira* isolates at our disposal, future targets can be identified to allow for a greater congruence between molecular and serological techniques.

Definitive factors contributing to virulence have somewhat eluded researchers with regards to *Leptospira*. Arising from the multiple PCR assays developed in recent years that target pathogenic strains, there have been several gene targets suggested that contribute to infection and colonisation. However, to date only seven proteins have been shown definitively to be virulence factors within *Leptospira* (Eshghi et al., 2012; Lambert et al., 2012; Liao et al., 2009; Lourdault et al., 2011; Murray et al., 2009b; Zhang et al., 2012).

An OmpA-family lipoprotein (*loa22*) was the first protein in *Leptospira* to be characterised that complied with the molecular Koch's postulates (Falkow, 1988; Ristow et al., 2007). The insertion of the *Himar1* transposon into *loa22* attenuated the pathogenic strain *L. interrogans* Lai 56601 within guinea pig and hamster models, including an absence of an inflammatory response compared to the wild type. Complemented strains restored *loa22* expression following re-isolation from the guinea pig model. Comparison of genes encoding for functional proteins across pathogenic, intermediate and saprophytic strains could highlight further

potential factors. Typically, functional proteins present solely in pathogenic strains are consistent with a role in pathogenesis and would warrant further investigation.

As mentioned previously, *L. borgpetersenii* possesses a reduced genome that is thought to have resulted from its adoption of a parasitic lifestyle. Due to this, it is reasonable to assume that similar processes have shaped the genomes of other leptospiral species. This in itself may result in proteins becoming essential for the infection and colonisation in specific hosts. Host specific virulence factors have been demonstrated with other bacteria (Rashid et al., 2006; Sarkar et al., 2006; Uehlinger et al., 2009). Given the reported presence of particular *Leptospira* serovars within certain hosts, it may be practical to consider host specific virulence factors within other pathogenic strains.

Recombination events between pathogenic and saprophytic strains have been previously reported. Thirteen genes within plasmid p74 have orthologues on chromosome I in pathogenic strains, with suggestions of p74 obtaining such genes through recombination (Picardeau et al., 2008). Orthologues discovered include several hypothetical proteins and exodeoxyribonuclease V sub-units.

SNPs are single base changes for a query strain when compared to a reference genome (Brookes, 1999; Collins et al., 1998). A SNP can be classed as synonymous (also referred to as silent) with no change in the codon, or non-synonymous that can change the corresponding codon. Non-synonymous SNPs can be classed as missense (changes the codon) or nonsense (introduces a stop codon). A further downstream effect is seen with non-synonymous SNPs resulting in changes in the composition of proteins that may affect their function.

Analysing SNPs within a query genome can potentially demonstrate regions of high variability. Previous work has looked into their ability to identify *Escherichia coli* strains involved with an outbreak, and determined their divergence from a comparator strain (Sherry et al., 2013). The discovery of two separate *Enterococcus faecium* sub-populations was also achieved through genome analysis, including SNP investigation, of 21 strains (Galloway-Pena et al., 2012).

For a genus such as *Leptospira*, for which nomenclature is based on antigen recognition, high variability regions may contribute towards the difference in serovars and host specificity. As a knock on from identifying these regions, potential virulence traits may be observed and investigated (Alix et al., 2006; Manning et al., 2008). Having a wider panel of sequenced genomes available can aid in allowing a greater comparison to be drawn from parasitic strains and those adapted to exploit environmental niches. As data are currently limited for

Leptospira in regards to virulence, research in this area is crucial to gain a better understanding of infection mechanisms and developing a suitable intervention.

The Illumina MiSeq (Illumina) sequencer allows for a fast turn-around of sequence data. The sequencing machine itself uses sequencing by synthesis (SBS) technology (Bentley et al., 2008; Liu et al., 2012). The basis of SBS was developed by Dr Shankar Balasubramanian & Dr David Klenerman as an alternative approach to Sanger sequencing. In brief, the sequencing concept uses modified dNTPs that each include a unique fluorescent labelled terminator to block further nucleotide polymerisation (Fuller et al., 2009). The fluorescence from the terminator is then imaged and the terminator is cleaved, allowing the addition of the next dNTP along the DNA fragment. This process continues until each fragment has been fully complemented. This method produces high quality results as the DNA fragments are bound to a solid surface and amplified prior to dNTP addition. Typically read sizes from the MiSeq platform are up to 250bp.

As a contrast to the HiSeq 2000 (also produced by Illumina), the MiSeq platform is better suited to a low throughput environment while still producing a high yield per run compared to similar sequencers on the market (Glenn, 2011; Loman et al., 2012; Quail et al., 2012). Alongside this, the MiSeq platform returns raw data with a far lower error rate on average, reported to be below 0.4%, compared with up to 13% for other platforms (Quail et al., 2012). Ion Torrent sequencing has demonstrated a greater accuracy for calling SNPs, however MiSeq has a lower rate of false calls, meaning potentially fewer errors in read output. Alongside this, MiSeq was able to call a greater percentage of true SNPs when compared to the HiSeq platform. As the average GC content for *Leptospira* is around 35%, consideration of bias would be need to be considered during the sequencing process. A similar situation is seen with the AT rich genome (GC content under 20%) of *Plasmodium falciparum* (Gardner, 2001; Quail et al., 2012). A disadvantage of MiSeq is the shorter read length (up to 250bp), meaning *de novo* constructs are limited. However the platform produces paired-end reads which provide a greater level of information compared to single reads (e.g. 2x75bp paired compared to 1x150bp single) and therefore improves the usefulness for using MiSeq reads for *de novo* constructions (Morozova and Marra, 2008).

Given the lower run costs and greater yield from the same quantity of sample DNA, the MiSeq platform was utilised for this study. As it was also suitable for deriving SNP data for all strains sequenced during the study, the greater calling accuracy is of benefit.

The aim of this study was to obtain the sequence data for *Leptospira* strains not previously analysed. This data could then be mapped onto existing reference genomes to analyse variations between different strains. A broad range of species, including pathogenic, intermediate and saprophytic, were included to demonstrate the high level of variation within the genus. Alongside SNP variations, functional gene presence within *de novo* constructs was compared for differences potentially arising from either host specificity or environmental stresses.

6.2 Methods

In this study, ten *Leptospira* strains were chosen for full genome sequencing. All ten strains were acquired courtesy of the AHVLA (Addlestone, UK), the details of which are in table 6.1.

Species	Serovar	Serogroup	Strain	Origin	Status
L. alexanderi	Manzhuang	Hebdomadis	A23	Human	Pathogenic
L. borgpetersenii	Hardjo-Bovis	Sejroe	Sponslee	Cattle	Pathogenic
L. interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	Human	Pathogenic
L. interrogans	Bratislava	Australis	Jez Bratislava	Hedgehog	Pathogenic
L. interrogans	Pomona	Pomona	Pomona	Human	Pathogenic
L. interrogans	Prajitno	Sejroe	Hardjo Prajitno	Human	Pathogenic
L. kirschneri	Grippotyphosa	Grippotyphosa	Moskva V	Human	Pathogenic
L. weilii	Mengding	Celledoni	M606	Human	Pathogenic
L. fainei	Hurstbridge	Hurstbridge	BUT 6T	Unknown	Intermediate
L. biflexa	Andamana	Andaman	CH11	Unknown	Saprophytic

Table 6.1. Ten strains sequenced for this study, together with source of isolation and infectious status.

All strains were kept at as low a passage as possible. Pathogenic strains were chosen based on their ability to infect either humans or canines, with intermediate and saprophytic species also included to ensure coverage of all pathogenicity types within the genus. The recently discovered genomospecies *L. alexanderi* was also included.

6.2.1 Extraction of DNA from all ten strains

All isolates were grown in liquid EMJH for seven days prior to DNA extraction. A total 5ml of live culture was centrifuged at 16,000g for 5 minutes and DNA extraction was carried out on the resulting pellet once the supernatant had been removed.

Initial attempts to extract DNA from isolates were made using phenol-chloroform. A total of 3ml of culture for each strain was centrifuged at 13,000rpm for two minutes. The resulting pellet was re-suspended in 467µl Tris-EDTA and mixed with 30µl 10% SDS and 20mg/ml proteinase K. The samples were incubated at 37^{o}C for 1 hour and 500µl of phenol-chloroform-isoamilic acid (25:24:1) was added. The mixture was homogenised and centrifuged at

14,000rpm for 5 minutes. After transferring the supernatant, 500µl of chloroform-isoamilic acid (24:1) was added, mixed and centrifuged at 14,000rpm for another 5 minutes. The supernatant was transferred to a new tube and 1/10 volume Sodium Acetate (3M) was included along with 0.6 volume isopropanol. This was then mixed and stored at -20^{o}C for 1 hour followed by centrifugation at 14,000rpm for 5 minutes. The supernatant was then removed and washed by adding 1ml 70% ethanol to the pellet and centrifuged again for 5 minutes. The excess was removed and allowed to air dry, followed by another wash with 1ml 100% ethanol. The excess ethanol was removed and the pellet re-suspended within 50µl of sterile water.

However, using this approach, insufficient DNA for sequencing (a minimum of 50ng/µl is required for the MiSeq platform) was obtained. Thus, a commercial Wizard Genomic DNA Kit (Promega, Southampton, UK) was subsequently used according to manufacturer's instructions with the following amendments; to increase the recovery of high concentration DNA, steps for incubation on ice and subsequent centrifugation were increased to 30 minutes each.

Following extraction and an initial estimate of concentration using the Nano-drop 1000, the purity and amount of DNA was assessed using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) according to manufacturer's instructions.

6.2.2 Sequencing of strains

Leptospiral genomes were sequenced at the Centre for Genomic Research (CGR) (Liverpool, UK) using the Illumina MiSeq platform (Illumina, UK). Briefly, DNA libraries were prepared for each of the ten samples. The purified sample DNA is fragmented, and both 5' and 3' overhangs are converted to blunt ends. Adapter sequences are then ligated to each fragment, purified and amplified using PCR to establish a library for each sample. Following denaturation with NaOH, samples were diluted in hybridisation buffer. Each library was then loaded onto MiSeq reagent cartridges according to manufacturer's instructions. Libraries were then sequenced in a paired end run using primers for adapters at both ends on each fragment (150 cycles) to obtain raw data in the Fastq file format.

Following the successful sequencing of each strain, Dr Roy Chaudhuri at the CGR carried out initial construction and analysis of sequencing files. Adapter sequences from the raw data were removed using Cutadapt (Martin, 2011) and data was further trimmed using Sickle (https://github.com/najoshi/sickle) to remove low quality fragment reads. The trimmed reads were assembled into contig predictions, and subsequently into draft genomes using Velvet.

6.2.3 *De novo* construction and analysis of draft genomes

Gene annotation on *de novo* constructs was carried out using Prokka (Prokka: Prokaryotic Genome Annotation System - http://vicbioinformatics.com/).

Coding sequences (CDSs) for each draft genomes were analysed in Artemis (Carver et al., 2012) and BLAST analysis was carried out on genes not originally identified by Prokka to confirm correct annotation. DNAPlotter (Carver et al., 2009) was used to create circular maps for each draft genome (Appendix 3). Locations of tRNA, mRNA, CDSs and GC skew were also mapped for each strain.

6.2.4 Identification of the pan- and core-genomes within *Leptospira*

Using CMG-biotools, the pan- and core-genome was calculated using BLAST analysis for individual proteins (Vesth et al., 2013). A cut-off of 50% identity and 50% coverage was used to identify gene homologues within all strains. Matching proteins within the same genome were considered as part of the same protein family. The core was determined from the presence of similar genes across all strains.

6.2.5 Identification and comparison of functional roles

Genbank files for each draft genome were uploaded for RAST annotation (Aziz et al., 2008). This allowed for variations in functional pathways possessed by each strain examined to be determined.

6.2.6 Phylogenetic comparison of gene orthologues

Alignment of nucleotide sequences for orthologues was carried out using MEGA5 (Tamura et al., 2011). Maximum likelihood analysis was used to infer evolutionary trees of concatenated locus sequences were constructed using MEGA5 using the default settings with 1000 bootstrap re-samplings.

6.2.7 Variant identification within sequenced strains

Bowtie2 analysis (Langmead and Salzberg, 2012) initially identified which reference genomes shared the most synteny with those of the sequenced strains. SNP variations for mapped reads to reference genomes were identified using GATK (DePristo et al., 2011). SNPeff software (Cingolani et al., 2012) identified SNP details such as base/codon changes, synonymous and non-synonymous SNPs and SNP density. SNP positions in comparison to the genome, along with density were visualised using Circos (Krzywinski et al., 2009).

6.3 Results

6.3.1 Mapping of sequenced strains and comparative analysis

All ten strains were successfully sequenced using the Illumina MiSeq platform. Reads were mapped to existing genomes within the NCBI database using Bowtie2 to identify similarities within the read coverage (Table 6.2). Pathogenic strains demonstrated very little similarity with saprophytic reference strains.

Sequenced Strain	Reference Strain	% Match[1]
***L. interrogans* Icterohaemorrhagiae**	Leptospira interrogans serovar Copenhageni	93.85
Pathogenic	Leptospira interrogans serovar Lai IPAV	91.45
	Leptospira interrogans serovar Lai	91.19
***L. interrogans* Bratislava**	Leptospira interrogans serovar Lai IPAV	91.38
Pathogenic	Leptospira interrogans serovar Copenhageni	91.29
	Leptospira interrogans serovar Lai	91.05
***L. interrogans* Pomona**	Leptospira interrogans serovar Copenhageni	89.08
Pathogenic	Leptospira interrogans serovar Lai IPAV	88.8
	Leptospira interrogans serovar Lai	88.47
***L. interrogans* Prajitno**	Leptospira interrogans serovar Copenhageni	85.83
Pathogenic	Leptospira interrogans serovar Lai IPAV	85.76
	Leptospira interrogans serovar Lai	85.41
***L. alexanderi* Manzhuang**	Leptospira borgpetersenii serovar Hardjo-bovis L550	50.93
Pathogenic	Leptospira borgpetersenii serovar Hardjo-bovis JB197	50.46
	Leptospira interrogans serovar Copenhageni	2.22
***L. borgpetersenii* Hardjo-Bovis**	Leptospira interrogans serovar Copenhageni	87.5
Pathogenic	Leptospira interrogans serovar Lai IPAV	87.45
	Leptospira interrogans serovar Lai	87.11
***L. kirschneri* Grippotyphosa**	Leptospira interrogans serovar Copenhageni	55.01
Pathogenic	Leptospira interrogans serovar Lai IPAV	54.98
	Leptospira interrogans serovar Lai	54.81
***L. weilii* Mengding**	Leptospira borgpetersenii serovar Hardjo-bovis L550	41.52
Pathogenic	Leptospira borgpetersenii serovar Hardjo-bovis JB197	41.11
	Leptospira interrogans serovar Lai IPAV	2.44
***L. fainei* Hurstbridge**	Leptospira interrogans serovar Copenhageni	0.26
Intermediate	Leptospira interrogans serovar Lai IPAV	0.25
	Leptospira interrogans serovar Lai	0.25
***L. biflexa* Andamana**	Leptospira biflexa serovar Patoc Patoc 1 Paris	88.85
Saprophytic	Leptospira interrogans serovar Copenhageni	0.23
	Leptospira interrogans serovar Lai IPAV	0.23

Table 6.2. Bowtie2 mapping identifying sequence similarity of all ten sequenced strains against available reference strains within Genbank. [1]The greatest three reference strain matches are shown.

Following identification of similar genomes, GATK variant detection was carried out on the six strains showing >80% mapped sequence similarity with a reference genome. The results from GATK detection were analysed using SNPeff to produce a range of SNP analysis data (Table 6.3).

L. biflexa serovar Andamana demonstrated the greatest amount of variation from the compared reference genome (*L. biflexa* serovar Patoc) with a total of 55,303 SNPs whereas *L. interrogans* serovar Icterohaemorrhagiae had the fewest with 686 (mapped to *L. interrogans* serovar Copenhageni). This equated to *L. interrogans* serovar Icterohaemorrhagiae also having the lowest change rate indicating a closer phylogenetic relation to the reference strain compared to the other five strains tested.

L interrogans serovar Icterohaemorrhagiae demonstrated the highest percentage of nonsense (3.728%) and missense (62.305%) SNPs. All other pathogenic strains maintained a similar ratio, with the only saprophytic strain, *L. biflexa*, having a greater proportion of silent SNPs.

Figures 6.1-6.7 demonstrate the sites where SNPs occur between the five sequenced strains and reference genomes. All strains had relatively even coverage across both genomes (and plasmid p74 for *L. biflexa*). It is clear that several sites on *L. interrogans* serovar Copenhageni chromosome I are conserved within several other pathogenic species given the minimal SNP disruption. Figures 6.2, 6.4 and 6.6 suggests a lower variation within chromosome II for all mapped strains, according to the summary in table 6.3 it is confirmed to have a lower rate of variation in four of the five strains sequenced. Serovar Icterohaemorrhagiae demonstrates a very high congruence for chromosome II, with only seven SNPs and a change rate of 1 in every 17, 509 bases.

Despite the large level of variants within chromosome I and chromosome II of the *L. biflexa* strain, there is a large section of plasmid p74 that contains no SNPs.

	Sequenced Strain					
	L. borgpetersenii Hardjo-Bovis	*L. interrogans* Bratislava	*L. interrogans* Icterohaemorrhagiae	*L. interrogans* Pomona	*L. interrogans* Prajitno	*L. biflexa* Andamana
Comparison Strain	*L. interrogans* Copenhageni	*L. interrogans* Lai	*L. interrogans* Copenhageni	*L. interrogans* Copenhageni	*L. interrogans* Copenhageni	*L. biflexa* Patoc
Total Variants	49,828	30,707	686	48,985	26,569	55,303
Change Rate	1 every 92 bases	1 every 153 bases	1 every 6745 bases	1 every 94 bases	1 every 174 bases	1 every 71 bases
Chromosome Variants						
I	46,304	28,497	666	45,534	24,878	49,788
Change Rate	1 every 92 bases	1 every 152 bases	1 every 6422 bases	1 every 93 bases	1 every 171 bases	1 every 72 bases
II	3,524	2,210	20	3,451	1,691	4,168
Change Rate	1 every 99 bases	1 every 162 bases	1 every 17509 bases	1 every 101 bases	1 every 207 bases	1 every 66 bases
Plasmid 74	N/A	N/A	N/A	N/A	N/A	1,347
Change Rate	N/A	N/A	N/A	N/A	N/A	1 every 55 bases
Change Type						
Homozygous	44,186	26,167	129	43,880	22,274	54,004
Heterozygous	5,642	4,540	557	5,105	4,295	1,299
Functional Class Effect						
Missense (%)	8,933 (31.493)	5,529 (32.697)	200 (62.305)	8,845 (31.602)	4,936 (33.679)	11,597 (23.009)
Nonsense (%)	127 (0.448)	95 (0.562)	12 (3.728)	145 (0.518)	95 (0.649)	133 (0.264)
Silent (%)	19,305 (68.059)	11,286 (66.742)	109 (33.956)	18,999 (67.88)	9,617 (65.654)	38,673 (76.728)
Missense/Silent Ratio	0.4627	0.4899	1.8349	0.4656	0.5133	0.2999

Table 6.3. Summary of GATK variation detection for each strain when compared against the closest matching reference genome.

