Replacement of hamsters with physiochemical analytical methods for *Leptospira* vaccine batch potency testing

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Declaration of Authorship

I hereby declare that all the work presented in this thesis is the result of my own independent investigations unless otherwise stated. Animal work was assisted by Gavin Thomson and staff at the Animal Health and Veterinary Laboratories Agency (AHVLA). Histological processing and analysis of hamster tissue was performed by Dr Alejandro Núñez Castel (AHVLA). Microscopic agglutination test (MAT) was performed by Lee Smith (AHVLA). Genetic sequencing was performed by the AHVLA sequencing unit.

This work has not been previously accepted for any degree, and is not being concurrently submitted in candidature for any other degree.

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Abstract

The current batch potency test for *Leptospira interrogans* serovar Canicola vaccines requires the use of a large number of hamsters and has severe effects; whilst effective, a safer, cheaper, more ethical replacement is desired. The aim of this study was to determine the common components of commercially available serovar Canicola vaccines to aid development of an *in vitro* potency test; lipopolysaccharide and protein were chosen for analysis due to their known immunogenic properties. Analysis of five serovar Canicola vaccines (A-E) using the Limulus amebocyte lysate assay and silver stained sodium dodecyl sulfate polyacrylamide gels, indicated that lipopolysaccharide was not detectable in all vaccines tested preventing it from being a suitable biomarker for an *in vitro* test. Therefore the protein contents of vaccines A-E were determined by two dimensional liquid chromatography mass spectrometry (221±31, 9±8, 34±4, 21±5 and 34±17 proteins [mean ± 1 standard deviation] found respectively) to identify conserved proteins. The outer membrane protein LipL32 was shown to be common to vaccines A-E and to be present at a significantly higher ($p \leq 0.05$) relative spectral abundance in a batch of vaccine which passed the *in vivo* potency test, compared to one which failed. Quantitative analysis using multiple reaction monitoring determined that the concentration of the N terminus of LipL32 was significantly lower ($p \leq 0.01$) in failed batches (n=2) of vaccine compared to passed batches (n=2); the concentration of the C terminus was relatively uniform. The protective effect of LipL32 against serovar Canicola was subsequently investigated in hamsters. Decreased kidney invasion was observed in groups vaccinated with LipL32 prior to challenge suggesting that LipL32 may be an active component of vaccines A-E. With additional supportive data, beyond the scope of this study, quantitative analysis of N terminal LipL32 has the potential to form an *in vitro* vaccine potency test.

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Glossary of Abbreviations

ACDP	Advisory committee on dangerous pathogens
AHVLA	Animal health and veterinary laboratories agency
APCI	Atmospheric pressure chemical ionisation
APEX	Absolute protein expression measurements
AQUA	Absolute quantification
BSA	Bovine serum albumin
CAAT	Cross agglutination absorption test
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CTAB	Cetyltrimethylammonium bromide
DC	Direct current
DHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides
DTT	Dithiothreitol
EIC	Extracted ion chromatogram
ELISA	Enzyme linked immunosorbent assay
EMJH	Ellinghausen, McCullough, Johnson and Harris *Leptospira* Media
ESI	Electrospray ionisation
FA	Formic acid
FDR	False discovery rate
H&E	Hematoxylin and eosin
HAP	Hemolysis associated protein
His	Histidine
ICAT	Isotope-coded affinity tag
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IM	Inner membrane
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITRAQ	Isobaric tag for relative and absolute quantitation
LAL	Limulus amebocyte lysate
LB	Luria-bertani
LC	Liquid chromatography
LIT	Linear ion trap
LOD	Limit of detection
LOQ	Limit of quantification
LPS	Lipopolysaccharide
MALDI	Matrix assisted laser desorption ionisation
MAT	Microscopic agglutination test
MLST	Multilocus sequence typing
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MWCO	Molecular weight cut off
NCBI	National centre for biotechnology information
NIH	National institutes of health
NSAF	Normalised spectral abundance factor
OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein
OMSSA	Open mass spectrometry search algorithm
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PG	Peptidoglycan
PIR	Protein information resource
PMSF	Phenylmethanesulfonyl fluoride
QQQ	Triple quadrupole (mass spectrometer)
Q-TOF	Quadrupole time-of-flight (mass spectrometer)
RAPD	Random amplification of polymorphic DNA
RF	Radio frequency
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RP-HPLC	Reverse phase high pressure liquid chromatography
RSPCA	Royal society for the prevention of cruelty to animals
SCX	Strong cation exchange
SDS	Sodium dodecyl sulfate
SILAC	Stable isotope labelling by amino acids in cell culture
SRM	Single reaction monitoring
TFA	Trifluoroacetic acid
TIC	Total ion chromatogram
TMB	Tetramethylbenzidine
TMT	Tandem mass tags
ToF	Time of flight
UHPLC	Ultra-high pressure chromatography
UK	United kingdom
Uniprot	Universal protein resource

USA	United states of america
UV	Ultraviolet
VNTR	Variable number tandem repeat

Publications in support of this thesis

1. **HUMPHRYES, P. C**., WEEKS, M. E., GIELBERT, A., THOMSON, G. & COLDHAM, N. G. 2012. Analysis of multiple *Leptospira interrogans* serovar Canicola vaccine proteomes and identification of LipL32 as a biomarker for potency. Clin Vaccine Immunol, 19, 587-93.
2. **HUMPHRYES, P. C**., WEEKS, M. E. & COLDHAM, N. G. 2012. Characterisation of the proteome of *Leptospira interrogans* serovar Canicola reveals conserved immunogenic proteins with other serovars. (Submitted-Analytical & Bioanalytical Techniques)

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Chapter 1 Introduction

1.1 History and Clinical importance of Leptospirosis Infection

1.1.1 Discovery of Leptospirosis

The discovery of Leptospirosis is generally ascribed to Adolf Weil who, in 1886, accurately identified the clinical symptoms of the disease (Alston and Brown, 1937). Weil's name is now so synonymous with the disease that the term 'Weil's disease' is often erroneously used to describe infection with *Leptospira interrogans (L. interrogans)*, regardless of the severity of the symptoms, when in fact it should only be used following the presentation of jaundice (Hill and Sanders, 1997). A disease matching the symptoms of Leptospirosis was described prior to 1886 (Levett, 2001) suggesting the disease had been prevalent for some time before its official 'discovery'. A recent paper (Marr and Cathey, 2010) has even suggested that Leptospirosis played a role in the demise of the Native American population of Massachusetts during the early 17th century.

It was noted early on that people who worked in close proximity to stagnant water such as in sewers or rice fields were prone to Leptospirosis. But it was not until 1915 that the bacteria were first isolated from the hepatic tissue of an infected guinea pig, which allowed *Leptospiras* route of infection and morphology to be determined (Inada et al., 1916).

1.1.2 Taxonomy

Currently serological differentiation is the most practical system used to categorise *Leptospira*. This involves using either the cross agglutination absorption test (CAAT) or

the microscopic agglutination test (MAT) to discriminate between *Leptospira* based on their antigen reactivity. Over 230 pathogenic serovars of *Leptospira* have been identified to date (Adler et al., 2011); antigenically related serovars are often assembled into serogroups.

In addition to this, a genetic classification system has also been developed based on DNA hybridisation studies; to date 20 species have been identified (Table 1) (Cerqueira and Picardeau, 2009). Some of these species can be clearly grouped as either pathogenic or non-pathogenic (Table 1); however a consensus regarding the pathogenicity of *L. broomii, L. fainei, L. inadai, L. licerasiae* and *L. wolffii* has yet to be determined resulting in their current 'intermediate' classification (Table 1).

Table 1: Current known species of *Leptospira* spp. (Cerqueira and Picardeau, 2009).

Pathogenic	Intermediate	Non- Pathogenic
L. alexanderi (genomospecies 2)	*L. broomii*	*L. biflexa*
L. alstonii (genomospecies 1)	*L. fainei*	*L. kmetyi*
L. borgpetersenii	*L. inadai*	*L. meyeri*
L. interrogans	*L. licerasiae*	*L. terpstrae (genomospecies 3)*
L. kirschneri	*L. wolffii*	*L. vanthielii (genomospecies 4)*
L. noguchii		*L. wolbachii*
L. santarosai		*L. yanagawae (genomospecies 5)*
L. weilii		

The new genetic classification system is of benefit as it puts *Leptospira* taxonomy in line with other species and thus allows more direct comparison in the literature. However, it was also reported that some serovars could belong to more than one species, thus making the two classification systems disparate (Levett, 2001).

1.1.3 Epidemiology

As a zoonotic, the primary route of infection for most humans is either through direct contact with infected animals or by exposure to water contaminated with their urine. Routes of entry into the host include ingestion, direct entry into the blood stream via cuts and inhalation of aerosols (Woodward, 2001).

The typical hosts of *Leptospira* include rodents, cattle, dogs, pigs, and sheep, although virtually any mammal is a potential carrier (Koizumi and Watanabe, 2009). Infection in dogs can often be asymptomatic (McDonough, 2001), which is of particular concern due to their status as companion animals as it increases the likelihood of human infection. Virtually all human infection occurs through contact with animals (Adler and de la Pena Moctezuma, 2010) which has led to its classification as a zoonotic, although transfer of *Leptospira* between humans can also occur in rare circumstances (Thornley et al., 2002). Transmission itself is dependent on a number of factors including climate (warmer, more humid environments provide better growth conditions), the population density and the frequency of contact with infected hosts (Levett, 2001). Global warming (Desai et al., 2009) alongside other ecological changes (Lau et al., 2012) has been associated with altering the epidemiology of the bacteria resulting in the emergence of previously unknown serovars.

Infection in dogs is closely monitored in the UK due to their status as companion animals; in 2010, 3407 canine serum samples were submitted to the Animal Health and Veterinary Laboratories Agency (AHVLA) for testing (Defra, 2010). Of these 1199 samples (35.2% of canine samples) were identified as positive for *L. interrogans* serovar Canicola. Incidence of human infection in the UK is typically fairly low (Table 2); although it should be noted that these figures only represent cases confirmed by the

Leptospirosis reference unit (Health Protection Agency, UK). Serotyping is required for accurate diagnosis, which many third world countries do not have access to; this in conjunction with *Leptospira*s limited public profile often leads to misdiagnosis and general underreporting of infection.

A higher prevalence of some serovars has been observed in particular species (Bharti et al., 2003), such as serovar Canicola in dogs and serovar Hardjo in sheep and cattle. This serovar-host predisposition can aid in tracking the spread of the disease and helps determine which serovar should be vaccinated against in particular animal populations.

Table 2: Confirmed reports of Leptospirosis in UK residents 1996-2010 (Defra, 2008, Defra, 2010).

Country	Year														
	1996	**1997**	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**
Scotland	0	1	1	0	0	0	3	0	2	4	3	6	13	2	3
England/Wales	22	39	29	41	54	48	54	28	29	41	44	74	62	52	39
N. Ireland	2	3	4	1	0	0	1	0	1	1	4	1	1	0	0
UK	24	43	34	42	54	48	58	28	32	46	51	81	76	54	42

1.2 *Leptospira* Spp.

1.2.1 Physical Characteristics

Leptospires are gram negative helically coiled spirochetes with a high motility due to the presence of two periplasmic flagella (Figure 1). They typically measure between 6-20 µm long and 0.1 µm wide (Levett, 2001) and have a 'hooked' end resembling a question mark. In addition to the hooked form a 'straight' form of the bacteria exists, this straight form is often associated with a loss of virulence and thought to be a result of laboratory culture; however, no clear link between morphology and virulence has so far been established (Woodward, 2001, Faine and Vanderhoeden, 1964).

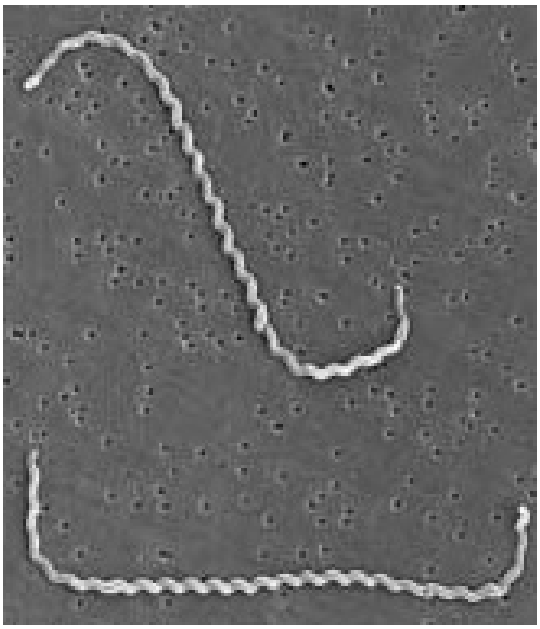

Figure 1: Electron micrograph of *Leptospira* spp. taken from Collins (2006).

Leptospira have an outer membrane principally consisting of lipopolysaccharide (LPS) and proteins; with LipL32, LipL36, LigA, LigB, Loa22 and OmpL1 being amongst the most abundant proteins (Figure 2). The diversity of LPS found on the outer membrane allows different variants of *Leptospira* to be categorised (de la Pena-Moctezuma et al.,

1999) based on their reaction to established serological tests resulting in the various different serovars.

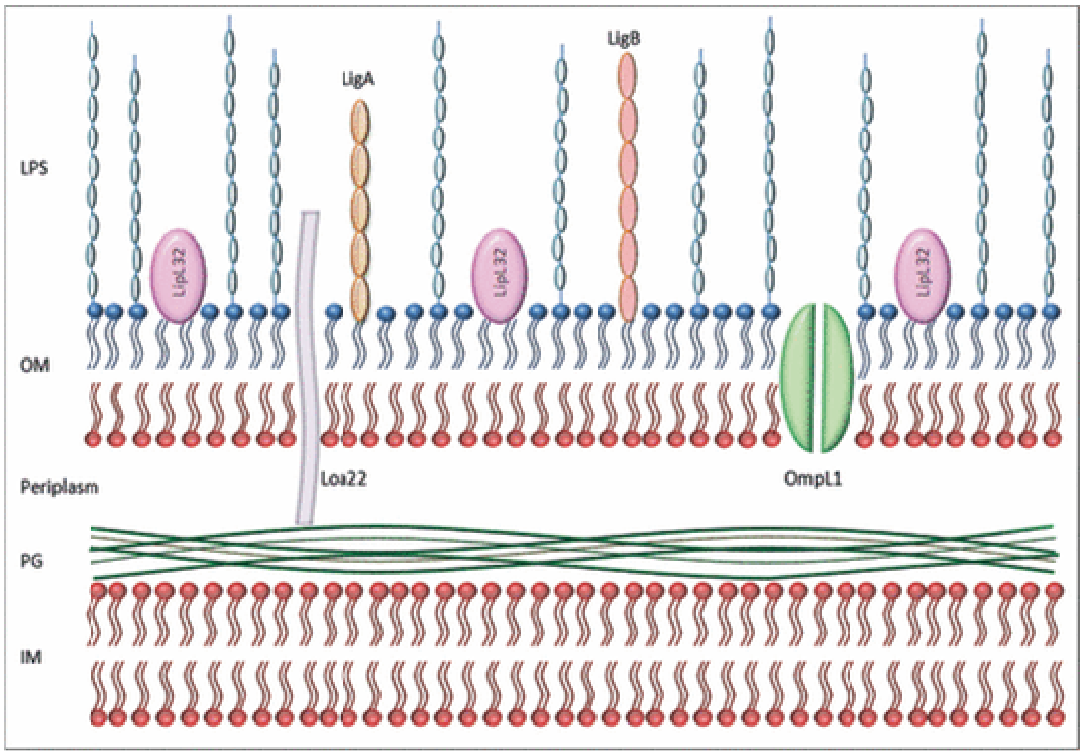

Figure 2: Diagram of the membrane architecture of *Leptospira* spp. (Fraga et al., 2011).

Key: LPS= Lipopolysaccharide, OM= Outer membrane, IM= Inner membrane and PG= Peptidoglycan. TonB dependant receptor systems, endoflagella and lipoprotein export apparatus have been omitted for simplicity.

Leptospira spp. requires very exact conditions in order to grow *in vitro* which can vary between serovars. However, broadly speaking, most pathogenic serovars can be grown at 30^{o}C in EMJH medium with a typical doubling time of approximately 14-18 hours and an optimum pH of 7.2-7.6 (Woodward, 2001); non-pathogenic strains are less fastidious and so are able to be grown at 13^{o}C (Levett, 2001), which allows easy distinction between the two (Johnson and Harris, 1967). The EMJH media currently used is based on the polysorbate 80 media, containing ammonium chloride, albumin, vitamin B12 and thiamine, first described by Ellinghausen and McCullough (1965); a

key component for culturing *Leptospira in vitro* is the presence of iron (Faine, 1959). *Leptospira* has a predisposition to lose virulence following *in vitro* culture (Haake et al., 1991) and liquid nitrogen storage (Reed et al., 2000), due in part to alterations in the expression of lipopolysaccharide and outer membrane proteins (Haake et al., 1991) such as LipL36 (Haake et al., 1998) and LipL45 (Matsunaga et al., 2002), and therefore has to be routinely passed (passaged) through small mammals which are its natural reservoir (Rahelinirina et al., 2010).

1.2.2 Genetic Characteristics

Genetic sequencing of *Leptospira* has resulted in the publication of six genomes to date (Adler and de la Pena Moctezuma, 2010), which indicate that the *Leptospira* genome size varies between 3.9-4.6 Mb with a guanine: cytosine ratio of 35-41%, dependant on the precise species/serovar. A core genome between *Leptospira interrogans, Leptospira borgpeteresenii* and *Leptospira biflexa*, comprising of 2050 genes (Picardeau et al., 2008), has also been identified using comparative genomics. Development of standard methods for genetic manipulation of *Leptospira* has proved difficult; however transposon mutagenesis has been successfully used to create mutants (Murray et al., 2009) resulting in the identification of LPS, Loa22, haem oxygenase and FliY proteins as virulence factors (Fraga et al., 2011).

1.2.3 Protein Characteristics

Protein analysis is a useful approach to quantitatively examine which proteins an organism is expressing at particular time points (referred herein as its proteome); this can aid in the understanding of biological processes in addition to providing target molecules for therapeutic and/or prognostic applications. To this end, the proteomes of *L. interrogans* serovars Copenhageni, Pomona and Lai have been determined using gel

and liquid chromatography based mass spectrometry approaches (Eshghi et al., 2009, Malmstrom et al., 2009, Vieira et al., 2009, Cao et al., 2010, Zhong et al., 2011b).

The proteomes of virulent and lab attenuated (avirulent) strains of serovar Lai (strains 56601 and IPAV respectively) have been directly compared to determine differences in protein expression (Zhong et al., 2011b). This concluded that differences in the expression of the outer membrane proteins OmpL1, LipL45, LipL48, LipL41 and LipL36 were responsible for the loss of virulence observed in the lab attenuated strain.

In addition, the *Leptospira* proteins LipL32 (Haake et al., 2000, Seixas et al., 2007b), LipL41 (Haake et al., 1999), LipL45 (Sakolvaree et al., 2007), OmpL1 (Dong et al., 2008), OmpA (Yan et al., 2010), LigA (Faisal et al., 2008) and LigB (Yan et al., 2009) have all been identified as potential vaccine targets and are actively being investigated for their immunological protective effects *in vivo*.

1.3 Infection and Treatment

1.3.1 Clinical features

Upon entering the body *Leptospira* incubate for 7-12 days before entering the septicaemic phase (Collins, 2006). This is often characterised as 'mild flu-like' symptoms which can include: Fever, headache, myalgia, nausea, cough, diarrhoea and vomiting. This septicaemic phase typically lasts about a week wherein the initial symptoms begin to dissipate.

After this preliminary phase the initial symptoms alleviate for a few days prior to the subject entering the immune phase of the disease. Common symptoms of this immune

phase (Howell and Cole, 2006), which can last in excess of 30 days, include meningitis, enlarged lymph nodes, hepatitis and cardiac arrhythmias. In extreme cases, the patient can go on to develop Weils disease which is characterised by hepatic and renal failure, fever, jaundice and/or pulmonary haemorrhage. In particularly rare cases, infection with *Leptospira* does not result in any obvious clinical symptoms (Ganoza et al., 2010) although the long term effects of this have yet to be determined. Presentation of Leptospirosis in animals can vary depending on the precise species. Renal and hepatic failure are particularly common in canines however pulmonary disorder such as dyspnoea are also prevalent (Kohn et al., 2010). Abortion of offspring is known to occur in cattle (Defra, 2008), swine (Ramos et al., 2006), sheep (Kingscote, 1985) and goats (Leon-Vizcaino et al., 1987) making the disease economically damaging to the farming industry.

1.3.2 Diagnostics

Due to the wide range of non-specific symptoms that *Leptospira* infection can induce it is often difficult to make an accurate diagnosis on clinical presentation alone. Typically the infection is detected in either a blood or urine sample but it is not uncommon for it to be found through a kidney biopsy.

Rapid confirmation of the presence of *Leptospira* can be achieved through direct visualisation using dark field microscopy (Adler and de la Pena Moctezuma, 2010) for fluids or silver staining for tissues (Skilbeck and Chappel, 1987). However, for accurate determination of the specific serovar, molecular or serological tests must be performed.

Molecular tests under development for more specific species, serovar and strain typing or identification include; multilocus sequence typing (MLST) (Ahmed et al., 2006),

real time polymerase chain reaction (Ooteman et al., 2006), insertion sequence typing (Cerqueira and Picardeau, 2009), 16s ribosomal RNA sequencing (Morey et al., 2006), pulsed field gel electrophoresis (PFGE) (Romero et al., 2009), restriction fragment length polymorphism (RFLP) (Turk et al., 2009), variable number tandem repeat (VNTR) (Majed et al., 2005), random amplification of polymorphic DNA (RAPD) (Ramadass et al., 1997), ribotyping (Kositanont et al., 2007) and more recently single nucleotide isolated polymorphism detection by denaturing high performance liquid chromatography (DHPLC) (Fenner et al., 2010).

Due in part to the disparity between genetic and serological taxonomy the MAT tends to be the preferred choice for *Leptospira* typing, although its usage in developing countries is often precluded by access to antisera. Other serological tests such as ELISA and CAAT tend to be less accurate (van de Maele et al., 2008) or too technically demanding in terms of time and expertise for routine diagnostic purposes (Cerqueira and Picardeau, 2009) respectively.

1.3.3 Infection and Immune Response

Following initial exposure to infection (section 1.1.3) *Leptospira* can bind to extracellular matrix components, such as fibronectin (Chirathaworn et al., 2007), and have also been shown to bind to endothelial cells, macrophages, and fibroblasts *in vitro* (Fraga et al., 2011). Further they are able to quickly pass through cell monolayers (Barocchi et al., 2002), allowing them to disseminate to multiple organs.

The involvement of the innate immune system against pathogenic *Leptospira* (Goris et al., 2011) has not been fully characterised; however it has been determined that *Leptospira* has the ability to evade the innate immune system of humans through

binding to the complement regulator C4BP (Barbosa et al., 2009). In addition the role of the cell mediated immune system against pathogenic *Leptospira* is also not fully understood (Fraga et al., 2011), although *Leptospira* is known to promote the apoptosis of macrophages (Jin et al., 2009). It is however well established that the principle immune response against *Leptospira* is humoral, due to the fact that immunity can be transferred from one host to another through serum (Adler and de la Pena Moctezuma, 2010). Immunoglobulin M (IgM) has been determined (Hartman et al., 1984) to be the initial response to first vaccination with a serovar Canicola vaccine in dogs, however subsequent booster vaccinations result in higher levels of immunoglobulin G (IgG) (Hartman et al., 1984).

1.3.4 Treatment

If Leptospirosis infection is identified at an early stage, before severe tissue damage, antibiotics are an extremely effective treatment which can completely cure the subject. Penicillin, doxycycline, ampicillin and ceftriaxone being the most commonly used (Pappas and Cascio, 2006). In addition to this, antibiotics can be administered as a prophylactic (Illangasekera et al., 2008) to inhabitants of higher risk areas (victims of floods for example) to help prevent the spread of infection. However, in some cases of human infection, antibiotic treatment of *Leptospira* spp can result in Jarisch–Herxheimer reactions (Maneewatch et al., 2009), whereby endotoxin is released due to the large number of organisms killed; this endotoxin causes a variety of additional symptoms including fever, making vaccination a preferable treatment strategy. Interestingly a recent study (Brett-Major and Coldren, 2012) was unable confirm that antibiotics statistically reduced the duration of infection. In the event of severe infection additional medical steps must be taken to improve the subject's condition (dialysis for example is used following kidney damage).

1.3.5 Vaccines

Although *Leptospira* infection can be treated (section 1.3.4), initial symptoms in cattle such as cessation of milk production and miscarriage (Defra, 2008), have often already occurred which are economically damaging to the farming community. Vaccination represents the most effective way of preventing infection and avoiding these symptoms. However, since *Leptospira* is predominantly found in wild animals, particularly rodents (Rahelinirina et al., 2010), it has not yet been possible to use vaccination to completely eradicate the bacteria from the environment. The strategy for disease prevention therefore relies heavily on the principle of herd immunity (Schultz et al., 2010), which relies on the vaccination of as many domestic and farm animals as possible to reduce the spread of infection. Dogs in particular are regularly vaccinated against *Leptospira* as part of their standard annual vaccination protocol.

The majority of *Leptospira* vaccines currently available on the market (Table 3) are derived from inactivated or killed bacteria. The specific methodologies used to generate these vaccines are commercially sensitive and not in the public domain. However, in general, *Leptospira* vaccine manufacture involves growing the bacteria in an albumin rich media, such as EMJH, and then killing/inactivating the bacteria using either formalin or heat. Live attenuated vaccines for *Leptospira* have also been developed (Kenzy et al., 1961); however they are not in widespread use, presumably due to the risk of the strains regaining their pathogenicity and causing clinical symptoms in the host (Srivastava, 2006). Subunit vaccines, engineered to contain specific antigens known to provide protection, have also been developed for *Leptospira* and are commercially available (Table 3). Historically *Leptospira* vaccines have been restricted to use in animals due to their high likelihood of causing adverse reactions

(reactogenicity) in humans (Adler and de la Pena Moctezuma, 2010). This reactogenicity of *Leptospira* vaccines has been associated with the presence of serum in the growth media (Koizumi and Watanabe, 2005); however vaccines derived from protein free media (Christopher et al., 1982) have not been shown to statistically decrease vaccine-associated side effects (Koizumi and Watanabe, 2005). Despite this, some human *Leptospira* vaccines have been successfully developed (Rodriguez-Gonzalez et al., 2004, Koizumi and Watanabe, 2005), although they are not currently in widespread usage. Unfortunately, all the vaccines currently available only provide immunity to specific serovars (Koizumi and Watanabe, 2005) and require regular boosters (Klaasen et al., 2003).

A number of other vaccines, such as recombinant outer membrane protein, recombinant lipoprotein, recombinant vector, LPS and DNA vaccines, have also been developed (Wang et al., 2007). A key problem associated with their development is a lack of information, both about *Leptospira*s route of infection and virulence factors. The majority of active research appears to concentrate on the development of subunit vaccines (Felix et al., 2011, Umamaheswari et al., 2012); however some success has also been reported in the creation of live attenuated LPS (Srikram et al., 2011) vaccines.

Table 3: Table of commercially available *Leptospira* vaccines available worldwide

Manufacturer	Commercial Name	Formulation	Target Species	Immunises against Serovars	Route(s) of Administration
Wyeth (Fort Dodge)	Triangle + Type II BVD	Killed Bacteria	Cattle	Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona	Intramuscular Subcutaneous
	Duramune Max 5	Subunit purification of live bacteria	Dog	Canicola, Grippotyphosa, Icterohaemorrhagiae and Pomona	Subcutaneous
	LeptoVax 4	Subunit purification of live bacteria	Dog	Canicola, Grippotyphosa, Icterohaemorrhagiae and Pomona	Subcutaneous
	Kavak L	Inactivated Bacteria	Dog	Data not available	Intramuscular Subcutaneous
Novartis	Lepto Shield 5	Inactivated Bacteria	Cattle, Swine	Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona	Intramuscular
Pfizer	Lepto-Eryvac	Inactivated Bacteria	Swine	Pomona and Tarassovi	Subcutaneous
	Spirovac	Inactivated Bacteria	Cattle	Hardjo	Subcutaneous
	Leptoferm-5	Inactivated Bacteria	Cattle	Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona	Intramuscular
	Leptoshield	Data not available	Cattle, Sheep, Goats, Deer	Hardjo and Pomona	Subcutaneous
	Vanguard Lepto ci	Inactivated Bacteria	Dogs	Data not available	Intramuscular Subcutaneous
	Ultravac	Data not available	Cattle	Hardjo and Pomona	Subcutaneous
Schering-Plough	Leptavoid 2	Inactivated Bacteria	Cattle, Sheep, Pigs, Deer	Hardjo and Pomona	Subcutaneous
	Leptavoid 3	Inactivated Bacteria	Cattle, Deer	Pomona, Hardjo and Icterohaemorrhagiae	Subcutaneous
Intervet	Nobivac Lepto 2	Inactivated Bacteria	Dog	Icterohaemorrhagiae and Canicola	Subcutaneous
	VL5 SQ	Inactivated Bacteria	Cattle	Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, and Pomona	Subcutaneous
	Procyon	Inactivated Bacteria	Dog	Icterohaemorrhagiae and Canicola	Intramuscular Subcutaneous
Virbac	Lepto 2 way	Inactivated Bacteria	Cattle	Hardjo and Pomona	Subcutaneous
	Lepto 3 way	Inactivated Bacteria	Cattle	Hardjo, Pomona and Copenhageni	Subcutaneous
	Canigen Lepto 2	Inactivated Bacteria	Dog	Canicola and Icterohaemorrhagiae	Subcutaneous
Merial	Eurican L	Inactivated Bacteria	Dog	Canicola and Icterohaemorrhagiae	Intramuscular Subcutaneous
Agvax	Trilepto	Inactivated Bacteria	Cattle	Hardjo and Pomona	Subcutaneous

Note: whilst this table is comprehensive it is not exhaustive and should not be taken as such.

1.4 *Leptospira* vaccine batch potency testing

The current *Leptospira* vaccine batch potency test used for serovar Canicola dog vaccines has been identified as severe, and in need of replacement, by the royal society for the prevention of cruelty to animals (RSPCA) (Cooper and Jennings, 2008) as it involves a high number of animal deaths following challenge from live organisms which produces a corresponding high degree of suffering (as euthanasia is not typically used to avoid skewing the results). In addition, the serovar Canicola strain used in the test must be regularly passed through hamsters (passaged), to prevent any loss of virulence associated with *in vitro* culture (Haake et al., 1991), resulting in further suffering and loss of life. Furthermore the test is time consuming, expensive and puts personnel at risk of exposure to an ACDP (advisory committee on dangerous pathogens) hazard group II organism.