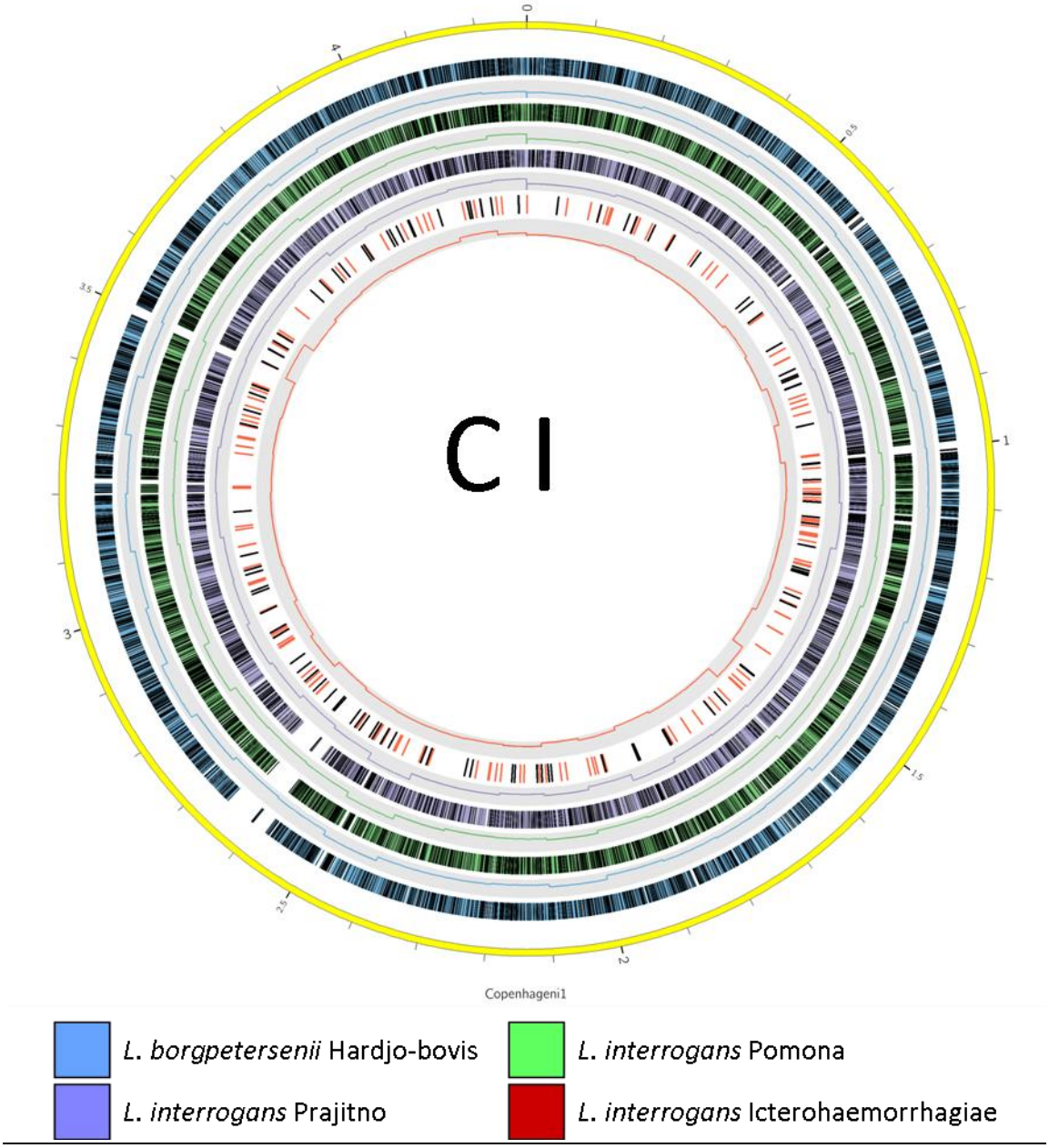

Figure 6.1. Circos diagram indicating SNP positions for four of the strains sequenced in this study mapped against the reference strain *L. interrogans* Copenhageni str Fiocruz L1-130 (NC_005823). **CI:** Chromosome 1 (4,277,158bp) **Outer track:** Reference strain (nucleotide position given in Mb). **Track 2:** SNP locations for *L. borgpetersenii* Hardjo-bovis. **Track 4:** SNP locations for *L. interrogans* Pomona. **Track 6:** SNP locations for *L. interrogans* Prajitno. **Track 8:** SNP locations for *L. interrogans* Icterohaemorrhagiae. **Tracks 3, 5, 7 & 9**: Histogram detailing relative SNP density (100kb windows) for each sequenced strain. Coloured lines indicate synonymous SNPs, Black lines indicate non-synonymous SNPs.

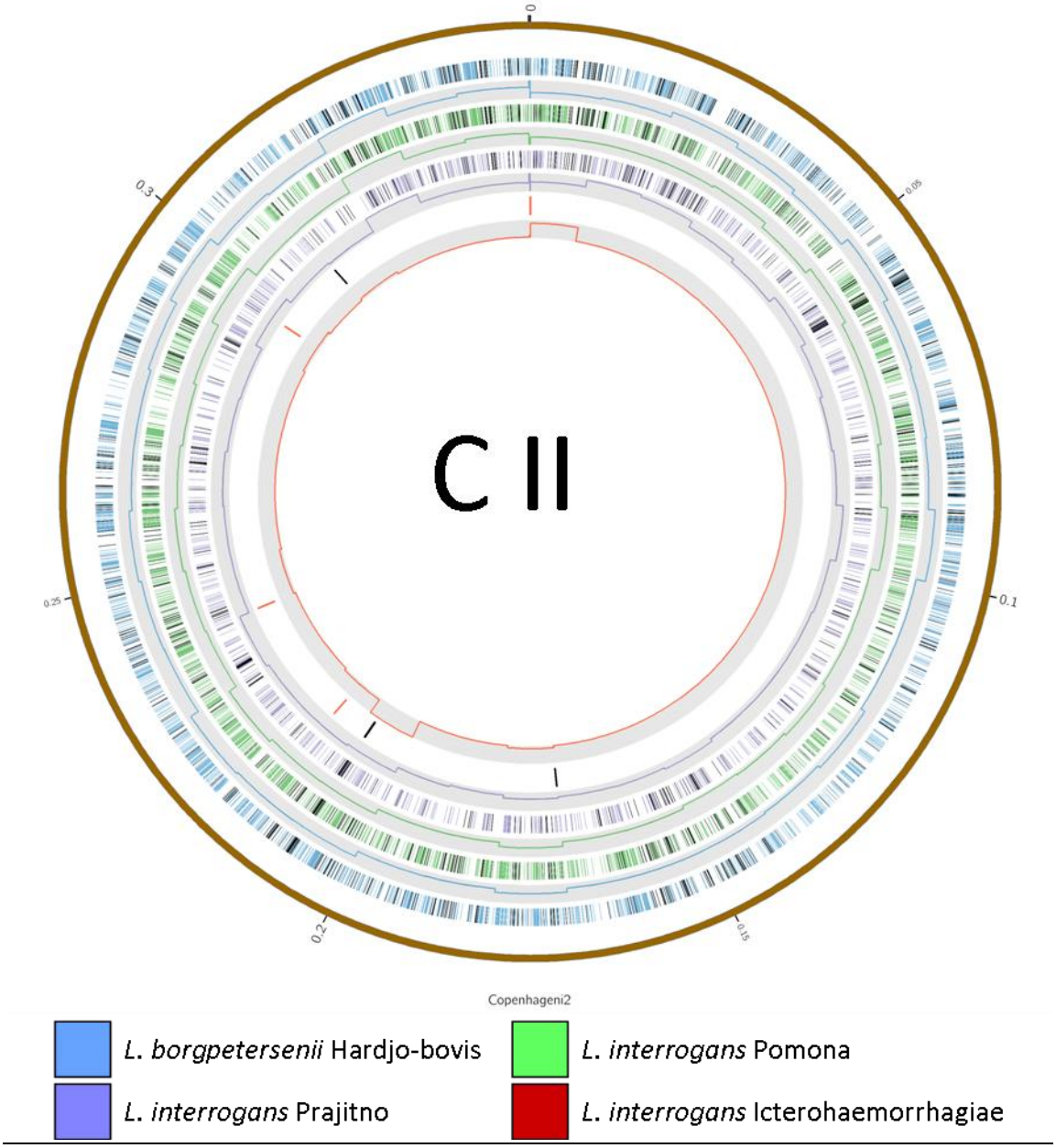

Figure 6.2. Circos diagram indicating SNP positions for four of the strains sequenced in this study mapped against the reference strain *L. interrogans* Copenhageni str Fiocruz L1-130 (NC_005823). **CII:** Chromosome 2 (350,181bp). **Outer track:** Reference strain (nucleotide position given in Mb). **Track 2:** SNP locations for *L. borgpetersenii* Hardjo-bovis. **Track 4:** SNP locations for *L. interrogans* Pomona. **Track 6:** SNP locations for *L. interrogans* Prajitno. **Track 8:** SNP locations for *L. interrogans* Icterohaemorrhagiae. **Tracks 3, 5, 7 & 9**: Histogram detailing relative SNP density (10kb windows) for each sequenced strain. Coloured lines indicate synonymous SNPs, Black lines indicate non-synonymous SNPs.

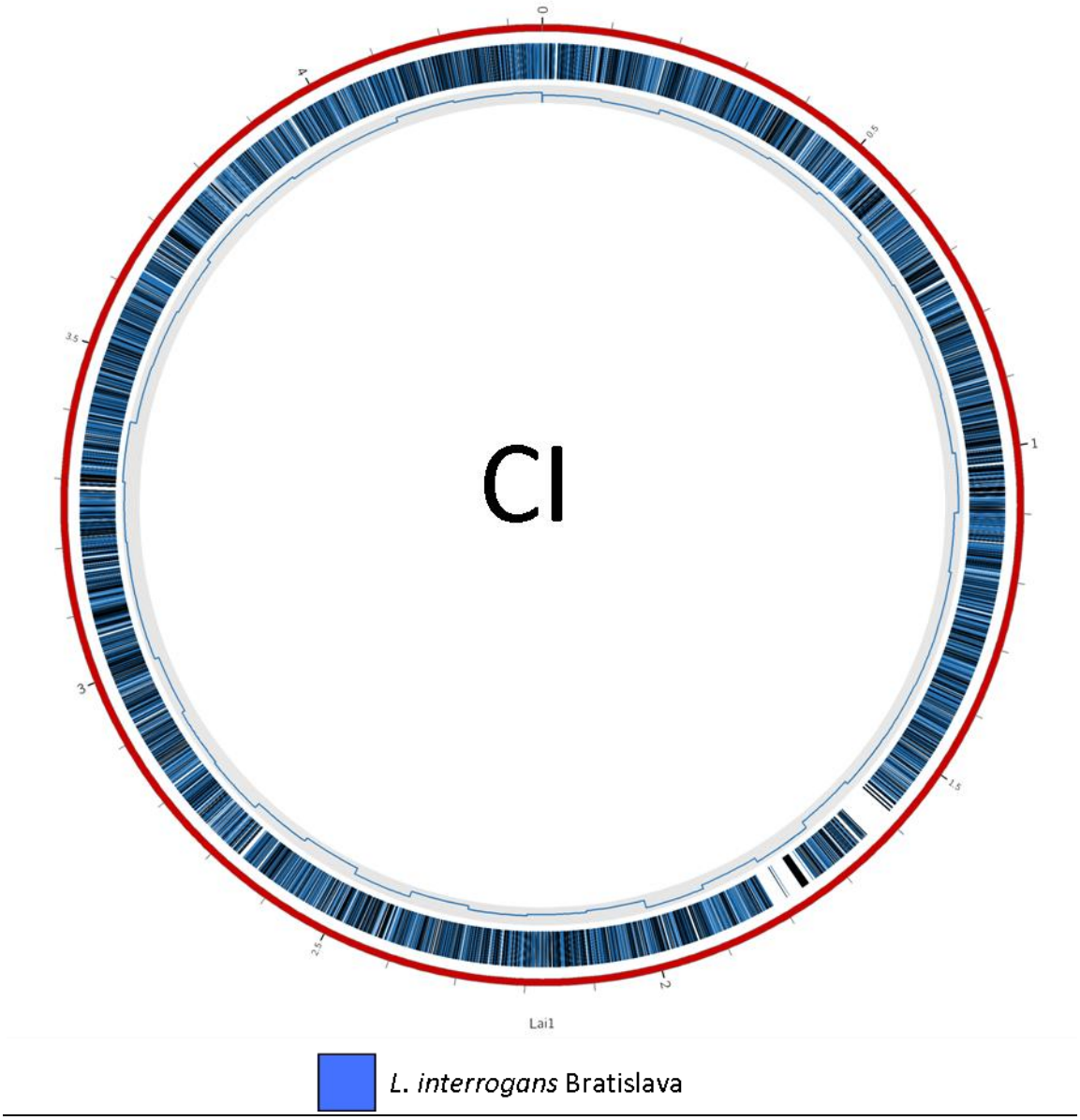

Figure 6.3. Circos diagram indicating SNP positions for *L. interrogans* Bratislava mapped against the reference strain *L. interrogans* Lai IPAV (NC_017551). **CI:** Chromosome 1 (4,349,158bp). **Outer track:** Reference strain (nucleotide position given in Mb). **Track 2:** SNP locations for *L. interrogans* Bratislava. **Track 3**: Histogram detailing relative SNP density (100kb windows). Coloured lines indicate synonymous SNPs, Black lines indicate non-synonymous SNPs.

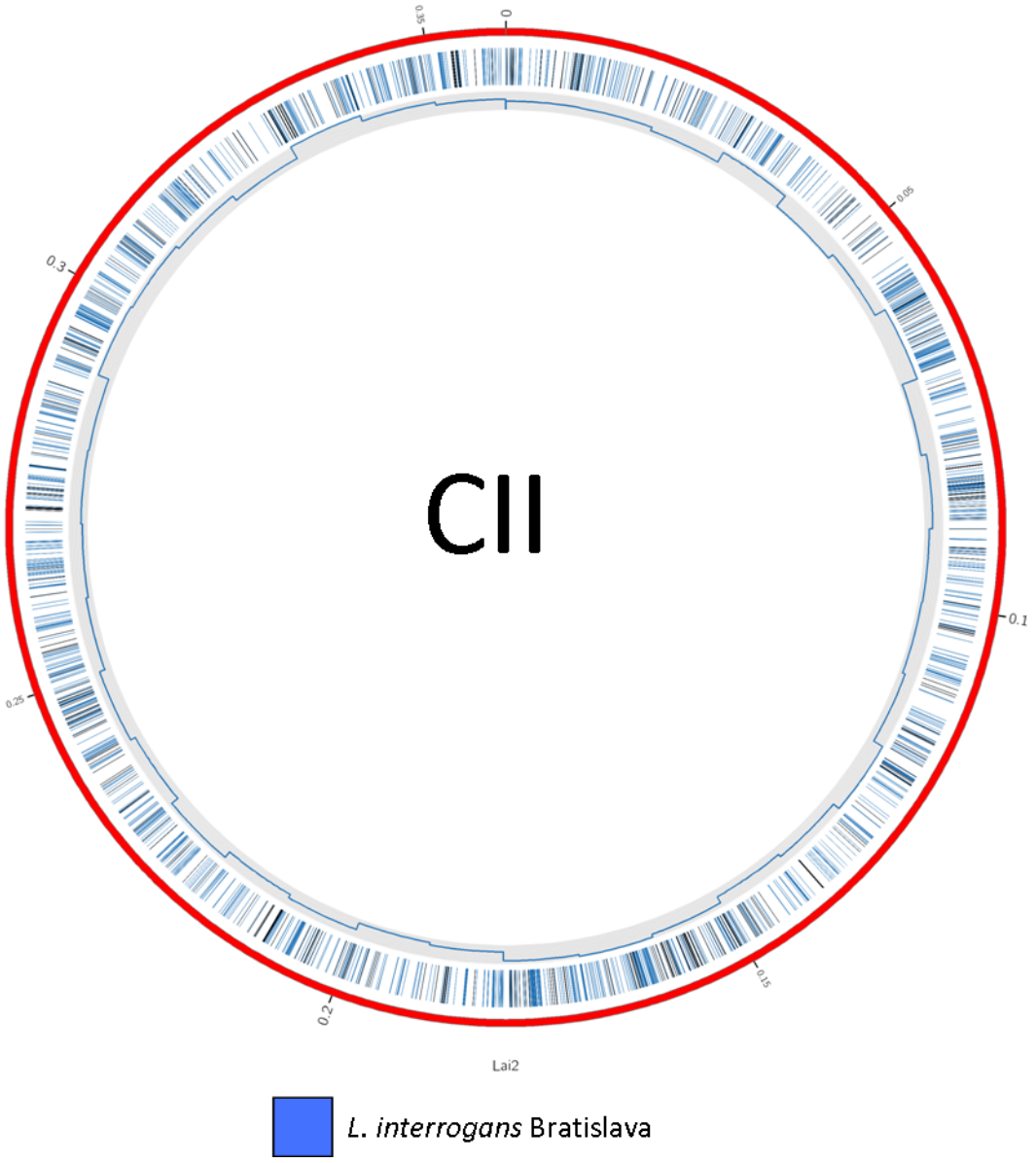

Figure 6.4. Circos diagram indicating SNP positions for *L. interrogans* Bratislava mapped against the reference strain *L. interrogans* Lai IPAV (NC_017551). **CII:** Chromosome 2 (359,372bp). **Outer track:** Reference strain (nucleotide position given in Mb). **Track 2:** SNP locations for *L. interrogans* Bratislava. **Track 3**: Histogram detailing relative SNP density (10kb windows). Coloured lines indicate synonymous SNPs, Black lines indicate non-synonymous SNPs.

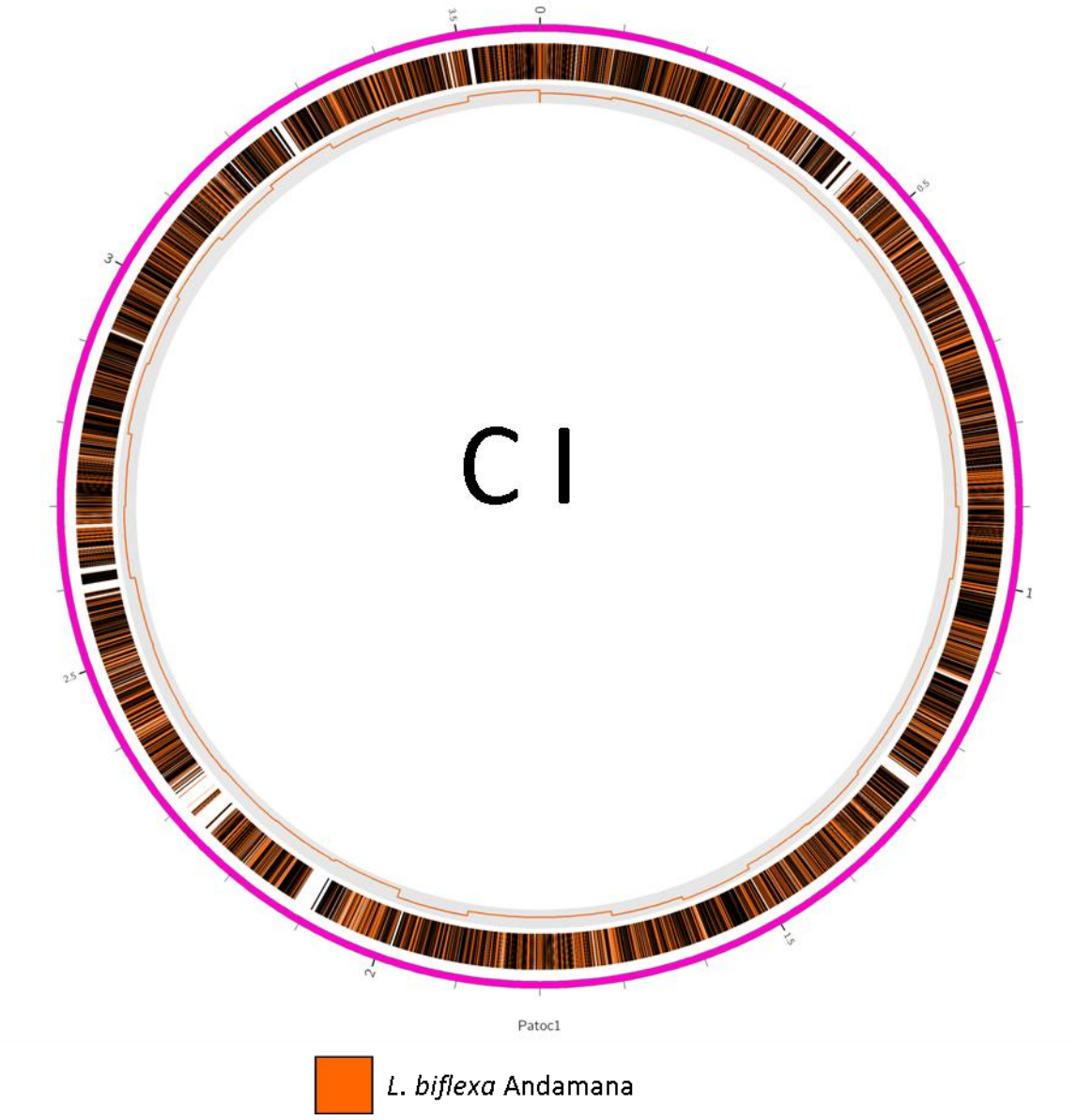

Figure 6.5. Circos diagram indicating SNP positions for *L. biflexa* Andamana mapped against the reference strain *L. biflexa* Patoc strain Patoc 1 (Paris) (NC_010602). **CI:** Chromosome 1 (3,599,677bp). **Outer track:** Reference strain (nucleotide position given in Mb). **Track 2:** SNP locations for *L. biflexa* Andamana. **Track 3**: Histogram detailing relative SNP density (100kb windows). Coloured lines indicate synonymous SNPs, Black lines indicate non-synonymous SNPs.