The current requirements for assessing the potency of serovar Canicola vaccines, as laid out in the most recent European monograph (1997), specifies the use of 10 hamsters no more than three months old and from the same stock. Five of the hamsters are inoculated with the vaccine (at a 40 times dilution) by subcutaneous injection, the other five act as a control group and are unvaccinated. Fifteen to twenty days following vaccination all 10 hamsters are challenged by inoculation with a virulent strain of serovar Canicola intraperitoneally.

The vaccine is considered appropriately potent if a minimum of 4 hamsters from the control group die within 14 days of infection (precise dosage of the bacteria varies depending on the strain virulence) and if a minimum of 4 hamsters from the vaccinated group are healthy 14 days after the death of the control animals. Although this test is

established to be effective the use of 5 hamsters per group is not statistically ideal. A power analysis would enable an optimal sample size to be derived; however any statistical benefits resulting from increasing the number of animals used per group would have to be carefully considered by the European pharmacopoeia prior to implementation.

1.4.1 *In vitro* alternatives to animal testing

Various monoclonal antibody based ELISA techniques have been developed to both assess potency and quantify specific *Leptospiral* antigens in vaccines. Whilst this has been successful for some serovars, such as Pomona (Ruby et al., 1992), similar attempts using serovar Canicola have had mixed success (Ebert, 1999); possibly due to the effect of adjuvant variation between batches. ELISA potency tests for certain specific serovar Canicola vaccines have been developed in the USA (Ruby et al., 1996); however, these require that adjuvants be removed from the vaccines prior to testing which is not practical for widespread implementation due to the wide variety of adjuvants used by different manufacturers. It has been clearly established however (Guerreiro et al., 2001, Ruby, 1999) that both protein and lipopolysaccharide (LPS), derived from *Leptospira*, are capable of eliciting an immune response.

Use of the MAT as an alternative to the hamster challenge test was first proposed in 1986 (Goddard et al., 1986). A subsequent study (Ebert, 1999), using *Leptospira borgpetersenii* serovar Hardjo vaccines and guinea pig serum substantiated Goddard *et al's* results. However, this line of research has seemingly been abandoned in favour of true *in vitro* alternatives (such as the ELISA). This may be due to the fact that the MAT, although requiring lesser numbers than the hamster challenge test, still involves animal infection (to obtain the serum).

1.4.2 Current Situation

As noted in the recent RSPCA report (Cooper and Jennings, 2008) the existing *in vitro* methods outlined above have largely not been put into widespread usage; primarily due to the effects of adjuvant interference with ELISA based techniques (Personal communication). Current work in this area focuses predominantly on the improvement of the existing *in vivo* test (Stokes et al., 2011), rather than the development of novel approaches for determining vaccine potency *in vitro*.

1.5 Principles and Applications of Proteomics

1.5.1 Protein characterisation of Vaccines

Many vaccines currently available, particularly those derived from heat/chemically killed bacteria, lack a defined mechanism for their ability to confer protective immunity; which hinders the development of specific *in vitro* potency tests and the design of more efficacious vaccines. Mass spectrometry based analysis of immune proteins (termed immunoproteomics) can often be utilised to characterise the immune response to foreign organisms (Purcell and Gorman, 2004); however proteomic analysis of vaccines is not widely used and the characterisation of *Leptospira* vaccines has not been previously reported.

The majority of research to date has concentrated on the proteomic identification of novel vaccine candidates. Proteomic 'vaccine' analysis has also been reported (Tsolakos et al., 2010), although this generally concentrates on characterising the bacterial strain used to manufacture the vaccine (Uli et al., 2006), rather than the final vaccine preparation (containing adjuvant and preservative) released for commercial sale.

Despite this, gel based mass spectrometry techniques for vaccine characterisation have been developed (Hennessey et al., 1999, Vipond et al., 2005) and a method for the desorption of aluminium phosphate adjuvant from anthrax vaccines has also been described (Whiting et al., 2004). In addition mass spectrometry based analysis of peptide vaccines has been reported for the purposes of quality control (Metz et al., 2002).

1.5.2 Sample Preparation

The majority of proteomic techniques require that the sample of interest be prepared prior to analysis to optimise downstream separation and mass analysis. Preparation methods can vary depending on the sample and the separation/analysis techniques used and are usually independently validated for each study. However, in general, sample preparation aims to increase the solubility of proteins (often through the use of denaturing agents) whilst reducing contamination (such as salts, highly abundant proteins etc) which could affect downstream analysis. Common techniques used include solid (Callesen et al., 2009) and liquid (Peng et al., 2001) phase extraction, protein precipitation (Fic et al., 2010) and molecular weight filtration (Greening and Simpson, 2010).

1.5.3 Protein separation

Separation of complex protein samples is usually required for identification and isolation of proteins of interest. Proteins can be separated out by their charge, size, isoelectric point and/or hydrophobicity depending on the precise needs of the experiment; common analytical separation techniques include gel electrophoresis, isoelectric focusing and chromatography. Separation is often done in conjunction with mass spectrometry which usually requires the use of proteolytic enzymes (for example

trypsin, chymotrypsin and proteinase K) either prior to separation when using liquid chromatography or after separation when using gel electrophoresis (often referred to as 'in gel digestion'(Weeks, 2010)). Complete enzymatic digestion of the samples can be impeded by the presence of contaminants and/or the incorrect pH making sample preparation (section 1.5.2) vitally important; in addition, samples have to be heat and/or chemically denatured prior to the addition of the proteolytic enzyme to break down the tertiary and quaternary structure of the protein, allowing the enzyme access to its cleavage site (lysine and arginine residues in the case of trypsin).

Gel based protein separation is achieved by performing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In brief the sample is mixed with a loading buffer, containing dye and SDS, and heat denatured causing the proteins to become charged. An electric field is then applied to the gel whereby the charged proteins will travel through the gel, with smaller proteins migrating quicker as they can fit through the pores of the gel easier, thus allowing a complex protein mixture to be separated out by size; a protein ladder comprising of known molecular weights is often run alongside samples to allow direct estimation of sample size. A variation of this is 2D-PAGE (Issaq and Veenstra, 2008) which allows further resolution of the protein sample by separating the samples based on their isoelectric point prior to SDS-PAGE. Western blotting can be used in conjunction with SDS-PAGE and 2D-PAGE allowing the identification of proteins based on their reactivity to antibodies (Burnette, 1981). For identification protein 'spots' separated by electrophoresis can be excised from the gel and then analysed using mass spectrometry (Weeks, 2010). However, whilst this is a valid and useful strategy it requires a large amount of sample handling, introducing the potential for sample loss (Staudenmann et al., 1998); additionally, it cannot effectively

separate certain proteins, such as those that are strongly alkaline/hydrophobic and/or membrane proteins (Beranova-Giorgianni, 2003).

Liquid chromatography is an alternative to gel based separation methods which involves allowing the samples to interact with a stationary phase (contained within a column) and then elution by passing an appropriate mobile phase over the stationary phase at a high pressure. The use of smaller particle sizes in the stationary phase can increase the resolution (Novakova et al., 2006), i.e. the ability to distinguish between peaks, of the separation. However, a higher flow pressure through the column is also then required (due to the Van Deemter equation) this is commonly referred to as ultra-high pressure chromatography (UHPLC). Multiple combinations of stationary and mobile phases can be used according to which chemical property the sample of interest is to be separated out by. A common example of liquid chromatography separation is reverse phase high pressure liquid chromatography (RP-HPLC), which separates molecules based on their hydrophobicity (through interaction with a non-polar stationary phase and polar mobile phase). Ion exchange chromatography is also routinely used whereby the stationary phase is either negatively (cation exchange) or positively (anion exchange) charged and the mobile phase is the reverse; mixed bed columns are also available (Motoyama et al., 2007) which retain both anions and cations. Another common option is size exclusion chromatography which uses a non-reactive stationary phase containing different sized pores; smaller molecules in the sample pass through the pores which retards their progress, whereas larger molecules do not and therefore elute from the column earlier. An affective combination for maximising protein separation utilises strong cation exchange in conjunction with RP-HPLC, which is often performed prior to mass spectrometry (Coldham and Woodward, 2004).

1.5.4 Mass Spectrometry

Following chromatographic or electrophoretic separation of the sample, it has to be ionised prior to entering into the mass spectrometer, typically either through electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI). This approach of enzymatically digesting proteins prior to separation and analysis on a mass spectrometer is commonly referred as a 'bottom up' approach. A popular alternative to this is termed 'top down' proteomics which has no protein digestion allowing intact proteins to be analysed on the mass spectrometer. These top down approaches can provide better overall protein coverage however they cannot achieve the separation and sensitivity of bottom up proteomics (Yates et al., 2009). Tandem mass spectrometers (de Hoffman and Stroobant, 2007) are arguably amongst the more widespread instruments used for both quantitative and shotgun based proteomics. Although hybrid mass spectrometers using ion traps in conjunction with an orbitrap (Kalli and Hess, 2012) are increasingly being used in place of Q-ToF based apparatus (Table 4).

After analysis the resultant mass spectra data can then be interrogated against a protein database (generally predicted from the genome for the organism of interest), using a suitable search program, to determine the protein content of the analysed sample. Search programs can be set to take into account known post translational modifications to ensure accuracy of identifications.

Table 4: Advantages and disadvantages of different types of mass spectrometer

Mass Spectrometer	Description	Advantage	Disadvantage
QQQ	Comprised of 3 quadrupoles for filtering/fragmenting ions	Highly sensitive quadrupoles can be used to select known product/precursor ions enabling accurate quantitation of peptides	Prior knowledge of product/precursor ions and collision energies is required.
Q-ToF	As QQQ with the addition of a time of flight tube for accurate mass detection	Allows for the identification of previously unknown proteins/peptides and their product/precursor ions	Limited number of uses compared to modern orbitraps
Linear ion trap (LIT) Orbitrap	LIT can be used to store, isolate and/or fragment ions independently or in conjunction with the Orbitrap analyser	Greater mass accuracy and sensitivity than Q-ToF resulting in more identifications	Not as accurate as QQQ for small molecule quantitation. Expensive

1.5.5 Protein quantitation

Quantitation of protein concentrations within samples is vitally important for the interpretation of proteomic datasets into usable biological observations. Existing approaches for quantitation can be broadly categorised as either being labelled or label free; each method has its disadvantages which should be carefully considered when planning experiments (Table 5).

Table 5: Comparison of different quantitation techniques used in mass spectrometry

Technique	Description	Advantage	Disadvantage
Stable isotope labelling with amino acids in cell culture (SILAC)	Measures relative proteomic differences in metabolism using labelled amino acids in the growth media	Excellent labelling efficiency leading to good protein coverage	Limited to cell culture experiments
Isotope coded affinity tags (ICAT)	Utilises a thiol specific affinity tag for protein quantitation	Rapid	Non- cysteine peptides residues not detected.
Tandem mass tags (TMT)	Mass tags attached to peptides allowing determination of the relative abundance of proteins	Allows for the quantitation & comparison of proteins in up to 6 different groups in 1 experiment	Underestimation of abundance
Isobaric tags for relative & absolute quantitation (iTRAQ)	As TMT however up to 8 channels can be labelled	As TMT but can compare up to 8 different groups	As TMT
Absolute quantification of proteins (AQUA)	Uses a labelled internal standard for quantification	Highly accurate	Expensive. Can only quantitate known proteins/peptides
Spectral counting	Equates the number of detected spectra for a protein to its abundance	Low cost per sample. Rapid	Results can be skewed due to the inability of 2D-LC/MS to detect all of a proteins constituent peptides
Absolute protein expression measurements (APEX)	Variation of spectral counting which uses machine learning to adjust the relative spectral abundance	As spectral counting	As spectral counting
LC-MS[e]	Equates signal intensity with peptide/protein abundance	Low cost per sample. Rapid	Reliant on the accuracy and reproducibility of the liquid chromatography
Single/multiple reaction monitoring (SRM/MRM)	Compares the abundance of the sample against a calibration curve of a synthetic peptide	Highly accurate	Can only quantitate known proteins/peptides

1.6 Aims and Objectives

The hypothesis of this study is that one or more protein/LPS potency biomarkers can be identified in commercially available *Leptospira interrogans* serovar Canicola vaccines and that these biomarkers may be used to replace the existing hamster challenge potency test. The primary objective of this study is the identification of protein and/or LPS potency biomarker(s) which may be suitable for the development of an *in vitro* vaccine batch potency test, to differentiate between efficacious and non- efficacious batches of serovar Canicola vaccine. The secondary objective of this study is to provide proof of principle of the feasibility for such an *in vitro* potency test using the potency biomarker(s) identified herein.

Chapter 2 Materials and Methods

2.1 Statistical Analysis

All results are presented as means and standard deviations of the mean; with the exception of proteomics data where proteins not common to all three replicates have been excluded from analysis. Comparison of the concentration and relative abundance of proteins between vaccines was performed using a Student's t-test; a P value of ≤ 0.05 was taken to be statistically significant. Comparison of the survival of hamster groups 1-6 was performed using Fisher's exact test; a P value of ≤ 0.05 was taken to be statistically significant. The limit of detection (LOD) was calculated (Armbruster and Pry, 2008) as $LOD = LOB + 1.645(SD_{\text{lowest concentration sample}})$, where $LOB = \text{mean}_{\text{blank}} + 1.645(SD_{\text{blank}})$. Whereas the limit of quantification (LOQ) was defined as the lowest concentration of the standard with a coefficient of variation lower than 20% (Armbruster and Pry, 2008).

2.2 Ethical approval

All animal procedures in this study were covered under the Animals (Scientific Procedures) Act 1986 by Home Office Project Licence No. PPL 70/7249 and were approved by the Animal Ethics Committee at the Animal Health and Veterinary Laboratories Agency (AHVLA) where all of this work was performed.

2.3 Bacterial Methodology

2.3.1 Bacterial strains

Leptospira interrogans serovar Canicola strain Hond Utrecht IV was obtained from the Animal Health and Veterinary Laboratories Agency (AHVLA; UK). *L. interrogans* serovar Canicola strain Kito was donated by the Pasteur Institute (France). *Escherichia coli* chemically competent cells, strain BL21(DE3) pLysS, were obtained from Promega (Southampton, UK).

2.3.2 Bacterial culture

Starter cultures of *L. interrogans* serovar Canicola (strains Hond Utrecht IV and Kito) were prepared by inoculation of Ellinghausen McCullough Johnson Harris (EMJH) media (20 ml; Becton Dickinson, USA) with 1 ml of pure culture (1 x 10^8 cell/ml) and incubated for 7 days at 30^o C with orbital agitation at 50 rpm. Larger working cultures (n=3) of strain Hond Utrecht IV for proteome extraction were initiated by inoculation of EMJH media (400 ml) with starter culture (10 ml) and incubated at 30^o C with orbital agitation at 50 rpm for 7 days.

The recombinant LipL32 *E. coli* strain (section 2.6.4) was grown on Luria-Bertani (LB) agar plates (35 μg/ml chloramphenicol and 50 μg/ml ampicillin) at 37^o C overnight to screen for transformant colonies. For expression of LipL32 protein the recombinant *E. coli* strain was grown to an optical density (OD_{600}) of 0.4 at 37^o C with orbital agitation at 225 rpm in LB broth (500 ml) containing 35 μg/ml chloramphenicol and 50 μg/ml ampicillin; isopropyl β-D-1-thiogalactopyranoside (IPTG) was added, to a final concentration of 2 mM to induce LipL32 expression and cultures were incubated for a further 4 hours before harvesting of bacterial cells (section 2.6.5).

2.3.3 Assessment of Bacterial Growth

The growth of *L. interrogans* serovar Canicola (strains Hond Utrecht IV and Kito) were assessed by counting cells with a dark field microscopy using a Thoma counting chamber (0.1 mm depth, 1/400 m^2). The growth of *E. coli* strains, for expression of recombinant LipL32, was assessed using a Spectronic Unicam Helios Gamma spectrophotometer (ThermoFisher, USA) at an OD of 600 nm.

2.3.4 Serotyping of Leptospira interrogans

L. interrogans serovar Canicola (strains Hond Utrecht IV and Kito) were serotyped using the MAT prior to use to confirm their serovars. Serovar Canicola specific antiserum (AHVLA, UK) was serially diluted (1/25 to 1/3200) in 0.9% (w/v) physiological saline to a final volume of 25 µl and added (in duplicate) to the wells of a flat bottomed 96 well plate (Nunc, UK). Aliquots (25 µl) of strains Hond Utrecht IV and Kito, grown to ~5 x 10^8 cells/ml, were then passed through a 0.8 µm filter syringe (to remove any bacterial aggregates that may have formed which might skew results) and added to the diluted antisera; known *L. interrogans* serovar Canicola and serovar Copenhageni strains were used as positive and negative controls respectively. The 96 well plates were incubated at 30^o C for 2 hours and wells were then assessed for agglutination using a dark field microscope at 40 times magnification.

2.3.5 Bacterial Protein Extraction for 2D-LC/MS^n Analysis

L. interrogans serovar Canicola (strain Hond Utrecht IV), cultured as previously described (section 2.3.2), was harvested during the logarithmic growth phase (~5 x 10^8 cells/ml). Cultures were cooled on ice for 30 minutes prior to centrifugation using a Sorvall RC 6 plus centrifuge (ThermoFisher, UK) at 4000 x g for 20 minutes at 4^o C. The bacterial cells were washed by suspension in chilled phosphate buffered saline (100

ml; PBS; 200 mM, pH 7.2) and centrifuged (4000 x g; 20 min, 4^{o} C). Bacterial cell pellets were suspended in chilled PBS (10 ml) containing PMSF (100 µM) and lysed by 6 second pulses of probe sonication (amplitude 60) using a Vibra-Cell ultrasonic processor (Sonics and Materials, USA) for 3 minutes on ice. Cell debris was removed by centrifugation at 3000 x g and the supernatant retained. A cytosolic extract was produced from the supernatant by centrifugation at 32000 x g for 30 minutes. The pellet was retained and the supernatant (cytosol extract) was diluted by the addition of 2.5 mM ammonium bicarbonate (2 ml; pH 8.0) and centrifuged in 5 kDa molecular weight cut off filters (MWCO; Sartorius Stedim, France) to desalt and concentrate to a final volume of 0.5 ml; this was stored at -20^{o} C.

The retained pellet was then washed by suspension in chilled PBS (200 mM, pH 7.2) and collected by centrifugation (32000 x g). The washed pellet was re-dissolved in lysis buffer (3 ml; Urea 5 M, Thiourea 2 M, DTT 100 mM, CHAPS 2% (w/v), 3-(Decyldimethylammonio) propanesulfonate inner salt 2% (w/v), Tris Base 0.48% (w/v)) and centrifuged at 32000 x g for 30 minutes. The protein extract was precipitated in a 4-fold excess of ice cold acetone and incubated at -20^{o} C for 48 hours prior to centrifugation (3000 x g for 30 minutes at 4^{o} C). The resulting pellet (precipitated extract) was desalted and concentrated as described above to remove excess acetone.

2.3.6 Standard Protein Assay

The protein concentration of all samples (unless otherwise stated) was determined using the Bradford method (Bradford, 1976). Vaccines A-E (before and after MWCO washing) and the bacterial extracts were determined by Bradford. Essentially samples were diluted 1/20 and 1/100, with 2.5 mM Ammonium bicarbonate (pH 8.0), in duplicate to a final volume of 1 ml; a range of protein calibration standards (1- 0.05

mg/ml) were also created in duplicate using bovine serum albumin (BSA; Sigma, UK) diluted with 2.5 mM Ammonium bicarbonate (pH 8.0). Aliquots (100 µl) of diluted sample and protein standards were added in duplicate to Bradford reagent (2 ml; Sigma, UK), briefly vortexed and left to incubate in the dark at room temperature for 30 minutes. Protein concentration was assessed using a Spectronic Unicam Helios Gamma spectrophotometer (ThermoFisher, USA) at an optical density (OD) of 600 nm. Calibration curves for the BSA standards were constructed using Graphpad Prism 4 software (Graphpad, USA) over the tested concentration range (1- 0.05 mg/ml) allowing determination of sample concentration by interpolation.

2.3.7 Low concentration Protein Assay

Due to the low sample volume obtained from recombinant cells the protein content of purified LipL32 protein (section 2.6.5) was determined using a Nanodrop ND-1000 (Thermo Scientific, UK).

2.4 Vaccine Methodology

2.4.1 Vaccines

Bivalent vaccines (giving protection against serovars Canicola and Icterohaemorrhagiae) which had passed the *in vivo* vaccine batch potency test and had been released for commercial sale were purchased from six different manufacturers (designated A-F) for analysis. Vaccine C was of subunit manufacture comprising outer membrane proteins whereas vaccines A, B, D, E and F were derived from heat inactivated bacteria. Untested batches of vaccine C (n=5) were obtained from the manufacturer and assessed during this study for their protective effect using the vaccine

batch potency test; untested batches of vaccines A, B, D, E and F were unavailable for testing.

2.4.2 Vaccine batch potency testing

Untested batches (n=5) of vaccine C were assessed for potency according to the guidelines laid out in the most recent European monograph (Marbehant, 1999). For each batch analysed five female hamsters (≤120 g; Charles River, Germany) were inoculated subcutaneously with 0.5 ml of vaccine (diluted 1/40 with 0.9% w/v physiological saline), five unvaccinated hamsters were kept as control animals. Fifteen days following vaccination all the hamsters were challenged by intraperitoneal inoculation with 1 ml of virulent *Leptospira interrogans* serovar Canicola (~1 x 10^8 cells/ml) strain Kito. For the test to be valid at least four of the five unvaccinated controls had to succumb to infection or be euthanased (according to a clinical score sheet, Table 6) within 14 days of infection. Vaccine batches were deemed potent if at least four of the five vaccinated hamsters survived for 14 days longer than the unvaccinated controls. Hamsters were routinely monitored and their condition assessed using a clinical score sheet (Table 6) developed at AHVLA. Hamsters with a score of 3 or higher were humanely euthanased using halothane; all surviving hamsters at the end of the test were also humanely euthanased.

Table 6: Clinical Score sheet used to assess condition of hamsters

Clinical Signs	Score	Action
Normal behaviour	0	None required
Arched back with slightly rough coat	1	Observe again in 2 hours.
Dull sunken eyes Moderately rough coat Subdued but will respond when stimulated	2	Observe again in 2 hours. If symptoms at the end of the day, consult the Named Veterinary Surgeon (and study director if possible)
Unstable on feet Subdued, will not respond when stimulated Nasal bleeding Blood in urine	3	Euthanise (= killed in extremis)
Prostration Permanently closed eyes	4	Euthanise (= killed moribund)
Found dead	5	None required

2.4.3 Quantitation of LPS

Limulus amebocyte lysate (LAL) assay was performed using an Endochrome K kit (Charles River, UK) according to the manufacturer's instructions. The concentration of LPS in unknown samples was determined by interpolation, using Graphpad Prism 4 software (Graphpad, USA), against a calibration curve (0.33-3333 ng/ml) prepared from LPS (*E. coli* derived; Charles River, UK).

2.4.4 Protein and LPS Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on X cell surelock system (Invitrogen, USA) using precast 4-12% gradient NuPAGE gels (Invitrogen, USA) run at 150 W for 80 minutes. For assessment of the protein and LPS content present in the vaccines, gels contained a 3.5- 260 kDa protein ladder (Invitrogen, USA), two concentrations of *E. coli* derived LPS (10 µg and 500 ng; Sigma, UK) and aliquots of untreated vaccine (A-E). Protein was visualised in gels by staining with EZ run Coomassie (Thermo Fisher, USA) used according to the manufacturer's instructions. LPS was detected in the gels using the silver staining method described by Tsai (Tsai and Frasch, 1982). For validation that the silver stain method was specific to LPS, vaccines A-E (72 µl) were also digested with 16 µg proteinase K (to digest the protein content; resuspended in 500 mM Tris HCl, 50 mM $CaCl_2$, pH 7.5) at 37^{o} C overnight. The vaccine digests A-E were equally divided (2 x 36 µl), one aliquot was loaded onto a gel stained with the silver stain and the other aliquot was loaded onto a gel stained with EZ run Coomassie to confirm that complete digestion had taken place.

2.4.5 Vaccine preparation for 2D-LC/MS^n Analysis

Vaccines A, C, D and E (1 ml) were concentrated to 0.5 ml using 5 kDa molecular weight cut off filters (MWCO; Sartorius Stedim, France), washed once with 2.5 mM ammonium bicarbonate (2 ml; pH 8.0) and concentrated again to a final volume of 0.5 ml. Due to a low initial protein content the concentration of vaccine B could not be accurately determined after washing, therefore unprocessed unwashed vaccine was subjected to direct trypsin digestion and subsequent 2D-LC/MS^n analysis.

2.5 Proteomic Analysis of Bacteria and Vaccines

2.5.1 Trypsin digestion of proteins

Three replicates of each vaccine and bacterial extract, normalised by dilution in 2.5 mM ammonium bicarbonate (pH 8.0) to 100 µg, were heat denatured at 95^o C for 5 minutes and then digested overnight with 2 µg sequencing grade trypsin (Promega, Southampton) (Coldham and Woodward, 2004). Digestion was terminated by the addition of 25.2 M formic acid (1 µl; Fluka, USA). Due to the low protein content of vaccine B, 6 µg of protein was used for each replicate.

2.5.2 Proteome analysis by 2D-LC/MS^n

Tryptic digests were centrifuged (5000 x g for 1 minute) to remove particulates and 50 µl aliquots of supernatant was subjected to strong cation exchange (SCX) fractionation by high pressure liquid chromatography (HPLC). Tryptic peptides were fractionated on a Biobasic SCX HPLC (2.1 x 100 mm) column (Thermo Scientific, UK) using a Hewlett-Packard 1100 HPLC system at a flow rate of 0.25 ml/min. Mobile phases used were 75:25 2.5 mM ammonium acetate: acetonitrile pH 4.5 (A) and 75:25 250 mM ammonium acetate: acetonitrile pH 4.5 (B) with a binary gradient (t = 0 min, A 100%; t

= 5 min, A 100%; t = 18 min, 65% A; t = 20 min, B 100%; t = 22 min, A 100%; t = 32 min, A 100%). Eluted peptides were monitored at 280 nm and 15 fractions (0.25 ml) were collected between 8 and 23 min. The SCX fractions were taken to dryness at 60^{o} C under vacuum using an Eppendorf 5301 centrifugal concentrator (Eppendorf, UK).

Dried SCX fractions were resuspended in 0.1% v/v formic acid (20 µl) and analyzed on an Agilent 6520 Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Agilent Technologies, UK) with an HPLC chip cube source. The chip consisted of a 40 nl enrichment column (Zorbax 300 SB- C18; 5 µm) and a 75 µm x 150 mm analytical column (Zorbax 300 SB- C18; 5 µm) driven by the Agilent Technologies 1200 series nano/capillary HPLC system. Both pumps (nano and capillary) were controlled by Masshunter Workstation Data Acquisition for Q-TOF (Version B.02.00, Patches 1, 2; Agilent Technologies). Tryptic peptides (1 µl injection volume) were loaded onto the enrichment column of the chip and washed with eight column volumes of 0.1% v/v Trifluoroacetic acid (TFA). Tryptic peptides were separated on the analytical column and eluted directly into the mass spectrometer. Mobile phases used were 0.1% v/v TFA (A) and 90:10 acetonitrile: 0.1% v/v FA (B) with a binary gradient (t = 0 min, A 95%; t = 5 min, A 95%; t = 40 min, A 60%; t = 41 min, A 20%; t = 45 min, A 20%; t = 47 min, A 95%) at a flow rate of 0.6 µl/min. The mass spectrometer was run in positive ion mode, and MS survey scans were run over a range of m/z 250 to 3000 and at five spectra per second. Precursor ions were selected for auto MS/MS at an absolute intensity threshold of 2000 and a relative threshold of 0.01, with a maximum of 5 precursors per cycle, and active exclusion set at 1 spectra and released after 3 minutes. Precursor charge state selection and preference were set to 2+ and then 3+. The mass spectrometer was calibrated to within a residual error of 2 ppm prior to each batch using ES-TOF Tuning Mix (Agilent, UK). A synthetic peptide mix (10 ng/ml; MRFA, AVDQLNEQSSEPNIYR, VTALYEGFTVQNEANK, ARPQELPFLASIQNQGR,

ISVNNVLPVDFNLMQQK and NYINQYSEVAIQMVMHMQPK) was analysed prior to each batch (defined as 15 SCX fractions) for quality assurance of both chromatography and mass spectrometry. The m/z values of the tuning mix calibrants were added to an exclusion list to ensure only sample derived peptides were subsequently analysed in full MS/MS mode. Two blank samples (comprising of 0.1% formic acid and 95:5% acetonitrile: H_2O) were analysed at the end of each batch to normalise and flush the column and system prior to subsequent analytical injections.

Initial data for vaccine proteome analysis showed high albumin concentration, therefore the most abundant albumin precursor ions were excluded from further analysis by creating a specific precursor ion exclusion list method (ions specified were not analysed).

2.5.3 Proteome analysis by 1D PAGE-LC/MS^n

In-gel trypsin digestion of vaccine samples was carried out according to the method described by Weeks (2010); LC/MS^n mass analysis was performed as previously described (section 2.5.2) using an Agilent 6520 Q-TOF. Mobile phases used were 0.1% v/v TFA (A) and 90:10 acetonitrile: 0.1% v/v FA (B) with a binary gradient (t = 0 min, A 96%; t = 15 min, A 50%; t = 16.5 min, A 10%; t = 18.5 min, A 95%; t = 21 min, A 95%) at a flow rate of 0.6 µl/min.