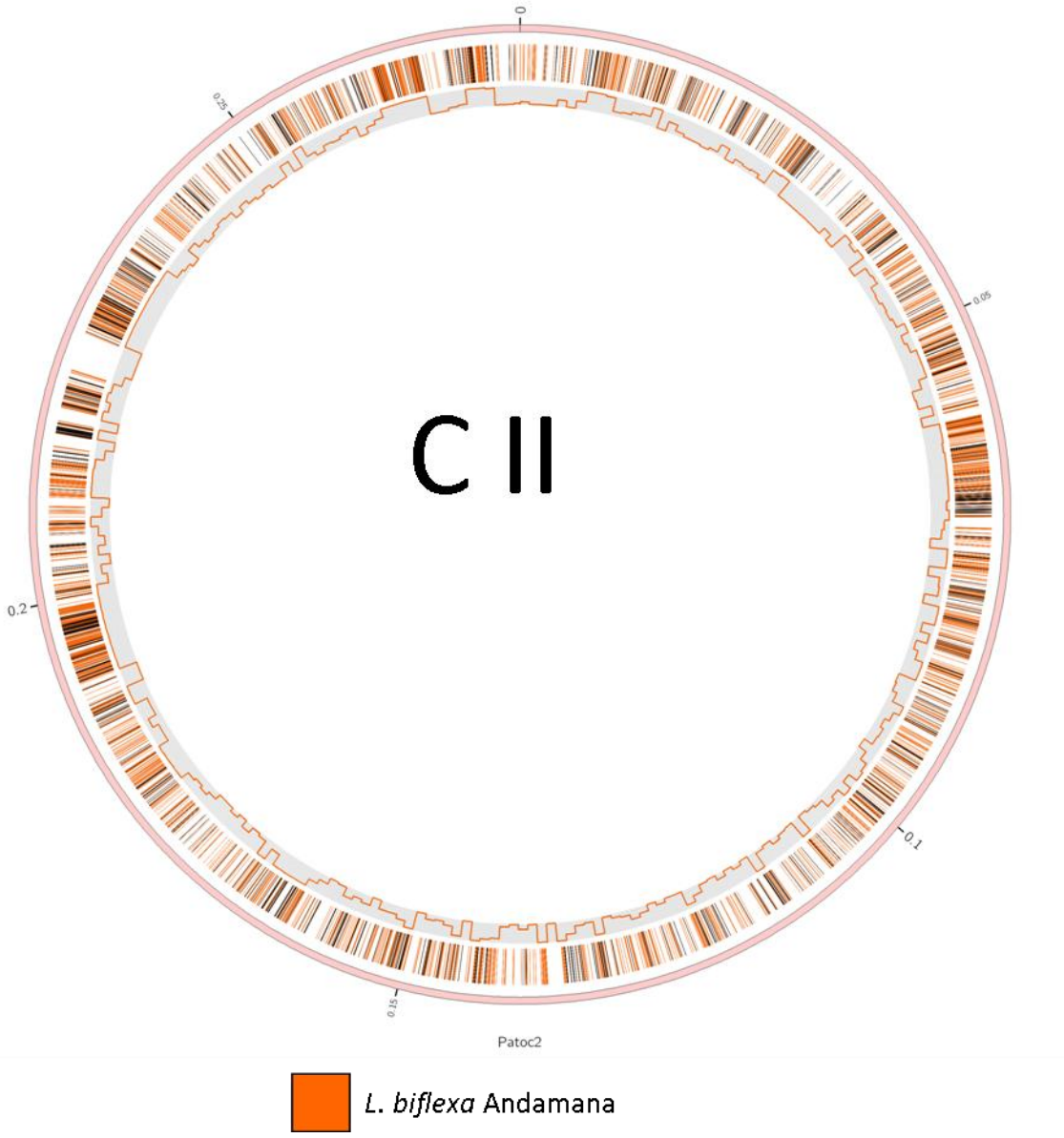

Figure 6.6. Circos diagram indicating SNP positions for *L. biflexa* Andamana mapped against the reference strain *L. biflexa* Patoc strain Patoc 1 (Paris) (NC_010602). **CII:** Chromosome 2 (277,655bp). **Outer track:** Reference strain (nucleotide position given in Mb). **Track 2:** SNP locations for *L. biflexa* Andamana. **Track 3**: Histogram detailing relative SNP density (1kb windows). Coloured lines indicate synonymous SNPs, Black lines indicate non-synonymous SNPs.

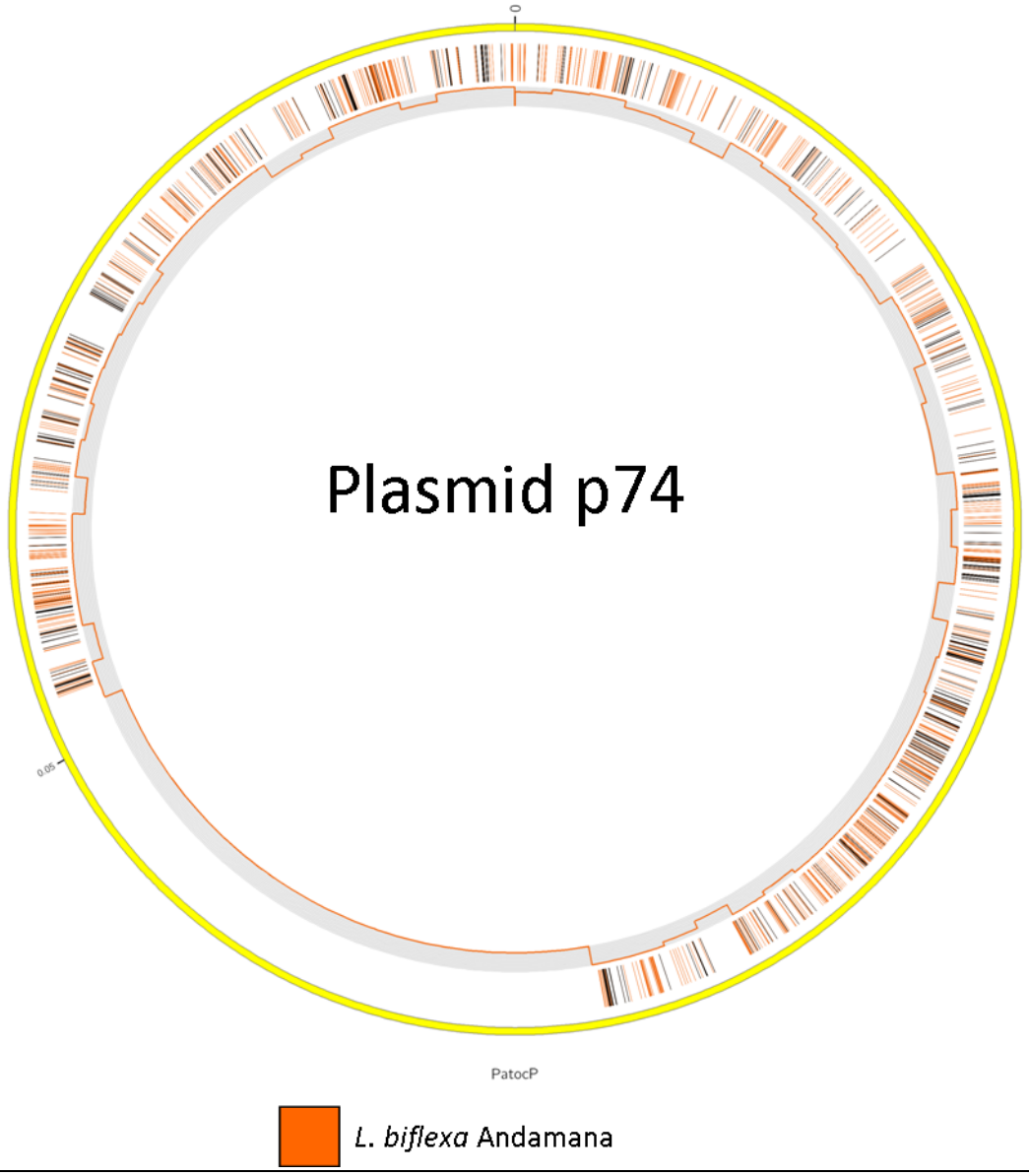

Figure 6.7. Circos diagram indicating SNP positions for *L. biflexa* Andamana mapped against the reference strain *L. biflexa* Patoc strain Patoc 1 (Paris) (NC_010602). **Plasmid p74** (74,116bp). **Outer track:** Reference strain (nucleotide position given in Mb). **Track 2:** SNP locations for *L. biflexa* Andamana. **Track 3**: Histogram detailing relative SNP density (1kb windows). Coloured lines indicate synonymous SNPs, Black lines indicate non-synonymous SNPs.

6.3.2 Annotation and analysis of *de novo* constructs for sequenced genomes

Following *de novo* construction of each sequenced genome using Velvet; all ten strains were passed through the automated annotation pipeline Prokka. Circular diagrams for each constructed sequenced strain (along with coding regions, tRNA and mRNA) can be seen in Appendix 3.

All ten strains were successfully annotated by RAST (Table 6.4) and the genes within 26 pre-determined functional subsystems could be compared (Figure 6.8).

	Assembled Genome Size (bp)	Number of Contigs	Subsystems[1]	Coding Sequences	GC Skew (%)	Number of Putitative RNA encoding genes	Number of tRNA encoding genes
***L. interrogans* sv. Icterohaemorrhagiae**	4,617,795	117	301	5111	34.83	40	37
***L. interrogans* sv. Bratislava**	4,659,159	104	298	5186	34.87	40	37
***L. interrogans* sv. Pomona**	4,558,815	165	295	5059	34.67	41	37
***L. interrogans* sv. Prajitno**	4,762,143	141	298	5386	34.81	47	37
***L. alexanderi* sv. Manzhuang**	4,045,279	294	285	4557	39.68	40	37
***L. borgpetersenii* sv. Hardjo-bovis**	4,695,933	153	295	5244	34.9	43	37
***L. kirschneri* sv. Grippotyphosa**	4,328,889	71	290	4315	35.77	42	38
***L. weilii* sv. Mengding**	4,379,309	350	282	4986	40.16	43	37
***L. fainei* sv. Hurstbridge**	4,961,646	1050	305	4928	41.25	59	50
***L. biflexa* sv. Andamana**	3,921,737	90	294	3894	38.73	43	35

Table 6.4. Summary of *de novo* genome constructs. Subsystems, coding sequences and RNA totals were calculated in RAST. [1] Pre-determined according to the RAST pipeline.

All assembled genomes were of similar size to the previously closed *Leptospira* genomes in Genbank. *L. borgpetersenii* serovar Hardjo-bovis was the only strain demonstrating any apparent difference, being roughly 700,000bp larger (across both chromosomes) than the two reference genomes of the same species (*L. borgpetersenii* serovar Hardjo-bovis, strains JB197 and L550). The GC skew was determined for each sequenced strain, demonstrating a skew between 34.67% and 41.25%.

Overall, the total amount of open reading frames (ORF) encoding for tRNA remained fairly consistent over all ten strains (n=35-38), with the exception of *L. fainei* that had 50. The previously sequenced strains of *L. interrogans* serovars Copenhageni and Lai both have 37.

6.3.3 Functional role analysis

The functional gene sub-systems were compared between each sequenced strain using RAST to identify the percentage of similarity across the seven different species (Figure 6.8). A high percentage of functions (at least 83.64%) are shared across all seven species investigated, and more importantly between pathogenic and saprophytic strains (Appendix 4).

The overall average of shared functional genes was at 91.89% (n=1,342), indicating a high majority of functional processes within all species of *Leptospira* are conserved within the genome. As expected, both the intermediate and saprophytic strains had the lowest percentage of shared functions.

Along with identifying the percentage of shared functional roles the actual total number of differences between each strain's functions was explored (Figure 6.8).

L. biflexa contains 31 genes relating to functional nitrogen subsystems whereas all pathogenic species investigated in this study only contain six. One major difference within *L. biflexa* and *L. fainei* is the presence of denitrification process genes (n=17 and 5, respectively). All ten strains contain a selection of the genes involved with producing glutamate synthase (GltS), although the two non-pathogenic strains contain a slightly higher number.

The pathways for potassium acquisition and homeostasis are the same across eight of the species investigated. Similarly, the number of genes for processing the histidine pathway are roughly equal across the ten species investigated in this study (n=10 – 13). Six of the eight pathogenic species contain greater numbers of functional subsystems for arginine biosynthesis (n=15 compared to n=8). In contrast, *L. biflexa* and *L. fainei* only contain eight and seven respectively.

The intermediate species investigated here was observed to contain genes for a partial pathway of methionine uptake and the degradation into homocysteine.

According to RAST analysis, the intermediate and saprophytic strains contained more genes within the virulence, disease and defence sub-system than any other strain (51 and 70, respectively). This is due to the higher number of antibiotic resistance genes, with *L. biflexa* containing 37 genes for resistance to antibiotics and toxic compounds. This includes resistance to fluoroquinolones, vancomycin, copper tolerance and beta-lactamases.

Low numbers of genes relating to iron acquisition were recognised within all strains, particularly within pathogenic strains. Only one gene was identified to relate to iron acquisition within each pathogenic strain, three in the saprophytic and none in the intermediate.

For each strain, low numbers of phage or transposable elements were identified using an automated pipeline. Further manual investigation for insertion sequences revealed no additional presence, particularly IS122 within pathogenic strains.

Overall, pathogenic species demonstrated a relatively similar functional difference, whereas the intermediate and saprophytic strains presented a much higher level of difference (Appendix 4).

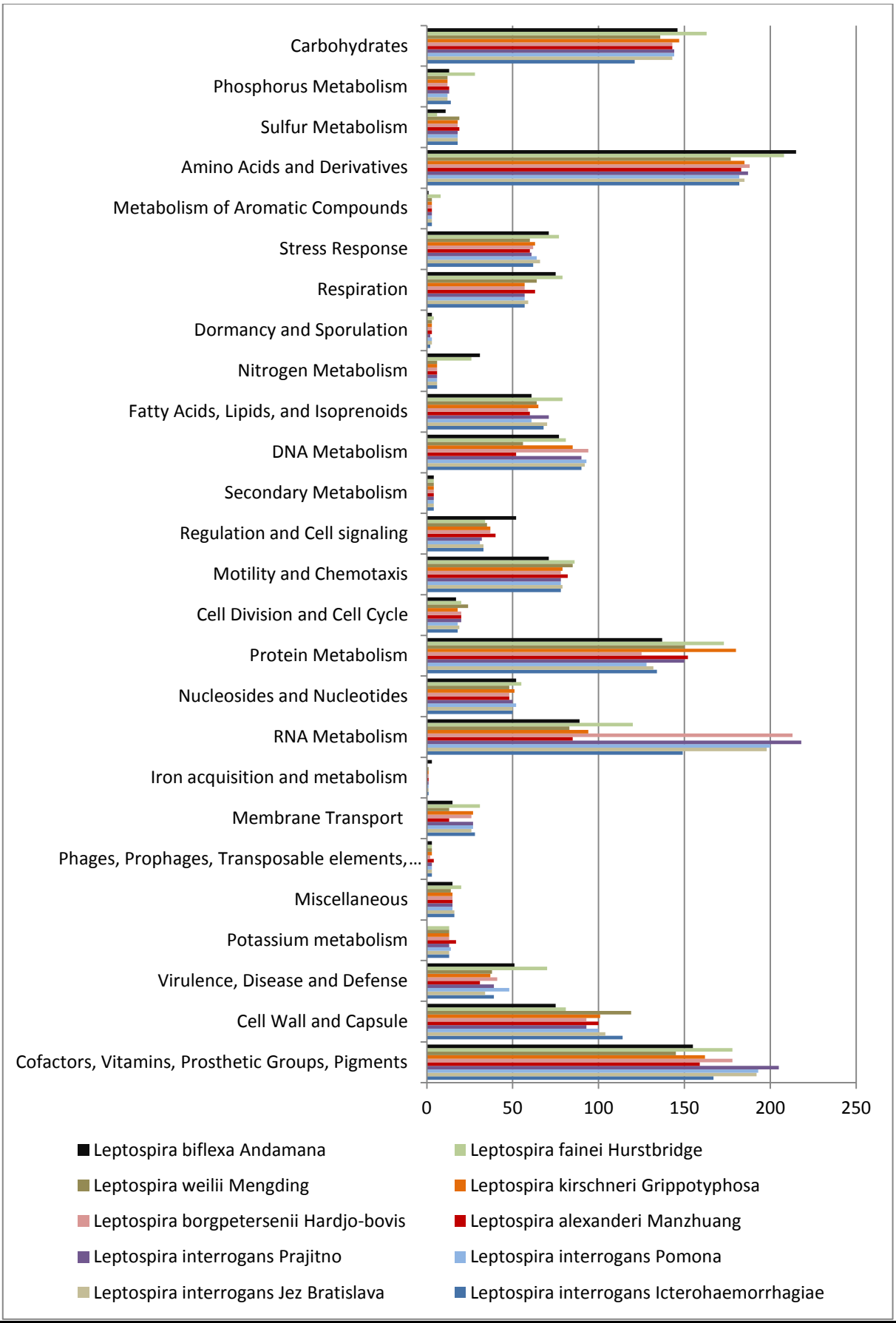

Figure 6.8. Comparison of the total number of genes within each functional sub-systems as determined by RAST annotation.

6.3.4 Identification of a 'core' genome within all strains sequenced in the study

All ten strains were subjected to BLAST analysis against themselves and the seven reference strains currently within the NCBI database. This allowed both the pan- and core-genomes amongst 17 strains of *Leptospira* (Figure 6.9) to be determined. Within 17 strains, there were 10,488 unique genes (pan-genome) and a core-genome represented by 1,095 genes (28.76% of the total genome) across different serovars and species. As expected, the core genome comprises of housekeeping genes involved with cell maintenance, DNA replication and repair, and cell motility. In keeping with the overall *Leptospira* genetic make-up, a proportion of core genes were hypothetical with no known role.

Within four strains of a single species, the core genome retained a higher percentage of the overall composition (Figure 6.10a). *L. interrogans* strains had a core of 3,153 genes (roughly 84.40% of the total genome). However as CMG-Tools only outputs total amounts, it was not possible to determine the identity of unique genes for each strain using this method.

The core for the eight pathogenic strains made up a much greater percentage of the overall genome when compared to all 17 strains (n=2,859), contributing 69.30% on average of the total gene pool.

One representative strain for the pathogenic, intermediate and saprophytic pathogenicity groups were then analysed, demonstrating a shared core-genome of 1,104 genes (roughly 28% of the total genome) (Figure 6.10b).

6.3.5 Presence of confirmed, or genes alluding to, virulence factors within *Leptospira*

The presence of 36 genes previously discussed as potential virulence factors or utilised in previously described PCR assays were investigated within each strain (Appendix 5). The presence of such genes within all eight pathogenic strains further confirms their role for determining *Leptospira* detection.

Four genes that have been strongly linked (or confirmed) to virulence, or were only identified in the pathogenic species, were aligned and compared. Inclusion of intermediate and saprophytic strains with the comparison demonstrates the nucleotide variation and their usefulness for species differentiation (Figure 6.11).

The gene first identified as a *Leptospira* virulence factor, *loa22,* was present in all eight pathogenic strains; however analysis of the *de novo* constructs found no presence within either *L. biflexa* or *L. fainei*. The nucleotide sequence itself was found to be highly conserved,

particularly within serovars of the same species. The gene encoding for GyrB was the only one out of the four analysed to be present within all sequenced strains.

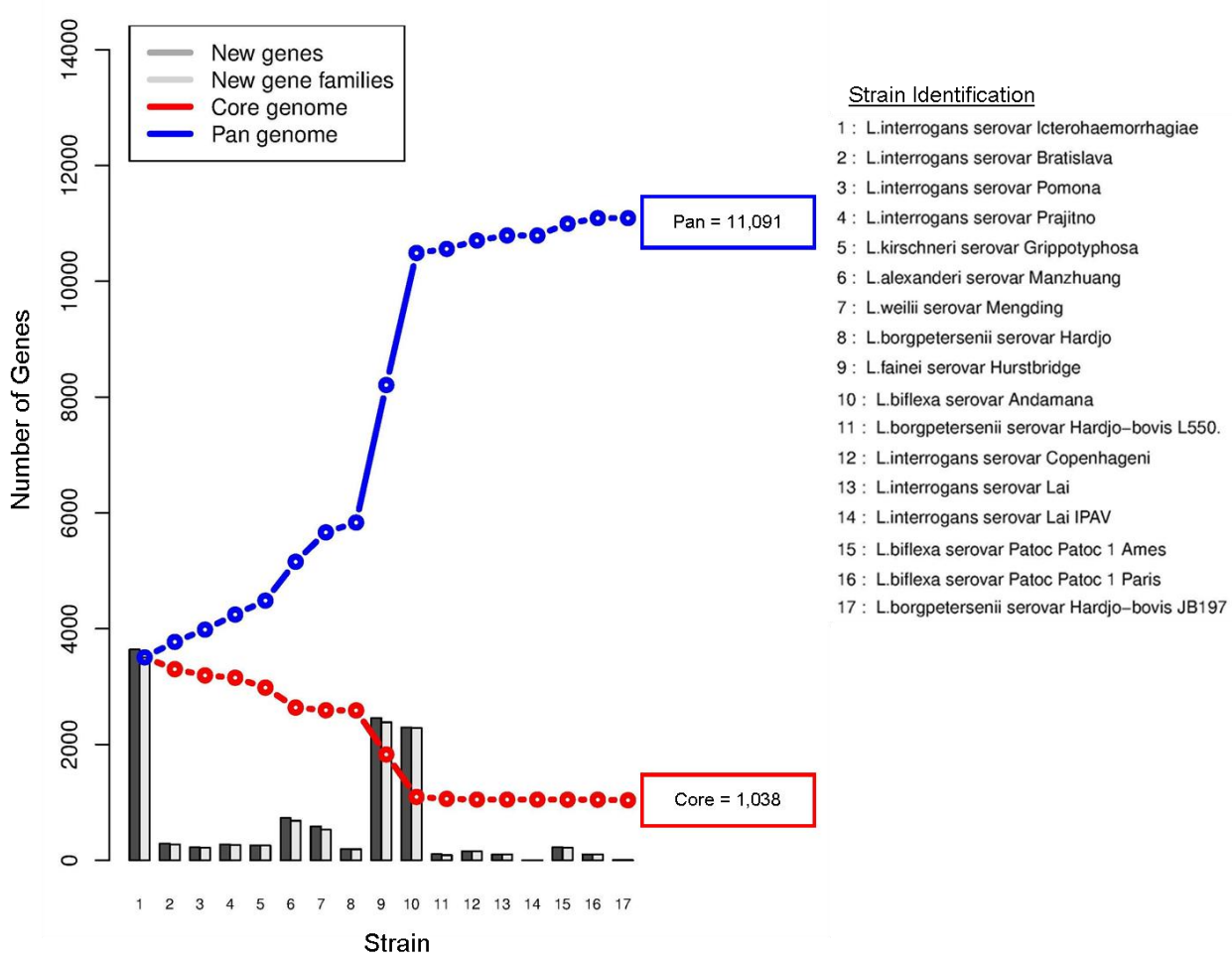

Figure 6.9. The pan- and core-genome as calculated using BLAST (parameters of 50% identity and 50% coverage). If two proteins matched within a genome they were included as one gene family. A gene was considered as core if present within all 17 strains. The pan-genome is the total number of unique genes present within all ten strains. **Strains 1-10:** Ten strains sequenced during this study. **Strains 11-17:** Seven closed and fully annotated strains from the NCBI database (Accession numbers: NC_004342, NC_005823, NC_008510, NC_008508, NC_017551, NC_010602, NC_010842).

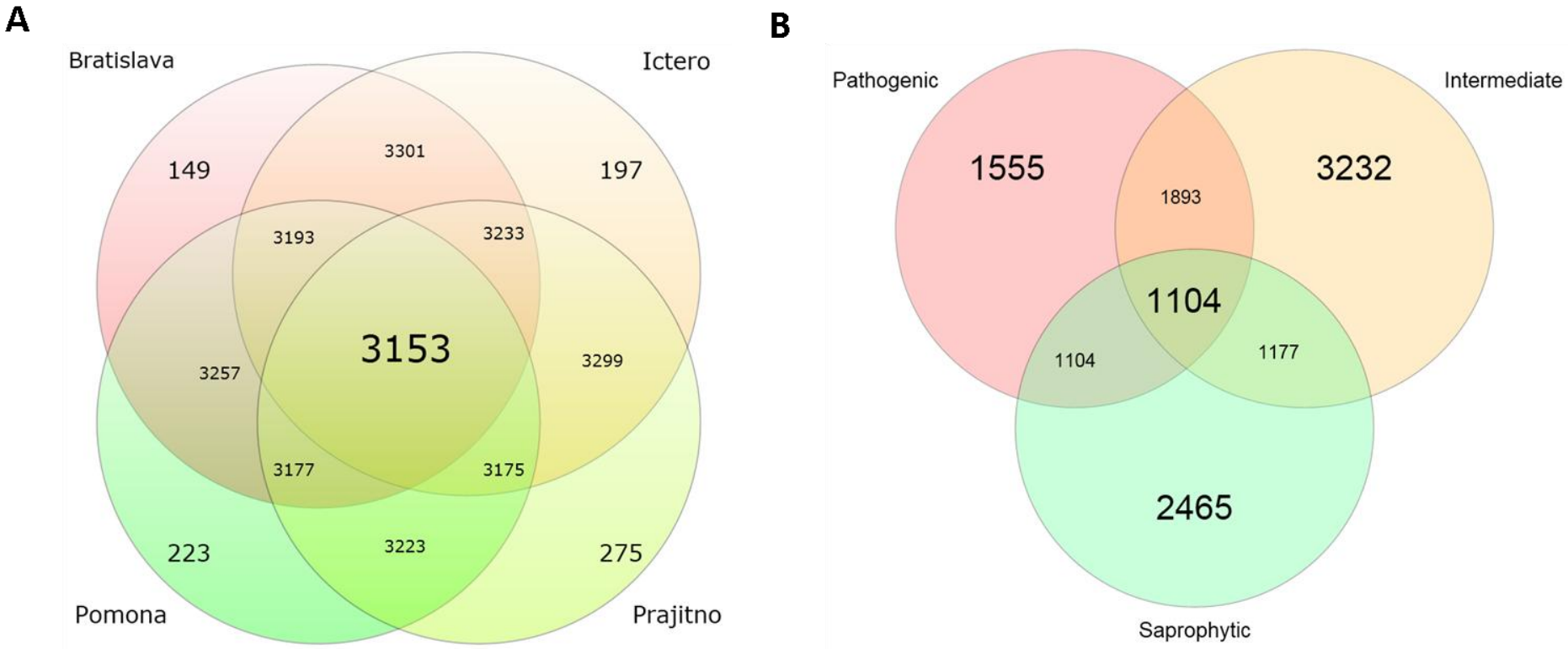

Figure 6.10. A: Unique and shared individual genes amongst the four *L. interrogans* serovars sequenced during this study. **B:** Unique and shared individual genes amongst strains representing each pathogenicity group; *L. interrogans* Icterohaemorrhagiae (pathogenic), *L. biflexa* serovar Andamana (saprophytic) & *L. faniei* serovar Hurstbridge (intermediate).

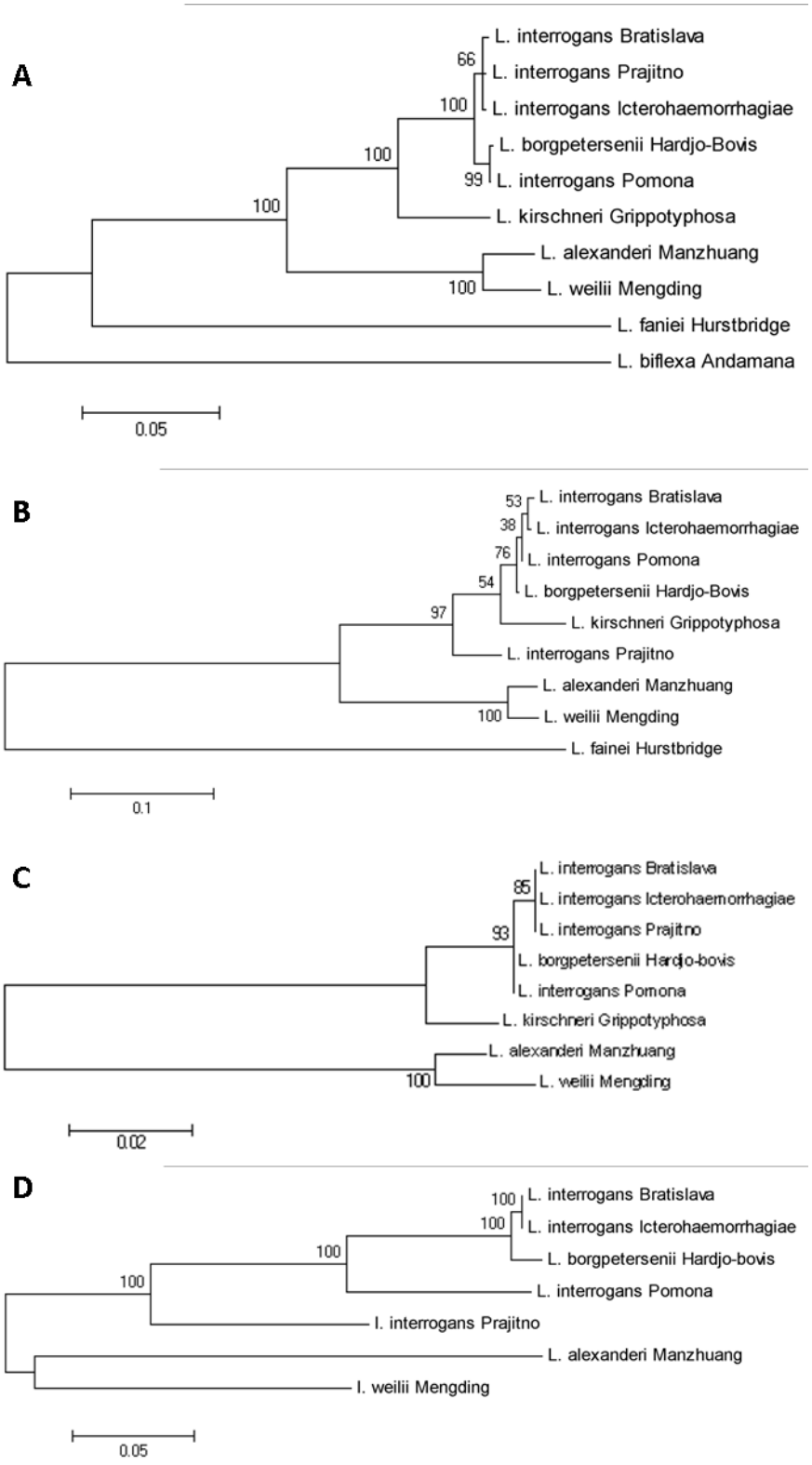

Figure 6.11. Maximum likelihood analysis for aligned sequences for four gene orthologues found within strains sequenced within this study. Carried out using the default settings with 1000 bootstrapping re-sampling. **A:** *gyrB* (DNA gyrase sub-unit B) **B:** *ompL1* (outer membrane protein) **C:** *loa22* (OmpA-family lipoprotein) **D:** *lipl36* (outer membrane lipoprotein).

6.4 Discussion

This study involved the sequencing of the genomes of ten *Leptospira* strains. From this, the variation between sequenced strains and seven previously sequenced reference genomes was investigated. A high level of variation was discovered, even within serovars of the same species. The individual functional genes were also compared, which further emphasised a diverse population of strains within the *Leptospira* genus.

Part of this study was to sequence isolated strains acquired during the overall project. However, due to issues encountered during the isolation process it was not possible to generate cultured isolates for genome sequencing. As a result the strains had to be acquired from other sources. This meant that I was not able to obtain strains specifically of rodent or canine origin which would have been ideal. Instead it was decided to sequence strains covering a range of species (pathogenic, intermediate and saprophytic) to allow for comparison between species that may have adapted to a range of different hosts.

Picardeau and colleagues (2008) previously determined by comparison of three species (pathogenic, intermediate and saprophytic), a core genome of 2,052 (61%) orthologous genes (Picardeau et al., 2008). This study determined a core genome of 1,095 (28.76%) genes amongst the ten sequenced strains. This figure was further reduced to 1,038 when seven reference strains were also included (for a total of 17 strains).

Given the core comprises of a much greater percentage between pathogenic strains, the reduction caused by inclusion of both the saprophytic and intermediate strains may reflect the difference in pathways utilised for survival within external environmental conditions.

This relatively large difference may be a culmination of several factors including sequencing methodology (MiSeq is not ideal for *de novo* constructs), annotation pipeline and parameters used during the BLAST search. Alongside this a larger amount of 17 strains were included in this analysis, compared to the three used by Picardeau and colleagues (2008). For a direct comparison, the strains examined by Picardeau were analysed using CMG-biotools, which identified a core of 1,070 genes. This finding is more in-line with the results of this study, indicating that the methodology may be the defining factor behind the large discrepancy.

As previously described, the 16S and 23S rDNA are not linked as with other spirochetes (Woo et al., 1996). Instead they are distributed within chromosome I, a situation seen in all strains sequenced within this study. Previous conflicting reports have discussed how the relatively low number of tRNA affects growth rates (Picardeau et al., 2008; Ren et al., 2003). This project

found a similar situation as Picardeau and colleagues (2008) were the saprophytic *L. biflexa*, despite previously known for growing faster than their pathogenic counterparts (Ganoza et al., 2006), have the same level of putitative tRNA coding genes.

Previous studies have described a genome reduction of approximately 700kb within *L. borgpetersenii* compared to *L. interrogans*, as it tends towards host specialisation (Bulach et al., 2006). However in the current study, the *L. borgpetersenii* strain sequenced had a total assembly size 700kb greater than the other pathogenic species. This may be due to the initial sequencing approach taken, and that the genomes in this study were primary constructs, not closed genomes. The MiSeq reads in this study were predominantly intended for SNP recognition, and so full *de novo* assemblies may have had a greater accuracy from a sequencing approach such as HiSeq. In comparison, the HiSeq outputs a greater maximum number of paired end reads (>1 billion compared to 50 million) and overall total data obtained (>90 Gb compared to 15 Gb).

6.4.1 SNP variation between sequenced and reference strains

Even though *L. biflexa* serovar Andamana was mapped to a serovar within the same species (*L. biflexa* serovar Patoc) it still presented with the greatest level of genetic variation within its genome. This difference may highlight the extent to which environmental pressures exert on the evolution of bacteria that exist exclusively outside of a host.

Survival within a range of environments may also expose the bacterium to a greater degree of genetic variation through insertion sequence (IS) acquisition or other mobile elements (Heuer and Smalla, 2007). Despite that however, no presence of IS1533, previously described as a prominent IS within *Leptospira* (Zuerner et al., 1995), was found in the strains sequenced for this study. This finding is a stark contrast from that witnessed by Picardeau and colleagues (2008) who described large numbers of IS elements, particularly within pathogenic strains (Picardeau et al., 2008). The discrepancy may be due to the sequencing and annotation process. However as a manual search also found no presence then it may be further evidence for using HiSeq over MiSeq to allow for greater genome coverage.

The region within the p74 plasmid that shows no SNP presence may be highly conserved and crucial for coding processes involved with the survival and transmission of the plasmid; however synonymous SNPs may still be present should this be the case.

Another explanation could be a lack of coverage (or complete absence) for the region by the sequence reads for *L. biflexa* serovar Andamana. If the region was not present within the

sequences obtained then no SNPs would be detected by GATK. Given the density of SNPs within the remaining regions of chromosome I, chromosome II and p74, then it seems that a lack of coverage resulting from sequencing is the greater possibility.

The greater number of SNPs seen within *L. borgpetersenii* can be somewhat explained by the reference genome (*L. interrogans* serovar Copenhageni) being of a different species completely. Initially, either of the two *L. borgpetersenii* genomes previously sequenced may have been chosen, however due to neither showing a close match from Bowtie2 mapping (both 2.75%) then they were not selected for the GATK variation pipeline.

The only strain that shows a lower level of SNP variation is *L. interrogans* serovar Icterohaemorrhagiae (mapped against *L. interrogans* serovar Copenhageni). The genetic similarities between serovars Icterohaemorrhagiae and Copenhageni have been previously described (Salaun et al., 2006), and so the low SNP presence is not unexpected. The SNP data in the present study further suggests that both serovars Icterohaemorrhagiae and Copenhageni have a closely related phylogeny. This is especially true for chromosome II. With only seven SNPs mapped within the chromosome a high level of similarity is seen.

However, despite the lower SNP count, RAST analysis recognised only 92.79% of functional roles shared when compared to the same reference strain. Icterohaemorrhagiae had 46 unique functions and Copenhageni demonstrated 54 unique functions. Of the pathogenic strains sequenced in this study, five showed a higher proportion of orthologues, with only *L. weilli* serovar Mengding demonstrating a lower level of similarity. Given the previous reported links between the two serovars (Icterohaemorrhagiae and Copenhageni), including similar reservoir hosts, the difference in functional roles is somewhat surprising. The higher nonsense/silent SNP ratio within Icterohaemorrhagiae suggests that protein disruption may suggest the discrepancy with translated proteins between the two.

The major difference for SNPs seen within serovar Icterohaemorrhagiae was the greater percentage coding for nonsense or missense variants. The introduction of nonsense codons will disrupt the reading of coding regions and may result in truncated proteins. Alterations of amino acid constituents from missense variations may cause multiple downstream effects such as the folding and structure of proteins. This change could disrupt pathways and may be a possible reason for such a difference within the functional roles as observed with RAST.

Between the four strains (*L. borgpetersenii* serovar Hardjo-bovis, *L. interrogans* serovar Icterohaemorrhagiae, *L. interrogans* serovar Pomona and *L. interrogans* serovar Pajitno)

mapped against serovar Copenhageni it is clear that there are three regions within chromosome I with a high congruence between each sequenced strain. There were no SNPs between nucleotides 992,109-1,005,189 suggesting a highly conserved region between the serovars and species. However within the region, only five hypothetical proteins have previously been identified on serovar Copenhageni. The conserved nature suggests towards an important role within the bacterium, however further investigation is needed to correctly identify the proteins within the region. The genes may also carry the potential for species identification through PCR assays.