2.5.4 Protein identification

The search engine Spectrum Mill (Agilent, UK) was used to extract MS/MS data from Masshunter acquisition files and proteins were subsequently identified by comparison of tryptic peptide product ion mass spectra against those generated *in silico* from a protein database. Search parameters included selection of trypsin as the proteolytic

enzyme with up to two missed cleavage sites and a variable modification for oxidation of methionine residues; precursor and product mass tolerances were set to 20 and 50 ppm respectively. Identified protein lists (and associated information) with a Spectrum Mill protein score higher than 11 were exported as tab separated files for bioinformatic analysis; protein identifications were accepted if at least two distinct different tryptic peptides were present in all three technical replicates. To date *Leptospira interrogans* serovar Canicola has not been fully genome sequenced therefore a custom made database derived from chromosomes I and II of *L. interrogans* serovar Copenhageni (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/Leptospira_interrogans_serovar_Copenhageni accessed on 12/6/10) was used for protein identification. To determine optimal database identifications vaccine D was also interrogated against the NCBI non-redundant database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz accessed on 12/6/10), and the Uniprot database (ftp://ftp.uniprot.org/pub/databases/uniprot/knowledgebase/uniprot_sprot.fasta.gz accessed on 12/6/10) using Spectrum Mill (Agilent, UK). To determine the optimal search engine vaccine D was interrogated against the custom *L. interrogans* serovar Copenhageni database using the open mass spectrometry search algorithm (OMSSA; NCBI) and Mascot (Matrix Science). The false discovery rate (FDR) (Elias et al., 2005), defined as the percentage of false positive identifications, was calculated by searching the three replicates of vaccine D against a reverse decoy database, created from the *L. interrogans* serovar Copenhageni database using the Perl script decoy.pl available from (http://www.matrixscience.com/help/decoy_help.html accessed on 5/8/10).

Access software (Microsoft Office 2003, USA) was used to identify proteins common to all technical repeats (n=3) for each sample, these common proteins are referred herein as a samples proteome; proteins not present in all three technical replicates were

discarded from further analysis. Conserved proteins in the precipitated and cytosol bacterial extracts were reassembled into a *L. interrogans* serovar Canicola proteome using Access (Microsoft Office 2003, USA). Comparison of the proteomes for vaccines A-E and serovar Canicola was also performed using Access (Microsoft Office 2003, USA).

The relative abundance of proteins present in samples (in all three technical repeats) was determined through spectral counting (Zybailov et al., 2005). The normalised spectral abundance factor (NSAF; Figure 3) was utilised to account for differences in protein length, allowing comparison of protein abundance between samples. This requires (Figure 3) that for each protein (k), the number of detected spectra for that protein (SpC) be divided by that proteins predicted length in amino acids (Length); this figure is then further divided by the sum of SpC/L for all the detected proteins (N). For expediency the NSAF was calculated using a novel program, written in R, developed at AHVLA.

$$(\mathrm{NSAF})k = \frac{(\mathrm{SpC}/\mathrm{Length})k}{\sum_{i=1}^{N} (\mathrm{SpC}/\mathrm{Length})i}$$

Figure 3: Equation used to determine the NSAF for a protein (k) from (Zybailov et al., 2007). Where SpC is the number of detected spectra that correspond to the protein and length is the number of predicted amino acids in the protein (translated from its gene sequence).

2.5.5 Quantification of LipL32 using LC-MRM

LipL32 was analysed by LC-MRM analysis with quantitation against synthetic peptides, corresponding to N and C terminal tryptic peptides found in this protein. LipL32 synthetic peptides were obtained at a purity of 98% (Peptides Synthetics, UK) and used

to make a range of calibration standards (0.01-100 fmol/µl). Vaccines A-E were prepared, quantified, and digested as described previously (sections 2.4.5). Recombinant LipL32 protein (0.5 µg; section 2.6.5) was digested with trypsin (in triplicate); half of this digest (0.25 µg of total protein) was subjected to SCX separation. SCX separation was performed as described previously (section 2.5.2), to clean up the sample, however the 15 fractions of each replicate were recombined prior to being taken to dryness, dissolved in 0.1% v/v formic acid (50 µl) and analyzed on an Agilent 6410 triple quadrupole mass spectrometer (Agilent, UK) with an HPLC chip cube source. The chromatography chip consisted of a 160-nl enrichment column (Zorbax 300 SB-C18; 5 µm) and a 75µm x 150 mm analytical column (Zorbax 300 SB- C18; 5 µm) driven by the Agilent Technologies 1200 series nano/capillary liquid chromatography system. Both pumps (nano and capillary) were controlled by Masshunter Workstation Data Acquisition for Triple Quadrupole (Version B.02.01; Agilent Technologies). Tryptic peptides (1 µl injection volume) were loaded onto the enrichment column of the chip and washed with eight column volumes of 0.1% v/v TFA. Peptides were then separated on the analytical column and eluted directly into the mass spectrometer. Mobile phases used were 0.1% v/v TFA (A) and 90:10 acetonitrile: 0.1% v/v FA (B) with a binary gradient (t = 0 min, A 95%; t = 1 min, A 95%; t = 16 min, A 60%; t = 20 min, A 20%; t = 21 min, A 0%; t = 24 min, A 0%; t = 25 min, A 95%) at a flow rate of 0.6 µl/min. The mass spectrometer was run in positive ion mode, with the electrospray voltage set to 1900 V and gas temperature at 300^{o} C. Optimal transitions and conditions for the peptides of interest were obtained using the MS and MS/MS data from previous Q-ToF analysis of vaccine C. The acquired data was quantified using the calibration standards (0.01-100 fmol/µl) with Agilent Masshunter Quantitative Analysis software (Version B.03.01; Agilent Technologies).

2.5.6 Functional annotation of *Leptospira* Proteomes

Functional annotation of proteins present in the proteomes of serovars Canicola, Copenhageni and Pomona was determined using the Protein Information Resource (PIR; http://pir.georgetown.edu accessed on 30/01/12).

2.6 Cloning and expression of LipL32

2.6.1 DNA extraction

Genomic DNA from *L. interrogans* serovar Canicola strain Kito was extracted for sequencing (section 2.6.3) using the modified cetyltrimethylammonium bromide (CTAB) method described by Ausubel (Ausubel, 1994). Plasmid DNA from recombinant *E. coli* strains (section 2.6.4) was extracted using a QIAGEN plasmid mini kit as per the manufacturer's instructions (Qiagen, UK). Estimation of DNA concentration of all samples was performed using a Nanodrop ND-1000 (Thermo Scientific, UK) at 260 nm.

2.6.2 DNA Gel Electrophoresis

Agarose gel electrophoresis was performed for the analysis of PCR products, restrictions digestions and to check DNA integrity/size using a 1% w/v TAE agarose gel at 70 V for 90 minutes using a 1 Kb ladder (Promega, UK) and 6 x loading dye (Promega, UK). Gels were soaked in 0.5 µg/ml ethidium bromide for 30 minutes, destained in water for 30 minutes, and then visualised under UV radiation using Gene Genius (Syngene, UK).

2.6.3 Sequencing

Genomic DNA (15 ng) from *L. interrogans* strain Kito was amplified using polymerase chain reaction (PCR) with primers 1 and 2 (20 pmol; Sigma, UK; Table 7); which annealed approximately 1305.8 kilobase pairs upstream and downstream of the LipL32 gene respectively to give a 2612.4 kilobase fragment which included the LipL32 gene. PCR was performed in a 50 µl total reaction volume containing 10 x buffer (5 µl; Clonetech, USA), 100 x deoxyribonucleotides (0.5 µl; dNTPs; Promega, UK) and taq polymerase (1 µl; Clonetech, USA). After an initial denaturation at 95° C for 2 minutes, the DNA was amplified by 25 cycles of 95° C for 30 seconds, 55° C for 30 seconds, and 68° C for 1 minute, with a final elongation at 68° C for 10 minutes on the GeneAmp PCR system 9700 (Applied Biosystems, UK). Amplified DNA was sequenced by the AHVLA sequencing facility (AHVLA, UK) using primers 3 and 4 (Table 7; Sigma, UK) which annealed to the LipL32 gene at base pairs 148 and 62 respectively (5'-3' orientation) and primers 5 and 6 (Table 7; Sigma, UK) which annealed to the LipL32 gene at base pairs 670 and 740 (5'-3' orientation) respectively. Resulting sequence files were amalgamated into a consensus sequence for LipL32 using Lasergene (DNASTAR, USA). The LipL32 gene sequence for strain Kito (determined in this study) was compared, using Lasergene (DNASTAR, USA), against the published complete gene sequences (from http://www.ncbi.nlm.nih.gov accessed on 12/01/12) of LipL32 from strains Hond Utrecht IV (GI: 33589193), RTCC 2805 (GI: 358357257) and Lin (GI: 48526297).

Table 7: List of primers used during this study to detect LipL32

Primer	Sequence*
1	GGAAACTACCGCAAAGTC
2	CACCACCGGACTCTAAAA
3	GTTGATCACAGATCCGTA
4	TTAGGCTTGGCAGACCAC
5	GTTGCATCTGTTGGTCTG
6	TGATCCACTCAAATCCTG
7	GGGGTACCGTGCTTTCGGTGGTCTGC
8	CGGAATTCTTACTTAGTCGCGTCAGAAG
9	CAGATCCGGATATAGTTC

*** All primers listed are in the 5'-3'orientation. All primers obtained from Sigma (UK); restriction endonuclease sites for KpnI and EcoRI underlined in primers 7 and 8 respectively.**

2.6.4 Cloning

Cloning of the LipL32 gene into an expression vector, downstream from a Polyhistidine tag, was performed using a method modified from Haake *et al* (2000). Whereby *L. Kirschneri* genomic DNA was substituted with *L. interrogans* serovar Canicola genomic DNA, which also necessitated that the restriction enzymes XhoI and SmaI be substituted with KpnI and EcoRI (and associated primers changed accordingly); in addition the expression strain BLR(DE3)/pLysS was substituted for BL21(DE3)/pLysS as it was more readily available. PCR was used to amplify the portion of the LipL32 gene encoding the mature protein beginning with the first residue after the amino terminal cysteine. The forward primer, primer 7 (20 pmol; Table 7), contained a nucleotide sequence coding for the amino acids following the amino terminal cysteine of mature LipL32, including a KpnI restriction endonuclease site (underlined; Table 7). The reverse primer, primer 8 (20 pmol; Table 7) contained the nucleotide sequence coding for the carboxy-terminal amino acids and the LipL32 stop codon, including an EcoRI restriction endonuclease site (underlined). PCR was performed in a 50 µl total reaction volume containing 10 x buffer (5 µl; Clonetech, USA), 100 x deoxyribonucleotides (0.5 µl; dNTPs; Promega, UK) and Taq polymerase (1 µl;

Clonetech, USA); genomic DNA (15 ng) from *L. interrogans* strain Kito was used as template. After an initial denaturation at 95° C for 2 minutes, the DNA was amplified by 25 cycles of 95° C for 30 seconds, 53° C for 30 seconds, and 68° C for 1 minute, with a final elongation at 68° C for 10 minutes on the GeneAmp PCR system 9700 (Applied Biosystems, UK). Assessment of PCR product was performed using gel electrophoresis (section 2.6.2), product was cleaned using a QIAquick PCR Purification Kit (Qiagen, USA) as per the manufacturer's instructions and DNA concentration estimated using a Nanodrop (section 2.6.1).

The amplified *lipL32* gene (15.8 µl of cleaned PCR product) and the pRSET C (Invitrogen, UK) expression vector (1.5 µg of DNA) were digested with Kpn1 (Promega, UK), as per manufacturer's instructions. Reaction mixtures were cleaned using a QIAquick PCR Purification Kit (Qiagen, USA) and digested again with EcoRI (Promega, UK), as per manufacturer's instructions. Reaction mixtures were then cleaned again using a QIAquick PCR Purification Kit (Qiagen, USA) and quantified using a Nanodrop (section 2.6.1). Finally the double digested insert (*lipL32* gene) and vector (pRSET C) were ligated together using T4 DNA ligase (Promega, UK) as per manufacturer's instructions. The ligation mixture was then transformed into chemically competent *E. coli* BL21 (DE3) pLysS cells, as per manufacturer's instructions, and transformants were selected as described previously (section 2.3.2).

PCR was used to confirm the presence and orientation of the *lipL32* insert within the pRSET C vector. Plasmid DNA from the recombinant *E. coli* strain (section 2.6.1) was used as template DNA (1 ng) with primers 7 and 9 (20 pmol; Sigma, UK; Table 7) which annealed to the 5' end of LipL32 and pRSET C vector respectively (shown in figure 21). PCR was performed in a 20 µl total reaction volume containing Hotstart

master mix (10 µl; Promega, UK). After an initial denaturation at 96° C for 15 minutes, the DNA was amplified by 25 cycles of 95° C for 30 seconds, 52° C for 30 seconds, and 72° C for 1 minute, with a final elongation at 72° C for 10 minutes on the GeneAmp PCR system 9700 (Applied Biosystems, UK). PCR product was run on a 1% w/v agarose gel for confirmation of size (section 2.6.2).

Plasmid diagram of the LipL32-pRSET C construct (Figure 21) was generated using SECentral (Sci-Ed, USA).

2.6.5 Expression and purification of LipL32

For purification of the LipL32 protein; recombinant *E. coli* was cultured (500 ml) and induced with IPTG (section 2.3.2). Cells were centrifuged (6000 x g) for 15 minutes at 4^{o} C and resuspended in lysis buffer (10 ml; 100 mM sodium phosphate, 6 M guanidine hydrochloride, pH 8). Cells were lysed by 6 second pulses of probe sonication (amplitude 60) using a Vibra-Cell ultrasonic processor (Sonics and Materials, USA) for 6 minutes on ice and centrifuged (4000 x g) for 10 minutes at 4^{o} C to remove cellular debris. The Polyhistidine tagged LipL32 protein was then purified from the retained supernatant using PureProteome Nickel Magnetic Beads (Millipore, UK), a wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, pH 8) and an elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 300 mM imidazole, pH 8) according to the manufacturer's instructions. Eluted protein was washed once with phosphate buffered saline (2 ml; 0.1 M PBS, pH 7.2) using a 5 kDa MWCO filter (Sartorius Stedim, France) and resuspended to a final volume of 200 µl in PBS.

2.7 Hamster Model Methodology

2.7.1 Preparation of protein samples and controls

Test samples 1-4 (described below) were prepared for assessment of their protective immunogenicity in hamsters; five aliquots of each were prepared. Test sample 1 comprised purified LipL32 protein (380 fmol; quantitation against the N terminal of LipL32), from the recombinant *E. coli* strain (section 2.6.5), resuspended in 0.9% (w/v) physiological saline to a final volume of 0.5 ml. Test sample 2 comprised purified LipL32 protein (380 fmol of N terminal LipL32), from the recombinant *E. coli* strain (section 2.6.5), resuspended in 0.9% (w/v) physiological saline to a final volume of 0.25 ml and mixed with Imject Alum adjuvant (0.25 ml; Thermo Scientific, USA). Test sample 3 comprised vaccine F diluted 1/40 with 0.9% (w/v) physiological saline to a final volume of 0.5 ml as a positive control. Test sample 4 comprised 0.5 ml 0.9% (w/v) physiological saline as a negative control.

2.7.2 Immunisation of hamsters with test products

Test samples 1-4 (section 2.7.1) were analysed in four separate treatment groups, each comprising five hamsters; a fifth untreated group, comprising 5 hamsters was used as a control. The hamsters in treatment groups 1-4 were inoculated subcutaneously (as previously described in section 2.4.2) with the relevant test sample (0.5 ml; section 2.7.1); control group 5 did not receive an inoculation. Treatment groups 1-5 were then challenged with virulent *L. interrogans* serovar Canicola (strain Kito) as previously described (section 2.4.2). A sixth group, comprising an additional three hamsters which were not vaccinated or challenged, were also used as a healthy control for histological comparison. All surviving animals in groups 1-6 were euthanised (section 2.4.2) on day 24, which was 14 days after the fourth hamster in the negative control (group 5) died.

2.7.3 Culture of *Leptospira* from hamster kidneys

Kidneys were excised from all hamsters at post mortem and dissected for assessment of infection. Half were retained for histological processing (section 2.7.4) and half were disrupted with a 10 ml syringe and cultured in EMJH media (as in section 2.3.1); presence of *Leptospira* was assessed by eye using a dark field microscope.

2.7.4 Histology

Samples from liver, spleen and kidney were collected from all hamsters at post mortem and fixed in 10% buffered formalin. Subsequent histological processing and analysis were performed by a veterinary pathologist (AHVLA, UK). Tissue samples were routinely processed and embedded in paraffin wax using a Hypercentre XP tissue processor (Thermo Shandon, UK). Consecutive 4 micron thick sections were cut using a Leica RM2025 (Leica, Germany) rotary microtome. Sections were stained with haematoxylin and eosin, for histopathological examination, and Warthin-Starry silver impregnation, for the visualization of leptospires in the tissues (Bancroft and Stevens, 1996). Renal lesions are indicative of infection and if present were graded according to their severity (0 as normal, 1 as minimal, 2 as mild, 3 as moderate and 4 as severe). The number of leptospires present on the various tissues were also graded with 0 as absent, 1 as rare, 2 as few, 3 as numerous and 4 as profuse. Slides were examined in a Leica DM4000B microscope (Leica, Germany). Pictures were taken using a Leica DFC480 digital camera (Leica, Germany) and Leica Application Suite software (Leica, Germany); Adobe Photoshop Elements 4.0 (Adobe, USA) was used to adapt images for publication.

2.7.5 Enzyme-linked immunosorbent assay

Total blood content of hamsters was extracted at post mortem (section 2.7.2) using cardiac puncture; serum was prepared from blood and stored at -20^{o} C. Assessment of circulating antibodies in the serum was performed using enzyme-linked immunosorbent assays (ELISA). Two antigens were chosen for assessment, purified LipL32 (section 2.6.5) and whole cell *L. interrogans* serovar Canicola (strain Kito). LipL32 antigen was prepared by diluting purified LipL32 protein (section 2.6.5) in antigen coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6; Sigma, UK) to a final concentration of 1 µg/ml (total protein). Whole cell antigen was prepared by culturing (section 2.3.2) *L. interrogans* serovar Canicola (20 ml, strain Kito; ~5 x 10^{8} cells/ml) and collection of cells by centrifugation at 4000 x g for 20 minutes at 4^{o} C. Cells were resuspended in 0.1 M PBS (100 µl; pH 7.2), heat denatured at 95^{o} C for 15 minutes, and diluted in antigen coating buffer to a final concentration of 5 µg/ml (total protein). Diluted antigens (100 µl) were added to the wells of a microtitre plate (Polysorb; Nunc, USA) and incubated overnight at room temperature. Plates were washed three times for 1 minute with wash buffer (400 µl; 0.1 M PBS pH 7.2, 0.05 % (v/v) Tween-20) and blocked (blocking buffer 200 µl; 3 % [w/v] dried skimmed milk in washing buffer) by incubation for 30 minutes at 37^{o} C. Plates were then washed three more times. Hamster serum was serially diluted with buffer (1 % (w/v) dried skimmed milk in washing buffer) from 1/100 to 1/12800; each dilution (200 µl) was added to the microtitre wells in duplicate (serum was added to both antigens). A polyclonal LipL32 antibody from Dr Jarlath Nally (UCD, Dublin) was used at a 1/1000 dilution as a positive control for the antigen; negative controls including serum with no antigen and empty wells were also used. Plates were incubated at 37^{o} C for 1 hour with orbital agitation (50 rpm) prior to washing (x3). The protein A/G (ThermoFisher, USA; conjugated to the enzyme horseradish peroxidase) was diluted 1/50000 in diluting buffer, and added (200 µl) to

the wells of the microtitre plate prior to incubation at 37° C for 30 minutes with orbital agitation (50 rpm). Plates were washed (x3) prior to the addition of Tetramethylbenzidine (100 µl; TMB) substrate (Sigma, UK); plates were incubated at room temperature in the dark for 10 minutes and the reaction stopped by the addition of 10 % (v/v) H_2SO_4 (50 µl) to all wells. Plates were read in a MRX Revelation (Dynex Technologies, USA) spectrophotometer at a wavelength of 450 nm. A diagram showing the ELISA protocol used is shown in Figure 4.

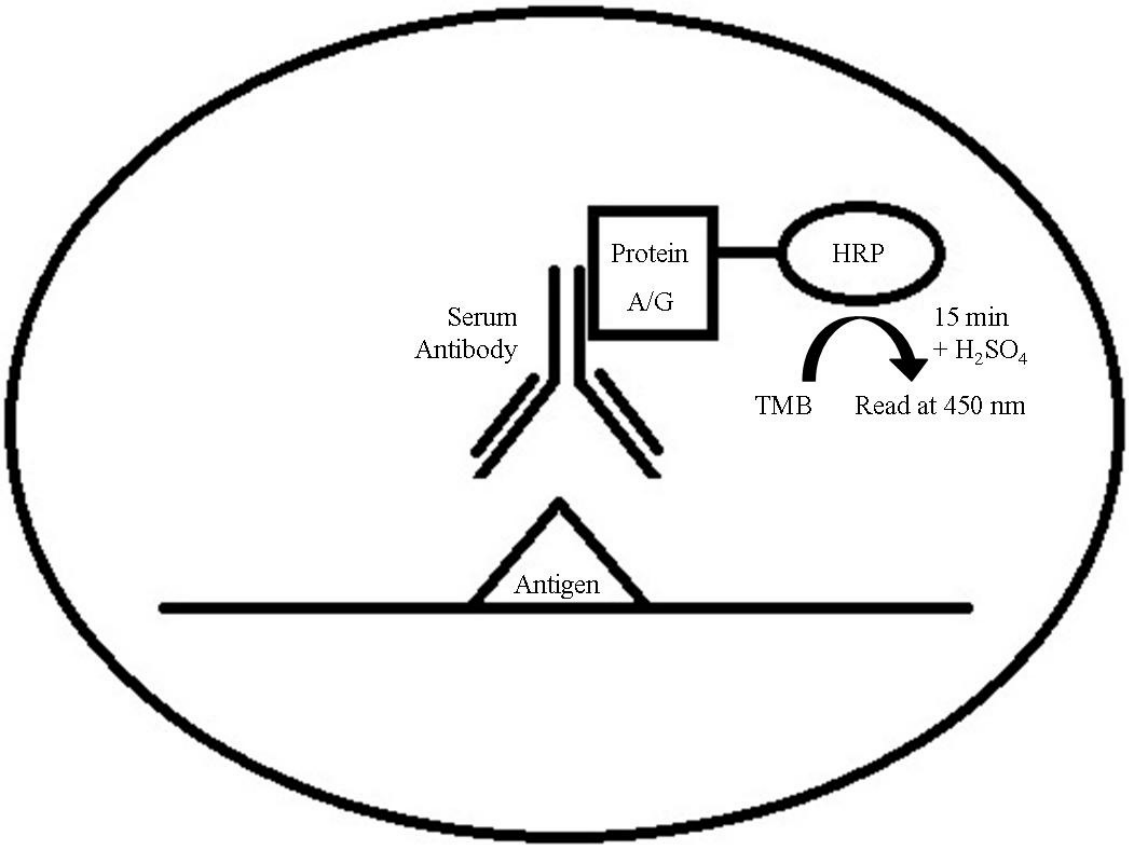

Figure 4: ELISA protocol used for the assessment of circulating antibodies in serum

Chapter 3 Analysis of *Leptospira* vaccines

3.1 Introduction

Vaccination is widely used to protect against *Leptospira* infection in animals; however the majority of vaccines currently available are serovar specific (Koizumi and Watanabe, 2005) and require regular boosters to maintain immunity (Klaasen et al., 2003). Bi and multivalent vaccines have been developed by blending multiple heat killed serovars of *Leptospira* together during vaccine formulation; however the protective effects of such vaccines are limited to the serovars from which they are comprised (Koizumi and Watanabe, 2005). In the past the epidemiology of *Leptospira* was such that particular serovars had a higher prevalence in certain species, for example serovar Canicola infecting dogs, making it easier to choose which vaccines to administer. However it has been observed that these trends can change (Brown et al., 1996) and with over 230 known pathogenic serovars in circulation (Adler et al., 2011) the question arises as to the extent of protection in vaccinated animals.

ELISA based potency assays (section 1.4) for some *Leptospira* serovars (Ruby et al., 1992, Goddard et al., 1986) have been developed; however the majority of *Leptospira* vaccines, particularly those providing protection against serovars Canicola and Icterohaemorrhagiae, have to be tested in a hamster model prior to being released onto the market. Aside from the ethical and financial implications of testing in this way it is not uncommon for batches of vaccine to fail resulting in higher costs for the manufacturer and therefore higher prices for the consumer. The biggest problem for the industry is that the mechanism of action for these vaccines is poorly defined, hindering attempts at new vaccine development and accurate quantification of efficacy.

The majority of *Leptospira* vaccine research in the literature (Wang et al., 2007) concentrates on identification of new vaccine targets from the causative organism. The proteome and genome of *Leptospira interrogans* have been determined (Sakolvaree et al., 2007, Malmstrom et al., 2009, Nascimento et al., 2004a) and a number of potential vaccine candidates have been identified (section 1.2.3). A recent publication has also identified lipopolysaccharide (LPS) as a good vaccine candidate and suggests that a LPS mutant may be able to provide cross protection against heterologous *Leptospira* serovar challenge (Srikram et al., 2011).

This approach to vaccine development can be affective; however it is also time consuming, costly and to date has failed to produce a longer lasting, cross serovar protective *Leptospira* vaccine. A more effective approach would be to investigate how existing *Leptospira* vaccines function. A number of previous proteomic studies have investigated how vaccines provide immunological protection (Ceccarini et al., 2000, Vaughan et al., 2006); however, comparative analysis of the protein content of multiple *Leptospira* vaccines has not been previously reported.

This chapter focuses on analysing the LPS and protein components of five *L. interrogans* serovar Canicola vaccines, the primary objective being to identify common elements that may be used for the development of an *in vitro* potency test. In addition, a definitive proteomic analysis of vaccine protein content may offer insights into how existing vaccines work and how they could be made more efficacious.

3.2 Results

3.2.1 Evaluation of vaccine LPS and protein content

Five vaccines (A-E) which had passed the *in vivo* vaccine batch potency test and been released for commercial sale, were purchased from different manufacturers for analysis (Table 8; *in vivo* potency data not available). The protein content of vaccines A-E was determined using the Bradford assay (Figure 5); the limit of detection for the Bradford assay was calculated (section 2.1) as 0.01 mg/ml, whereas the limit of quantification was calculated (section 2.1) as 0.05 mg/ml. The protein content of vaccine B was very low (0.07 ± 0.01 mg/ml; mean ± 1 SD) making 2D-LC/MS analysis difficult as only 6 µg of protein could be tryptically digested and analysed for each replicate, compared to the 100 µg of protein used for the other vaccines. Gel electrophoresis (Figure 6) of vaccines A-E, with Coomassie staining for protein, was consistent with the data from the Bradford assay confirming that vaccine B had a lower protein content than the other vaccines analysed; the maximum well volume (36 µl) was loaded for vaccine B which equates to 2.5 µg. Gel spots were cut out and analysed on the Q-ToF (section 2.5.3); proteins detected in the visible bands (Figure 6) were all identified as albumin (Appendix 1), which is likely to be left over from the growth media used to culture *Leptospira* (section 1.3.5). A complete table detailing all the proteins identified in the vaccine gel spots is listed in Appendix 1.

Table 8: Composition and route of administration for five vaccines (A-E) derived from the National Office of Animal Health (NOAH) Compendium (Accessed on 17/11/11).

Vaccine*	Formulation	Adjuvant	Preservative	Immunises against Serovars	Route(s) of administration
A	Inactivated Bacteria	Not Known	Thiomersal	Canicola & Icterohaemorrhagiae	Subcutaneous
B	Inactivated Bacteria	Aluminium Hydroxide	Not Known	Canicola & Icterohaemorrhagiae	Subcutaneous or Intramuscular
C	Outer Membrane Coat Protein	Not Known	Not Known	Canicola & Icterohaemorrhagiae	Subcutaneous
D	Inactivated Bacteria	Not Known	Not Known	Canicola & Icterohaemorrhagiae	Subcutaneous
E	Inactivated Bacteria	Not Known	Thiomersal	Canicola & Icterohaemorrhagiae	Subcutaneous

***Vaccine and manufacturer names excluded for confidentiality reasons. Note all vaccines listed are for use in dogs.**

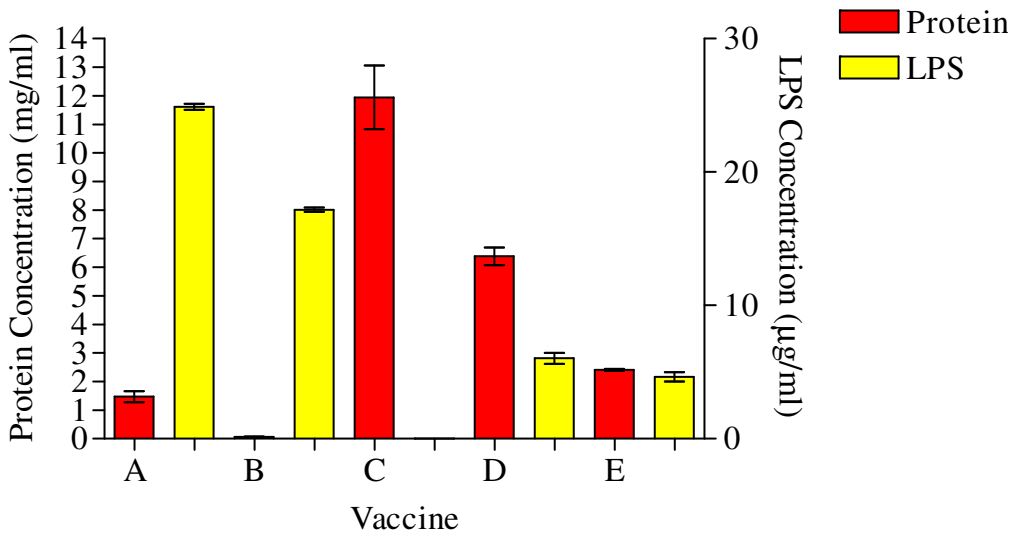

Figure 5: Concentration of protein and LPS in vaccines A-E (as sold) as determined by the Bradford and LAL assays respectively.

Note: Two replicates of each vaccine were analysed for each assay; mean and standard error of the mean are shown.

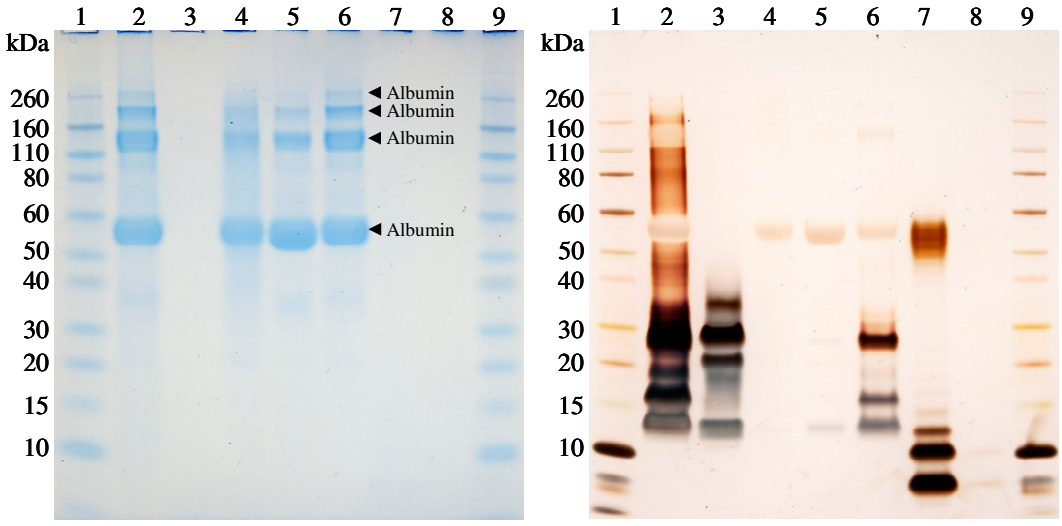

Figure 6: Vaccines A-E (lanes 2-6; 20 µg. Lane 3 contains 2.5 µg) run on a 4-12% NUPAGE gel stained with Coomassie blue to detect protein (left) and Silver to detect LPS (right).

Note: Lanes 1 and 9 contain a 3.5 kDa protein ladder and lanes 7-8 contain 10 µg and 500 ng *E. coli* LPS respectively as positive controls.

The LPS content of vaccines A-E was evaluated by PAGE using a LPS specific silver stain (Figures 5-6) which showed that vaccine C did not contain any detectable LPS. *E. coli* LPS was purchased (Sigma, UK) for use as a control as *Leptospira* derived LPS could not be obtained; the upper and lower limits of *E. coli* LPS detection were determined to be 10 µg and 500 ng respectively by systematically testing a range of concentrations.

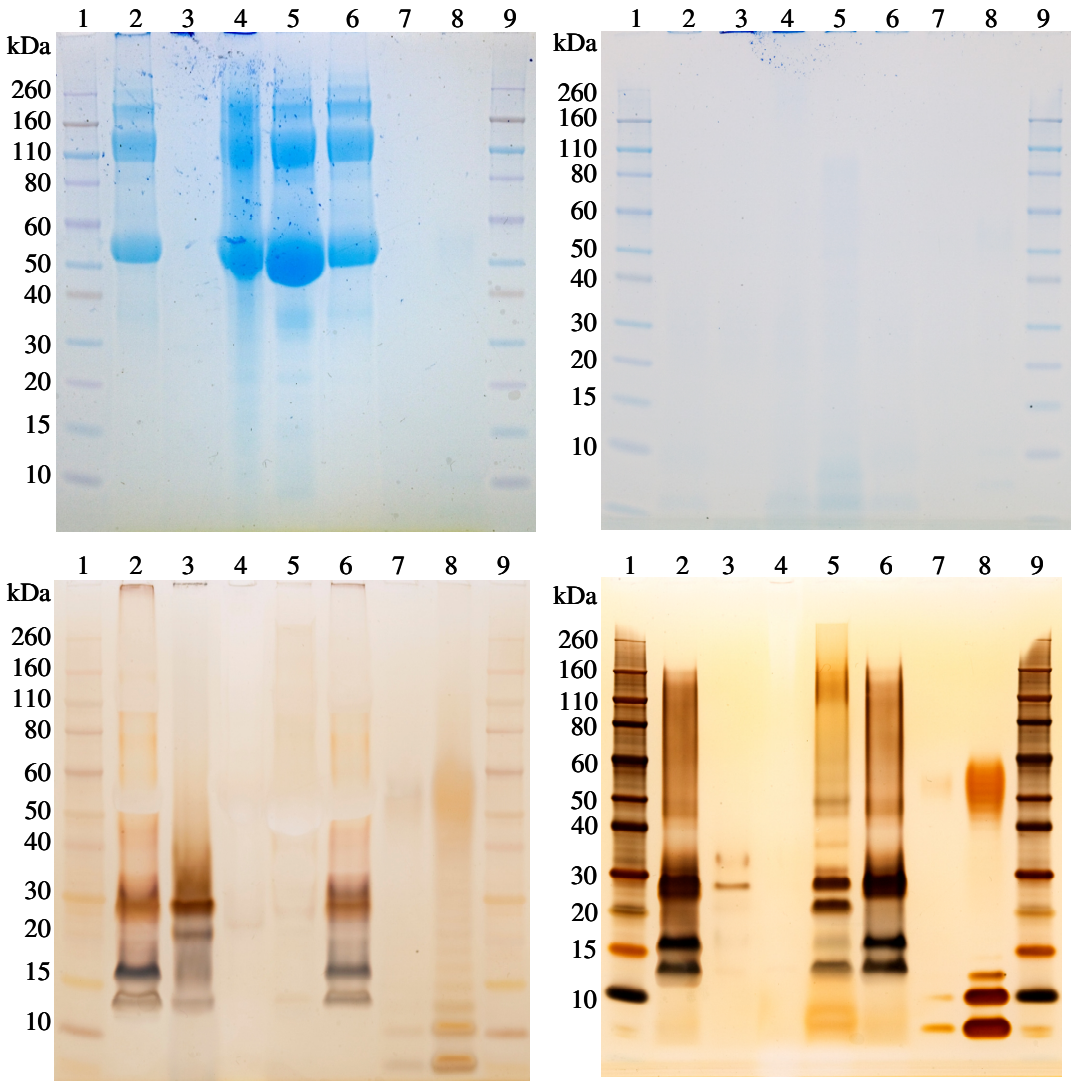

Figure 7: Evaluation of specificity of silver staining for LPS.

Note: Undigested (left) and proteinase K digested (right) aliquots of vaccines A-E (lanes 2-6; 36 µl; lanes 5 and 3 in right hand gels contain vaccines B and D respectively) run on a 4-12% NUPAGE gel stained with Coomassie blue to detect protein (top) and Silver to detect LPS (bottom). Lanes 1 and 9 contain a 3.5 kDa protein ladder and lanes 7-8 contain 500 ng and 10 µg *E. coli* LPS respectively as positive controls.

To determine the specificity of the silver stain used for LPS, proteinase K digested aliquots (36 µl) of vaccines A-E were subjected to gel electrophoresis and stained for both protein (Coomassie stain) and LPS (silver stain) and compared to non-proteinase K digested aliquots (Figure 7). Proteins were observed in the non-digested aliquots of the vaccines, when stained with coomassie (Figure 7), but not in the digested aliquots suggesting that complete protein digestion had occurred. The pattern of banding seen in the silver stained gels however was substantially the same between the digested and non-digested vaccines indicating that the silver stain used is specific to LPS.

For corroboration, the LAL assay (Charles River, UK) was also performed on vaccines A-E; a calibration curve (Figure 8) using *E. coli* derived LPS (Charles River, UK) was constructed using Prism 4 (Graphpad, USA) over the tested concentration range (0.33-3333 ng/ml) allowing determination of sample concentration (Figure 5) by interpolation. The limit of detection for the LAL assay was calculated (section 2.1) as 0.08 ng/ml, whereas the limit of quantification was calculated (section 2.1) as 0.33 ng/ml. LPS was not detectable in vaccine C which is in agreement with the results from the silver stained gels (Figures 5-6).

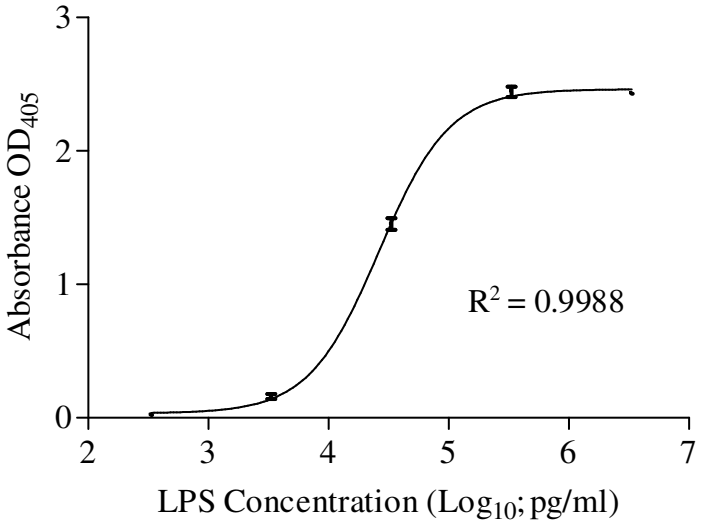

Figure 8: Nonlinear regression of LPS calibration standards using the limulus amebocyte lysate assay detected at 405 nm.

Note: Two replicates of each standard were analysed for each concentration; mean and standard error of the mean are shown.

3.2.2 Vaccine Preparation

Removal of interfering agents from vaccines A-E (Table 8), such as adjuvant and preservative, was required prior to proteomic analysis. Molecular weight cut off filters (MWCO; 5 KDa) were therefore used to reduce the concentration of low molecular weight contaminants such as Aluminium hydroxide (78 Da) and Thiomersal (405 Da). Strong cation exchange chromatography was subsequently used to assess the effect of MWCO washing on peak resolution and intensity (Figure 9); vaccine D was arbitrarily chosen for assessment as a larger volume of this vaccine was available for analysis. Separation of vaccine D was shown to be improved by prior washing of the vaccine once in a molecular weight cut off concentrator (Figure 9) with an additional peak observed at 13.81 minutes. However additional washes led to an overall decrease in peak intensity and separation. Acetone precipitation of protein from vaccine D was also assessed however no improvement of peak resolution was observed (Figure 9).

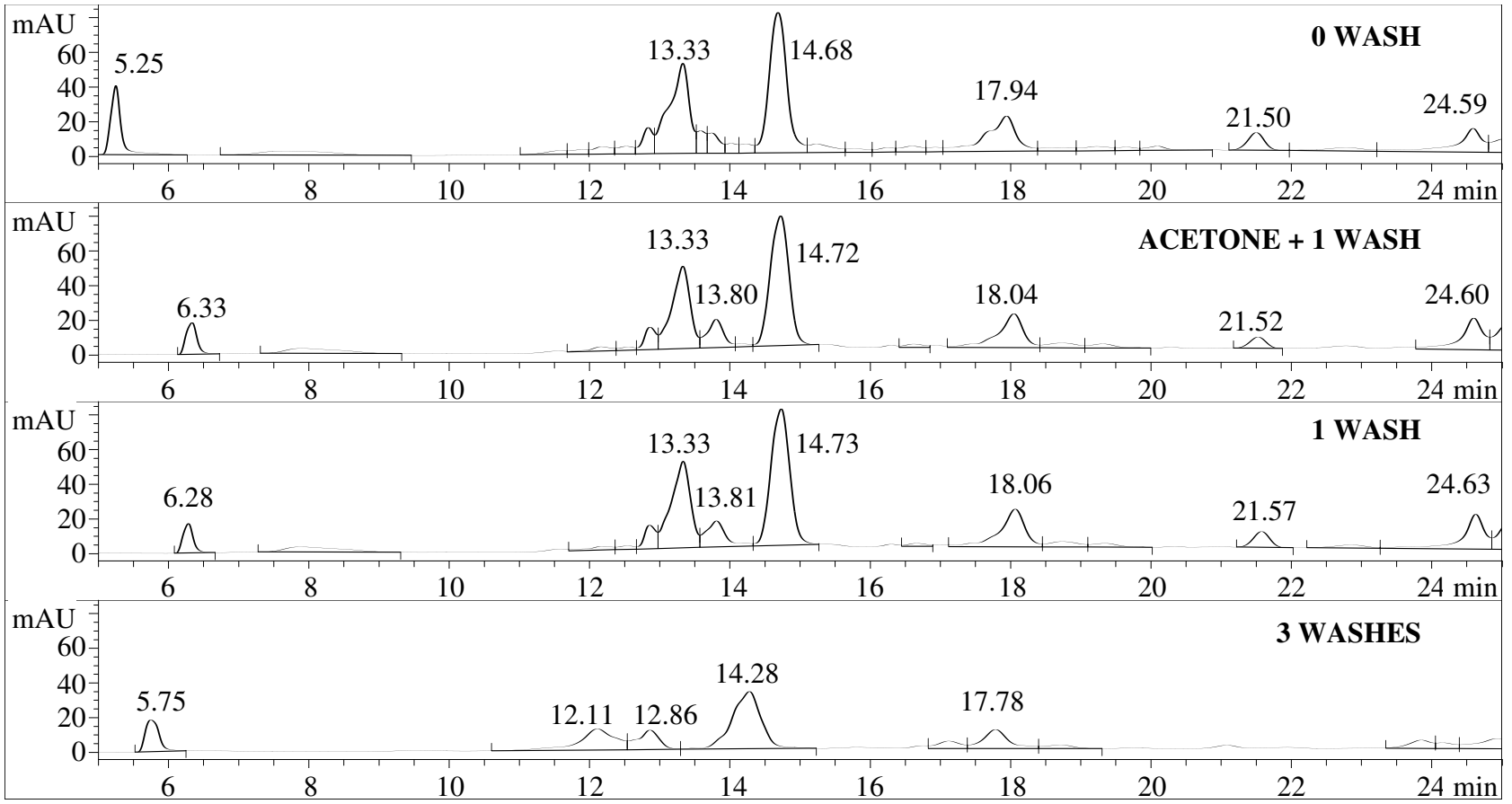

Figure 9: Strong cation exchange separation of trypsin digested vaccine D when prepared using different conditions.

Note: Chromatograms recorded at 280nm.

3.2.3 Strong Cation Exchange Chromatography

Separation of tryptic peptides from vaccines A-E showed a high degree of reproducibility between the chromatograms of the three technical replicates, an example of which can be seen in Figure 10 for vaccine D. Direct comparison of the different vaccines however, revealed considerable disparity (Figure 11); this can most likely be attributed to variation in the manufacturing process used to create the different vaccines however a distinct peak with a retention time of ~14 minutes was observed in vaccines A, C, D and E. Note each vaccine has a different scale in figure 11 due to the high intensity of peaks in vaccine C.

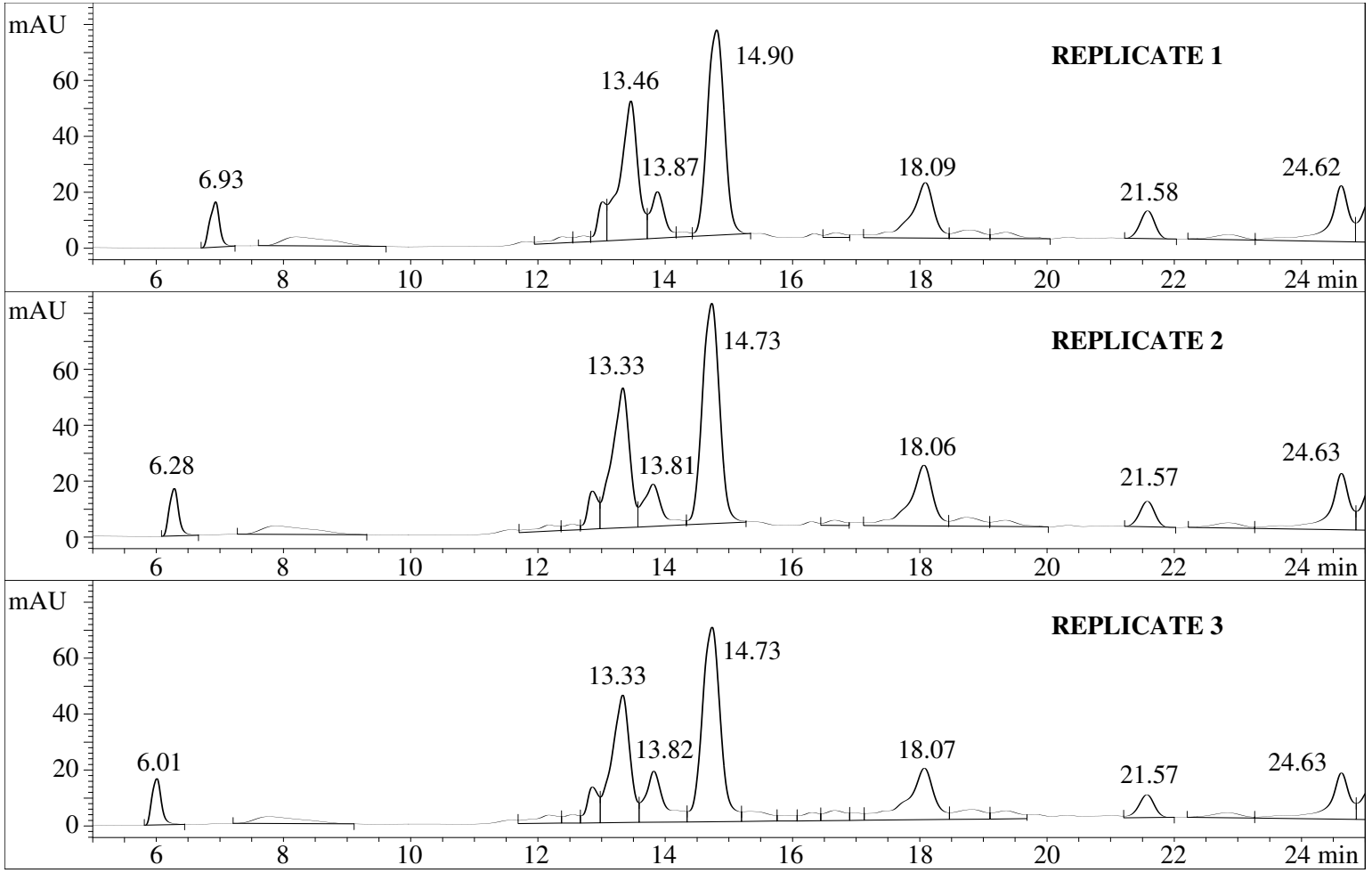

Figure 10: Strong cation exchange separation of three technical replicates of vaccine D.

Note: Chromatograms recorded at 280nm.

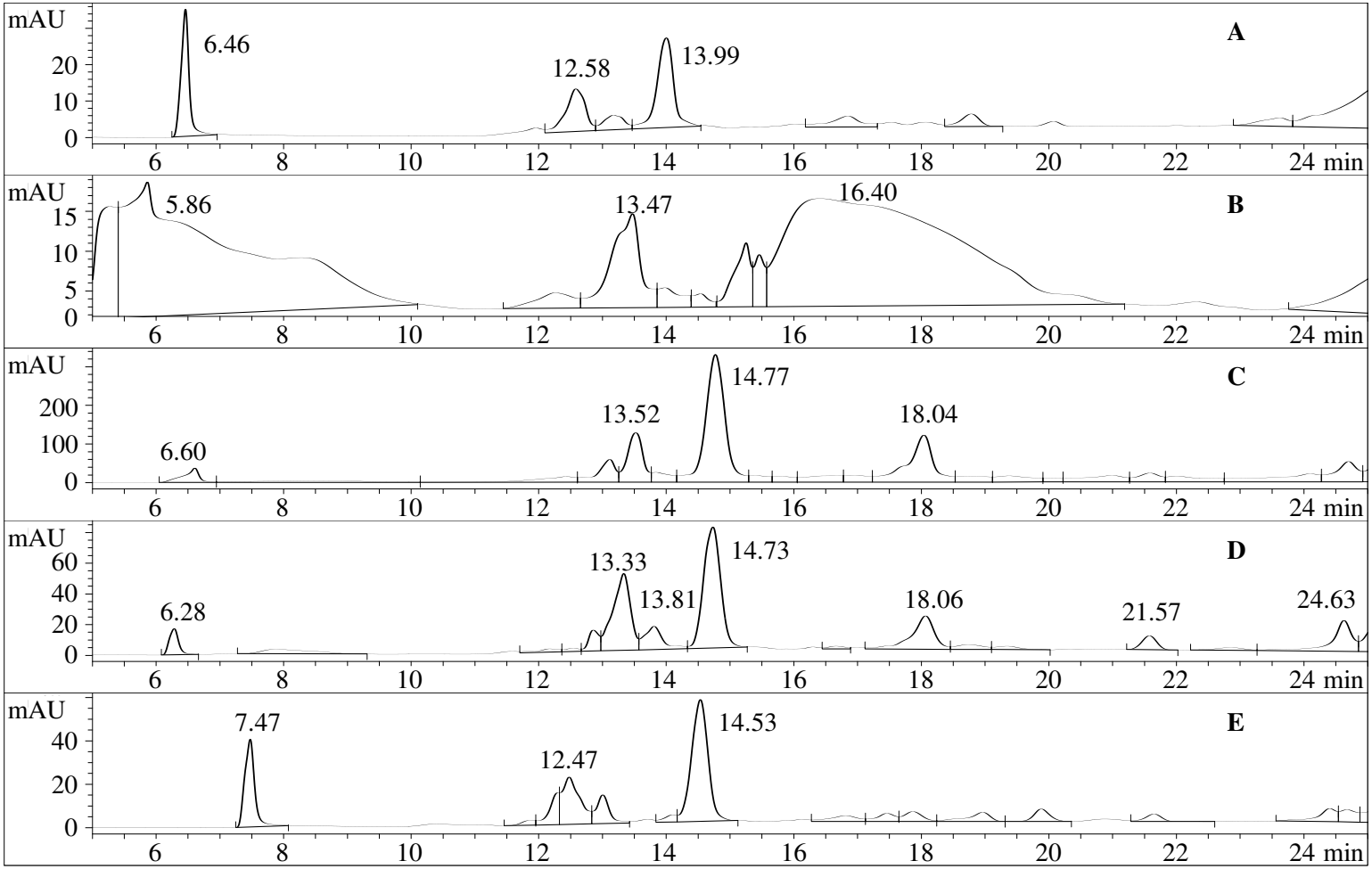

Figure 11: Strong cation exchange separation of vaccines A-E.

Note: Chromatograms recorded at 280nm

3.2.4 Reverse phase HPLC-MS^n

SCX fractions were analysed by reversed phase HPLC-MS^n with reproducibility being assessed by the analysis of a known synthetic peptide standard in each assay (Table 9, figure 12). Blanks before and after each run of samples (a run being defined as 15 SCX fractions) were used to prevent sample carryover and to confirm that no buffer or other equipment issues were occurring.

Table 9: Synthetic peptide controls used to assess the performance of RP-HPLC separation and Q-ToF detection.

Number*	Peptide Sequence	Molecular Mass (Da)	Precursor ion (m/z)	Retention Time (min)
1	MRFA	523.26	262.64	12.36
2	AVDQLNEQSSEPNIYR	1861.88	931.95	19.29
3	VTALYEGFTVQNEANK	1782.88	892.45	24.48
4	ARPQELPFLASIQNQGR	1924.03	642.35	29.75
5	ISVNNVLPVDFNLMQQK	1958.03	980.02	36.95
6	NYINQYSEVAIQMVMHMQPK	2423.14	808.72	38.47

***Peptide numbers correspond to ion chromatograms shown in figure 12.**

An example of ion chromatograms for SCX fractions 1-15 (Vaccine D, replicate 1), collected at one minute intervals between 8 and 22 minutes, is shown in Figure 13. The intensity of the fractions is consistent with the corresponding peaks observed in the SCX chromatograms of vaccine D (Figures 9-10).

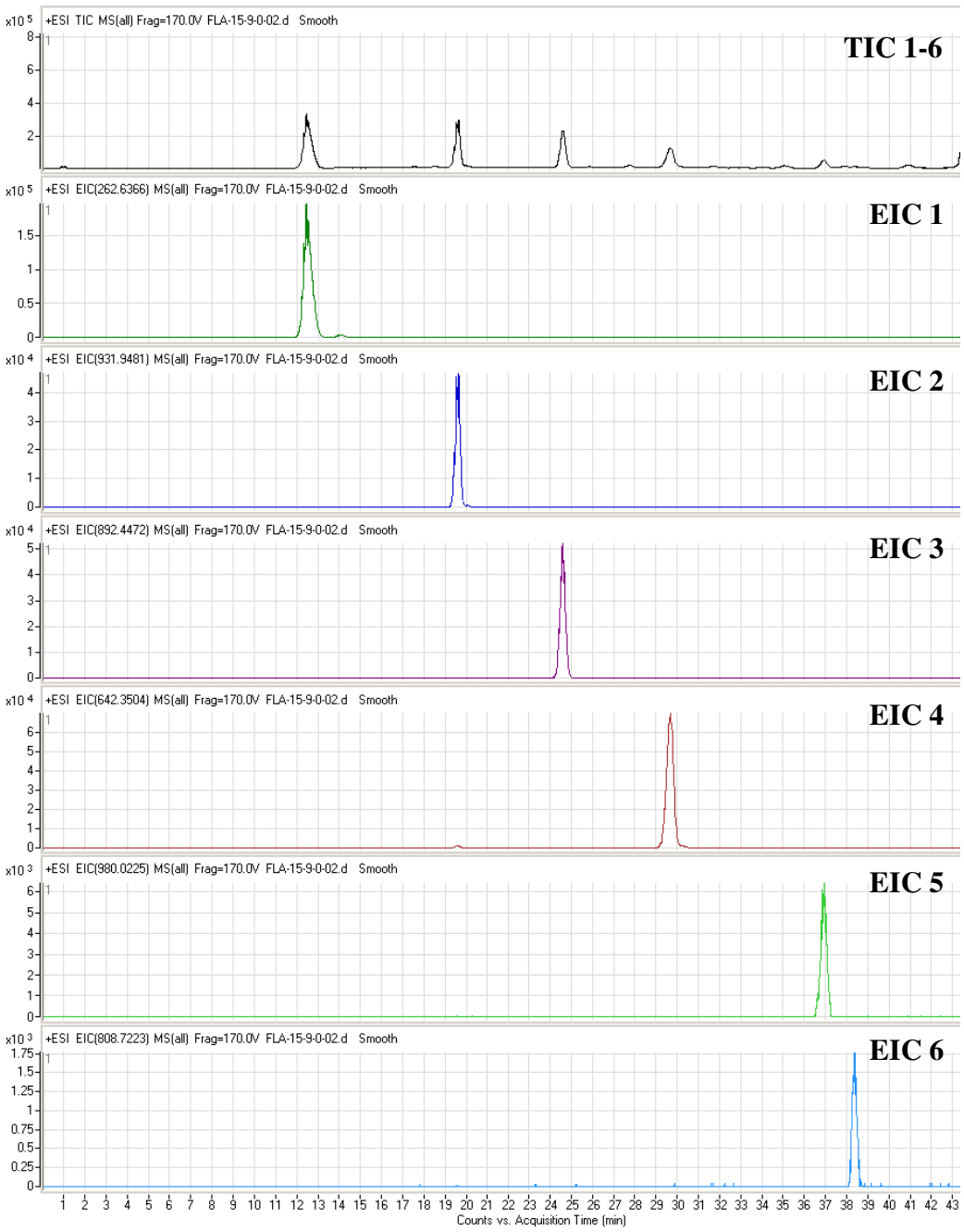

Figure 12: Total (TIC) and extracted (EIC) ion chromatograms (smoothed) of a synthetic quality control peptide mix (100 mg/ml; comprising synthetic peptides 1-6, Table 9).

Note: Peptides were separated by reversed phase chromatography and detected on a Q-TOF mass spectrometer; amino acid sequences, masses, precursor ions and retention times for peptides 1-6 are given in Table 9.

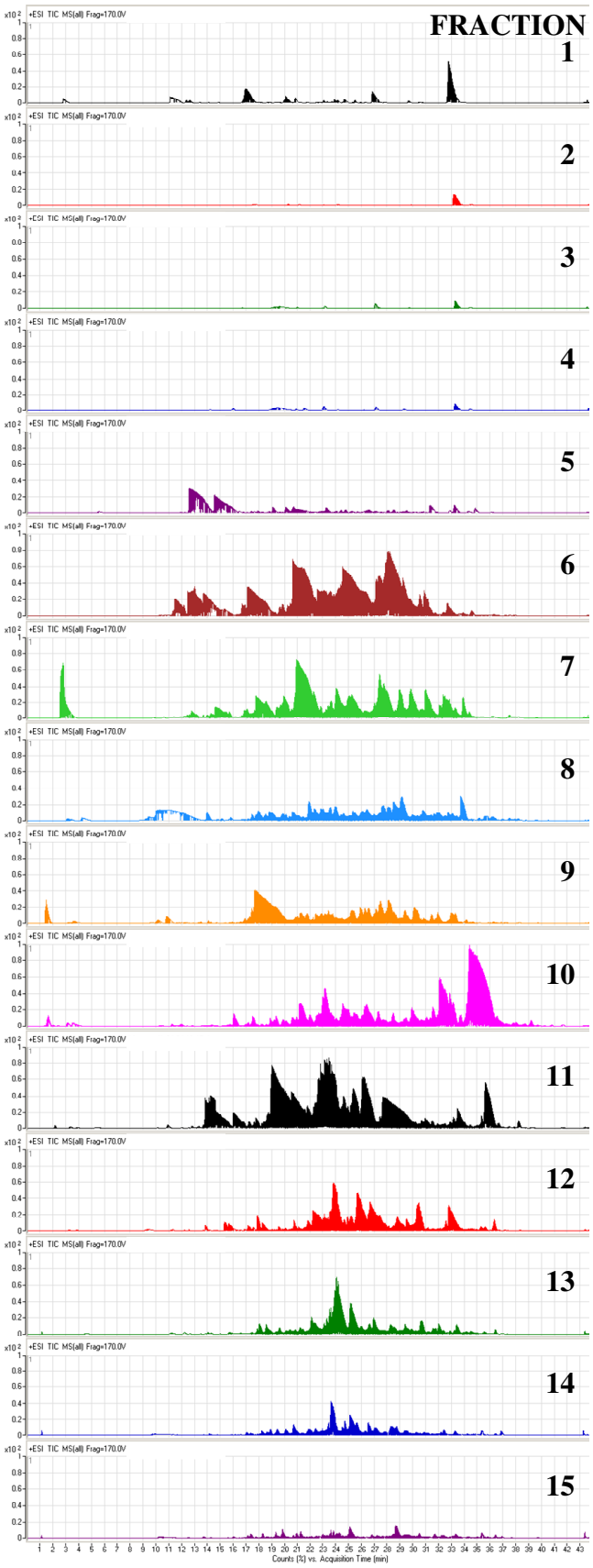

Figure 13: Total ion current chromatograms (TIC) of tryptic peptides from SCX fractions 1-15 (vaccine D, replicate no. 1) detected on a Q-ToF mass spectrometer.

3.2.5 Database comparison

Leptospira interrogans serovars Copenhageni and Lai are the only *L. interrogans* serovars to have been genome sequenced to date (Nascimento et al., 2004b, Ren et al., 2003); comparative analysis has revealed 99% sequence identity between protein coding genes (Nascimento et al., 2004a).