Nucleotides 2,577,755-2,658,246 also show very little SNP presence between the *L. interrogans* strains. As with nucleotide region 992,109-1,005,189, there are also a very high proportion of hypothetical proteins. However this region does contain *mutT* (mutator protein), and the absence of the gene can lead to increase spontaneous A/T to C/G mutations (Bhatnagar et al., 1991). Therefore its involvement with maintaining correct base sequence may be crucial for the bacteria, causing the low level of nucleotide variation.

Following analysis of SNP density within set kb frames it is possible to identify regions with a possible increased likelihood for base changes. The change in base (and subsequent possible amino acid and protein difference) may present one explanation for the antigenic differences causing certain serovars that may be closely genetically linked (such as serovars Canicola and Icterohaemorrhagiae) tend to favour different reservoir hosts.

A section amongst the genes mapped to Copenhageni (2.4-2.5Mb) showed an increase in SNP density within three of the four strains. For serovar Copenhageni, this region contains genes encoding for dehydrogenases (e.g. *serA* involved in serine biosynthesis and *sdhA/B/C,* which are three of the four subunits within succinate dehydrogenase), GTP-binding proteins (e.g. *lepA*), tRNA synthases (e.g. *asnS*) and 56 hypothetical proteins. Given the large proportion of hypothetical proteins in the region, it is entirely feasible that the SNPs occur within these and not genes typically involved with housekeeping roles.

A disadvantage of SNP analysis for the sequence data in this study is the limited range of fully sequenced reference genomes. As a result of this, a number of strains are mapped against genomes that they may not be closely phylogenetically related. This can be seen in figures 6.1-6.7 as there is a high proportion of SNPs within four strains.

6.4.2 Acquiring nutrient components from environmental sources

Between the species investigated here, there is a difference in the ability to acquire and utilise nitrogen compounds. Despite the saprophytic strain containing 31 genes within nitrogen subsystems (six of which relate to ammonia assimilation), only six genes are present within pathogenic strains for acquiring nitrogen. This finding emphasises the host specificity, given that pathogenic species colonise host kidneys and so require less genes involved with acquisition pathways that may be essential for environmental survival. As previously stated, genes involved in the metabolic pathway for GltS are present within all ten strains; however a great number are present within non-pathogenic strains. GltS is involved with nitrogen assimilation and breakdown of compounds into their individual nitrogen components (Vanoni and Curti, 2008).

Although denitrification is an anaerobic reduction, the process is witnessed in aerobic bacteria such as *Pseudomonas* (Carlson and Ingraham, 1983). The presence of several genes involved with this stage of the nitrogen cycle could give both the non-pathogenic species from this study a survival advantage within the environment over pathogenic species.

Similarly, genes for the process of ammonification of nitrites and nitrates are seen in the non-pathogenic species. The ability to convert organic nitrogen compounds back into ammonium plays an important role for respiration under low-oxygen conditions, and generates considerable levels of energy for the bacteria (Strohm et al., 2007). As well as providing more evidence for their growth advantage within environmental sources such as soil or small bodies of water, it may also explain in part why saprophytic species can out-grow pathogenic when maintained on commercial growth media.

Given the importance of potassium for cell membrane potential regulation during changes in pH and osmolarity (Torres et al., 1997), it is of no surprise that related subsystems are stable between all species. The recently defined genomospecies *L. alexanderi* contains an additional four genes relating to both the process of maintaining potassium homeostasis and flagellar structure and motility. Although no reservoir host has been defined to date, the impact of the additional processes would need to be further investigated. The key aspect to focus on however is the absence of any potassium homeostasis genes within *L. biflexa*. While the ability to adapt to changes in pH within a host would impart greater benefit to pathogenic strains, adapting to osmolarity changes would benefit an environmental bacterium so their absence is somewhat unusual.

The absence of iron acquisition or uptake genes is surprising, particularly in relation to pathogenic strains. Iron plays a crucial role in bacterial processes and once within a host, acquiring iron from sources requires specialised pathways. Previous work has eluded to a putitative protein demonstrating low similarity in both sequence and structure to FepA in *E. coli* (Sritharan et al., 2006). Given that no genes were identified during this study that are similar to those utilised by other bacteria, it then suggests towards potential involvement from hypothetical proteins. Investigation into genes actively transcribed during iron uptake would be required to determine if this is the case for *Leptospira*.

6.4.3 Amino acid processes and their derivatives

The highly conserved histidine pathway amongst all strains indicates a level of stability during the bacteria's evolution and a basic requirement for survival.

It is interesting to note that *L. biflexa* contains two genes involved with a putrescine utilisation pathway. The enzyme 4-aminobutyraldehyde dehydrogenase (ABADH) oxidises gamma-aminobutyraldehyde into gamma-aminobutyrate (GABA). Once converted, GABA is then further converted into succinate semialdehyde by GABA aminotransferase (*gabaT*). Both genes are absent from all other species in this study. Despite this however, the pathway is incomplete as no other enzymes (such as putrescine aminotransferase) are encoded for within the genome. The remaining pathway may be compensated for by the hypothetical genes not yet characterised, however further work would be required to confirm if this was the case.

As well as the additional putrescine proteins, *L. biflexa* also contains nine genes for polyamine metabolism only present within this species. Spermidine synthase (SPDS) synthesises spermidine from putrescine and aids cell growth and proliferation which may further contribute to their ability for prolonged environmental survival compared to pathogenic strains. The pathways for polyamine metabolism have been a target for therapeutics, and particularly to counter cancer cell growth (Bergeron et al., 1988; Marton and Pegg, 1995). However their potential for disrupting *Leptospira* colonisation and proliferation rates would need further investigation.

Homocysteine is an amino acid that has been demonstrated as a pre-cursor to both hydrogen sulphide in oral bacteria (Yoshida et al., 2009) and methionine (Or-Rashid et al., 2001). Due to only a partial processing pathway being presented within *L. fainei*, and its presence in just a single species, acquisition via horizontal gene transfer could explain one possible origin. The

advantages over other species for survival or infection that the pathway confers would need to be further investigated.

One external stress that may result in differences arising within the genome is the level of passage a culture goes through before sequencing. The effects of which are not yet known for *Leptospira*. Strains used during this study were kept to low passage numbers to prevent potential disruptions. How the genome is disrupted following repeated sub-culturing would need further investigation, with sequence analysis carried out and SNP counts compared between passage numbers for the same strain.

Overall, the average number of shared functional genes (n=1,342) is of a similar level to the core genome discovered for the same strains (n=1,095). The high presence of hypothetical proteins may factor into the discrepancy between the two methods of annotation.

6.4.4 Previously identified virulence factors

Certain members of the *lipL* gene family have been put forward as potential virulence factors, however to date only *lipL32* has been investigated. As a result, it was found that *lipL32*$^{-}$ mutants were able to infect hamster models (Murray et al., 2009c). This could suggest a degree of functional redundancy as other genes may compensate for the loss of *lipL32*.

Following manual comparison in the present study, another *lipL* gene family member, *lipL36*, was found to only be present in the pathogenic strains sequenced. However, despite its exclusive presence in pathogenic serovars, previous work has eluded to its role being tailored towards environmental survival rather than virulence. When challenged with a host-adapted strain it was demonstrated that an absence of LipL36 antibody response suggests a down-regulation during infection when compared to a culture-adapted strain (Haake et al., 1998). The study by Haake and colleagues (1998) examined a strain of *L. kirschneri*; however I was not able to locate the gene within the strain sequenced from the same species. The presence in pathogenic strains, but reported down-regulation, may suggest a role in the transmission of the bacteria.

The gene encoding for LipL36 has not yet been the subject of PCR assays and the variation presented here demonstrates the ability to not only distinguish only pathogenic strains, but the ability to potentially determine individual serovars. Whether or not this is possible would need further investigation. However, phylogenetic analysis presented here of other genes such as *gyrB* and *ompL1* seem better suited for such diagnostic abilities. This finding echoes

previous work which propose the use of *gyrB* for identifying pathogenic species (Slack et al., 2006).

A lipoprotein belonging to the OmpA family, loa22, was the first gene to be successfully demonstrated as an essential virulence factor (Ristow et al., 2007). The *loa22* gene was analysed within all pathogenic strains for this present study, and comparison revealed low nucleotide variation, particularly within the same species. This restricted variation emphasises the importance towards host infection and survival. The evidence presented here is in line with that found by Ristow and colleagues (2007).

Six further genes (*katE*, *flaA*, *fliY*, *clpB*, *hemO*, *mce*) previously described as crucial for pathogenesis were investigated for their presence in the ten strains sequenced in this study (Eshghi et al., 2012; Lambert et al., 2012; Liao et al., 2009; Lourdault et al., 2011; Murray et al., 2009b; Zhang et al., 2012). I found no presence of any of the six genes associated with pathogenicity within the saprophytic strain. However, the *clpB* gene was present in the intermediate strain, leading to suggest that while it may be essential for virulence; other contributing factors may also be required. A situation similar to that is described for *lipL41*, which although not required for virulence, requires the chaperone *lep* for expression (King et al., 2013). Further investigation would be required to confirm if this is also true for *clpB*, however I found no CDS present within close proximity to *clpB* for any strain sequenced in this study.

Another protein previously suspected to confer virulence was LigB. LigB is one of three leptospiral immunoglobulin-like repeated (Lig) proteins that mediate host cell attachment. The gene *ligB* was only present in the eight pathogenic strains for this study, which does suggest a role in virulence. Despite this however, *ligB*$^{-}$ mutants have been shown to still infect rodent models (Croda et al., 2010) with a suggestion of other proteins in similar roles compensating for the loss. Given the large gene pool within pathogenic *Leptospira*, it is entirely feasible that this could be the case.

In summary, this study successfully sequenced the genomes of ten *Leptospira* strains not previously analysed. Strains representing pathogenic, intermediate and saprophytic groups were included to allow comparison between strains inhabiting different ecological niches. Genomic analysis demonstrated a high level of SNP variation, particularly amongst serovars of the same species. The high level of non-synonymous SNPs could play a vital role in host-specific adaption and lend itself to the variety of reservoir animals that play host to the bacteria.

Chapter Seven

General Discussion & Future Work

7.1 General Discussion

Current and accurate data regarding both infection levels and serovars causing those infections is lacking in the UK. A high number of pathogenic serovars have proven their ability to infect a wide range of both reservoir and incidental hosts (Babudieri, 1958; Jimenez-Coello et al., 2010; Tubiana et al., 2013) and a limited awareness of circulating serovars may affect both diagnostic and vaccination practices. Further to this, with the advancement of molecular typing techniques, there is an on-going issue with relating results to the traditional nomenclature based on serological antigen recognition. With the successful sequencing of the full *Leptospira* genome, it has been possible to identify several putative virulence factors. However, as some strains have seemingly become host dependant (Bulach et al., 2006) further investigation is required into other potential factors that may contribute to pathogenesis.

This thesis aimed to identify the current situation regarding *Leptospira* within the UK. To this end, both reservoir hosts and retrospective cases arising within the vet-visiting dog population were examined. To gain a better insight into molecular identification, this study aimed to apply highly discriminative multi-locus sequence typing (MLST) to establish the identity of serovars circulating within UK wild rodents. Following this, the genome sequences of ten strains encompassing a range of pathogenicity traits were compared and analysed against seven strains previously published. This allowed for identification of single nucleotide polymorphisms (SNPs) and translated functional proteins. SNP data was compared between sequenced strains to identify regions containing potential virulence factors and housekeeping genes.

The canine vaccination status for *Leptospira* in the UK is currently unknown. Details of a bivalent vaccine were first published in 1960 (Jull and Heath, 1960) and recently, aspects of a novel tetravalent vaccine (Klaasen et al., 2013) were announced for the UK. Currently in the UK, human leptospirosis is no longer a notifiable disease (HPA, 2012), resulting in confirmed cases not being reported. Further to this, due to the perceived low levels of infection in the UK, it is not identified as a 'core' vaccine for dogs despite annual vaccination required to maintain immunity. A combination of the two may result in a lack of both accurate published information and knowledge within communities outside of the veterinary profession.

A questionnaire based survey was undertaken to determine vaccination rates and the frequency of suspected or confirmed canine leptospirosis cases. The study described how vaccination against the disease is carried out for a high percentage (60.37% on average) of canines seen by practices. A lack of consideration for canine contact with potential

environments with a known association for leptospirosis infections during a diagnosis was highlighted, and demonstrated that clinicians are typically more likely to consider icteric signs. As the anicteric signs are typically vague and can be associated with a range of diseases, linking rodent and/or water contact may aid an early diagnosis. Identifying and treating leptospirosis at an earlier stage increases survival likelihood (Lau et al., 2010), and prevents infection from progressing into the later stages of disease were renal and hepatic failure can be common (Winearls et al., 1984; Yang et al., 2001).

Confirming previous published results, no relationship was seen with dog age or breed (Oliveira Lavinsky et al., 2012). The practice size was also not deemed important in regards to witnessing a case of leptospirosis. Conclusions drawn here are limited however due to the low numbers of returning practices, and the low number reporting a recent leptospirosis case. An increased compliance rate would provide greater clarity for potential links to associated risk factors in the UK.

Future work guided towards increased uptake of diagnostic testing for canines presenting with relevant anicteric clinical signs would be of great benefit. Serum sampling at the point of first contact would provide a better indication of infection levels within a wider population and provide accurate information on circulating serogroups over a prolonged period. Due to the issues regarding timing and cost of implementing a system of this nature, it wasn't possible within the scope of this project. While culturing *Leptospira* from potential cases of infection would be ideal, several issues contribute towards difficulty with doing so. The bacteria itself is notoriously fastidious and inoculation from sample material may take several months before growth is visible under dark field microscopy (Doern, 2000). During this prolonged growth stage, given the rich composition of EMJH, contamination can also affect culture attempts. Molecular typing of infecting strains provides a rapid and accurate alternative. While recent work has helped link molecular typing (such as MLST and VNTR) with serological results to an extent, continuous work in the field is still needed to provide a method that has complete homology with serological testing.

In chapter four, the extent of *Leptospira* presence was described within wild rodents populating multiple sites of the North West. Studies carried out by Hathaway and colleagues in 1983 demonstrated the presence of *Leptospira* within wild rodents, particularly serogroup Australis (Hathaway et al., 1983b). Following this, Webster and colleagues identified the prevalence within rats on farmland (Webster et al., 1995). Since then however, no studies have carried out further investigation. As new serovars are continually being discovered (Lau

et al., 2012; Paiva-Cardoso et al., 2013; Roberts et al., 2010), there is a need for current identification of infectious strains within UK local environments.

Following trapping at fifteen sites over a period of 24 months, it was determined that *Leptospira* are being maintained and potentially shed within wild rodents. The range of rodent species identified as acting as reservoirs for the same pathogenic serovar opens up a greater possibility for both human and canine infections. As distinct rodent species occupy differing habitats, the likelihood to shed into local environments, potentially causing infection within incidental hosts increases. An increased awareness of typical environmental conditions that the bacteria occupy would aid in preventing future infections to both humans and dogs.

A major observation with this study was a lack of infection witnessed within urban rat samples obtained. Despite the disease being historically linked with rat exposure, no presence of *Leptospira* was identified from 81 rat samples. However, the wood mouse samples from the same urban locations were also negative; leading to suggestions that rural leptospirosis may be a bigger threat in the UK. This finding provides further evidence of an absence of *Leptospira* infecting urban samples despite a presence within rural samples (Blakelock and Allen, 1956; Brockie, 1977).

Building on previous molecular studies, results gained during this project emphasised the usefulness of molecular approaches for identifying both the presence and identity of strains. Comparisons to serological testing and histopathology results demonstrated that PCR assays were of the same standard, if not better, than the MAT. Not only were they more accurate and effective, but the results from molecular testing are more favourable for a diagnosis. While the MAT can only provide information regarding the serogroup and requires a paired sample for serogroup confirmation (Ooteman et al., 2006), PCR has the ability to potentially identify the serovar from a single sample via direct sequencing (Slack et al., 2006).

Histopathology was shown to be the least discriminative and informative testing method. As such it is not used for routine diagnostic testing and is only of benefit for research purposes to visualise tissue infections. Despite the disadvantages, clear images of individual and aggregates of leptospires were obtained. The lack of inflammation witnessed via H&E staining at the same sites bacteria were noted at, emphasised a rodent's role as a reservoir animal for *Leptospira*.

Further to work investigating the presence of pathogenic *Leptospira* in wild rodents, this project aimed to explore the possibility of identifying the serovar using molecular techniques.

For this, several techniques were initially outlined. Methods identified from previously published work included MLST, macro-restriction pulsed-field gel electrophoresis (PFGE) and variable number tandem repeat (VNTR) analysis (Ahmed et al., 2011; Boonsilp et al., 2013; Galloway and Levett, 2010; Pavan et al., 2011; Salaun et al., 2006; Thaipadungpanit et al., 2007).

As described in chapter three, due to difficulties in obtaining cultures of isolates from rodent kidney tissue, it wasn't feasible to utilise PFGE for this study. Both MLST and VNTR are better suited as it is possible to apply either technique to tissue DNA extracts (Arvand et al., 2010; Henriksen et al., 2009; Santos et al., 2012). The VNTR protocol previously described by Picardeau and colleagues (2008) was applied to two positive controls (*L. interrogans* serovar Australis and *L. kirschneri* serovar Grippotyphosa). However, despite repeated testing it was not possible to successfully amplify all loci required to gain interpretable data.

A combination of preliminary testing from this study and previously published work describing the success for MLST (Perez and Goarant, 2010; Romero et al., 2011; Thaipadungpanit et al., 2007), demonstrated its ability as the technique most suited for samples obtained during this project. The work in chapter four further demonstrates the typing ability of the scheme, particularly in regards to DNA extracted from kidney tissue material, which has never been described before in regards to *Leptospira*.

Sequence types were obtained for eleven samples of which all were ST-24. A further five samples offered interpretable data from at least four loci that also suggests association with ST-24. This demonstrated the presence of a unique ST within wild rodents populating the North West of England. This ST has been identified within small rodents and humans within several European countries, including Germany and the Czech Republic. Within ST-24 there are currently three serovars *L. interrogans* serovar Lora, *L. interrogans* serovar Jez Bratislava and *L. interrogans* serovar Jalna. All three belong within the Australis serogroup.

The presence of only a single unique ST is of high interest given that multiple rodent species were sampled at multiple sites. In the original study for the *Leptospira* MLST scheme; a dominant ST was present, however a number of other strains were found to infect also (Thaipadungpanit et al., 2007). The results from PCR assay sequencing in chapter three correlated with those found via MLST in chapter four. Direct *gyrB* amplicons sequencing identified all samples to belong within *L. interrogans* following BLAST analysis. Despite only identifying the species, *gyrB* sequencing provided further evidence that a single ST was found to be infecting wild rodents.

The serovars belonging to ST-24 have previously been shown to confer the ability to infect both humans and canines (Arent et al., 2012; Hathaway et al., 1983a; Renaud et al., 2012; Wasinski et al., 2012). The results from MLST analysis in this study confirms the need for the additional serogroups (such as Australis) included along with those in the previous UK bivalent vaccine. Canines covered by the bivalent vaccine may succumb to infection should they encounter a challenge from the other serovars identified during this study. Continuous investigations into serovars maintained within reservoir populations would aid both research and vaccine effectiveness. A combined approach with sentinel vet practices sampling for the presence of infection within canines would also help to ensure vaccine coverage was both sufficient and effective.

All histopathology samples demonstrating signs of infection were identified using conventional PCR and MLST. MLST identified the infecting sequence type within a further sample found negative by histopathology. In contrast, MAT only detected antibodies within four samples previously shown to be positive using MLST. This direct comparison of results emphasises the greater typing ability that MLST offers in contrast to either histopathology or MAT testing. While visualisation of the bacteria is beneficial for research purposes, it offers little in terms of diagnostic advantages. Similarly, the antibody skew that previous infections or vaccinations can have on MAT testing also hinders its capabilities. MLST does not suffer from this limitation as it directly identifies bacteria present using DNA extracts.