Since serovar Canicola has not been sequenced, three protein databases were selected for comparison in this study (Table 10) to determine which yielded optimal identifications when interrogated with mass spectra from vaccine D using Spectrum Mill (Agilent). The NCBI non-redundant database and the Uniprot database were chosen as they were both known to contain multiple proteomes from different species. A custom database derived from chromosomes I and II of *Leptospira interrogans* serovar Copenhageni, was arbitrarily chosen due to its phenotypic similarity to serovar Canicola [both serovars are reported to infect dogs (Defra, 2010)]

Table 10: Comparison of proteins identified in vaccine D when searched against different databases.

Accession Number	Protein identification	Species	Database		
			NCBInr	UniProt	Serovar Copenhageni
P92916	Bifunctional 6(G)-fructosyltransferase/2,1-fructan:2,1-fructan 1-fructosyltransferase	*A. cepa*	-	+	-
121704634	RSC Complex Subunit (RSC1), Putative	*A. clavatus*	+	-	-
P42246	Uncharacterized ABC transporter ATP-binding protein ycbN	*B. subtilis*	-	+	-
27806789	Transthyretin Precursor	*B. taurus*	+	-	-
30794280	Serum Albumin Precursor	*B. taurus*	+	-	-
94966811	Alpha-1-Acid Glycoprotein Precursor	*B. taurus*	+	-	-

160332365	RecName: Full=Serpin A3-1: AltName: Full=Endopin-1A: AltName: Full=Muscle endopin-1A: Short=mEndopin-1A: Flags: Precursor	*B. taurus*	+	-	-
O46375	Transthyretin	*B. taurus*	-	+	-
P02769	Serum albumin	*B. taurus*	-	+	-
P41361	Antithrombin-III OS=Bos taurus GN=SERPINC1 PE=1 SV=2	*B. taurus*	-	+	-
Q3SZR3	Alpha-1-acid glycoprotein	*B. taurus*	-	+	-
66806199	MAP Kinase Phosphatase	*D. discoideum*	+	-	-
20129847	CG1371	*D. melanogaster*	+	-	-
28590	Unnamed Protein Product	*H. sapiens*	+	-	-
116328028	Endoflagellar Filament Core Protein	*L. borgpetersenii* serovar Hardjo-bovis	+	-	-
45645172	Major Outer Membrane Protein	*L. interrogans*	+	-	-
48995769	Major Outer Membrane Protein	*L. interrogans* serovar Canicola	+	-	-

45656096	Peptidoglycan Associated Cytoplasmic Membrane Protein	*L. interrogans* serovar Copenhageni	-	-	+
45656175	Cell Wall Hydrolase	*L. interrogans* serovar Copenhageni	-	-	+
45656272	Putative Lipoprotein	*L. interrogans* serovar Copenhageni	+		+
45656311	Hypothetical Protein LIC10411	*L. interrogans* serovar Copenhageni	-	-	+
45656396	Putative Lipoprotein	*L. interrogans* serovar Copenhageni	-	-	+
45656890	LipL71	*L. interrogans* serovar Copenhageni	-	-	+
45657043	Dihydrolipoamide Dehydrogenase	*L. interrogans* serovar Copenhageni	-	-	+
45657230	LipL32	*L. interrogans* serovar Copenhageni	-	-	+
45657753	Flagellin Protein	*L. interrogans* serovar Copenhageni	-	-	+
45658793	LipL41	*L. interrogans* serovar Copenhageni	-	-	+
45658988	Hypothetical Protein LIC13166	*L. interrogans* serovar Copenhageni	-	-	+
24212922	OmpA Family Lipoprotein	*L. interrogans* serovar Lai	+	-	-
24213171	Hypothetical Protein LA_0471	*L. interrogans* serovar Lai	+	-	-
Q0W6M2	Probable deoxyribose-phosphate aldolase	*Methanogenic Archaeon*	-	+	-
57619174	Ceruloplasmin Precursor	*O. aries*	+	-	-
Q9XT27	Ceruloplasmin	*O. aries*	-	+	-

57335404	Type III Vimentin	*P. aethiopicus*	+	-	-
34540298	Hypothetical Protein PG0482	*P. gingivalis W83*	+	-	-
39933667	Putative Carboxyl-Terminal Protease	*R. palustris*	+	-	-
21218825	Transcriptional Repressor Protein	*S. coelicolor A3(2)*	+	-	-
136429	RecName: Full=Trypsin: Flags: Precursor	*S. scrofa*	+	-	-
P00761	Trypsin	*S. scrofa*	-	+	-
A8F866	Alanyl-tRNA synthetase	*T. lettingae*	-	+	-
	Total Conserved Proteins		21	10	11

Note + indicates protein detection.

The NCBI database identified 21 proteins in vaccine D whereas the Uniprot and custom databases identified 10 and 11 proteins respectively (Table 10). However, only 6 of the 21 proteins identified using the NCBI database were of *Leptospira* origin and none of the proteins identified using the Uniprot database were specific to *Leptospira*; the custom database was therefore utilised for subsequent searches as it resulted in a higher number of *Leptospira* specific identifications.

3.2.6 Search Engine Comparison

Identification of vaccine proteins required that mass data be interrogated using a suitable search engine. For the purposes of this study Spectrum Mill (Agilent), OMSSA (NCBI) and Mascot (Matrix Science) were chosen for comparison (Table 11) to determine which gave the most reliable identifications. Mass spectra for vaccine D was interrogated against the custom serovar Copenhageni database using equivalent conditions for each search engine (section 2.5.4) and results were exported as excel files for bioinformatic analysis.

Table 11: Comparison of the number of proteins identified in vaccine D and its percentage of false discoveries using different search engines

Search Engine	Mean Proteins ± 1 SD	% False Discovery Rate ± 1 SD
Spectrum Mill	21 ± 5	2.9 ± 1
OMSSA	44 ± 14	23.8 ± 7
Mascot	48 ± 8	12.8 ± 3

The number of proteins identified using OMMSA and Mascot were higher in vaccine D than in Spectrum Mill; however, the false discovery rates calculated for the two search engines were also substantially higher. Since the same search parameters were selected across the three programs it is apparent that Spectrum Mill is more stringent, under these conditions, which results in more reliable data.

3.2.7 Exclusion List Comparison

Previous gel electrophoresis (section 3.2.1) revealed albumin to be present at a high abundance in vaccines A-E (Figure 6, Appendix 1). In an effort to increase the identification of *Leptospira* proteins an exclusion list (section 2.5.2) method, to exclude known albumin precursor ions from being subjected to MS/MS, was trialled using vaccine D (which had been washed 3 times with a molecular weight cut off concentrator). The resulting mass spectra was searched against the NCBInr database in addition to the serovar Copenhageni databases to allow identification of albumin (Table 12). The mean percentage coverage of albumin increased by 9% when the exclusion list was used however no significant difference was observed in the number of proteins detected compared to when not using an exclusion list (Table 12).

Table 12: Comparison of the number of proteins identified in vaccine D when using an albumin exclusion list when searched against different databases.

Database	Albumin Exclusion	Mean Total Proteins ± 1 SD	P Value
NCBInr	+	53 ± 25	0.91
	-	50 ± 16	
Serovar Copenhageni	+	6 ± 2	0.77
	-	5 ± 3	

Note + indicates that the exclusion list was utilised.

Further experimentation using albumin exclusion lists was precluded due to the low scan speed of the mass spectrometer used; subsequent analysis of vaccines was therefore performed without the use of an exclusion list.

3.2.8 Vaccine Proteome Analysis

The protein content of vaccines A-E were analysed using 2D-LC/MS; proteins present across all three technical repeats were then compiled using Microsoft Access to give the proteome of each vaccine (Appendix 2).

Proteins present in all five of these vaccine proteomes were determined using Microsoft Access (Table 13) and their respective abundances calculated using the normalised spectral abundance factor. Although the total number of proteins in each varied considerably (Table 13) two proteins, LipL32 and flagellin, were found to be common to all five vaccines. Due to the low number of proteins identified in vaccine B and their correspondingly low coverage a separate comparison of vaccines A and C-E was also performed, which identified two additional conserved proteins (shaded area, Table 13) LipL41 and hypothetical protein LIC10411.

Table 13: Conserved proteins present in commercially available vaccines from five different manufacturers (A-E).

Accession Number	Protein Identification	Relative Abundance (Mean ln(NSAF)				
		A	B	C	D	E
45657230	LipL32	-4.11 (27)	-2.64 (6)	-2.48 (27)	-2.22 (24)	-2.88 (19)
45657753	Flagellin Protein	-3.41 (31)	-2.44 (5)	-3.01 (17)	-2.35 (26)	-2.59 (21)
45658793	LipL41	-3.35 (33)	ND	-2.09 (31)	-2.08 (31)	-2.39 (27)
45656311	LIC10411 (Hypothetical)	-2.45 (39)	ND	-3.13 (15)	-2.96 (15)	-3.03 (15)
Total Proteins (mean ± 1 SD)		221±31	9±8	34±4	21±5	34±17

Higher ln(NSAF) values indicate greater abundance. ND = Protein not detected. Figures in brackets represent mean percentage protein coverage. Note: Shaded areas represent additional conserved proteins identified when vaccine B is excluded from comparison.

3.3 Discussion

An ELISA based approach was previously developed to determine the potency of *Leptospira* vaccines *in vitro* (Ruby, 1999). Though the ELISA format was ultimately unsuccessful due to issues with adjuvant interference (section 1.4.1) the monoclonal antibodies developed for the assay were established to be reacting to a specific LPS-like moiety; further it was also established that these monoclonal antibodies were serovar specific. Since it is theorised that the microscopic agglutination test (MAT) used for identification of *Leptospira* serovars reacts specifically against LPS (Guerreiro et al., 2001) and that *Leptospira* vaccines are known to be serovar specific (Koizumi and Watanabe, 2005) it appeared reasonable that the active component of the vaccines, responsible for conferring protective immunity might be LPS based.

LPS was therefore investigated as a possible biomarker for potency in vaccines A-E. Extraction of the LPS component of the vaccines and analysis using a MALDI-ToF (Yi and Hackett, 2000) mass spectrometer was planned; however initial evaluation of vaccine LPS content using PAGE with silver staining suggested that it was not common to all vaccines, which was confirmed through subsequent analysis using the LAL assay. This absence of detectable LPS in vaccine C suggests that, at least in this vaccine, it may not be necessary to induce protective immunity against *Leptospira*; further work is required to elucidate its function in the other vaccines analysed which is outside the remit of this project.

The amount of validation required for an *in vitro* potency test to be approved by the European pharmacopeia, which would be a prerequisite prior to implementation by vaccine manufacturers, would be both time consuming and costly. Therefore it would not be practical to develop an LPS based *in vitro* potency test if it could not be

universally applied to all *Leptospira* vaccines and as a result further work into the analysis of the vaccines LPS content was not performed.

Since protein derived from *L. interrogans* is known to be highly immunogenic and capable of providing cross protection against different serovars (Sonrier et al., 2000) a method to characterise the protein content of the *Leptospira* vaccines was sought. A 2D-LC/MS approach was chosen, as good results had been previously reported (Coldham and Woodward, 2004, Vaughan et al., 2006, Chao et al., 2007) in the proteomic analysis of bacteria using a similar method, and a technique for cleaning the samples prior to analysis developed. Subsequent interrogation of mass spectra for protein identity was optimised through evaluation of multiple protein databases and search programmes resulting in a de novo approach for the analysis of *Leptospira* vaccine proteomes using 2D-LC/MS.

The total protein content of vaccine B was found to be much lower than that found in the other vaccines when assessed using the Bradford assay. It was therefore necessary to analyse a smaller protein content of vaccine B using 2D-LC/MS which could account for the lower number of proteins identified (Table 13). Subsequent proteomic analysis of vaccines A and C to E has revealed four conserved proteins. Of these it is LipL32, LipL41 and the flagellin protein that are of interest as they are known to be potential immunogens (Haake et al., 1999, Hauk et al., 2008). When vaccine B was included in the comparison the only proteins identified as being conserved were flagellin and LipL32.

Flagellin is a constituent part of the flagellum and is therefore a highly abundant protein in bacteria. Bacterial flagellin typically stimulates the innate immune response (Hayashi

et al., 2001) however it can also play a role in the adaptive immune system (Letran et al., 2011). The immunogenic potential of flagellin is such that it makes an excellent adjuvant candidate (McSorley et al., 2002, Cuadros et al., 2004) when constructing vaccines; though its use in the creation of *Leptospira* vaccines has not been widely reported.

LipL32, also known as hemolysis associated protein-1 (Hap-1), is a major surface expressed outer membrane protein (Cullen et al., 2005) found in pathogenic *Leptospira* species (Haake et al., 2000). It is known to provide cross protection against *Leptospira interrogans* (Branger et al., 2001) in the gerbil model and various different methods for presenting it as a vaccine have been trialled (Branger et al., 2005, Seixas et al., 2007b). A recent study (Lucas et al., 2011) has reported that recombinant LipL32 does not confer protective immunity against leptospirosis in hamsters; however this study was limited to three serovars and did not include the Canicola serovar.

In conclusion 2D-LC/MS has been confirmed to be an effective tool for vaccine analysis and a technique for the analysis of vaccines proteomes has been demonstrated. Vaccines from five major manufacturers have been assessed and two conserved proteins, LipL32 and flagellin, have been identified. Both of these conserved proteins are known to be highly immunogenic and may present good targets for the development of an *in vitro* potency test. Further work is required to ascertain if there are any differences in these proteins between batches of vaccine which pass the hamster challenge test and batches that fail.

Chapter 4 Identification of LipL32 as a biomarker for *L. interrogans* serovar Canicola vaccine potency

4.1 Introduction

Proteomics has been used as a tool for biomarker discovery for some time in a diverse range of fields including (but not limited to) bacterial identification (Welker, 2011), cancer (Zeng et al., 2011), autoimmune (De Franceschi et al., 2011), cardiac (Zhang et al., 2011), respiratory (O'Neil et al., 2011) and neurologic diseases (Dudley et al., 2011). Identified biomarkers can be used both for therapeutic purposes, such as the discovery of novel drug targets, and prognostically allowing better identification and stratification of disease states (which in turn can lead to more personalised disease treatment with obvious health benefits). To date however its only application in *Leptospira* vaccinology has been in the development of novel therapeutic agents.

Our previous studies (Chapter 3) established that 2D-LC/MS could be used to analyse the protein content of serovar Canicola vaccines. This chapter focuses on applying label free, quantitative proteomic techniques to characterise the range and abundance of proteins present in batches of a *L. interrogans* serovar Canicola vaccine. The primary aim of this chapter is to prove that protein biomarkers for vaccine potency are present in both passed and failed vaccine batches and can be quantitatively identified using mass spectrometry.

4.2 Results

4.2.1 Bacterial culturing conditions

Growth was assessed by dark field microscopy (section 2.3.3) in triplicate for the Hond Utrecht IV strain of *Leptospira interrogans* serovar Canicola (AHVLA, UK) over a 9 day period, to determine the optimum point at which cell numbers peaked. A nonlinear regression growth curve was then created using the program Graphpad Prism 4 (Figure 14).

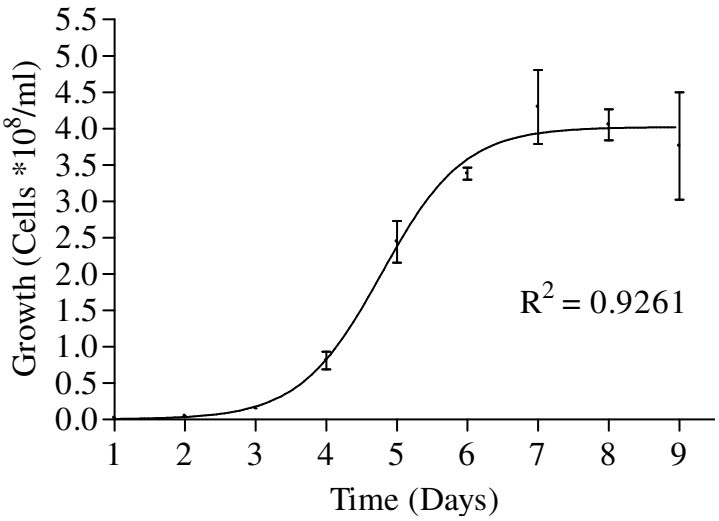

Figure 14: Nonlinear regression growth curve for *Leptospira interrogans* serovar Canicola.

Note: Results are presented as the mean and the standard error of the mean.

The end of log phase, day 7, was chosen as the optimal time point for maximal protein extraction due to the high concentration of cells present. Two fractions, approximately corresponding to the cytosol (soluble fraction) and cell membrane (precipitated insoluble fraction) were extracted from the bacteria (section 2.3.5), and assessed for protein content using the Bradford assay (Table 14).

Table 14: Concentration of protein in cellular extracts of *Leptospira interrogans* serovar Canicola.

Extract	N	Concentration (mg/ml; mean ± 1SD)
Soluble	3	4.77 ± 1.18
Precipitated	3	60.53 ± 6.26

4.2.2 *Leptospira* Proteome Characterisation

The protein content of the soluble and precipitated extracts of serovar Canicola were analysed using 2D-LC/MS (see section 2.5.2) and identified by searching against the serovar Copenhageni protein database. Proteins present across all three technical repeats were then compiled using Microsoft Access to give the proteome of each extract; 952 ± 59 and 666 ± 8 proteins (mean ± 1 SD) were detected between the cytosol and precipitated extracts respectively, the combined fractions yielded 1015 unique proteins (Appendix 3). The 25 top most abundant proteins (according to their NSAF score) in each extract are shown in tables 15 and 16; the flagellin and LipL32 proteins previously identified in vaccines A-E (Chapter 3) were present in both extracts. Detection of identical proteins in both the soluble and precipitated extracts can be attributed to overlap in the extraction process, due to incomplete fractionation of the two extracts. The total serovar Canicola proteome (i.e. the consensus of proteins identified in the two extracts) was characterised through grouping the proteins by their functional annotation (Figure 15) as determined using PIR (section 2.5.6). The serovar Canicola proteome determined in this study was subsequently compared to other *Leptospira* proteomes available in the literature (Table 17). A venn analysis of serovar Canicola with serovars Pomona (Vieira et al., 2009) and Copenhageni (Eshghi et al., 2009) was also performed (Figure 16), LipL32 was present in all three serovars analysed; protein lists for the other studies listed in table 17 were unavailable online precluding them from analysis.

Table 15: Top 25 most abundant serovar Copenhageni proteins in the cytosol extract of serovar Canicola.

Accession Number	Protein identification	Primary Protein Function	Protein Length (Amino Acids)	Mean No. of peptides	Mean % Coverage	Mean ln(NSAF)*
45657213	Chaperonin Groel	Nucleotide binding	546	307	63.33	-3.95
45658705	Elongation Factor Tu	Hydrolase activity	401	206.33	55.67	-4.04
45656891	Anti-Sigma Factor Antagonist	Protein binding	112	42.67	51	-4.34
45657831	Thioredoxin	Electron carrier activity	104	38.33	46.67	-4.37
45657930	Cysteine Synthase	Catalytic activity	309	83.33	56.33	-4.68
45657429	Rna-Binding Protein	Nucleic acid binding	76	17	28.33	-4.88
45656311	Hypothetical Protein LIC10411	Binding	157	31.67	47.67	-4.99
45657838	Rna-Binding Protein	Nucleotide binding	92	17.33	72.33	-5.05
161621774	Malate Dehydrogenase	Catalytic activity	326	62	56.33	-5.05

45656263	Electron Transfer Flavoprotein Alpha-Subunit	Cofactor binding	319	56.67	41	-5.11
45659145	Nucleoside Diphosphate Kinase	Nucleotide binding	137	22.67	44.33	-5.17
45656303	Riboflavin Synthase Beta Chain	Transferase activity	151	25.33	48.33	-5.22
45658676	Dna-Directed Rna Polymerase Subunit Alpha	Transferase activity	325	51	52	-5.23
45657230	LipL32	Unknown	272	39.67	44.33	-5.3
45657300	Peptidyl-Prolyl Cis-Trans Isomerase	Isomerase activity	129	18.67	52	-5.31
45657237	Hypothetical Protein LIC11359	Oxidoreductase activity	392	53.33	41.67	-5.37
45657363	Chemotaxis Protein	Unknown	154	20.33	22.33	-5.4

45659065	Isocitrate Dehydrogenase	Ion binding	398	53	44.33	-5.4
45657214	Co-Chaperonin Groes	Nucleotide binding	96	12.67	45.33	-5.41
45658176	Putative Lactoylglutathione Lyase	Lyase activity	152	19.33	40.33	-5.44
45657753	Flagellin Protein	Structural molecule activity	282	34	42	-5.49
45658793	LipL41	Binding	355	43	32	-5.49
45655803	Hypothetical Protein LIC20223	Unknown	143	17	11	-5.5
45656176	Elongation Factor G	Hydrolase activity	706	84.33	41.33	-5.5
45658048	UDP-Glucose 4-Epimerase	Catalytic activity	330	40	35	-5.5

***Higher ln(NSAF) values indicate greater abundance.**

Table 16: Top 25 most abundant serovar Copenhageni proteins in the precipitated extract of serovar Canicola.

Accession Number	Protein identification	Primary Protein Function	Protein Length (Amino Acids)	Mean No. of peptides	Mean % Coverage	Mean ln(NSAF)*
45656311	Hypothetical Protein LIC10411	Binding	157	53.67	67.33	-3.63
45657230	LipL32	Unknown	272	58.67	52.67	-4.09
45657489	Arsr Family Transcriptional Regulator	Nucleic acid binding	99	20.33	59	-4.18
45658793	LipL41	Binding	355	68.67	59.33	-4.2
45657213	Chaperonin Groel	Nucleotide binding	546	82.33	52	-4.45
45656006	Hypothetical Protein LIC10095	Unknown	118	15.33	18	-4.61
45657363	Chemotaxis Protein	Unknown	154	19.67	22	-4.61
45659005	Methyl-Accepting Chemotaxis-Like	Unknown	127	17	54.67	-4.61
45656891	Anti-Sigma Factor Antagonist	Protein binding	111	13.33	37	-4.7

45656096	Peptidoglycan Associated Cytoplasmic Membrane Protein	Unknown	195	22	66.33	-4.74
45657753	Flagellin Protein	Structural molecule activity	282	31.33	48.67	-4.75
45656767	Hypothetical Protein LIC10876	Unknown	183	19	34.67	-4.82
45657214	Co-Chaperonin Groes	Nucleotide binding	96	8.67	50.33	-4.96
45658705	Elongation Factor Tu	Hydrolase activity	401	36.33	48.67	-4.96
45657123	F0F1 Atp Synthase Subunit Delta	Transporter activity	186	15	28.33	-5.07
45657432	50S Ribosomal Protein L19	Structural molecule activity	138	11.33	30.67	-5.07
45658702	50S Ribosomal Protein L4	Structural molecule activity	211	17	33.67	-5.07

45657374	Hypothetical Protein LIC11499	Unknown	80	6.33	53	-5.09
45656646	50S Ribosomal Protein L7/L12	Structural molecule activity	127	9.67	35	-5.14
45657429	Rna-Binding Protein	Nucleic acid binding	76	5.67	21	-5.16
161621777	F0F1 Atp Synthase Subunit Alpha	Nucleotide binding	503	36	41.67	-5.19
45656645	50S Ribosomal Protein L10	Unknown	177	12	29	-5.25
45658988	Hypothetical Protein LIC13166	Unknown	306	20.67	34.67	-5.25
45655941	Hypothetical Protein LIC10027	Unknown	234	18	18	-5.26
45656741	30S Ribosomal Protein S2	Structural molecule activity	294	19.67	47.67	-5.27

***Higher ln(NSAF) values indicate greater abundance.**

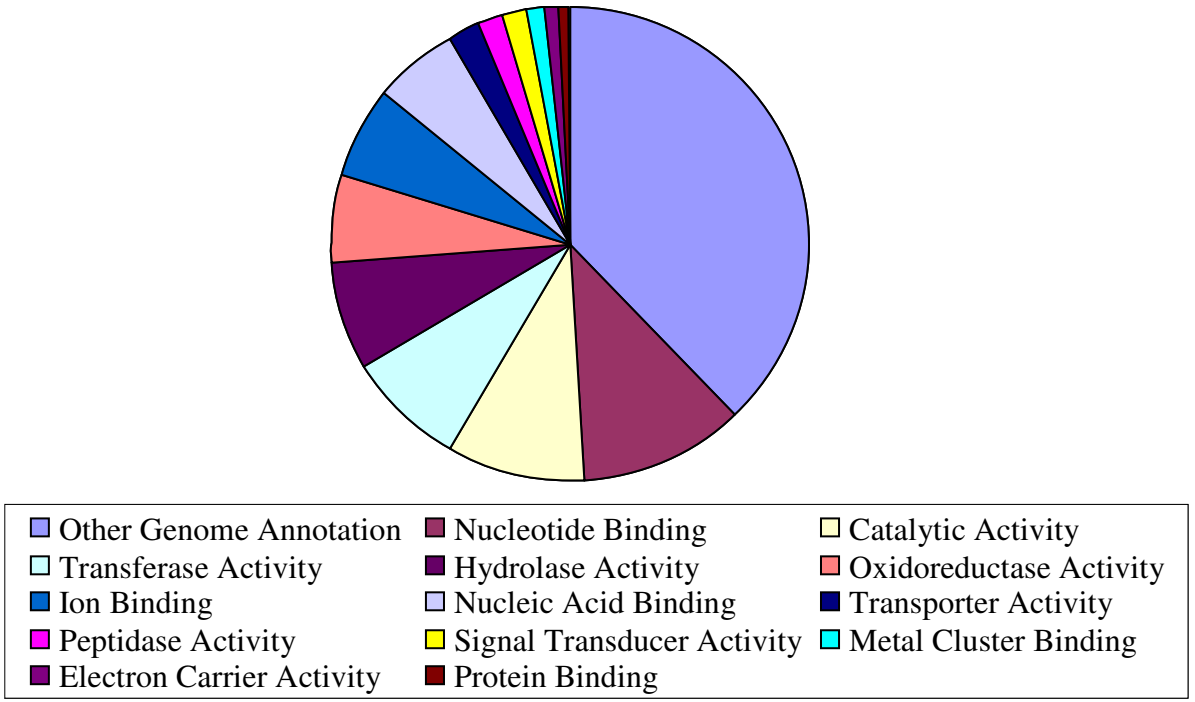

Figure 15: Number of proteins identified in the total *L. interrogans* serovar Canicola proteome within different functional groups as determined by gene annotation using PIR.

Table 17: Comparison of the total number of *Leptospira* proteins identified in this project with previous studies.

Reference	Species	Strain	*Leptospira* Proteins Identified
(Eshghi et al., 2009)	*L. interrogans* serovar Copenhageni	Fiocruz L1-130	563
(Vieira et al., 2009)	*L. interrogans* serovar Pomona	LPF	108
(Malmstrom et al., 2009)	*L. interrogans* serovar Copenhageni	Fiocruz L1-130	2221
(Cao et al., 2010)	*L. interrogans* serovar Lai	56601	2540
(Zhong et al., 2011a)	*L. interrogans* serovar Lai	IPAV	2608
(Zhong et al., 2011a)	*L. interrogans* serovar Lai	56601	2673
This Study	*L. interrogans* serovar Canicola	Hond Utrecht IV	1015

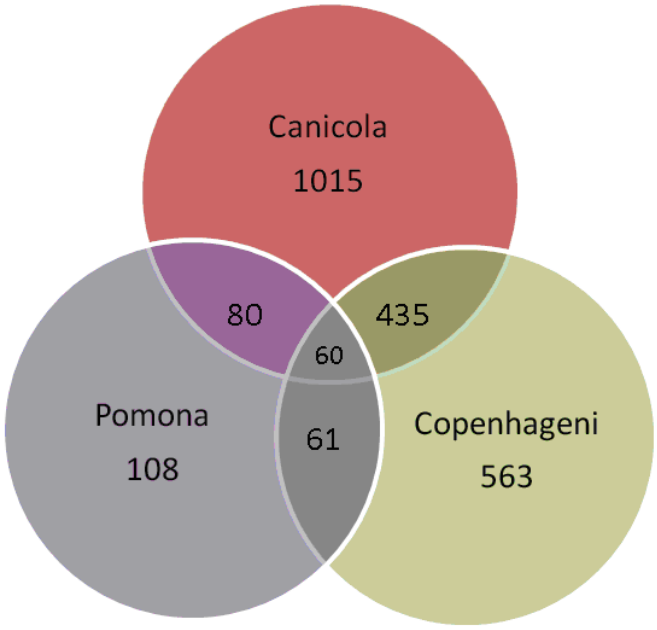

Figure 16: Venn comparison of total number of proteins identified in this study against previous studies by Eshghi et al (2009) and Vieira et al (2009).

4.2.3 Identification of LipL32 as a biomarker for potency in serovar Canicola vaccines

Five batches of vaccine C were assessed (section 2.4.2) for their protective effects against *L. interrogans* serovars Canicola and Icterohaemorrhagiae (Table 18), further batches were unavailable for testing.

Table 18: Hamster potency test performed for five batches of Vaccine C against two serovars of *Leptospira interrogans*.

Vaccine C	Serovar Canicola		Serovar Icterohaemorrhagiae	
Batch Name	Hamsters Died	Test Outcome	Hamsters Died	Test Outcome
PH1	0	Pass	1	Pass
PH2	0	Pass	1	Pass
PH3	2	Fail	1	Pass
PH4	2	Fail	1	Pass
PH5	0	Pass	2	Fail

Note at least four hamsters had to survive the test for it to pass.

Serovar Copenhageni proteins present in batches of vaccine C which had passed (PH1) and failed (PH3) the *in vivo* potency test (when challenged against serovar Canicola) were identified using 2D-LC/MS. Proteins present across all three technical repeats were then compiled using Microsoft Access to give the *Leptospira* proteome of each vaccine batch (Tables 19 and 20). Proteins present in both of these batches were then determined (Table 21) and their respective abundances calculated via spectral counting using the normalised spectral abundance factor (NSAF). The LipL32 and flagellin proteins identified in vaccines A-E (Chapter 3) and the serovar Canicola proteome (section 4.2.2) were present in both batches of vaccine C. Based on the NSAF LipL32 and Histidine Kinase Sensor Protein were both shown to be present at a higher abundance ($p \leq 0.05$) in the batch of vaccine C which had passed the *in vivo* potency test

(PH1) compared to the failed (PH3) batch (Table 21); however no statistical difference was observed in the abundance of the flagellin protein.

Table 19: Serovar Copenhageni proteins detected in a passed batch (PH1) of vaccine C.