Previous MLST work has shown the discriminative power of the technique (Agampodi et al., 2013; Li et al., 2013; Li et al., 2012) and the results gained from this study provided further evidence for this. Given the success of the scheme's ability to identify infecting STs from tissue DNA extracts, it would be of benefit to the database overall if the scheme was adopted for similar studies in the future. Not only would this assist research into serovar presence within reservoir animals, but identifying and locating new alleles would help further the application of the scheme.

Following on from the success of molecular typing strains actively infecting wild rodents, the next stage of the project was to investigate the genomes of additional *Leptospira* strains belonging to serovars not currently in the NCBI database. Currently there are seven fully sequenced genomes; two *L. biflexa* serovar Patoc, two *L. interrogans* serovar Lai, two *L. borgpetersenii* serovar Hardjo-bovis and *L. interrogans* serovar Copenhageni. Due to issues regarding the culturing of isolates as explained in chapter four, the sequencing and analysis of ten strains of differing virulence traits were described in chapter six; *L. alexanderi* (serovar

Manzhuang), *L. borgpetersenii* (serovar Hardjo-Bovis), *L. interrogans* (serovars Icterohaemorrhagiae, Bratislava, Pomona and Prajitno), *L. kirschneri* (serovar Grippotyphosa), *L. weilii* (serovar Mengding), *L. fainei* (serovar Hurstbridge) and *L. biflexa* (serovar Andamana).

Previous work has alluded to several putitative and confirmed virulence factors (Eshghi et al., 2012; Lambert et al., 2012; Liao et al., 2009; Lourdault et al., 2011; Murray et al., 2009b; Zhang et al., 2012), of which their presence within each strain was described. Variation and functional analysis was utilised to identify genes encoding potential virulence factors that may be essential for pathogenicity.

Variation analysis using identified SNPs showed a high level of variability spread amongst both chromosomes (and the additional plasmid p74 for *L. biflexa*). This raises an interesting point in regards to serovars within the same species. The high level of variation suggests the extent to which molecular differences affect host specification, possibly in regards to environmental pressures for survival. The greatest variation was seen within the saprophytic strain *L. biflexa* serovar Andamana, which had 55,303 SNPs from the reference strain of the same species. Due to the extensive pathways utilised for varied environmental conditions, this is somewhat unsurprising, giving rise to the higher SNP total.

Density values for SNPs were analysed to identify common regions of both high and low variation. High variability may indicate the location of antigenic genes responsible for differing serological reactions, whereas low variability may highlight genes essential for survival or virulence. Unfortunately, due to the poor characterisation of a large proportion of *Leptospira* genes, the majority of those within such regions were hypothetical. This limitation emphasises the need for further investigation and research into the functional roles of hypothetical genes within the given regions. A result of which may highlight future potential virulence factors and targets for both vaccinations and therapeutics.

Annotated *de novo* constructs provided the opportunity to compare genes present within all ten strains (and a further seven reference strains). Using previously published methodology (Vesth et al., 2013) it was possible to identify the 'core' genome present within all 17 strains as being roughly 28.76%. Compared to the 61% previously described, the core identified by this study was relatively low (Picardeau et al., 2008). For Picardeau and colleagues (2008), genomes for only three species were available at the time; *L. borgpetersenii* (serovar Hardjo), *L. interrogans* (serovars Copenhageni and Lai) and *L. biflexa* (serovar Patoc). All three were included for analysis during this study. Further investigation showed that the difference in methodology may be responsible for the discrepancy. Despite this, when excluding *L. biflexa*

and *L. fainei*, the pathogenic strains retained a relatively high core, making up 69.30% of total genes. As explained, the difference in both the sequencing method and analysis methodology will have an effect on annotations and downstream analysis.

Aside from the limitations regarding gene annotation, the sequencing method chosen for this study (MiSeq, Illumina) is better suited for SNP analysis rather than *de novo* constructs. Chapter six outlines the reasoning for utilising MiSeq for this study, however for the *de novo* constructs, a platform offering greater coverage such as the HiSeq (Illumina) may have produced more accurate data.

Following on from the success of identifying potential regions containing virulence factors, further work to characterise the function and potential virulence of hypothetical proteins is needed. Making use of transposon mutagenesis as previously described (Murray et al., 2009c), may allow additional exploration of the dependency for virulence. Similarly, the effects on colonisation ability would also be worth investigating, particularly with certain species, such as *L. borgpetersenii*, being observed to preferentially infect specific hosts.

In summary, the work here identified the serovars infecting multiple species of wild rodents acting as reservoirs for *Leptospira*. Given the nature of the host-dependency, it is entirely feasible to presume they may be shedding live bacteria into the local environment. Application of a highly discriminative molecular technique has allowed for identification of infecting strains down to the serovar level. Further to this, the advantage of using MLST allowed for direct comparisons to previous studies utilising the scheme. Increased use of MLST will aid both research and diagnostic procedures. Finally, this thesis identified the high level of nucleotide variation within a range of pathogenic serovars. Further work into coding regions for hypothetical proteins within highly variable regions may yield targets for diagnostic testing and aid future therapeutic protocols.

7.2 Future Work

- Extensive practice sampling for suspected leptospirosis cases within vet visiting canines at the point of first contact, particularly for cases presenting with mild clinical signs.
- Increased rodent sampling within urban areas for all local species including molecular, serological and histopathology testing. Where possible, culturing and isolation from blood and/or tissue would be of benefit. Further sampling within more regions of

England would also be of benefit to identify any geographic differences of circulating serovars.

- Extended sampling to identify the presence of pathogenic *Leptospira* within animal species previously reported as having a known association with leptospirosis such as squirrels or bats.
- Knock-out studies targeting genes within conserved regions as identified within this study. This may result in the identification of virulence or essential genes for pathogenesis within pathogenic strains.
- Further characterisation of hypothetical genes potentially encoding for functional proteins within regions of high nucleotide variability.
- Extended species testing for *ompL1* as a potential candidate for serovar identification through direct sequencing.

Appendix One

UNIVERSITY OF LIVERPOOL

Prevalence of Leptospira cases in the UK vet visiting dog population

Dear Colleague,

We are carrying out a study to investigate the occurrence of Leptospira spp. in the UK. Over the course of this study we will be investigating the prevalence and the different types of Leptospira within vet visiting dogs.

We would ask you to assist us by completing the accompanying questionnaire on Leptospira cases that you have seen in the previous 12 months within your practice. Based on data gathered from this, we may wish to contact some practices for recruitment into a follow up study.

The questionnaire is completely voluntary and you may choose to not answer any of the questions. Information will remain strictly confidential and only accessible by the researchers involved in the study. Privacy will be maintained in any published data. Once completed please return in the pre-paid envelope supplied. Alternatively you can return either by fax or email to the address at the bottom of the page.

What benefit will this study contribute to veterinary medicine?
To date there have been no widespread studies investigating leptospira infection in dogs in the UK. As Leptospira is considered by many to be a re-emerging pathogen it is important to have a full understanding of it's likely prevalence, and of which serovars/strains are thought to be involved to ensure effective treatment and vaccination strategies.

We ask if you could please complete and return the questionnaire, even if you have not seen any cases over the past 12 months as any response will aid us in this study.

Thank you for your participation and if you have any further questions or comments regarding this study then please don't hesitate to get in touch on 0151 7946017 or cball88@liverpool.ac.uk.

Yours sincerely,

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Phone: 0151 7946017 Fax: 0151 7946005

Appendix 1.1 Covering letter for questionnaire survey of UK veterinary practices.

Ref ☐☐☐☐

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UNIVERSITY OF LIVERPOOL

Leptospirosis Questionnaire—Veterinary Practices

As a part of research investigating the prevalence of canine leptospirosis in the UK, the University of Liverpool are conducting a survey of veterinary practices about cases from the past 12 months. Your participation in this survey will help us gain a better understanding of the current canine leptospirosis situation within the UK.

This questionnaire asks about your practice and any suspected or confirmed canine leptospirosis cases that have been seen in the last 12 months.

If you are uncertain of any question at any point please indicate and you may decline to answer any of the questions. Once completed either post, email or fax it back, addresses are at the bottom of the page.

Even if you have not seen any cases over the past 12 months please still complete the questionnaire accordingly as this will be valuable information for the for the purposes of the study.

We may wish to contact you again concerning this, or possible future studies. Please tick this box if you do not wish to be contacted. ☐

Answering the Questions

Please complete all questions by either placing an X in the box provided or in block capitals when a text response is required. Where multiple boxes are provided please write only one number per box.

1. Is your Practice: Small Animal ☐ Mixed ☐

2. Number of Small Animal Vets at the Practice? (Full time and Part time) ☐☐

3. Estimated average number of dogs seen per day in your practice? ☐☐☐

4. Number of Leptospira dog vaccine doses administered by the practice over the past 12 months? ☐☐☐

5. Estimated proportion of Dogs 'up to date' (i.e. vaccinated within the last 12 months) with Leptospirosis Vaccine? ☐☐☐%

6. Date of last canine Leptospirosis case seen by the practice? ☐☐ *Day* ☐☐ *Month* ☐☐ *Year*

Was the diagnosis: Suspected ☐ Lab Confirmed ☐

Please continue on the next page

7. Has anyone in your practice seen any suspected or confirmed canine Leptospirosis cases in last 12 months?

Yes ☐ No ☐ Don't Know ☐

If yes please fill in the table below with as much information you can provide

	Lab Confirmed		Up to Date Vaccination					
Case	Yes	No	Yes	No	Don't Know	Age (Years)	Breed (If Known)	Outcome
1								
2								
3								
4								
5								
6								
7								

Please continue on a blank piece of paper if you have more than seven cases

8. When making a presumptive clinical diagnosis on suspected Leptospirosis cases, would you be able to list below the main signs you look for?

Thank you for taking the time to complete this questionnaire

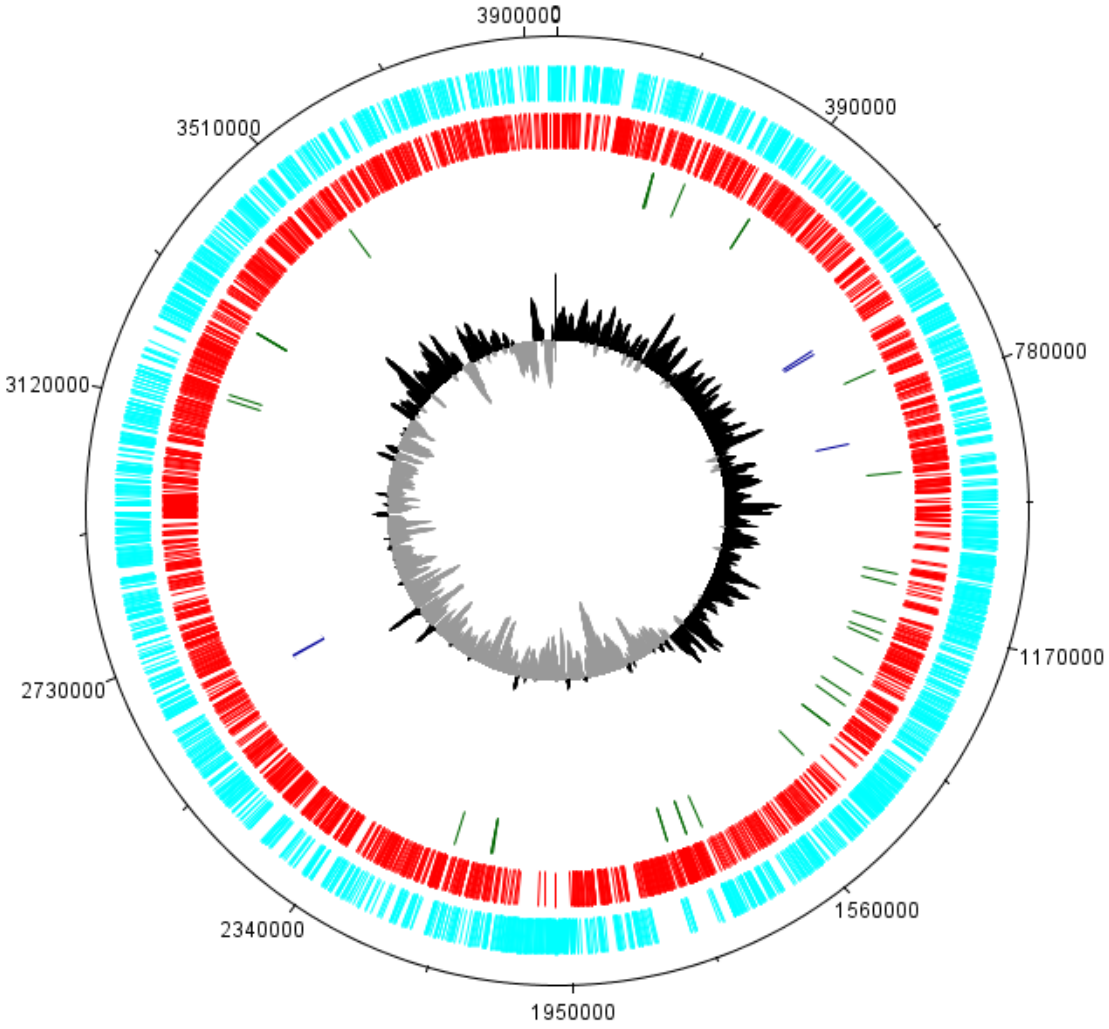
Appendix 1.2 Questionnaire utilised for the veterinary practice survey in Chapter three.

Appendix Two

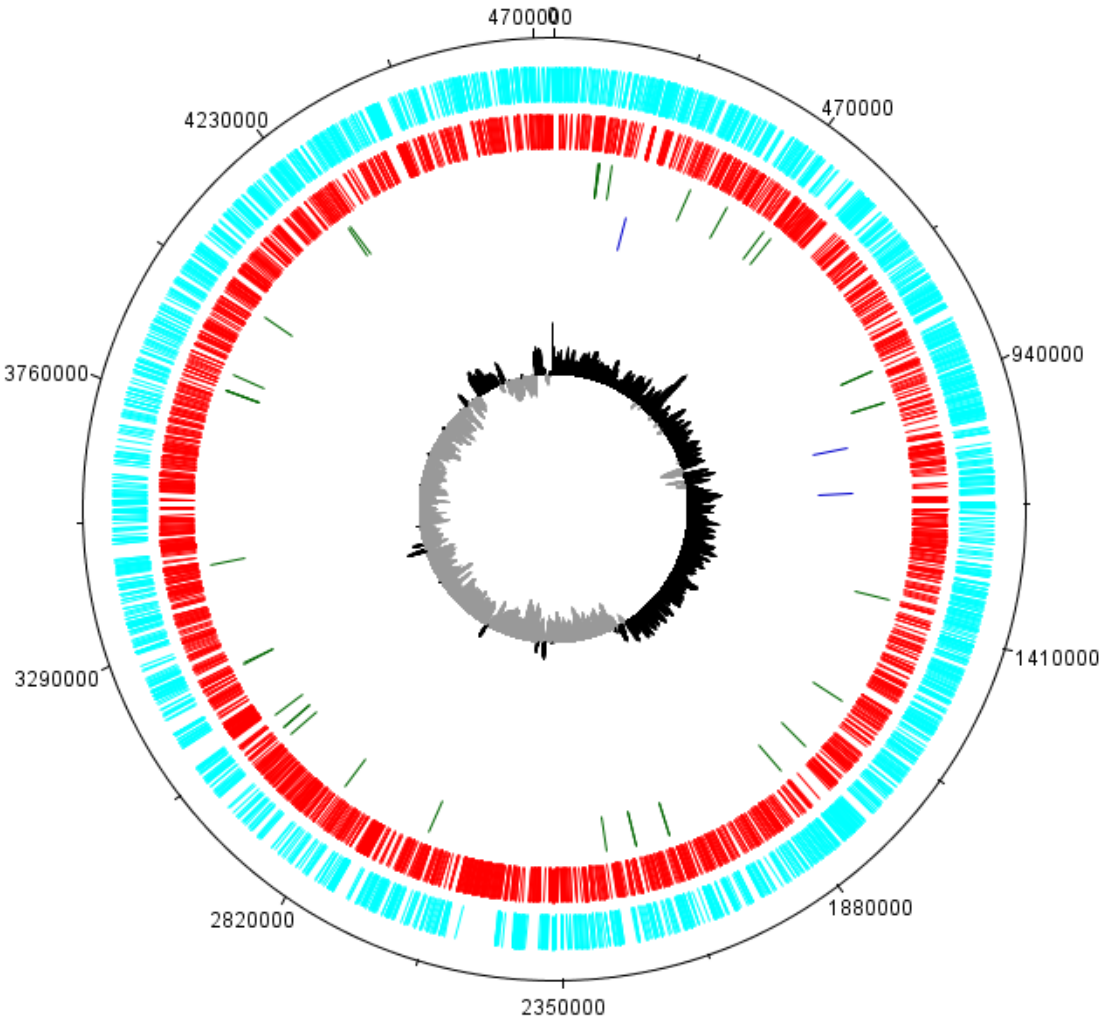
Name	Sequence	Reference	Purpose	Product Size (bp)
*rrs*F	GGAACTGAGACACGGTCCAT	Tansuphasiri et al., 2006	Leptospira Screening	430
*rrs*R	GCCTCAGCGTCAGTTTTAGG			
*lipL32*F	GTCGACATGAAAAAACTTTCGATTTTG	Cheemaa et al., 2007	Pathogenic Leptospira Screening	756
*lipL32*R	CTGCAGTTACTTAGTCGCGTCAGAAGC			
2For	TGAGCCAAGAAGAAACAAGCTACA	Slack et al., 2006	Pathogenic Leptospira Screening	502
504Rev	MATGGTTCCRCTTTCCGAAGA			
glmU-F	AGGATAAGGTCGCTGTGGTA	Thaipadungpanit et al., 2007 Boonsilp et al., 2013	MLST	650
glmU-R	AGTTTTTTTCCGGAGTTTCT			
pntA-F	TAGGAAARATGAAACCRGGAAC			621
pntA-R	AAGAAGCAAGATCCACAAYTAC			
sucA-F	TCATTCCACTTYTAGATACGAT			640
sucA-R	TCTTTTTTGAATTTTTGACG			
tpiA-F	TTGCAGGAAACTGGAAAATGAAT			639
tpiA-R	GTTTTACRGAACCHCCGTAGAGAAT			
pfkB-F	CGGAGAGTTTTATAARAAGGACAT			588
pfkB-R	AGAACACCCGCCGCAAAACAAT			
mreA-F	GGCTCGCTCTYGACGGAAA			719
mreA-R	TCCRTAACTCATAAAMGACAAAGG			
fadD-F	AGTATGGCGTATCTTCCTCCTT			576
fadD-R	TTCCCACTGTAATTTCTCCTAA			
caiB-F	CAACTTGCGGAYATAGGAGGAG			650
caiB-R	ATTATGTTCCCCGTGAYTCG			
CytB-For	TCATCMTGATGAAAYTTYGG	Schlegel et al., 2012	Rodent Species Identification	946
CytB-Rev	ACTGGYTGDCCBCCRATTCA			
fad2-F	ACGTGATCTCCCTTATGCCAAGCA	Caimi et al., 2012	MLST - Alternate Fad Primers	
fad2-R	ATCCAACCGACAGAAGTATGGCGT			
VNTR4-F	AAGTAAAAGCGCTCCCAAGA	Salaun et al., 2006	VNTR	425+34n
VNTR4-R	ATAAAGGAAGCTCGGCGTTT			
VNTR7-F	GATGATCCCAGAGAGTACCG			299+46n
VNTR7-R	TCCCTCCACAGGTTGTCTTG			
VNTR10-F	GAGTTCAGAAGAGACAAAAGC			420+46n
VNTR10-R	ACGTATCTTCATATTCTTTGCG			
PSBF	ACWRVHVHRGYWDCCTGGTCYTCTTC	Cerqueira et al., 2009	ligB Detection	380
PSBR	TARRHDGCYBTAATATYCGRWYYTCCTAA			
flab-F	TCTCACCGTTCTCTAAAGTTCAAC	Krishna et al., 2008	flaB Detection	793
flab-R	CTGAATTCGGTTTCATATTTGCC			