Accession Number	Protein identification	Primary Protein Function	Protein Length (Amino Acids)	Mean No. of peptides	Mean % Coverage	Mean ln(NSAF)*
45656175	Cell Wall Hydrolase	Hydrolase activity	221	17.33	3	-1.97
45658793	LipL41	Binding	355	14.33	32.33	-2.65
45655648	Acyl Carrier Protein	Transporter activity	77	3	16.33	-2.71
45657230	LipL32	Unknown	272	9	21.33	-2.83
45657213	Chaperonin GroEL	Nucleotide binding	546	12.33	22	-3.22
45657102	Peroxiredoxin	Antioxidant activity	193	3.67	9	-3.4
45658988	Hypothetical Protein LIC13166	Unknown	306	5	15.33	-3.54
45657748	Putative Lipoprotein	Unknown	412	5.67	16	-3.78
45657753	Flagellin Protein	Structural molecule activity	281	3.67	9.33	-3.79

45656504	DNA-Binding Stress Protein	Ion binding	172	2.33	13.33	-3.88
45656611	Putative Lipoprotein	Unknown	440	5	9.33	-3.94
45657078	Putative Citrate Lyase	Catalytic activity	330	3.67	18.67	-3.95
45656816	Putative Lipoprotein	Unknown	497	4.33	10	-4.17
45657930	Cysteine Synthase	Catalytic activity	309	2.33	7	-4.33
45658246	Isoleucyl-tRNA Synthetase	Catalytic activity	914	6	2	-4.46
45657869	ATP-Dependent Protease	Nucleotide binding	860	5	1	-4.58
45657309	Histidine Kinase Sensor Protein	Nucleotide binding	489	2	1	-4.92

45658253	Putative Glutamine Synthetase Protein	Catalytic activity	473	2	3.67	-4.98
45657926	Hypothetical Protein LIC12078	Catalytic activity	525	2	1	-5.09
45658471	Hypothetical Protein LIC12634	Binding	1125	2.33	0.33	-5.62
45656945	Cell Division Protein	Nucleic acid binding	948	2	0.33	-5.68
45656648	DNA-Directed RNA Polymerase Subunit Beta'	Transferase activity	1404	2	1.33	-5.98
Total Conserved Proteins			22			

***Higher ln(NSAF) values indicate greater abundance.**

Table 20: Serovar Copenhageni proteins detected in a failed batch (PH3) of vaccine C.

Accession Number	Protein identification	Primary Protein Function	Protein Length (Amino Acids)	Mean No. of peptides	Mean % Coverage	Mean ln(NSAF)*
45656175	Cell Wall Hydrolase	Hydrolase activity	221	13.67	3	-1.88
45655648	Acyl Carrier Protein	Transporter activity	77	3	20	-2.36
45658793	LipL41	Binding	355	12.33	31.33	-2.44
45657230	LipL32	Unknown	272	5	16.33	-3.07
45657213	Chaperonin GroEL	Nucleotide binding	546	7.67	15.33	-3.35
45656611	Putative Lipoprotein	Unknown	440	4.67	9	-3.63
45657043	Dihydrolipoamide Dehydrogenase	Oxidoreductase activity	490	5	2.67	-3.67
45658988	Hypothetical Protein LIC13166	Unknown	306	3	10	-3.7
45658246	Isoleucyl-tRNA Synthetase	Catalytic activity	914	7.67	2	-3.89
45657078	Putative Citrate Lyase	Catalytic activity	330	2.67	15.33	-3.91

45657753	Flagellin Protein	Structural molecule activity	281	2	6.67	-4.12
45657748	Putative Lipoprotein	Unknown	412	2.33	5	-4.27
45657309	Histidine Kinase Sensor Protein	Nucleotide binding	489	2	1	-4.57
45657022	Hypothetical Protein LIC11138	Unknown	501	2	1	-4.6
45657869	ATP-Dependent Protease	Nucleotide binding	860	3	1	-4.73
45656046	ABC Transporter ATP-Binding Protein	Nucleotide binding	606	2	1	-4.79
45655920	DNA Gyrase Subunit A	Nucleic acid binding	834	2	1	-5.11
45656945	Cell Division Protein	Nucleic acid binding	948	2	0	-5.24
Total Conserved Proteins			18			

***Higher ln(NSAF) values indicate greater abundance.**

Table 21: Abundance of conserved serovar Copenhageni proteins present in a passed (PH1) and failed (PH3) batch of vaccine C.

Accession Number	Protein identification	Primary Protein Function	Relative Protein Abundance (Mean ln(NSAF))*		P Value
			Passed	Failed	
45657309	Histidine Kinase Sensor Protein	Nucleotide binding	-4.92 (1)	-4.57 (1)	9.23E-03
45657230	LipL32	Unknown	-2.83 (21)	-3.07 (16)	3.20E-02
45658246	Isoleucyl-tRNA Synthetase	Catalytic activity	-4.46 (2)	-3.89 (2)	8.58E-02
45658988	Hypothetical Protein LIC13166	Unknown	-3.54 (15)	-3.70 (10)	9.39E-02
45656611	Putative Lipoprotein	Unknown	-3.94 (9)	-3.63 (9)	1.05E-01
45657748	Putative Lipoprotein	Unknown	-3.78 (16)	-4.27 (5)	1.70E-01
45658793	LipL41	Binding	-2.65 (32)	-2.44 (31)	1.90E-01
45656945	Cell Division Protein	Nucleic acid binding	-5.68 (0)	-5.24 (0)	2.04E-01
45657213	Chaperonin GroEL	Nucleotide binding	-3.22 (22)	-3.35 (15)	3.63E-01
45655648	Acyl Carrier Protein	Transporter activity	-2.71 (16)	-2.36 (20)	3.65E-01
45657869	ATP-Dependent Protease	Nucleotide binding	-4.58 (1)	-4.73 (1)	3.83E-01
45657753	Flagellin Protein	Structural molecule activity	-3.79 (9)	-4.12 (7)	4.66E-01

45656175	Cell Wall Hydrolase	Hydrolase activity	-1.97 (3)	-1.88 (3)	5.42E-01
45657078	Putative Citrate Lyase	Catalytic activity	-3.95 (19)	-3.91 (15)	8.55E-01
	Total Conserved Proteins		22	18	

***Higher ln(NSAF) values indicate greater abundance. Figures in brackets represent mean percentage protein coverage**

4.2.4 Quantitation of LipL32 in passed and failed batches of vaccine

Quantification of LipL32 in batches of vaccine C was determined using multiple reaction monitoring (MRM) mass spectrometry (section 2.5.5). Four tryptic peptides, identified as corresponding to LipL32 in serovar Canicola (section 4.2.2; Table 22) and vaccine C (section 4.2.3; Table 22), were chosen for quantitation; the full sequence of LipL32 and the relative positions of the tryptic peptides selected are shown below table 22. A number of different MRM transition states for these tryptic peptides were derived (Table 23) from the MS and MS/MS data acquired through previous 2D-LC/MS analysis of serovar Canicola (section 4.2.2); Masshunter (Agilent) was used to automatically determine which of these were optimal (Table 24).

Table 22: Comparison of LipL32 tryptic peptides detected in serovar Canicola protein extracts and two batches of vaccine C (PH1 and PH3).

Peptide Sequence	Serovar Canicola	Passed Vaccine	Failed Vaccine
SSFVLSEDTIPGTNETVK	+	+	-
AYYLYVWIPAVIAEMGVR	+	-	-
MISPTGEIGEPGDGDLVSDAFK	+	-	-
SMPHWFDTWIR	+	+	+
MSAIMPDQIAK	+	+	+
LDDDDDGDDTYK	+	-	-
IKIPNPPK	+	-	-
IPNPPK	+	-	-
ISFTTYKPGEVK	+	-	+

Note: + or – indicates the presence or absence of the tryptic peptide respectively. The full amino acid sequence of LipL32 and the locations of the tryptic peptides used for quantitation (grey) are shown below. The signal peptide is shown in red.

MKKLSILAISVALFASITACGAFGGLPSLKSSFVLSEDTIPGTNETVKTLLPYGSV
INYYGYVKPGQAPDGLVDGNKKAYYLYVWIPAVIAEMGVRMISPTGEIGEPGD
GDLVSDAFKAATPEEKSMPHWFDTWIRVERMSAIMPDQIAKAAKAKPVQKLD
DDDDGDDTYKEERHNKYNSLTRIKIPNPPKSFDDLKNIDTKKLLVRGLYRISFTT

YKPGEVKGSFVASVGLLFPPGIPGVSPLIHSNPEELQKQAIAAEESLKKAASDAT

K

Table 23: MRM transitions identified from the MS/MS analysis of the LipL32 tryptic peptides of interest.

Peptide Sequence	Precursor ion for MRM (m/z)	Product ion for MRM (m/z)	Collision Energy (V)
ISFTTYKPGEVK	457.30	529.30	12
	457.30	585.30	12
	457.30	628.80	12
	685.60	585.30	23
	685.60	820.50	23
	685.60	1022.60	23
SSFVLSEDTIPGTNETVK	962.50	421.20	30
	962.50	845.40	35
	962.50	1061.50	23
	962.50	1390.70	30
SMPHWFDTWIR	493.50	235.10	13
	493.50	629.80	13
	493.50	1024.50	13
	738.30	235.10	23
	738.30	629.30	23
	738.30	1024.50	23
AYYLYVWIPAVIAEMGVR	706.30	662.30	15
	706.30	775.40	15
	706.30	1042.60	15
	1058.80	773.40	28
	1058.80	1042.60	28
	1058.80	1342.70	28

Note: Collision energies calculated automatically by Masshunter (Agilent).

Table 24: Tryptic peptides and product ions selected for detection and quantification of LipL32 using multiple reaction monitoring (LC-MRM).

Number	Peptide Sequence	Position from N Terminus	Molecular Mass (Da)	Precursor ion for MRM (m/z)	Product ion for MRM (m/z)	Retention Time (min)
1	ISFTTYKPGEVK	212-223	1369.56	457.3	628.8	10.8
2	SSFVLSEDTIPGTNETVK	31-48	1924.07	962.5	845.4	13.16
3	SMPHWFDTWIR	126-136	1475.671	738.3	1024.5	13.301
4	AYYLYVWIPAVIAEMGVR	79-96	2114.516	706.3	1042.6	20.709

Calibration curves for all four tryptic peptides were constructed using Graphpad Prism 4 over the tested concentration range (0.01-100 fmol; Figure 17). Total ion chromatograms of the four tryptic peptides at the different concentrations are shown in figure 18. Tryptic peptides 3 and 4 were excluded from further analyses due to the low response rates observed in figures 15 and 16.

The LOD of tryptic peptides 1 and 2 was determined (section 2.1) as 0.018 and 0.025 fmol\µg respectively, whereas the LOQ were 0.05 and 0.02 fmol\µg respectively.

Batches of vaccine C that had passed (PH1 and PH2) and failed (PH3 and PH4) the hamster challenge test (Table 18), when tested against serovar Canicola, were subjected to MRM quantification. Initial analysis of the resulting chromatograms (Figure 19) showed that there was substantially less of the N terminus region of LipL32 in the failed batches than was present in the passed.

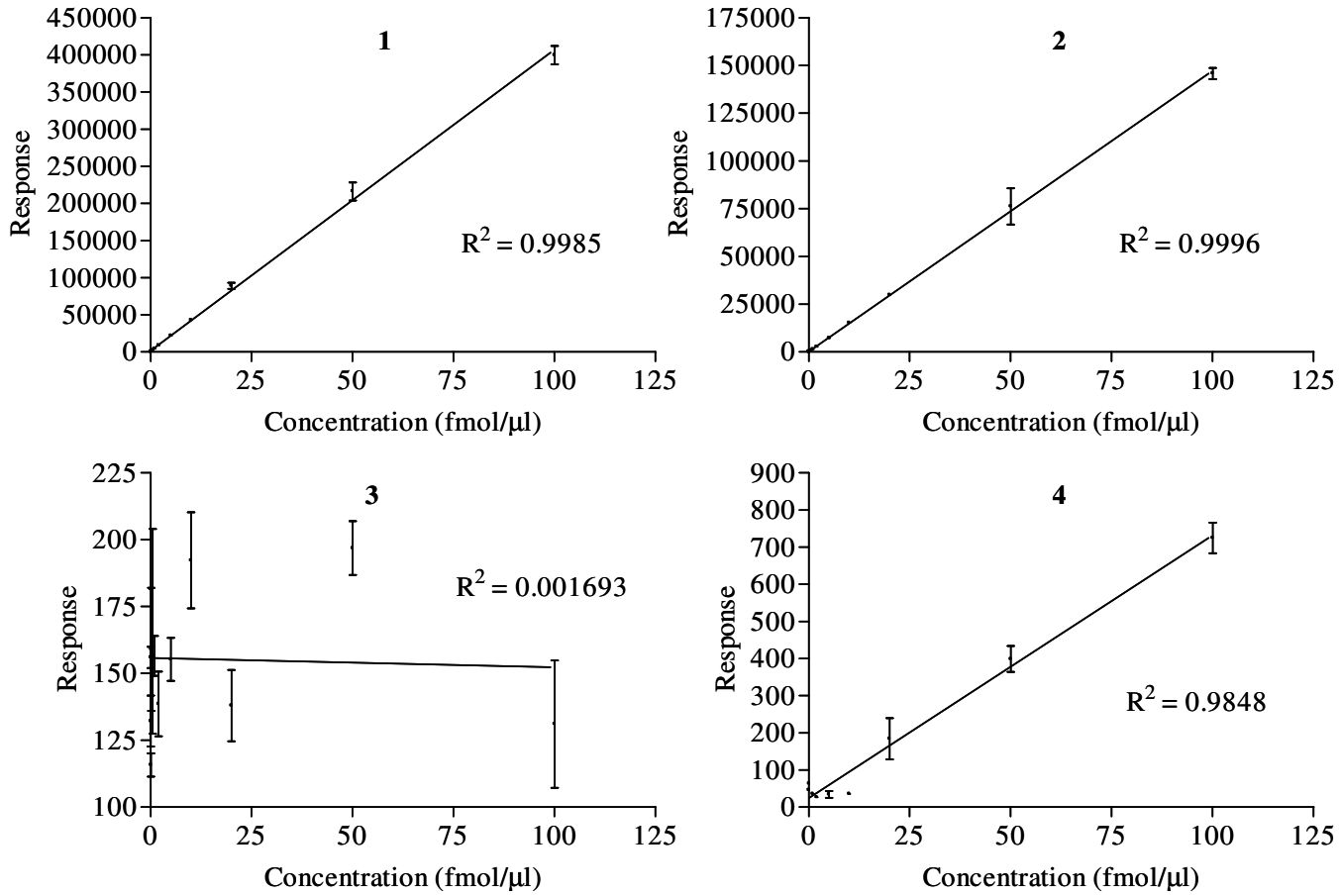

Figure 17: Linear regression of synthetic LipL32 peptides 1-4 (Table 24) standards using MRM.

Note: Two replicates of each standard were used for each concentration; mean and standard error of the mean are shown. Response is an arbitrary measure used by Masshunter (Agilent) and has no unit of measurement

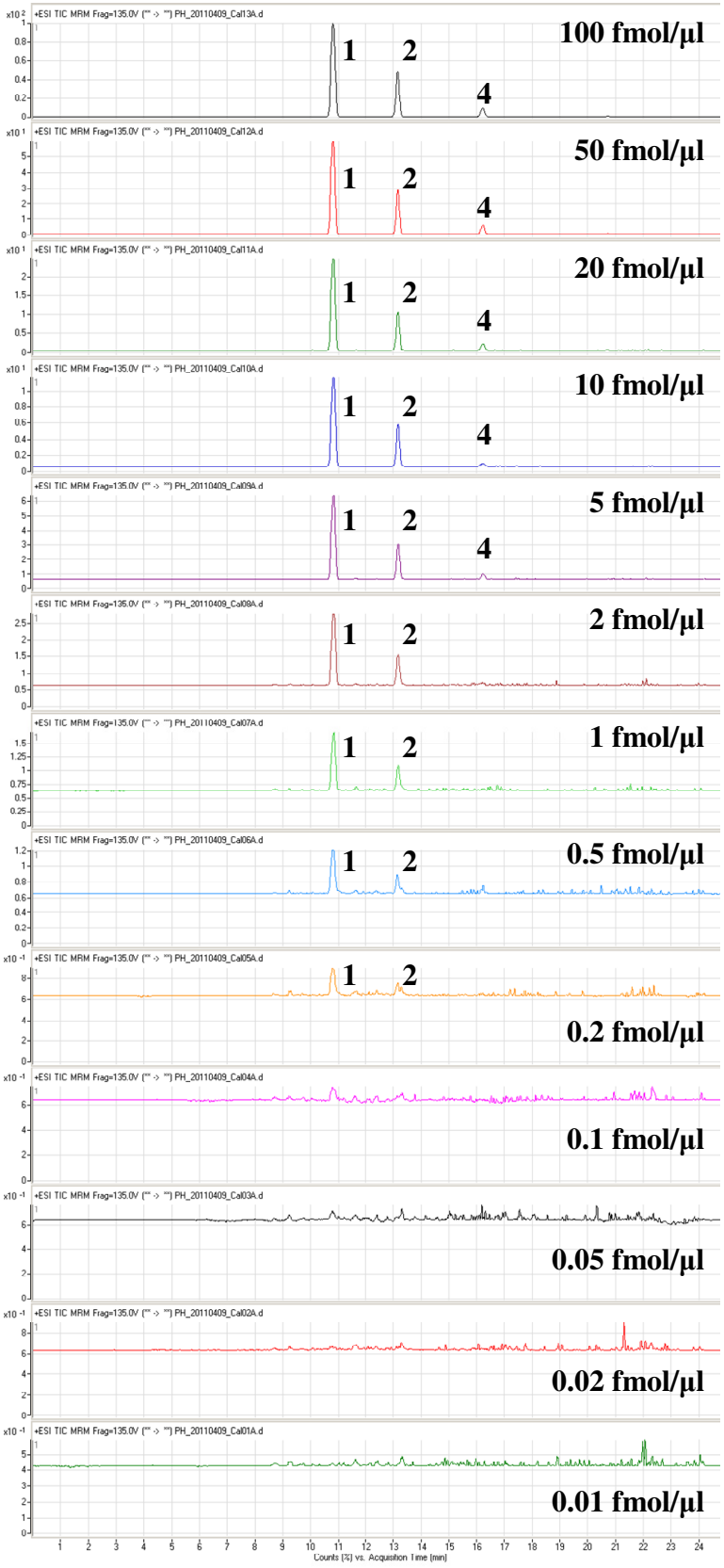

Figure 18: Total ion chromatograms (TIC) of synthetic LipL32 tryptic peptides used as calibration standards detected on a QQQ mass spectrometer.

Note: Peak numbers correspond to peptide sequences shown in Table 24.

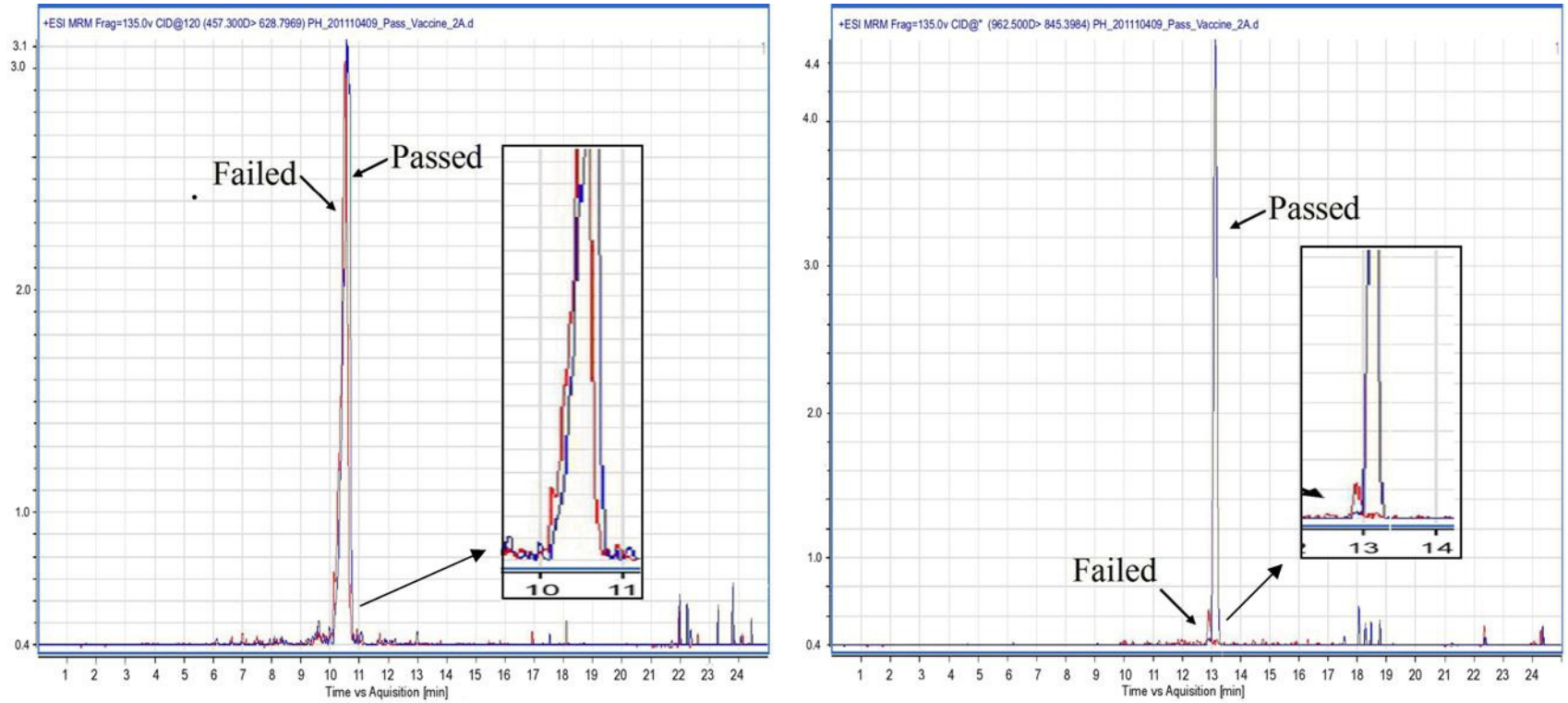

Figure 19: MRM chromatogram of passed (shown in blue) and failed (shown in red) batches of vaccine C.

Note: Transition selected on left is for the C terminus of the protein (Peptide 1; Table 24) and transition selected on right is for the N terminus (Peptide 2; Table 24).

Accurate determination of the concentration of the selected C and N terminal peptides in each vaccine batch was achieved through interpolation with the synthetic peptide calibration curve (Figure 17) using Masshunter (Agilent). The results presented herein clearly show that the concentration of the selected LipL32 N terminus peptide was significantly lower ($p \leq 0.01$) in vaccine batches that failed against serovar Canicola (n=2; Table 25) compared to the same LipL32 peptide in batches that passed against both serovars (n=2); no statistical difference was observed in the concentration of the peptide selected to represent the C terminus region. A vaccine batch that had passed against serovar Canicola but failed against Icterohaemorrhagiae (PH5) was selected to act as a control and the selected peptides were quantified for this sample (Table 25); a decrease in the concentration of the selected LipL32 N terminus peptide was not observed suggesting this to be specific to batches that fail against Canicola. Vaccines A, B, D and E were also analysed using the selected peptides to provide a point of comparison against vaccine C (Table 25); all four vaccines showed substantially decreased concentrations of the selected LipL32 N terminus peptide compared to that observed in batches PH1 and PH2 of vaccine C.

Table 25: Concentration of the C and N terminus tryptic peptides (peptides 1 and 2) of LipL32 in vaccines A-E.

Vaccine	Vaccine Concentration (fmol/µg); Mean ± 1 SD)		P Value
	C Terminus	N Terminus	
A	26.04 ± 5.56	0.10 ± 0.02	0.0149
B	39.40 ± 5.86	0.18 ± 0.01	0.0073
C- PH1*	1.14 ± 0.07	1.73 ± 0.71	0.2989
C- PH2*	0.62 ± 0.12	0.83 ± 0.19	0.1043
C- PH3	0.59 ± 0.06	0.06 ± 0.01	0.0051
C- PH4	1.00 ± 0.02	0.10 ± 0.01	0.0001
C- PH5	0.98 ± 0.08	2.27 ± 0.23	0.0103
D	2.43 ± 0.12	0.04 ± 0.02	0.0009
E	30.31 ± 1.99	0.12 ± 0.03	0.0015

Note: Three replicates of each batch were analysed. Passed batches denoted by *

4.3 Discussion

Proteomic analysis of serovar Canicola identified 1015 *Leptospira* proteins when searched against a serovar Copenhageni database; this represents a substantial improvement in coverage over the serovar Copenhageni and Pomona proteomes previously determined by Eshgi et al (2008) and Vieira et al (2009) respectively (Table 17). Whilst the studies performed by Malmstrom (2009), Cao (2010) and Zhong (2011) all identified a larger number of proteins (Table 17) it is important to note that all of these studies benefited from a species specific protein database which is currently unavailable for serovar Canicola; in addition the studies by Malmstrom (2009) and Cao (2010) did not report the use of biological replicates. Annotation of the serovar Canicola proteome (Figure 15) revealed that the proteins identified were representative of a large range of biological functions indicating that protein extraction (and resulting analysis) had not selectively favoured specific cellular components. More importantly 2D-LC-MS/MS analysis of serovar Canicola revealed which peptides, corresponding to *Leptospira* proteins, could be identified using this technique allowing a subsequent comparison (Table 22) which was used when choosing which peptides to quantify in the vaccines using MRM.

Initial estimation of the protein abundance of conserved proteins in passed and failed batches of vaccine C were performed using spectral counting (NSAF). The results achieved indicated that two proteins, LipL32 and Histidine Kinase Sensor protein, were present at a statistically ($p \leq 0.05$) higher relative abundance in the passed batches compared to the failed (Table 21). The low spectral abundance (NSAF score) of LipL32 in failed batches of vaccine C and the previously identified conservation of this protein in all vaccines tested (A-E; Chapter 3) suggested that this would be a good candidate protein for further investigation using a more quantitative approach (Ong and Mann,

2005); conversely the absence of Histidine Kinase Sensor Protein from the protein consensus of vaccines A-E (Chapter 3) suggest it to be a poor biomarker and so was excluded from further analysis. Multiple reaction monitoring (MRM), which quantitates proteins based on the mass detection of specific peptide sequences, was chosen as a quantification method due to its high sensitivity and specificity (Lange et al., 2008) and to ensure good coverage of LipL32 two peptide sequences, corresponding to the start (N terminus) and end (C terminus) of the protein, were selected for quantitation (section 2.5.5). The concentration of the C terminus peptides was approximately the same across PH1-PH4 ($p \leq 0.7$; Table 25); in contrast the concentration of the N terminus peptide was substantially lower ($p \leq 0.01$; Table 25) in batches that failed against serovar Canicola (PH3-PH4). This may be an indication that alteration of the LipL32 N terminus has taken place in batches of vaccine C that failed the *in vivo* potency test (when challenged against Canicola), the result of which is a detectable change in abundance of a specific N terminal peptide; whereas the C terminal peptide (extracted and quantified in the same experiment) remains relatively unchanged between sample sets. Interestingly, no decrease in the concentration of the same N terminal LipL32 peptide was observed in a vaccine batch (PH5) that failed when challenged against serovar Icterohaemorrhagiae (but passed against Canicola). While this suggests that the LipL32 N terminal truncation occurs in a serovar specific manner a far larger sample cohort is required to gain statistically relevant confirmation of these findings. However it is plausible to suggest that N terminal quantification of LipL32 could be used as a specific potency test for vaccine C.

It is interesting to note that substantially decreased concentrations of the N terminal LipL32 peptide, compared to the C terminal LipL32 peptide ($p \leq 0.01$), were observed in vaccines A, B, D and E. However failed batches of these vaccines were not available for

analysis, as the manufacturers were unwilling to release them due to their commercial sensitivity, making it impossible to do a proper comparison of the N and C terminal LipL32 peptides from different vaccines and batches. It is conceivable that lower levels of the N terminal LipL32 peptide in these vaccines is sufficient to elicit an immune response due to the presence of more efficacious adjuvants; likewise it is possible that other components of these vaccines (such as protein or LPS) could be having an immunostimulatory effect. Further analysis of passed and failed batches of these vaccines is required to determine if N terminal quantification of LipL32 could be applied to them as an *in vitro* potency test.

In contrast to the results achieved herein, a previous study (Hauk et al., 2008) identified the primary immunogenic domain of LipL32 as its C terminus region. Using a recombinant protein Hauk et al showed that a representative peptide of the N terminus of LipL32 did not react against antibodies raised in mice or humans (the N and C terminus regions used by Hauk et al encompass the N and C terminal LipL32 peptides used for quantification in this study). However there is insufficient evidence given that the mice and human sera contained protective antibodies against *Leptospira* as the mice used were not challenged with *Leptospira* after inoculation and there is no mention of the humans being vaccinated or having recovered without medical intervention (i.e. antibiotics). Therefore the study by Hauk et al may not accurately represent how LipL32 is recognised *in vivo* explaining the disparity found with this work. Interestingly two synthetic LipL32 peptides (AAKAKPVQKLDDDDDGDDTYKEERHNK and LTRIKIPNPPKSFDDLKNIDTKKL) have been previously identified (Lottersberger et al., 2009) as being highly immunogenic against serovars Copenhageni and Icterohaemorrhagiae. However as these sequences are located between the N and C LipL32 peptides used for quantitation in this study, and the additional two LipL32

peptides assessed (AYYLYVWIPAVIAEMGVR and SMPHWFDTWIR) could not be quantitated, it is impossible to ascertain if they were present in the failed batches of vaccines.

It is also conceivable that the role of the N terminus in stimulating protective antibodies is more indirect and consequently it may not be able to stimulate an immune response on its own. The crystal structure of LipL32 (Vivian et al., 2009) reveals that the N terminus region in question forms a β- hairpin structure which, when viewed in three dimensions, appears to wrap around the middle region of the protein. It is possible that the presence of the N terminus region of LipL32 confers conformational stability to the structure of the whole protein such that the antibody representing motif is recognised by the immune system resulting in the generation of protective antibodies. Thus any disruption of the LipL32 tertiary structure would impact on the ability of the protein to elicit an immune response preventing the generation of protective antibodies, as seen in batches that fail against serovar Canicola.

In conclusion this study has used 2D-LC-MS/MS, in conjunction with spectral counting, to identify LipL32 as a potential biomarker for potency in serovar Canicola vaccines. Further it has been shown that MRM based quantitation of specific N terminal peptides has the potential to act as a novel assay target for the determination of potency *in vitro*. Thorough detailed validation of this assay will require access to a larger sample cohort of passed and failed batches from multiple manufacturers to determine if it can replace the hamster challenge batch potency test.

Chapter 5 Evaluation of the immunoprotective effects of LipL32 against *Leptospira interrogans* serovar Canicola in Hamsters

5.1 Introduction

Our previous studies (Chapter 4) proposed that N terminal quantitation of LipL32 could be an effective *in vitro* biomarker assay for the determination of vaccine potency. However due to the commercially sensitive nature of the vaccines further passed and failed batches were unavailable, preventing more thorough validation of this assay. This chapter focuses on the evaluation of the protective effects of LipL32 (purified from a recombinant *E. Coli* expression system; section 2.6.5) against *Leptospira interrogans* serovar Canicola in the hamster model to determine if LipL32 is a suitable vaccine candidate. The primary aim was to test the hypothesis that LipL32 is a suitable vaccine candidate. This would be an important step in establishing if the shortening of LipL32 observed in failed vaccine batches (Chapter 4) was responsible for the inability of the failed vaccines to confer immunological protection following challenge with *L. interrogans* serovar Canicola.

5.2 Results

5.2.1 Genetic analysis of LipL32 from *Leptospira interrogans* serovar Canicola strain Kito

A highly virulent strain (Silva et al., 2008) of *Leptospira interrogans* serovar Canicola (strain Kito) was chosen as the challenge strain in this study. The gene sequence of LipL32, derived using DNA from strain Kito, was therefore determined (section 2.6.3) to ascertain commonalities with other serovar Canicola strains in the literature. The

resulting nucleotide sequence was translated into an amino acid sequence using Lasergene (DNASTAR Inc, USA) and compared (section 2.6.3) against three complete LipL32 sequences, from strains Hond Utrecht IV (GI: 33589193), RTCC 2805 (GI: 358357257) and Lin (GI: 48526297) (Figure 20).

The *in silico* translated amino acid sequence of strain Kito was identical to strain Lin (Figure 20). The sequence was highly conserved (compared to strains Kito and Lin) across strains RTCC 2805 and Hond Utrecht IV, each having only one amino acid substitution (at positions 73 and 165 respectively). The substitution of the amino acid valine for alanine, in strain RTCC 2805, is unlikely to represent a major structural change in the protein due to the shared chemical structure and properties of the two amino acid residues (both being non polar aliphatic compounds). The substitution of aspartic acid for asparagine in strain Hond Utrecht IV represents a similarly minor chemical change although a change in amino acid charge state would also occur.

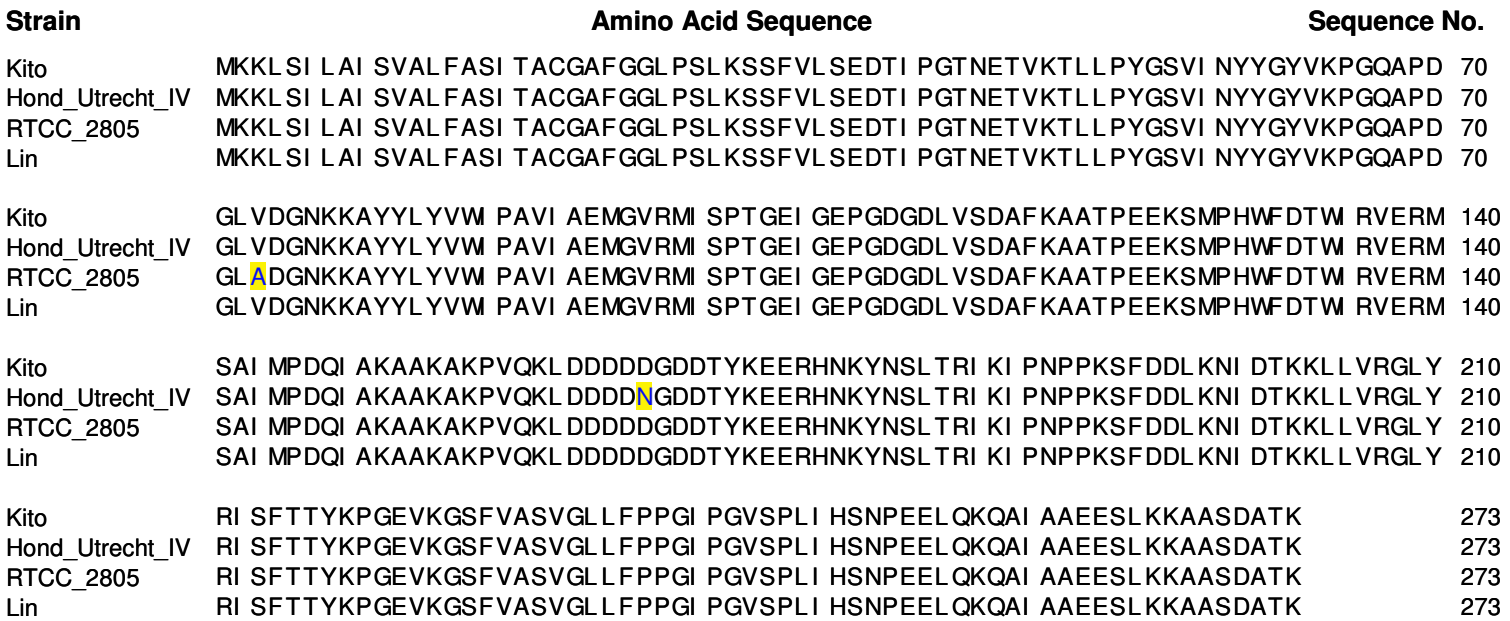

Figure 20: Sequence comparison of LipL32 from serovar Canicola strain Kito (characterised in this study) with LipL32 genes from other serovar Canicola strains found in the literature.

Note: Amino acid sequences shown are predicted from the nucleotide sequence; differences are highlighted in yellow.

5.2.2 Construction of a recombinant *E. coli* strain to express LipL32 protein

The LipL32 gene from serovar Canicola strain Kito was subsequently cloned (section 2.6.4) into a pRSET C vector, downstream from a polyhistidine tag and T7 expression promotor (Figure 21).

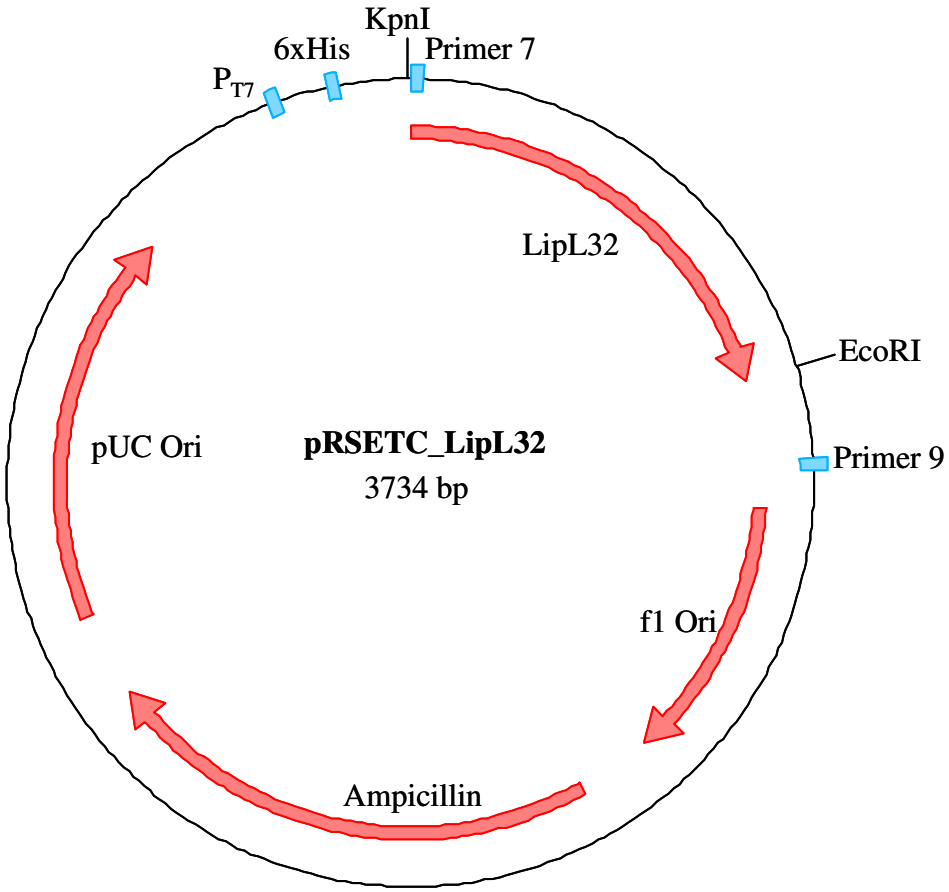

Figure 21: Plasmid schematic of pRSET C following insertion of LipL32 gene.

Note: Promotor (T7), Polyhistidine tag (6xHis) and primers used to confirm insertion shown in blue; KpnI and EcoRI restriction sites used to insert LipL32 gene are also shown.

Transformation into the *E. coli* expression strain BL21(DE3)pLysS (containing a chloramphenicol resistance gene) of the resultant recombinant vector, termed pRSETC_LipL32, was achieved chemically (section 2.6.4) resulting in an IPTG inducible expression strain. Successful transformants were screened using selective media (containing 50 µg/ml ampicillin and 35 µg/ml chloramphenicol; section 2.3.2) and polymerase chain reaction (PCR) was used to confirm the presence and orientation of the LipL32 gene within the strain (Figure 22).

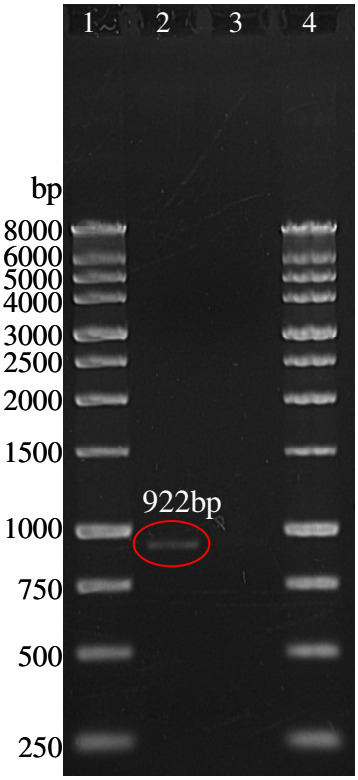

Figure 22: PCR of recombinant LipL32 expression strain (Lane 2) using primers 7 and 9 (Table 7) run on a 1% agarose gel.

Note: Lanes 1 and 4 contain a 1Kb ladder and lane 3 contains a negative control reaction without DNA.

Primers 7 and 9 (Table 7) were selected for PCR as they bind to the LipL32 gene (insert) and pRSET C (vector) respectively; a band approximately corresponding to the

predicted PCR product (922 base pairs) was observed following gel electrophoresis (Figure 22).

5.2.3 Purification of expressed LipL32 protein

Expression of LipL32 from the recombinant pRSETC_LipL32 *E. coli* strain was induced using IPTG (2 mM; section 2.6.5) and the resulting His tagged LipL32 protein was purified using nickel magnetic beads (section 2.6.5). The total protein contents of the purified LipL32 protein, and a passed vaccine (Vaccine F; positive control for subsequent *in vivo* experiments), were estimated using the Bradford assay and a nanodrop (280 nm; section 2.3.7) respectively; assay derived differences in protein concentration did not affect the final *in vivo* experiment as all samples were subjected to MRM quantitation prior to use. The LPS content of the purified protein and passed vaccine was determined using the LAL assay (Table 26) as previously described (section 2.4.3).

Table 26: Concentration of LipL32 (N and C termini using MRM), total protein and LPS in recombinant LipL32 protein and Vaccine F.

	Concentration (Mean ± 1 SD)		**Concentration of LipL32 (fmol/µg); Mean ± 1 SD**	
	Protein (mg/ml)	**LPS (µg/ml)**	**C Terminus**	**N Terminus**
Vaccine F	9.69±0.40	0.01±0.00	1.07±0.22	2.65±1.09
LipL32	21.75±0.49	0.47±0.01	3096.17±1449.91	7130.33±2649.79

As QQQ mass spectrometers are known to be more accurate, compared to a Q-ToF, for the analysis of small molecules (Wolf-Yadlin et al., 2007), MRM mass spectrometry was used to analyse (section 2.5.5) the C and N termini of LipL32 in the recombinant protein and passed vaccine. Since the selected peptides, and their MRM transitions, are specific to LipL32 this allowed accurate identification and quantitation of the protein in

a single experiment. Gel electrophoresis (Figure 23) of the purified LipL32 protein (10µg; 71.3 pmol, N terminus) with Coomassie staining, showed one band at the predicted size of mature length LipL32 (27.8 kDa). At this level of sensitivity the absence of additional bands indicated that, within the detection range of the stain (≥ 5 ng of protein; Fisher, UK), no other proteins were detected.

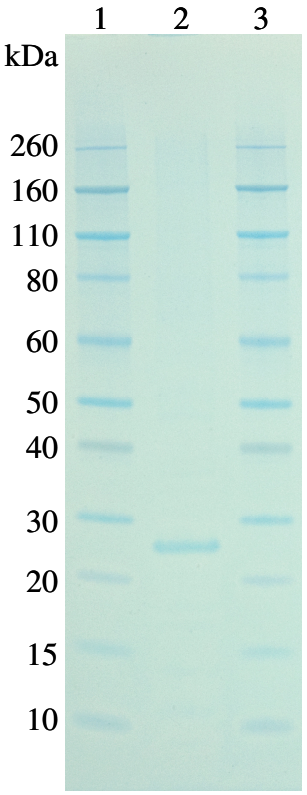

Figure 23: Purified LipL32 protein (Lane 2; 10µg) run on a 4-12% NUPAGE gel stained with Coomassie blue to detect protein.

Note: Lanes 1 and 3 contain a 3.5 - 260 kDa protein ladder.

5.2.4 Assessment of the protective effect of recombinant LipL32 in the hamster vaccine batch potency test model

The hamster vaccine batch potency test (section 2.4.2) was used to determine the protective effect of recombinant LipL32 against *L. interrogans* serovar Canicola. Six groups of hamsters (n=5/group) were used, each receiving a different treatment (Table

27); group 6, as a negative control, comprised of less hamsters (n=3) to reduce animal use. Treatment groups 1 and 2 comprised of recombinant LipL32 +/- adjuvant respectively to explore the potential adjuvant effects on efficacy; each group (1 and 2) received 868.43 pmol/hamster of N terminal LipL32 (section 2.6.5). Group 3, the positive control, comprised of a potent vaccine (vaccine F; Table 27), diluted 1/40 with saline (as described in the details for the potency test, section 2.4.2) representative of a final N terminal LipL32 concentration of 0.64 pmol/hamster. Groups 4-6 (Table 27) comprised of negative controls to confirm, respectively, that the saline diluent used had no effect (group 4), that the challenge used was appropriately virulent (group 5) and that the stock of hamsters used were otherwise healthy (in addition to providing tissues for histological analysis; group 6; section 2.7.4).

Table 27: Treatments protocols applied to hamster groups 1-6 (n=5) and number of survivors.

Group	Treatment	Survivors	P value
1	LipL32 (no adjuvant) + challenge	1/5	1.000
2	LipL32 + adjuvant + challenge	1/5	1.000
3	Vaccine F + challenge	4/5	0.048
4	Saline + challenge	0/5	ND
5	No treatment + challenge	0/5	1.000
6	No treatment + No challenge	3/3	0.018

Note: Group 6 comprised of 3 hamsters. P values are 2 sided and obtained through comparison with the negative control (group 4) using Fisher's exact test; ND indicates not determined.

Following challenge with virulent *L. interrogans* serovar Canicola hamsters were routinely monitored and their condition assessed as described previously (section 2.4.2). Groups 1 and 2 failed the vaccine potency test on days 10 and 12 respectively with 4/5 hamsters (Figure 24) either succumbing to infection or having to be euthanased; one hamster from each group survived until the end of the test (day 24), however this was not statistically significant (Table 27). Group 3, the positive control, passed the vaccine potency test with 4/5 hamsters surviving until day 24 ($p \leq 0.05$). As expected the

negative control groups 4 and 5 failed the test, at days 11 and 10 respectively; none of these hamsters survived until the end of the test. The hamsters in group 6 survived to day 24 confirming that the hamsters were from healthy stock ($p \leq 0.05$).

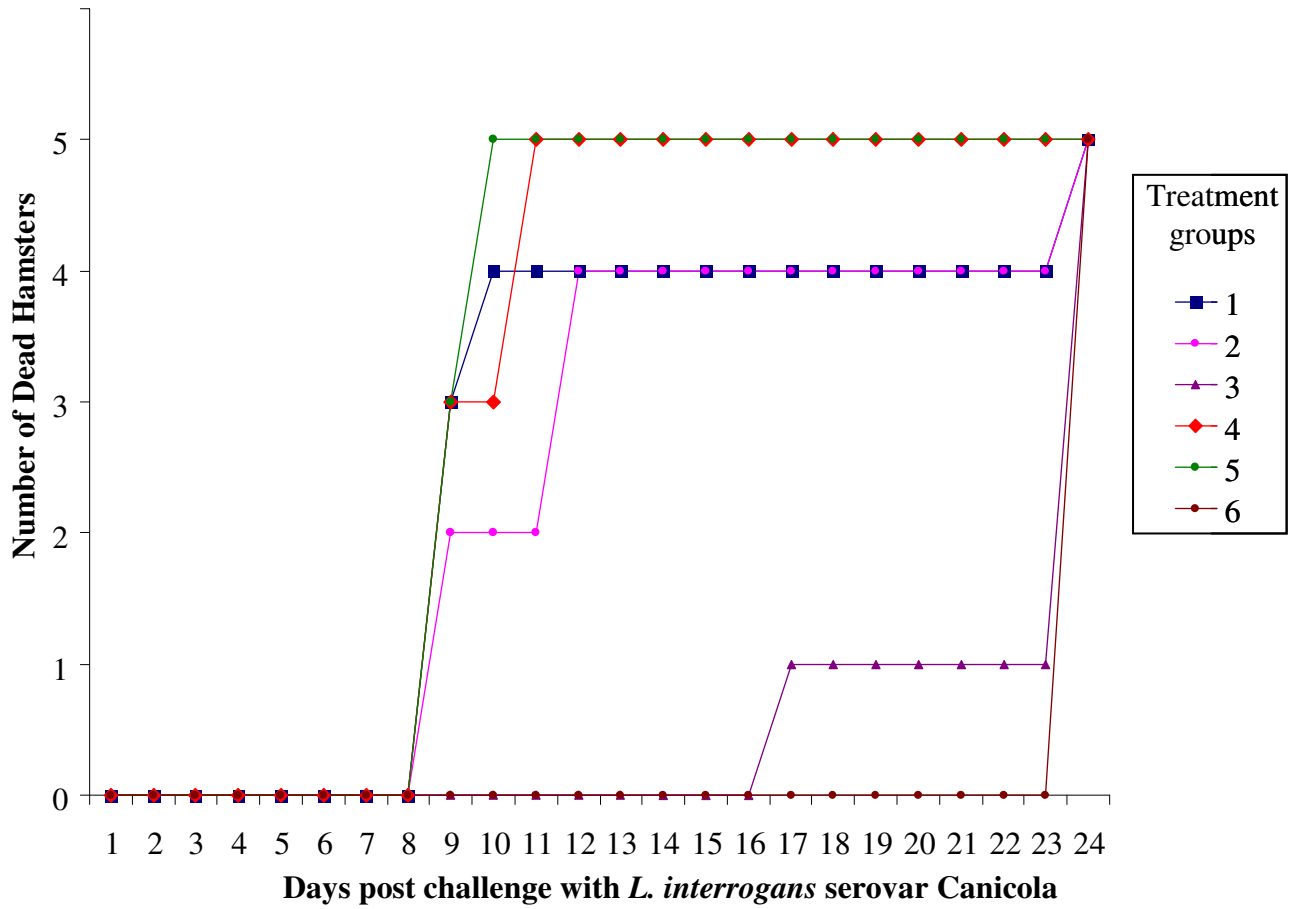

Figure 24: Survival of hamsters (days) in treatment groups 1-6 following infection with virulent *L. interrogans* serovar Canicola.

Note: Some hamsters were euthanased due to severe clinical conditions.

5.2.5 Assessment of circulating antibodies in hamsters following vaccination with recombinant LipL32

Serum taken from hamsters at post mortem (section 2.7.2) was analysed by ELISA (section 2.7.5) to determine the presence of antibodies specific to recombinant LipL32 and *L. interrogans* serovar Canicola (Figure 25). While animals were frequently monitored some hamsters succumbed to infection between monitoring points and were therefore excluded from ELISA analysis. Statistical comparison of ELISA results from groups 1-6 could therefore not be performed as serum could not be obtained from sufficient subjects of the negative controls (groups 4 and 5). Data gained from ELISA analysis was therefore not considered when assessing the effect of LipL32 *in vivo* and is only included herein to show diligence of the approach attempted.

Groups 1 and 2 displayed a higher antibody response to LipL32 than the negative control groups 4-5 (Figure 25); however a corresponding increase in response to *L. interrogans* serovar Canicola was not indicated. Group 3 displayed a higher response to both LipL32 and *L. interrogans* serovar Canicola (Figure 25), compared to groups 4 and 5. Group 6, which was not vaccinated or challenged (Figure 25), showed a higher response to LipL32 than groups 4 and 5; however this did not correspond to an increased response to *L. interrogans* serovar Canicola.

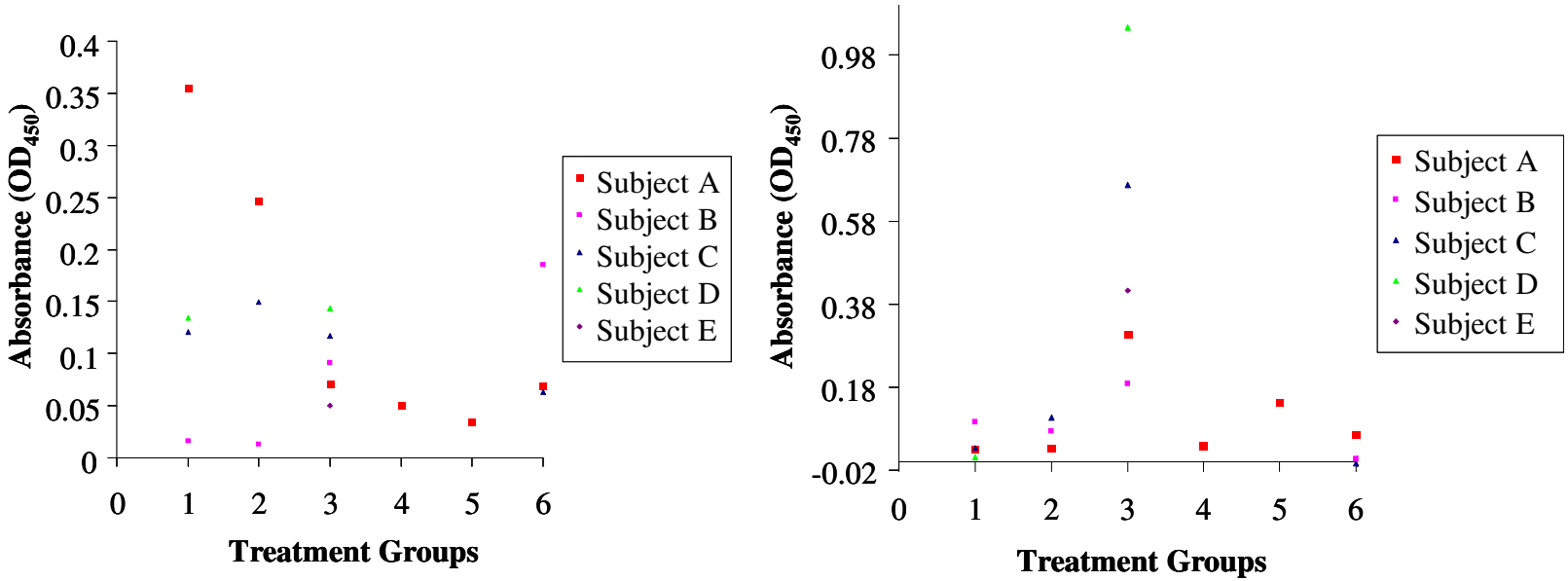

Figure 25: Antibody response of serum (1/100 dilution) derived from treatment groups 1-6 against recombinant LipL32 (left; 1 µg/ml total protein) and *L. interrogans* serovar Canicola (right; 5 µg/ml total protein).

5.2.6 Histopathological analysis of hamsters immunised with LipL32

Variable degrees of diffuse tubulointerstitial nephritis, consistent with *Leptospira* infection, were observed in the hamsters that died (or were euthanised according to the clinical score sheet; section 2.4.2) following challenge with *L. interrogans* serovar Canicola. The histological changes consisted of minimal infiltration of the interstitial spaces with lymphocytic cells and the frequent presence of strongly eosinophilic hyaline casts in the lumen of tubules, associated with attenuation of tubular epithelial cells and nephrosis. Occasional tubules displayed a mixture of sloughed cells and leukocytes in their lumen.

Renal lesions were scored (section 2.7.4) based on their severity from 0-4 (Figure 26) using a semiquantitative scoring system modified from Palaniappan *et al* (2006); one slide, containing approximately 100 nephrons, was assessed per animal. Lower scores were observed in groups 1, 3 and 6 (Table 28) compared to groups 2, 4 and 5 (Table 28); treatment group 1, which comprised LipL32 without adjuvant, had a significantly lower score ($p \leq 0.01$) compared to the negative control (group 4). Only one survivor from group 3 (euthanised on day 24) showed evidence of renal pathology as a minute focal lesion; no lesions were observed in group 6. The presence and number of leptospires in the kidney, liver and spleen was assessed using Warthin-Starry stain (Figure 27) and tissues were stratified using a semiquantitative scoring system (Table 28).

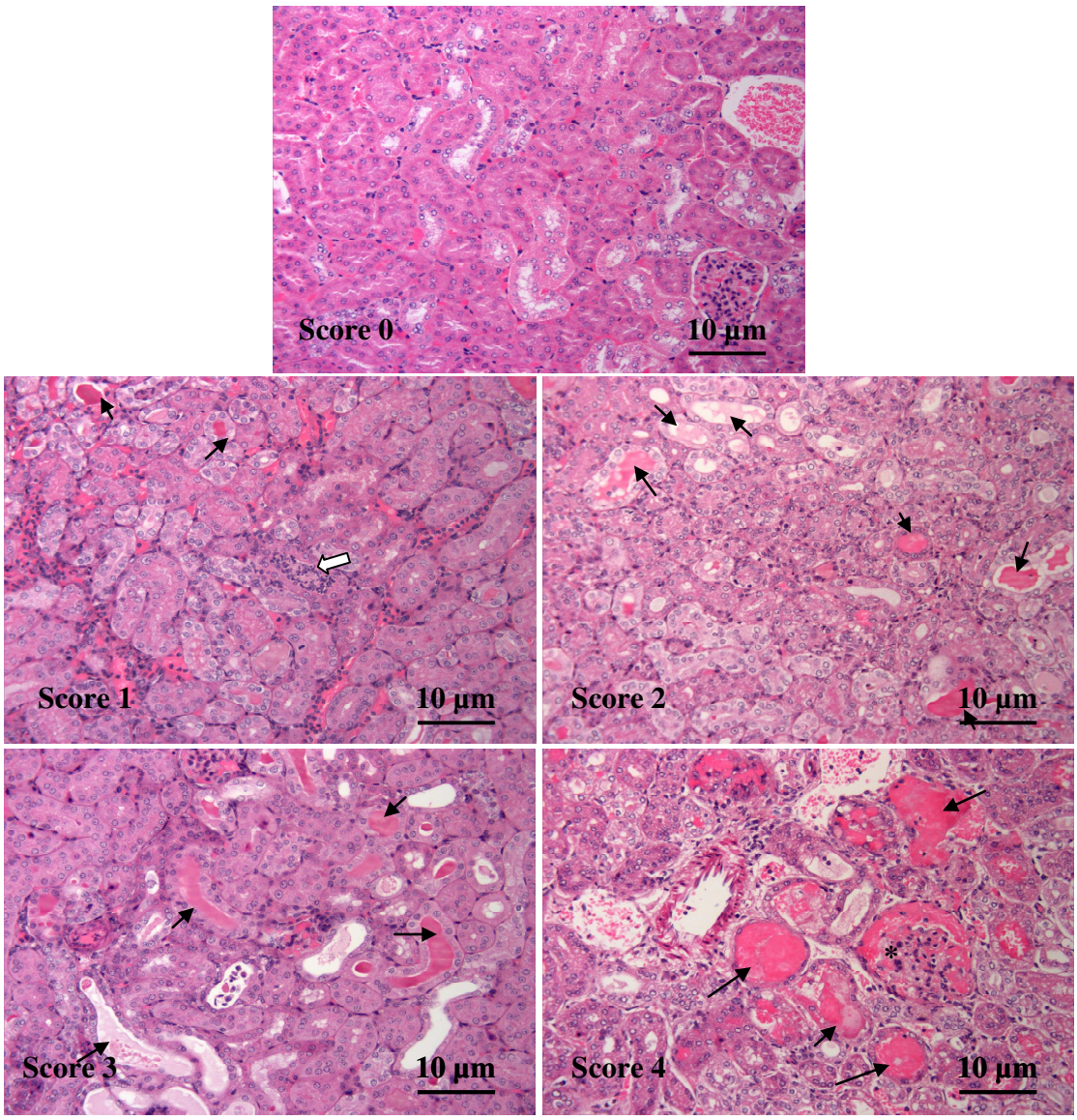

Figure 26: Scoring system used to assess major histopathological changes observed in hamster kidneys following infection with *L. interrogans* serovar Canicola.

Note: The increase in the number of tubules with eosinophilic protein casts in the lumen (black arrows) and intratubular inflammatory infiltration (white arrow); eosinophilic material is also present in the uriniferus spaces of Bowman's capsule (shown with *). Tissues stained with hematoxylin and eosin (H&E) and imaged using a 200x magnification.

Table 28: Severity of renal lesions and invasion of *Leptospira* in hamster tissues determined through staining with H&E and Warthin and Starry respectively.