Appendix 2.1 Full primer list and source for all published primers utilised throughout this thesis

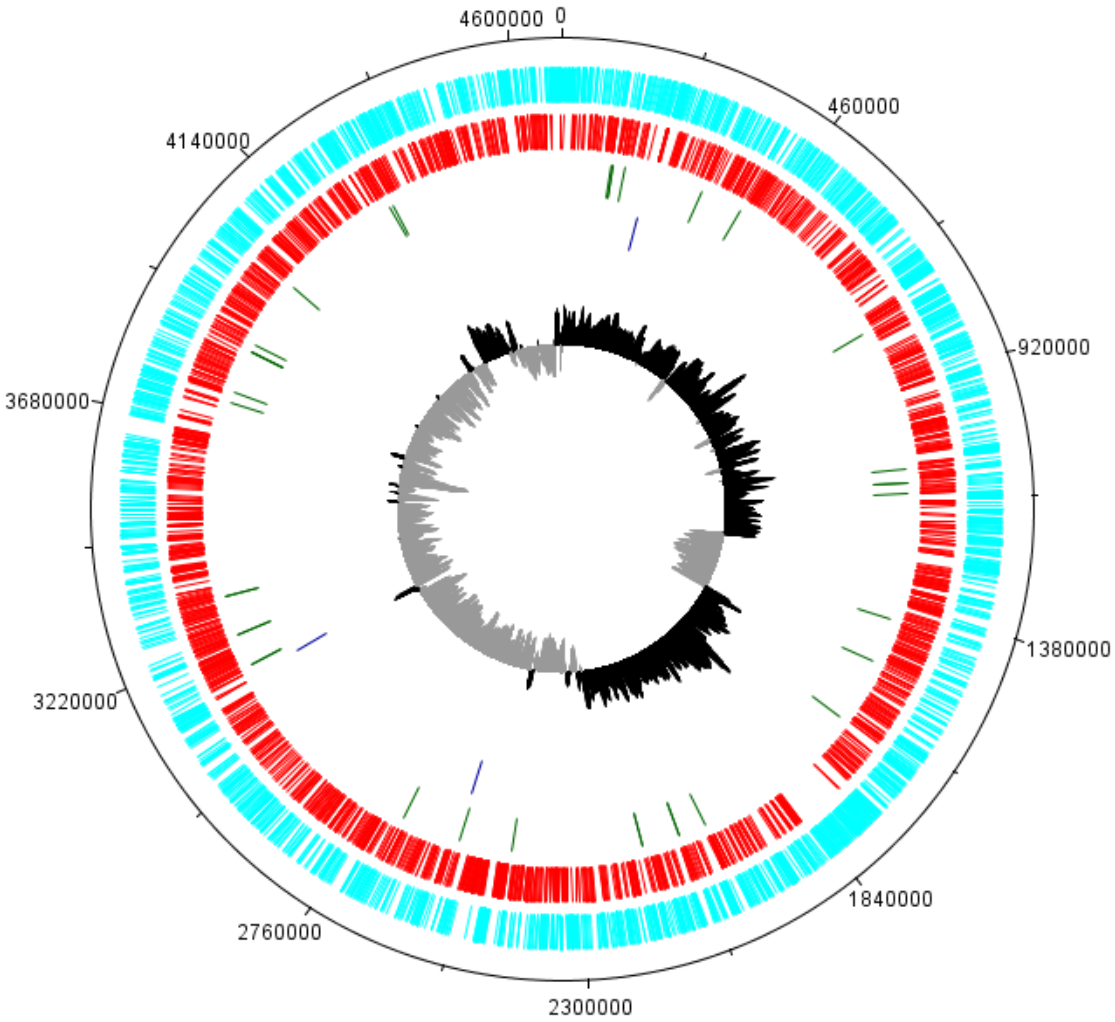
Appendix Three



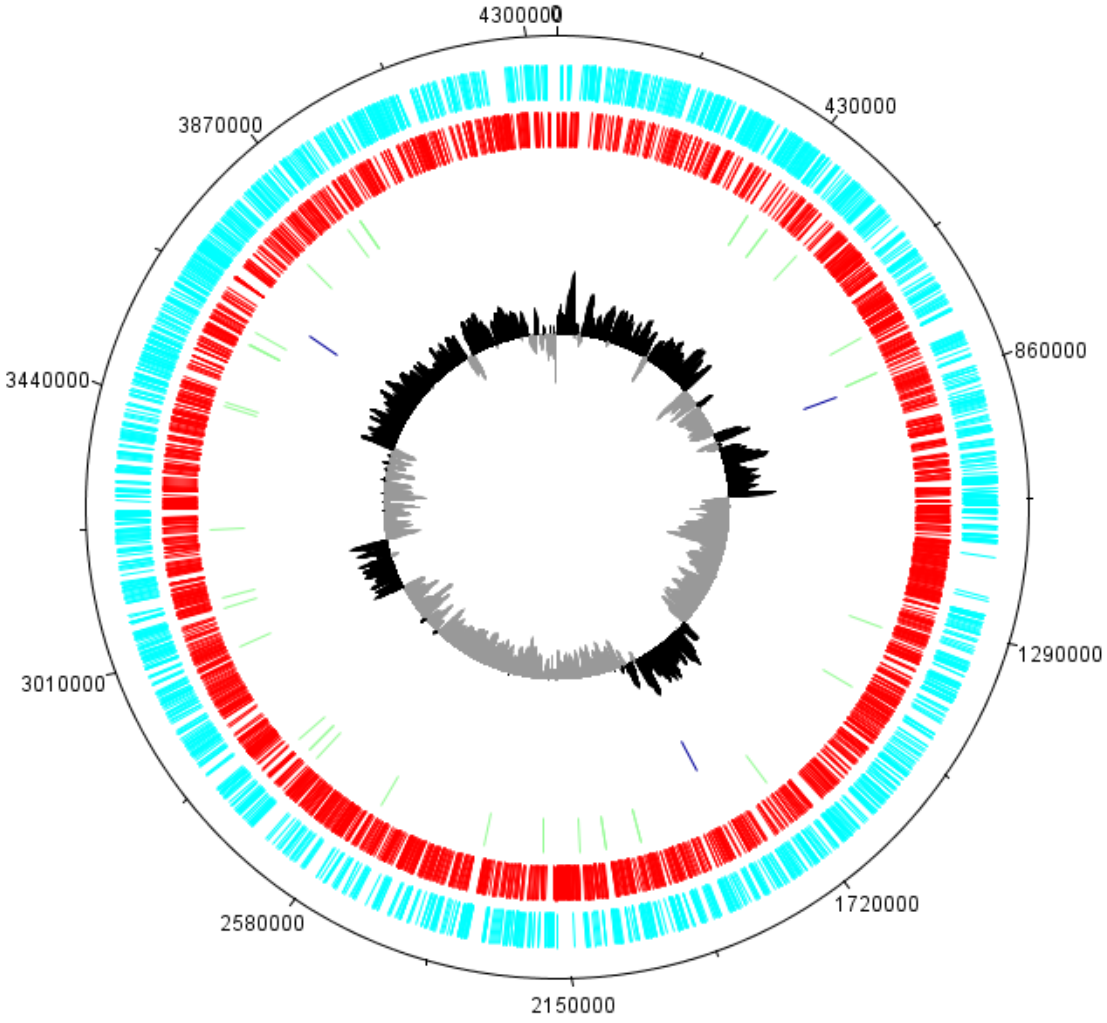
Appendix 3.1. Circular map of *L. biflexa* serovar Andamana. Track 1: Coding sequences on the forward strand. Track 2: Coding sequences on the reverse strand. Track 3: tRNA. Track 4: rRNA. Innermost track is GC skew with positive skew shown in black and negative in grey.



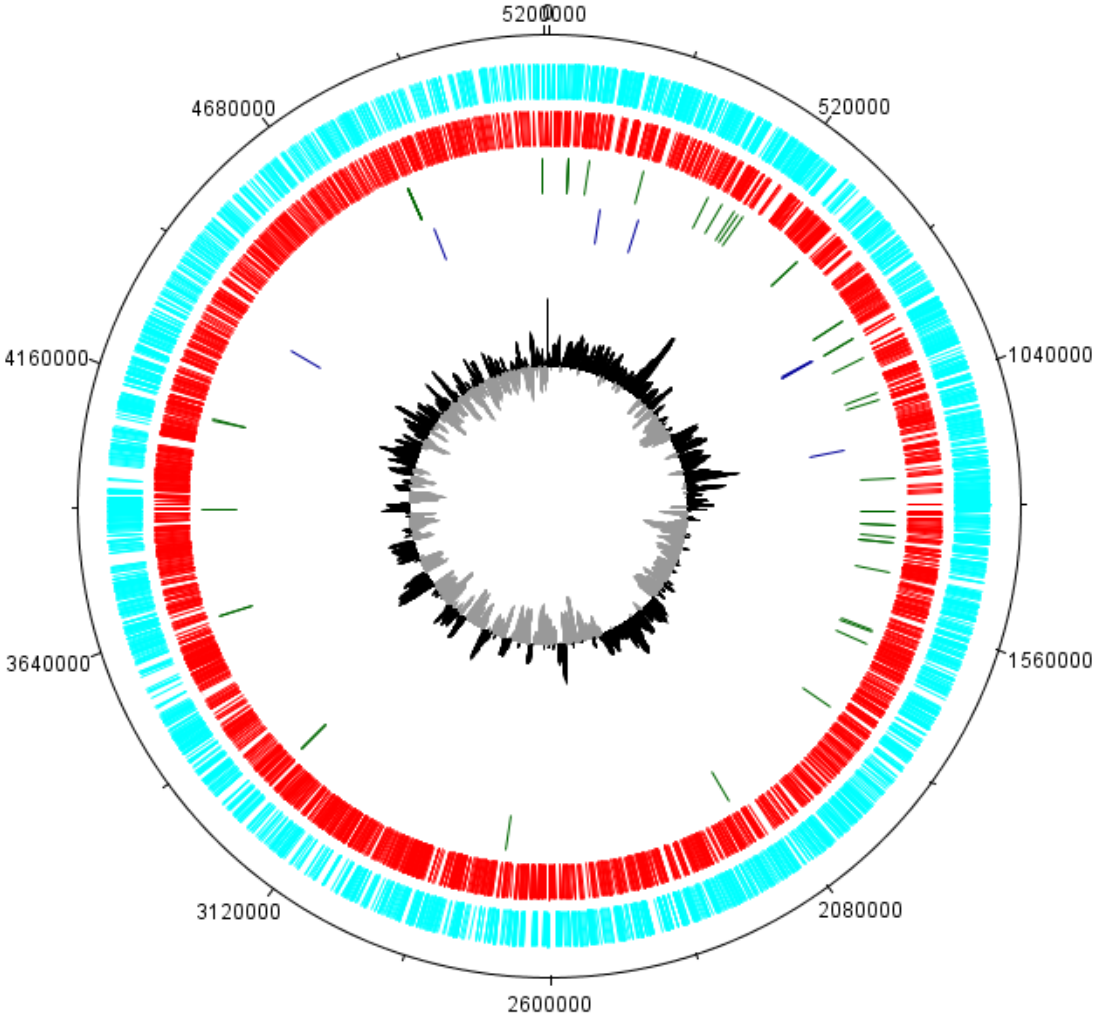
Appendix 3.2. Circular map of *L. borgpetersenii* serovar Hardjo-Bovis. Track 1: Coding sequences on the forward strand. Track 2: Coding sequences on the reverse strand. Track 3: tRNA. Track 4: rRNA. Innermost track is GC skew with positive skew shown in black and negative in grey.



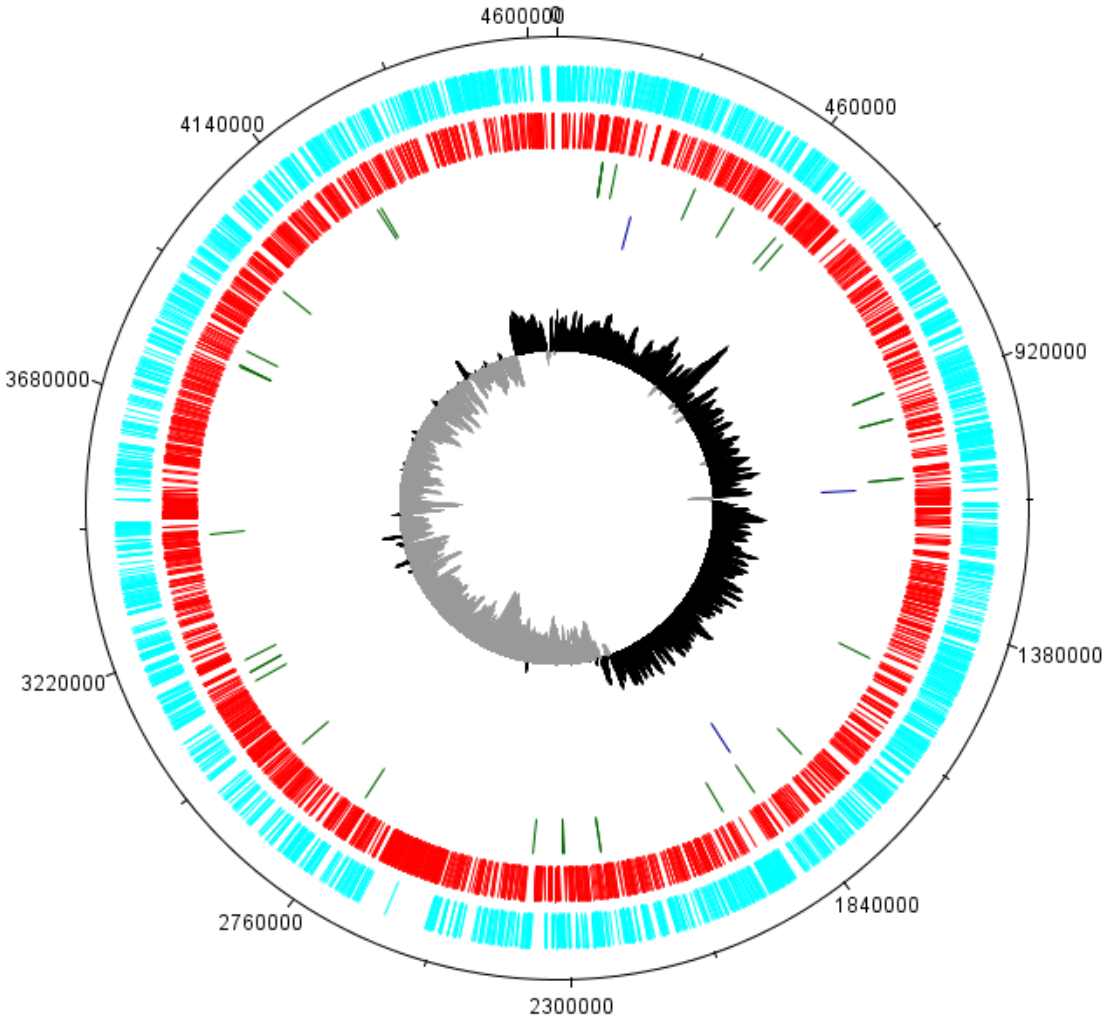
Appendix 3.3. Circular map of *L. interrogans* serovar Bratislava. Track 1: Coding sequences on the forward strand. Track 2: Coding sequences on the reverse strand. Track 3: tRNA. Track 4: rRNA. Innermost track is GC skew with positive skew shown in black and negative in grey.



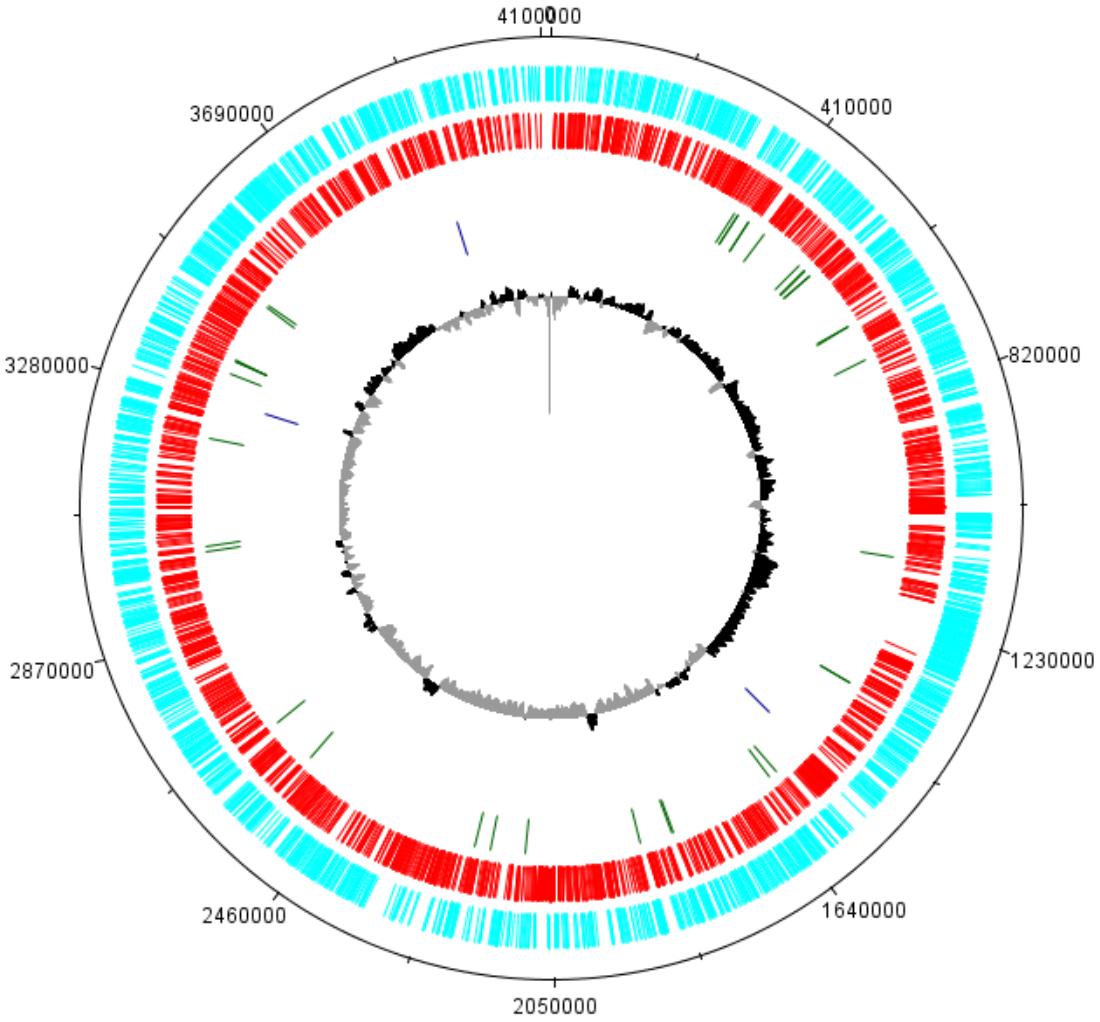
Appendix 3.4. Circular map of *L. kirschneri* serovar Grippotyphosa. Track 1: Coding sequences on the forward strand. Track 2: Coding sequences on the reverse strand. Track 3: tRNA. Track 4: rRNA. Innermost track is GC skew with positive skew shown in black and negative in grey.