Treatment Group	Severity of Renal lesions		Invasion of *Leptospira*					
			Kidney		Liver		Spleen	
	Mean ± 1 SD	P Value	Mean ± 1 SD	P Value	Mean ± 1 SD	P Value	Mean ± 1 SD	P Value
1	1.8±1.1	0.004	1.2±0.8	0.009	2.4±1.3	0.070	1.0±1.0	0.621
2	2.6±1.5	0.374	2.0±1.4	0.189	1.6±1.8	0.034	1.0±1.0	0.621
3	0.6±0.9	0.001	0.0±0.0	ND	0.0±0.0	ND	0.0±0.0	ND
4	3.0±0.7	ND	3.0±0.0	ND	3.6±0.5	ND	1.2±0.45	ND
5	2.6±0.5	0.178	2.8±0.4	0.374	2.8±1.6	0.242	1.2±0.84	1.000
6	0.0±0.0	ND	0.0±0.0	ND	0.0±0.0	ND	0.0±0.0	ND

Note: Scored using a semiquantitative scoring system modified from Palaniappan et al 2006 (Palaniappan et al., 2006). Mean and standard deviation of the mean for the observed scores are shown. P values obtained through comparison with the negative control (group 4) using a Student's t-test; ND indicates not determined.

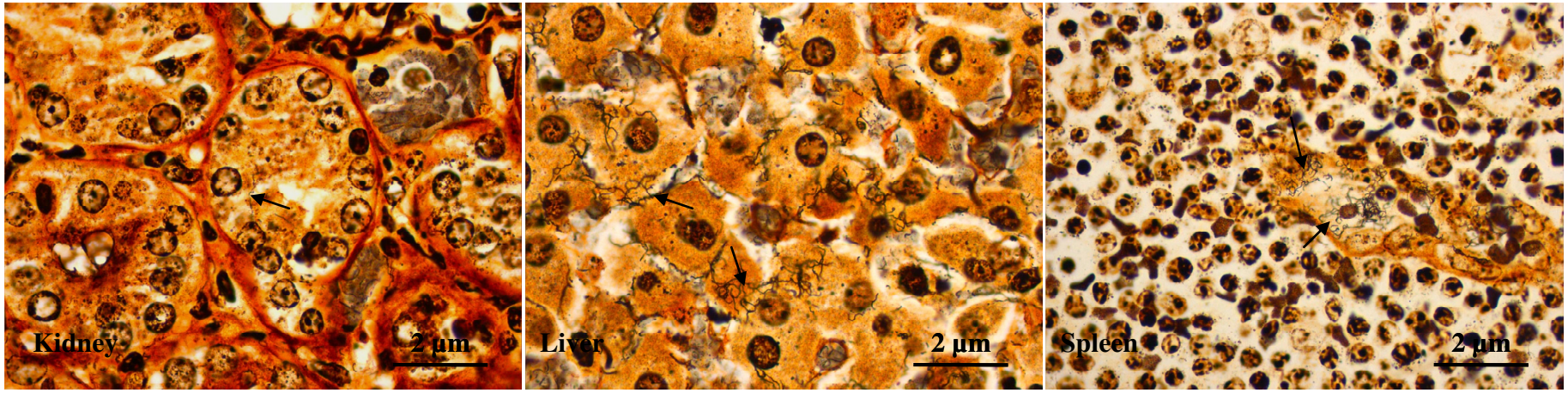

Figure 27: Visualisation of Leptospires in infected hamster tissues.

Note: Leptospires are highlighted with black arrows. Tissues stained with Warthin and Starry and imaged using a 1000x magnification.

Leptospires, when present in the kidneys, could be observed in the interstitial spaces and tubular lumina in the renal cortex and occasionally in intravascular locations or glomeruli. A significantly lower ($p \leq 0.01$) score for *Leptospira* kidney invasion was observed (Table 28) in group 1 compared to the negative control (group 4); no leptospires were observed in groups 3 or 6.

In addition to histological analysis of hamster kidneys at post mortem, culturing was also performed (section 2.3.2). Leptospires were not observed in kidney cultures of animals euthanased at day 24 (Figure 24; groups 1-3, 6), which is in agreement with the histological findings. No leptospires were observed, by histological staining, in the kidneys of the hamster from group 3 that died at day 17 (Figure 24); however confirmatory data could not be obtained for this animal using kidney culturing due to the detection of bacterial contamination during processing.

All hamsters that died (or had to be euthanased) following challenge with *Leptospira* displayed hepatodystrophy and liver plate disarray, with loss of the normal hepatic sinusoid architecture and multifocal infiltration by lymphohistiocytic cells. Some rare areas of minute necrosis were observed in two animals from group 4; no hepatic lesions were observed in the hamster from group 3 that died at day 17. Groups 1 and 2 showed reduced liver invasion scores (2.4 ± 1.3 and 1.6 ± 1.8 respectively; Table 28) compared to group 4 (3.6 ± 0.5) however only group 2 showed a significant ($p \leq 0.05$) difference. An example of the histophathological effects of *Leptospira* on hamster livers is shown in figure 28, where loss of normal structure of the tissue, disorganization of the hepatic cords and altered morphology/size of hepatocytes can be seen in the diseased state.

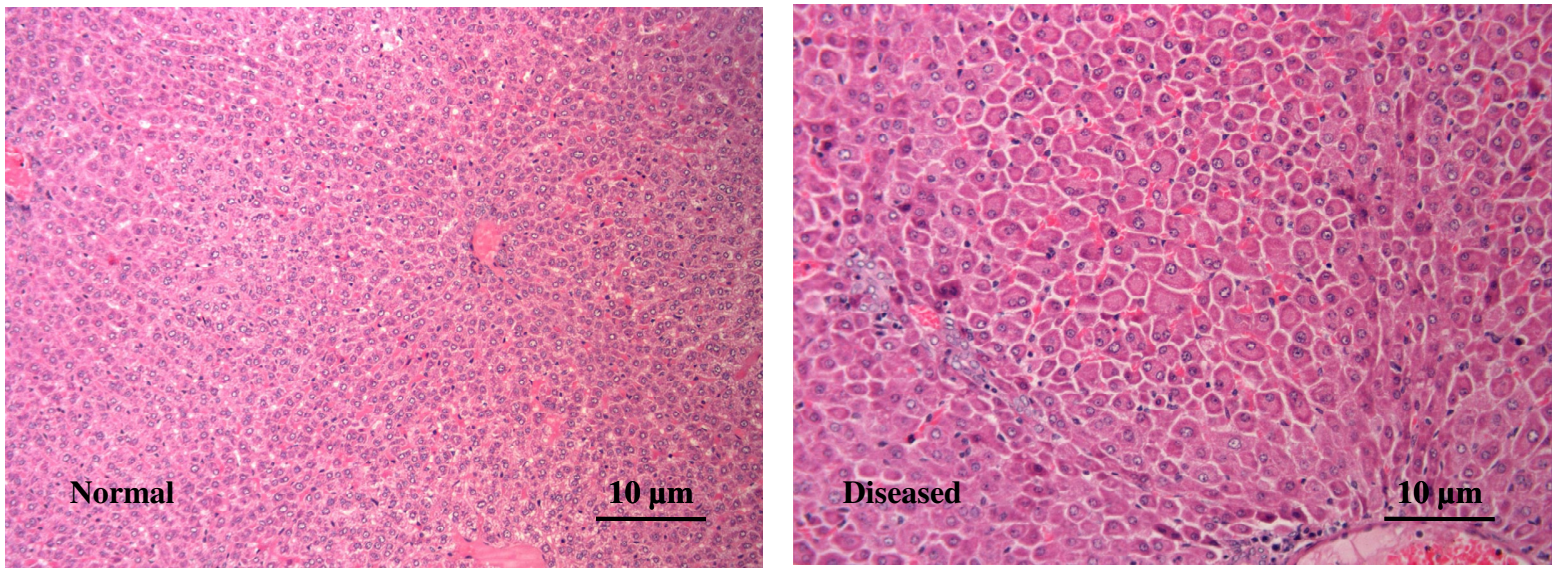

Figure 28: Histopathological changes to hamster liver following infection with *Leptospira*.

Note: Tissues stained with hematoxylin and eosin (H&E) and imaged using a 200x magnification.

The spleen of animals that died (or had to be euthanased), following challenge with *Leptospira*, showed marked hypertrophy and hyperplasia of macrophages of splenic cords in red pulp in the diseased state (Figure 29). Very few leptospires could be observed in the red pulp of hamsters showing splenic pathology; no significant difference in splenic invasion was observed between groups (Table 28).

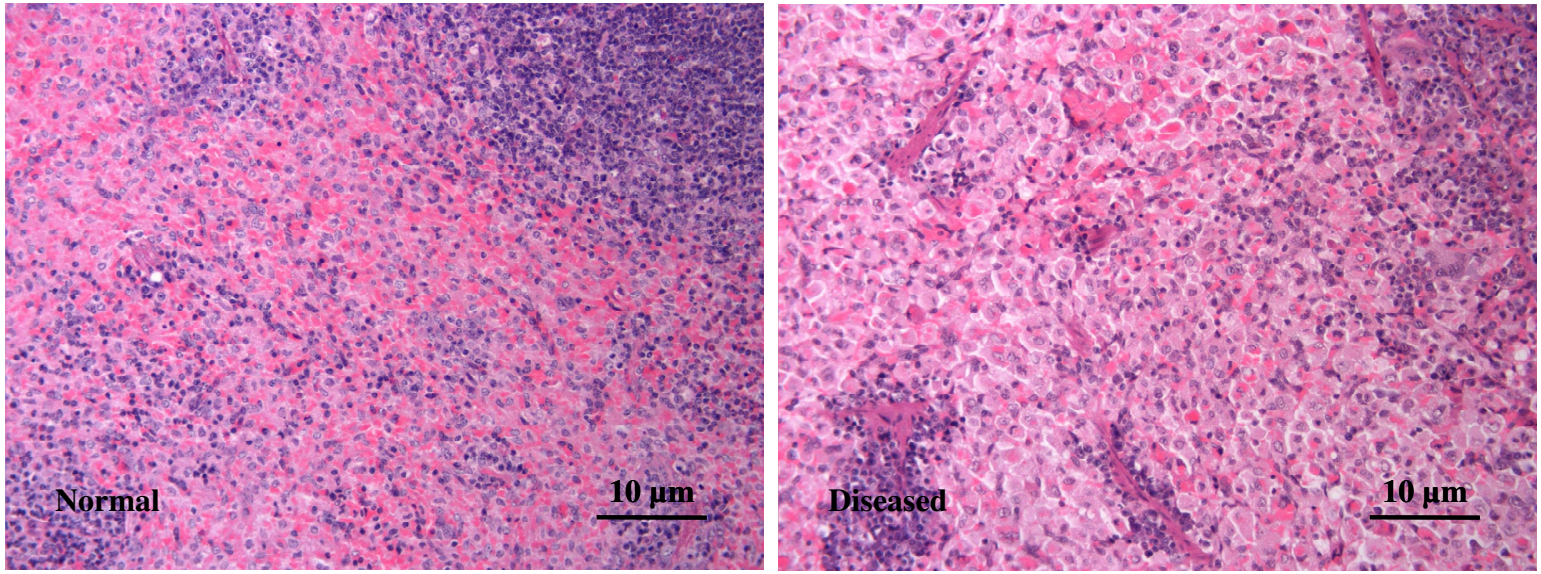

Figure 29: Histopathological changes to hamster spleen following infection with *Leptospira*.

Note: Tissues stained with hematoxylin and eosin (H&E) and imaged using a 200x magnification.

5.3 Discussion

Construction of a recombinant *E. coli* strain to express LipL32 was performed using a variation of the method developed by Haake (Haake et al., 2000). As noted by Haake processing of the LipL32 protein does not occur properly in *E. coli* therefore the first 19 amino acids, the signal peptide, was deliberately excluded from the recombinant strain created. Since the signal peptide is cleaved during processing of the protein (Haake et al., 2000) its absence from the recombinant strain is unlikely to have an effect on the resulting conformation of the protein, although conformation may be different compared with *Leptospira* derived LipL32; further confirmatory work is required to ascertain the exact conformation of *E. coli* derived LipL32. Glycosylation has been previously established to have no effect on the immunogenicity of LipL32 (Hartwig et al., 2010) therefore the effect of protein glycosylation was not investigated herein.

Although *E. coli* protein contaminants were not observed in the purified LipL32 protein, it is probable that some were present at levels below the detection limit of SDS-PAGE. However histological analysis of the recombinant LipL32 treated hamsters clearly established Leptospirosis as the cause of death, therefore any *E. coli* proteins present are not likely to have contributed significantly to the disease process. Similarly it can also be assumed that the comparatively high level of LPS (compared to vaccine F) observed in the recombinant LipL32 had no detrimental effects. The presence of *E. coli* proteins and/or LPS could however be providing an immunostimulatory effect, in a similar manner to an adjuvant. Whilst no evidence exists to support this assertion it should be taken into account when planning future experiments and additional controls used to discount it as a contributing factor.

As the vaccine batch potency test is the only test currently recognised for the assessment of vaccines against *L. interrogans* serovar Canicola it was used to determine the protective effect of LipL32. Insufficient quantities of vaccines A-E (analysed in chapters 3 and 4) were available at the time of performing this test therefore an additional passed vaccine (F) was obtained and utilised solely for the purpose of a positive control. The N terminal concentration of LipL32 in groups 1 and 2 was deliberately in excess of that calculated in vaccine F (group 3) to ensure a response; in depth experimental analysis with multiple concentrations of LipL32 was precluded by funding and time constraints. The precise constituents of each vaccine (A-F) were not revealed by manufacturers due to the commercial considerations, therefore the potential beneficial effects of specific adjuvants could not be tested alongside the recombinant LipL32 used in this study; however the adjuvant used in vaccine B, aluminium hydroxide, was accepted as a proxy for all testing.

The recombinant LipL32 used in this study did not show statistically increased survival against *L. interrogans* serovar Canicola which is in agreement with a previous study (Lucas et al., 2011). Subsequent antibody analysis of the hamster's blood, using ELISAs, was largely inconclusive as limited samples precluded in depth statistical analysis. However the response of groups 1 and 2 against LipL32 was predominantly either equal to or in excess of the response observed in group 3 confirming that an equivalent dosage of LipL32 had been administered. A decreased score of kidney invasion ($p \leq 0.01$) was observed in the groups treated with LipL32 (group 1) which corresponded to decreased scores of kidney lesions ($p \leq 0.01$); indicating that although LipL32 is unable to provide complete protection, it is still able to reduce the severity of infection in the hamsters. This is not in accord with Lucas *et al* (Lucas et al., 2011) who concluded that LipL32 in isolation was unable to stimulate protective immunity in

hamsters. However histological analysis was not performed in this study which may explain the discrepancy; it should also be noted that serovar Manilae was assessed in their study, instead of serovar Canicola.

Interestingly group 2, which received adjuvant in conjunction with LipL32, did not show significantly decreased kidney invasion/lesions when compared with the negative control (group 4). It is impossible to definitively explain this without a larger study however it is conceivable that aluminium hydroxide is either not suitable for presentation of a single protein against *L. interrogans* serovar Canicola or requires a larger dosage to be noticeably effective. As the concentration of LipL32 in groups 1 and 3 was approximately the same it is reasonable to assume that group 3 either contains additional components required to initiate protective immunity, or possesses an increased immunostimulatory effect (either through the use of an adjuvant or other naturally occurring bacterial components). A recent study (Grassmann et al., 2012) demonstrated that LipL32 could provide protective immunity against serovar Copenhageni in hamsters when coadministered with the B subunit of *E. coli* heat-labile enterotoxin (LTB) as an adjuvant. It is conceivable therefore that the immunogenic effect of LipL32 against serovar Canicola will also be increased through using LTB as an adjuvant. This chapter suggests that LipL32 may be the active component of the vaccines studied which would suggest that the approach for vaccine analysis used herein has successfully identified a potential potency biomarker for an *in vitro* test, confirming the main hypothesis of this study; further it would also suggest that LipL32 may be a suitable vaccine candidate confirming the chapter hypothesis. A larger study using a range of LipL32 concentrations, in conjunction with a range of adjuvants, is suggested to fully elucidate the role of LipL32 in the vaccines. In addition further hamster studies should consider increasing the number of hamsters used from five hamsters per group to

ten as it would increase the 80% power of the test to detect differences between the groups.

Chapter 6 General Discussion

The hypothesis of this study was to determine if biochemical analysis could be used to identify and quantify the common component(s) of commercially available *L. interrogans* serovar Canicola vaccines. The primary objective of which was to identify biomarker(s) of efficacy, present in *L. interrogans* serovar Canicola vaccines, which may be suitable for the development of an *in vitro* vaccine batch potency test as an alternative to the current hamster challenge test. The initial approach investigated the potential for a LPS based biomarker, as it had been previously reported to be a protective immunogen (Koizumi and Watanabe, 2003) and to have potential applications as a vaccine candidate (Wang et al., 2007, Srikram et al., 2011). Further an ELISA based test of serovar Canicola vaccines had been previously developed using LPS specific monoclonal antibodies (Ruby, 1999). However our experimental findings (Chapter 3) showed that LPS was not present at detectable levels in vaccine C. This suggested that biomarkers derived from this bacterial component would (at this level of sensitivity) be unsuitable for the development of a universally applicable *in vitro* potency test. Proteins derived from *L. interrogans* have also been reported to be immunogenic (Sonrier et al., 2000) and a number of protein based vaccines have previously been tested (Seixas et al., 2007a, Yan et al., 2009, Yan et al., 2010). The potential application of *Leptospira* derived proteins as biomarker(s) of efficacy for serovar Canicola vaccines was therefore also investigated. A novel method for the characterisation of the protein content of serovar Canicola vaccines, using 2D-LC/MS, was therefore developed and used to analyse the same five commercially available serovar Canicola vaccines (Chapter 3).

Although the use of proteomics for the analysis of *L. interrogans* serovar Canicola vaccines has not been previously reported in the literature, proteomic based approaches

for the analysis of non-*Leptospira* vaccines, such as meningococcal outer membrane vesicle (Vipond et al., 2005), *Actinobacillus pleuropneumoniae* (Buettner et al., 2011) and influenza vaccines (Creskey et al., 2010) have been described. Many proteomic vaccine studies, such as those performed for *Brucella melitensis* (Eschenbrenner et al., 2002) and *Neisseria meningitidis* (Uli et al., 2006), characterise the bacterial strain used to create the vaccine rather than the final fully formulated product used in the host. This approach is aided by the absence of interfering agents, present in the final vaccine formulation, such as adjuvant and preservative. Therefore the protein content determined using this approach is not entirely representative of the final vaccine product as it does not take into account protein losses/modifications that may occur during the formulation process. ELISA based methods for vaccine analysis and determination of potency have also been described in certain vaccines, such as poliovirus (Rezapkin et al., 2005) and bovine virus diarrhoea (Pecora et al., 2009) vaccines. Although the ELISA approach is quick and relatively cheap it is highly sensitive to contaminants and requires previous knowledge of the active vaccine component responsible for protective immunity.

The development of a 2D-LC/MS method for serovar Canicola vaccine analysis provides a more robust approach for the identification of protein biomarkers of efficacy, which could later be developed into an *in vitro* vaccine potency test, as it characterises the protein component of fully formulated vaccines. This allows for the identification of the precise subsets of bacterial proteins potentially available for presentation to the host immune system (referred herein as the vaccine proteome); such proteomes can be used to identify protein biomarkers of efficacy which, once validated, could be developed into an *in vitro* vaccine potency test. Proteomic analysis of serovar Canicola vaccines may confirm the presence, and therefore potential involvement, of previously described

immunogenic factors, such as LipL41 (Haake et al., 1999) and LipL32 (Haake et al., 2000); subsequent quantitation of their abundance in the vaccines may consequently increase our current understanding of how these serovar Canicola vaccines provide protection. Known immunogenic proteins identified in the serovar Canicola vaccines (A-E) may be suitable for development into novel subunit or recombinant protein vaccines; although further confirmatory work in animal models would be required to confirm this. Additional uses for vaccine analysis using mass spectrometry can be envisaged for the quality assurance stage of vaccine manufacture to ensure production consistency and shelf life. This could determine if the vaccine proteome differs between batches and that, if known, protein(s) responsible for vaccine potency are present at appropriate concentrations. Finally it is entirely feasible to expect that the proteomic vaccine analysis method described herein will be applicable to the analysis of other non-*Leptospira* vaccines. This could be of particular benefit for the characterisation of *Clostridium* vaccines as their existing challenge vaccine batch potency test has been recently identified (Kulpa-Eddy et al., 2011) as being in need of replacement due to the severity, and large number of animals required, of the current test.

Proteomic characterisation of pathogenic *Leptospira interrogans* serovars is not widespread and to date only serovars Copenhageni (Eshghi et al., 2009, Malmstrom et al., 2009), Pomona (Vieira et al., 2009) and Lai (Cao et al., 2010, Zhong et al., 2011b) have been characterised (Chapter 4). A variety of methodologies ranging from basic 2D PAGE/MALDI-ToF techniques (Vieira et al., 2009) to an advanced LTQ-Orbitrap based LC-MS/MS approach (Cao et al., 2010) have been employed. Whilst these previous studies are informative it is important to note that to date only 3 of the 230 known pathogenic serovars (Adler et al., 2011) have been characterised, making generalisations about *Leptospira interrogans* problematic. As the main focus of this

study was the analysis of vaccines that provide protection against *L. interrogans* serovar Canicola it was considered important to determine the proteome of bacterial extracts of serovar Canicola to increase our understanding of the bacteria and provide a point of comparison for vaccine analysis. The proteome of serovar Canicola, described herein (Chapter 4), is believed to be the first to be reported for this serovar.

By identifying 1015 proteins in serovar Canicola this study has confirmed that these proteins are being actively expressed in the bacteria, under the growth conditions and at the time point used. Comparison of the functional annotation of these 1015 confirmed proteins, with the protein content of other serovars, may increase our overall understanding of the pathogenicity of *Leptospira*. It is also conceivable that such proteomic comparison may reveal commonalities between serovars, allowing the identification of novel multi serovar drug and vaccine targets. In addition unique serovar specific protein biomarkers may be identified which would allow the development of novel diagnostic tests, using techniques such as MRM or ELISA, for serovar differentiation. Due to the quantitative nature of these techniques it could be suggested that they may be more accurate than the existing serology based test (MAT). Such improved accuracy would improve determination of the epidemiology of *Leptospira* and aid in preventative treatment strategies such as vaccination (Pol et al., 2009). Further optimisation of proteomic coverage may be achieved by selection of alternate growth conditions and/or protein extraction methods with associated further development of processing methods adopted herein. This could be considered in future studies to increase our understanding of how *Leptospira* responds to different environmental stimuli/stresses and increase the coverage of the serovar Canicola proteome.

In an analytical comparison of five serovar Canicola vaccine proteomes the outer membrane protein LipL32 was detected in all five vaccines analysed (Chapter 3). LipL32 had a lower relative spectral abundance in failed batches of vaccine C compared to passed batches (Chapter 4). Multiple reaction monitoring, which has a high sensitivity and specificity (Wolf-Yadlin et al., 2007), was subsequently used to quantitate the concentration of LipL32 in passed and failed batches of serovar Canicola vaccine (Chapter 4). This study is the first to apply 2D-LC/MS and MRM for biomarker discovery and quantitation in serovar Canicola vaccines; this approach could be successfully adapted for the analysis of other commercially available protein based killed vaccines. MRM quantitation of vaccines has a number of potential applications including, but not limited to, the development and/or improvement of *in vitro* vaccine potency tests; such as the aforementioned *in vitro* tests required for *Clostridium* vaccines. MRM analysis is a rapid analytical methodology with a high level of accuracy (Wolf-Yadlin et al., 2007), however it requires the use of synthetic peptides that match the protein/peptide of interest making it expensive thereby limiting its universal adoption.

A link between reduced concentrations of N terminal LipL32 with a reduction in serovar Canicola vaccine potency (Chapter 4) was determined following MRM quantitation of serovar Canicola vaccine C. Although the structure of LipL32 has been determined (Vivian et al., 2009), and a previous study has suggested that the N termini does not have a direct involvement in the protective immune response (Hauk et al., 2008), any indirect effect of the N terminus with the proteins conformational structure and immunogenicity has not been investigated. The results presented herein suggest that LipL32 may be a suitable target molecule for the development of an *in vitro* vaccine batch potency test for serovar Canicola which achieves the primary objective of this

work. Proof of principle for such a test, using N terminal amino acid quantitation of LipL32, has also been demonstrated thereby achieving the secondary objective of this work. The identification of LipL32 as a biomarker for potency and the proof of principle for it's used as part of an *in vitro* potency test answers the question of whether a protein potency biomarker can be identified in *Leptospira interrogans* serovar Canicola vaccines, raised in the hypothesis of this study.

Methodologies developed herein may be applicable to other *Leptospira* serovars. However for accurate measurement of the potency of multivalent vaccines against specific serovars it would be advisable to choose different biomarkers for each serovar being assessed. N terminal LipL32 quantification of serovar Canicola vaccines using MRM represents a highly accurate potential replacement for the current hamster vaccine potency test. However vaccine quantification using mass spectrometry may not be the most practical test for widespread usage, particularly for smaller laboratories, due to the large commitment required in terms of equipment and expertise. An ELISA based *in vitro* potency test using N terminal LipL32 specific monoclonal antibodies may be more appropriate for widespread usage as it is cheaper to perform and less technically demanding. However as ELISAs are known to be highly sensitive to adjuvant contamination both approaches would need to be fully evaluated before the existing hamster vaccine potency test could be replaced.

As additional failed batches of serovar Canicola vaccine were unavailable for analysis the use of N terminal quantitation of LipL32 as an *in vitro* potency assay could not be more extensively validated. Confirmatory evidence that LipL32 may be an active component of serovar Canicola vaccines was therefore sought by assessing the involvement of recombinant LipL32 in the protective immune response against *L.*

interrogans serovar Canicola in the hamster model. Recombinant LipL32 protein was extracted from an *E. coli* expression system using a modified version (Chapter 5) of the original method described by Haake (Haake et al., 2000). Comparison of the N and C termini of the recombinant LipL32 protein, against LipL32 derived from vaccines A-E, was achieved using MRM prior to its inoculation in the hamsters; the immunogenicity of the recombinant LipL32 protein was tested using ELISA with a polyclonal LipL32 antibody. This study is the first to demonstrate that vaccination with low doses of recombinant LipL32 results in decreased kidney invasion of serovar Canicola and reduced lesion severity. Although complete immune protection was not observed this reduction of kidney invasion is indicative that LipL32 is involved in the immune response suggesting that the presence of LipL32 in serovar Canicola vaccines may be contributing to their potency and immunoprotection.

Multiple studies into the usage of LipL32 as a vaccine candidate have been reported previously which have shown LipL32 to provide protection against serovars Canicola (Branger et al., 2005) and Copenhageni (Seixas et al., 2007a, Grassmann et al., 2012) but not against serovars Pomona (Cao et al., 2011) and Manilae (Lucas et al., 2011). The results presented herein provide further confirmatory evidence that LipL32 is involved in the protective immune response against serovar Canicola either on its own or in combination with other undetermined bacterial components.

6.1 Future Work

Prior to the existing hamster challenge vaccine potency test being replaced by an *in vitro* assay, such as N terminal LipL32 quantitation, approval will be required from the European pharmacopeia; obtaining such approval can be both time consuming and expensive. Further refinement and validation of the N terminal LipL32 quantitation

assay described must be performed, prior to it being submitted to the pharmacopeia for consideration, to increase its probability of gaining acceptance. Significant analysis of passed and failed vaccine batches, for every serovar Canicola vaccine currently available commercially, would therefore be required to enable statistical validation of the assay. In addition non serovar Canicola vaccine batches must be analysed to confirm that the reduction of N terminal LipL32 is specific to serovar Canicola vaccines. The inclusion of sequentially truncated N terminal LipL32 synthetic peptides in the MRM analysis of the vaccines could also be considered to determine the exact amino acid position that the protein has become modified. In addition the effect of N terminal LipL32 modification on the conformational structure of the protein could be investigated to ascertain how this differs from full length LipL32 and how differences may affect antibody binding. Refinement and validation of the LipL32 N terminal quantitation assay would require the cooperation of multiple vaccine manufacturers as well as a significant financial expenditure. The longer term benefits resulting from the implementation of an *in vitro* serovar Canicola vaccine potency test, such as a reduction in animal usage and concomitant costs, should therefore be carefully considered.

Without further information from the vaccine manufacturer it is difficult to speculate as to why LipL32 has a reduced concentration of N terminal LipL32 in failed batches of vaccine C. Several possibilities are conceivable however including genetic mutation of the stock strain, physical/chemical modification of the protein during the manufacturing process and/or some form of structural protein instability leading to degradation over time. All of these factors need to be thoroughly investigated and, if possible, alterations to the vaccine manufacture process need to be made to reduce the incidence of this modification occurring. Development of a simplified vaccine, using a targeted series of peptides capable of eliciting a protective immune response, should also be considered;

as it would remove the requirement for bacterial culture, and the associated usage of animals to maintain strain virulence, requisite to create current vaccines.

Whilst the work presented herein was comprehensive, within the remit of the study, it was not exhaustive and was necessarily limited by the technology and resources available. A number of additional optimisations are therefore possible which should be considered for future studies into this area.

- Chromatographic separation of the vaccine tryptic peptides could be improved in the first instance through the use of ultra-high pressure chromatography (UHPLC) which is reported (Plumb et al., 2004) to give faster separation as well as better peak resolution compared to standard RP-HPLC; this would enable more accurate sample fractionation, increasing reproducibility and potentially increasing identification of low abundant proteins downstream.
- The use of a more modern mass spectrometer, such as a linear ion trap orbitrap, could identify a larger number of proteins in the bacterial and vaccine proteomes due to an increased scan speed and mass accuracy (Olsen et al., 2009) compared to the Agilent 6520 Q-ToF.
- Certain software improvements, such as automated exclusion lists which allow identified proteins to be excluded from subsequent reanalysis, could also dramatically increase the identification of proteins with low abundance (Hiemstra et al., 2011).
- Similarly proteomic analysis of *Leptospira interrogans* serovar Canicola and derived vaccine products would also benefit from genetic sequencing of serovar Canicola. This would provide a serovar specific protein database against which resultant mass spectra could be searched.

In summary this study has achieved its aims and objectives by identifying LipL32 as a potential biomarker for efficacy in serovar Canicola vaccines and providing proof of principle for the use of N terminal LipL32 quantitation as a potential *in vitro* replacement for the existing hamster challenge serovar Canicola vaccine potency test. However further refinement and validation of this N terminal LipL32 assay will be required prior to replacement of the existing hamster challenge test.

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Appendices

Please see attached disk for appendices 1-3