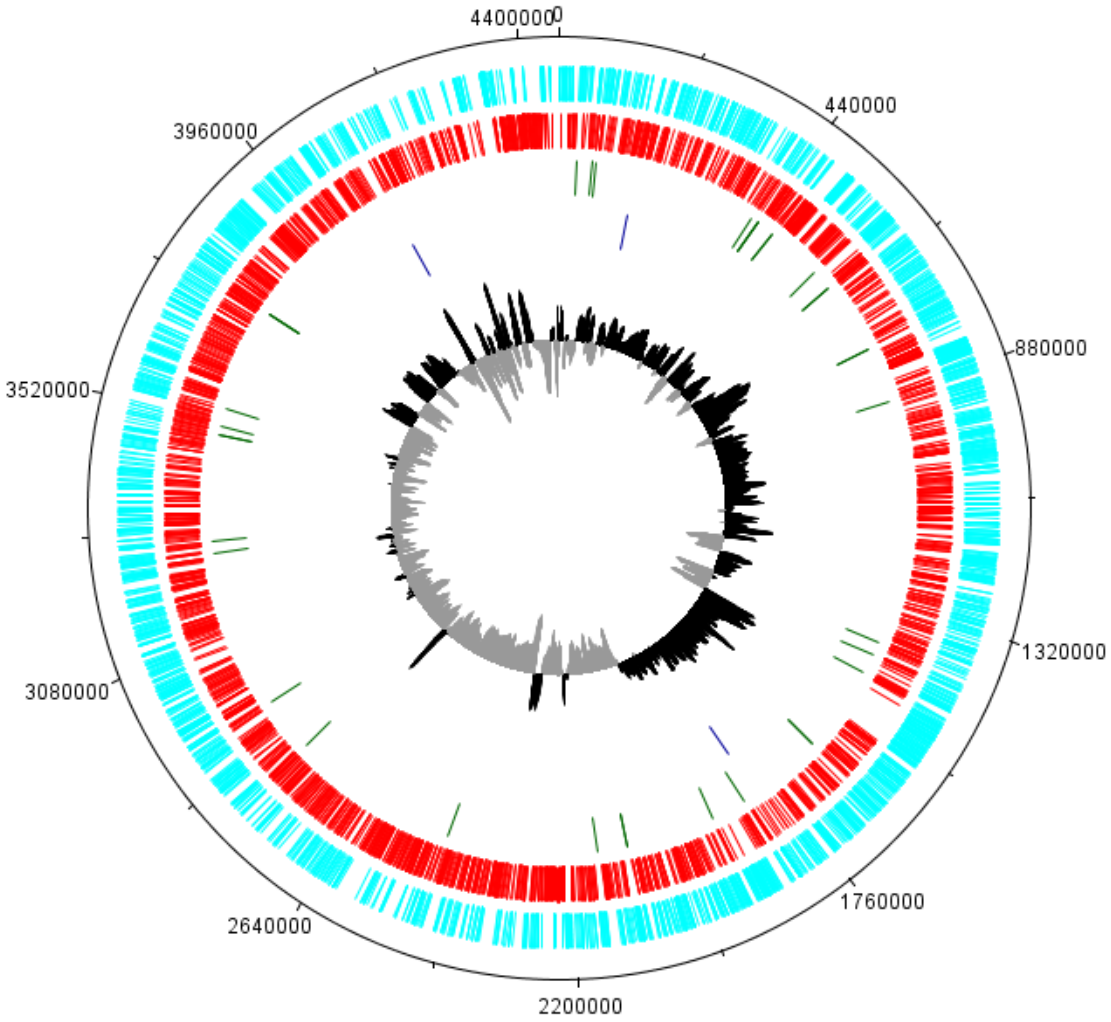
Appendix 3.5. Circular map of *L. fainei* serovar Hurstbridge. Track 1: Coding sequences on the forward strand. Track 2: Coding sequences on the reverse strand. Track 3: tRNA. Track 4: rRNA. Innermost track is GC skew with positive skew shown in black and negative in grey.



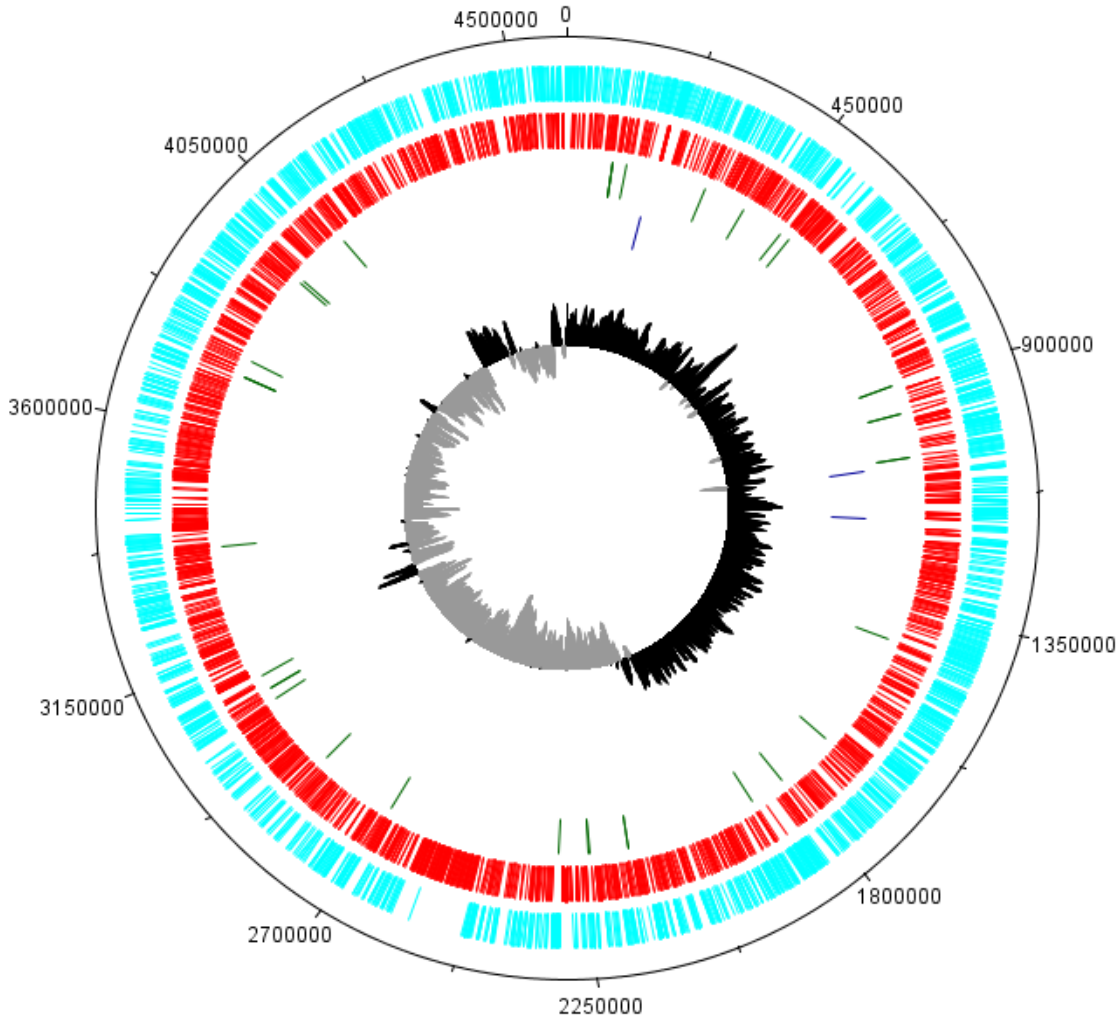
Appendix 3.6. Circular map of *L. interrogans* serovar Icterohaemorrhagiae. Track 1: Coding sequences on the forward strand. Track 2: Coding sequences on the reverse strand. Track 3: tRNA. Track 4: rRNA. Innermost track is GC skew with positive skew shown in black and negative in grey.



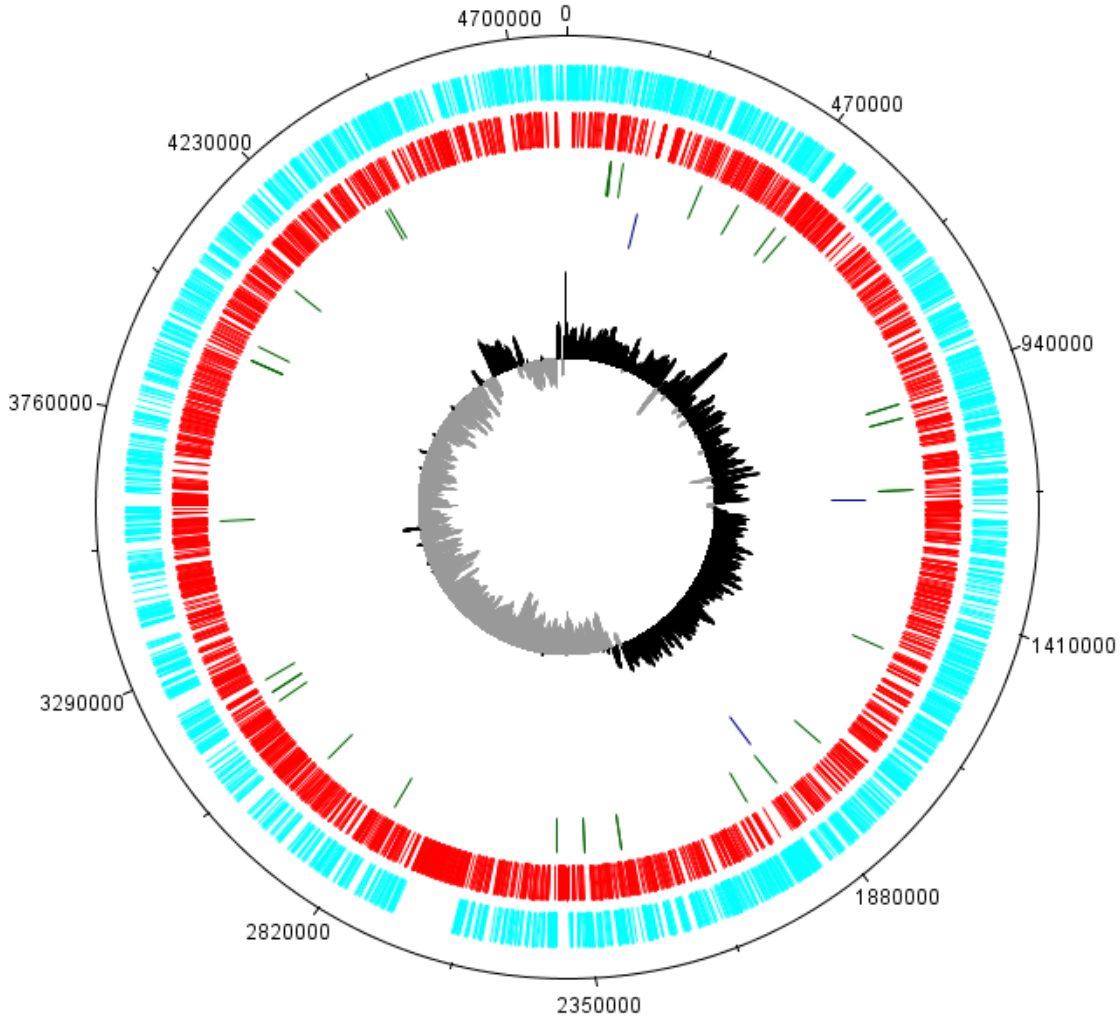
Appendix 3.7. Circular map of *L. alexanderi* serovar Manzhuang. Track 1: Coding sequences on the forward strand. Track 2: Coding sequences on the reverse strand. Track 3: tRNA. Track 4: rRNA. Innermost track is GC skew with positive skew shown in black and negative in grey.



Appendix 3.8. Circular map of *L. weilii* serovar Mengding. Track 1: Coding sequences on the forward strand. Track 2: Coding sequences on the reverse strand. Track 3: tRNA. Track 4: rRNA. Innermost track is GC skew with positive skew shown in black and negative in grey.



Appendix 3.9. Circular map of *L. interrogans* serovar Pomona. Track 1: Coding sequences on the forward strand. Track 2: Coding sequences on the reverse strand. Track 3: tRNA. Track 4: rRNA. Innermost track is GC skew with positive skew shown in black and negative in grey.



Appendix 3.10. Circular map of *L. interrogans* serovar Pajitno. Track 1: Coding sequences on the forward strand. Track 2: Coding sequences on the reverse strand. Track 3: tRNA. Track 4: rRNA. Innermost track is GC skew with positive skew shown in black and negative in grey.

Appendix Four

Comparison Strain	Sequenced Strain										Comparison Strain
	L. interrogans Bratislava	*L. interrogans* Ictero[1]	*L. interrogans* Pomona	*L. interrogans* Prajitno	*L. alexanderi* Manzhuang	*L. borgpetersenii* Hardjo-Bovis	*L. kirschneri* Grippotyphosa	*L. weilii* Mengding	*L. fainei* Hurstbridge	*L. biflexa* Andamana	
L. interrogans Bratislava		15 (1347)	12 (1354)	6 (1361)	36 (1302)	19 (1358)	20 (1338)	40 (1288)	149 (1411)	134 (1358)	*L. interrogans* Bratislava
L. interrogans Ictero[1]	8 (1347)		13 (1348)	4 (1352)	37 (1288)	15 (1308)	25 (1326)	38 (1274)	150 (1399)	136 (1345)	*L. interrogans* Ictero[1]
L. interrogans Pomona	13 (1354)	21 (1348)		14 (1363)	35 (1299)	9 (1359)	16 (1344)	37 (1284)	145 (1409)	132 (1357)	*L. interrogans* Pomona
L. interrogans Prajitno	10 (1361)	15 (1352)	17 (1363)		35 (1297)	17 (1361)	24 (1337)	42 (1286)	147 (1405)	133 (1353)	*L. interrogans* Prajitno
L. alexanderi Manzhuang	48 (1302)	57 (1288)	46 (1299)	43 (1297)		41 (1294)	36 (1323)	18 (1269)	140 (1372)	126 (1356)	*L. alexanderi* Manzhuang
L. borgpetersenii Hardjo-Bovis	22 (1358)	25 (1308)	11 (1359)	16 (1361)	31 (1294)		22 (1337)	36 (1282)	148 (1417)	135 (1357)	*L. borgpetersenii* Hardjo-Bovis
L. kirschneri Grippotyphosa	24 (1338)	36 (1326)	19 (1334)	24 (1337)	28 (1323)	24 (1337)		28 (1298)	140 (1424)	121 (1382)	*L. kirschneri* Grippotyphosa
L. weilii Mengding	61 (1288)	67 (1274)	58 (1284)	59 (1286)	26 (1269)	55 (1282)	45 (1298)		155 (1345)	127 (1322)	*L. weilii* Mengding
L. fainei Hurstbridge	88 (1411)	98 (1399)	86 (1409)	83 (1405)	69 (1372)	84 (1417)	79 (1424)	75 (1345)		113 (1430)	*L. fainei* Hurstbridge
L. biflexa Andamana	116 (1358)	127 (1345)	115 (1357)	112 (1353)	99 (1356)	115 (1357)	105 (1382)	89 (1322)	151 (1430)		*L. biflexa* Andamana

Appendix 4.1. Total number of functional gene variations for the ten sequenced strains when compared between themselves. The number of shared functions between species shown in brackets. [1] *L. interrogans* Icterohaemorrhagiae.

Comparison Strain	Sequenced Strain										Comparison Strain
	L. interrogans Bratislava	*L. interrogans* Ictero[1]	*L. interrogans* Pomona	*L. interrogans* Prajitno	*L. alexanderi* Manzhuang	*L. borgpetersenii* Hardjo-Bovis	*L. kirschneri* Grippotyphosa	*L. weilii* Mengding	*L. fainei* Hurstbridge	*L. biflexa* Andamana	
L. interrogans Bratislava		98.32	98.19	98.84	93.94	97.07	96.82	92.73	85.62	84.45	*L. interrogans* Bratislava
L. interrogans Ictero[1]	98.32		97.54	98.61	93.2	97.03	95.6	92.39	84.94	83.64	*L. interrogans* Ictero[1]
L. interrogans Pomona	98.19	97.54		97.78	94.13	98.55	97.46	93.11	85.91	84.6	*L. interrogans* Pomona
L. interrogans Prajitno	98.84	98.61	97.78		94.33	97.63	96.53	92.72	85.93	84.67	*L. interrogans* Prajitno
L. alexanderi Manzhuang	93.94	93.2	94.13	94.33		94.73	95.39	96.65	86.78	85.77	*L. alexanderi* Manzhuang
L. borgpetersenii Hardjo-Bovis	97.07	97.03	98.55	97.63	94.73		96.67	93.37	85.93	84.44	*L. borgpetersenii* Hardjo-Bovis
L. kirschneri Grippotyphosa	96.82	95.6	97.46	96.53	95.39	96.67		94.68	86.67	85.95	*L. kirschneri* Grippotyphosa
L. weilii Mengding	92.73	92.39	93.11	92.72	96.65	93.37	94.68		85.4	85.96	*L. weilii* Mengding
L. fainei Hurstbridge	85.62	84.94	85.91	85.93	86.78	85.93	86.67	85.4		84.42	*L. fainei* Hurstbridge
L. biflexa Andamana	84.45	83.64	84.6	84.67	85.77	84.44	85.95	85.96	84.42		*L. biflexa* Andamana

Appendix 4.2. Percentage of shared functional genes amongst all ten sequenced strains when compared between themselves. [1]*L. interrogans* Icterohaemorrhagiae

Appendix Five

Gene	Strain									
	L. borgpetersenii Hardjo-Bovis	*L. Interrogans* Bratislava	*L. kirschneri* Grippotyphosa	*L. interrogans* Icterohaemorrhagiae	*L. alexanderi* Manzhuang	*L. interrogans* Pomona	*L. interrogans* Prajitno	*L. biflexa* Andamana	L. weilii Mengding	*L. fainei* Hurstbridge
gyrB	+	+	+	+	+	+	+	+	+	+
fadD	+	+	+	+	B/H/L	+	+	+	-	B/H/L/M/R
caiA	2	2	2	2	5	-	-	5	-	5
caiB	+	+	+	+	+	+	+	+	-	+
pntA	+	+	+	+	+	+	+	+	-	+
glmU	+	+	+	+	+	+	+	+	-	+
sucA	+	+	+	+	+	+	+	+	-	+
tpiA	+	+	+	+	+	+	+	+	-	+
pfkA	+	+	+	+	+	+	+	+	-	+
pfkB	-	+	+	+	-	+	+	-	-	-
mreA	-	+	+	+	-	+	+	-	-	-
mreB, C, D	+	+	+	+	+	-	-	+	-	+
flaB (1/2/3)	+	+	+	+	+	-	-	+	-	+
lig	+	+	+	+	-	-	-	+	-	+
fliG	+	+	+	+	-	-	-	+	-	3
loa22	+	+	+	+	+	+	+	-	+	-
adk	+	+	+	+	+	-	-	+	-	+
icdA	+	+	+	+	+	-	-	+	-	+
rrs2	-	-	-	-	-	-	-	-	-	-
secY	+	+	+	+	+	-	-	+	-	+
lipL21	+	+	+	+	+	-	-	+	-	+

Gene	Strain									
	L. borgpetersenii Hardjo-Bovis	*L. Interrogans* Bratislava	*L. kirschneri* Grippotyphosa	*L. interrogans* Icterohaemorrhagiae	*L. alexanderi* Manzhuang	*L. interrogans* Pomona	*L. interrogans* Prajitno	*L. biflexa* Andamana	L. weilii Mengding	*L. fainei* Hurstbridge
lipL31	+	+	+	+	+	-	-	+	-	+
lipL32	+	+	+	+	+	+	+	-	+	+
lipL36	+	+	-	+	+	-	-	-	-	-
lipL41	2	2	+	2	+	-	-	-	-	2
lipL45	+	+	+	+	+	-	-	+	-	+
lipL48	+	+	+	+	+	-	-	+	-	-
lipL71	+	+	+	+	+	-	-	+	-	+
flaA (1/2)	+	+	+	+	+	-	-	+	-	+
ompL1	+	+	+	+	+	+	+	-	+	2

Appendix 5.1. Individual gene presence within all ten sequenced strains. Numbers correspond to presence of >1 copy of the gene.

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