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Molecular and immunological analysis of New Zealand isolates of *Mycoplasma ovipneumoniae*

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Abstract

Mycoplasma ovipneumoniae is a pathogen of caprinae expected to infect the majority of domestic sheep in early life. *M. ovipneumoniae* is suspected to be the primary cause of chronic non-progressive pneumoniae (CNP) in lambs. Currently there is no effective vaccine against *M. ovipneumoniae*, and many commonly used antibiotics have limited effect. Diagnosis of *M. ovipneumoniae* infections can be difficult, due in part to the limited overt symptoms seen in cases of CNP. Despite the significance of *M. ovipneumoniae* infections there is limited information on the molecular and immunological data of *M. ovipneumoniae*. Thus, the major focus of this thesis was to identify potential immunogenic proteins for a vaccine or diagnostic purposes and construct genomes from New Zealand isolates of *M. ovipneumoniae*.

Draft genomes were produced for three New Zealand isolates of *M. ovipneumoniae*, (isolates 16, 90, and 103), and annotated. Using antisera produced against these isolates, immunogenic proteins were identified from hydrophobic membrane protein fractions of *M. ovipneumoniae* using Western blotting. Those protein bands were subjected to mass spectrometry analysis, and by comparing mass spectrometry data to the genomes, six novel proteins, GAPDH, P146, MATE, hypothetical protein 1, hypothetical protein 2, hypothetical protein 3, and two previously described immunogenic proteins, EF-Tu and HSP70, were identified. Five of the proteins were produced as recombinant proteins for further study.

The nucleotide sequence of EF-Tu was edited using an overlap polymerase chain reaction (PCR) to convert the sequence to *Escherichia coli* codon preference. This sequence was then cloned into the pET22b (+) vector for expression of histidine-tagged fusion protein. Additionally, EF-Tu, GAPDH, P146, hypothetical protein 3 and an epitope of hypothetical protein 1 were produced as recombinant proteins by GenScript. Antisera were produced in sheep against each of the recombinant proteins for further studies. Enzyme-linked immunosorbent assay (ELISA) confirmed that each protein was immunogenic and elicited antibody responses in sheep. Immunofluorescence microscopy confirmed the localisation of native EF-Tu and GAPDH proteins on the surface of *M. ovipneumoniae* cells.

Antisera produced against whole cell antigens from *M. ovipneumoniae* cross-reacted with P146 and hypothetical protein 3 suggesting a potential role for these proteins to be used to detect animals infected with *M. ovipneumoniae*. Sheep vaccinated with *M. ovipneumoniae* produced weak IFN- γ responses but stronger IL-17 responses in whole blood cultures stimulated with whole cell antigens. Of the five proteins tested as recall antigens in the IL-17A assay, recombinant GAPDH produced a strong IL-17A response in sheep vaccinated with *M. ovipneumoniae* suggesting a potential role for GAPDH in a diagnostic assay for measuring IL-17A responses in *M. ovipneumoniae* infected sheep.

Mycoplasmas are known to adhere to host epithelial cells as part of the pathogenesis process. A bovine endometrial epithelial cell line was established as a model to study adherence of *M. ovipneumoniae* to epithelial cells and determine if antibodies against the surface proteins could interfere with adherence. *M. ovipneumoniae* cells adhered to the endometrial epithelial cells. Adherence was inhibited by antibodies directed against whole cells of *M. ovipneumoniae* but not against the recombinant proteins.

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List of Abbreviations

16s ribosomal RNA gene	16s
ATP binding cassettes	ABC
Acute pneumonia	AP
Antigen-presenting cells	APCs
B-cell receptors	BCR
Base pair	BP
Bovine serum albumin	BSA
Cell-mediated immune	CMI
Chronic non-progressive pneumonia	CNP
Chronic Pneumonia and Polyarthritis Syndrome	CPPS
Clusters of orthologous groups	COGs
Colour change units	CCU
Conserved domains database	CDD
Contagious bovine pleuropneumonia	CBPP
4',6-diamidino-2-phenylindole	DAPI
DNA gyrase subunit B	gyrB
Elongation factor thermo unstable	EF-Tu
Enhanced chemiluminescence	ECL
Enumeration of IFN- γ secreting T cells	ELISpot
Environmental Science and Research	ESR
Enzyme-linked immunosorbent assay	ELISA
Extracellular matrix	ECM
Fibronectin	Fn
Flow through assay	FTA
Fluorescein isothiocyanate	FITC
General secretory pathway	GSP
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Heat-shock protein 70	HSP70
Hematopoietic stem cells	HSC
High-throughput sequencing	HTS
Horseradish peroxidase	HRP
Hydrogen peroxide	H ₂ O ₂
Hydrophobic protein fractions	HPF
Hypothetical protein	HP
Immunoglobulin	Ig
Interferon gamma	IFN- γ
Interferon gamma receptor	IFNGR
Interferon gamma release assays	IGRA
16S-23S ribosomal RNA intergenic spacer	IGS
Interleukin	IL
Isoelectric focusing	IEF
Isopropyl β -D-thiogalactoside	IPTG
Kilobase pair	kbp
Kilodalton	kDa
Latent tuberculosis infection	LTBI
Lateral flow dipstick	LFD
Leukotoxin	lkt+

Liquid chromatography - tandem mass spectrometry	LC-MS-MS
Loop mediated isothermal amplification	LAMP
Luria-bertani	LB
Maedi-visna	MV
Marginal zone	MZ
Milli-q	MQ
Molecular weight	Mw
Multi-locus sequence typing	MLST
Mucin	Mn
Multidrug and toxin extrusion	MATE
Cpg oligodeoxynucleotides	ODN
One-dimensional	1D
Optical density	OD
Ovine progressive pneumonia	OPP
Paired end	PE
Phosphate-buffered saline	PBS
Phosphoenolpyruvate-dependent System	PTS
Pleuropneumonia like organisms	PPLO
Polyvinylidene difluoride	PVDF
Porcine enzootic pneumonia	PEP
Porcine reproductive and respiratory syndrome virus	PRRSV
Porcine respiratory disease complex	PRDC
Protein families	pfam
Regulatory T-cells	Treg
Restriction fragment length polymorphism	RFLP
RNA polymerase beta subunit	rpoB
Secondary lymphoid organs	SLO
Signal recognition particle	SRP
Single nucleotide polymorphisms	SNPs
Sodium dodecyl sulphate polyacrylamide gel electrophoresis	SDS-PAGE
Surrogate immunoglobulin light chain	SL
T helper	T _H
T-cell-dependent activation	TD
3,3',5,5'-tetramethylbenzidine	TMB
Transitional B cells of type 1	T1
Transitional B cells of type 2	T2
Transforming growth factor beta	TGF- β
Tris-buffered saline	TBS
Tris-EDTA	TE
Tris-Sodium chloride-EDTA	TNE
Tumour necrosis factor	TNF
Two-dimensional	2D
Ultraviolet	UV
Whole cell	WC
Whole cell lysates	WCL

Chapter 1 Literature Review

1.1 History and taxonomy of *Mycoplasma*

1.1.1 Mollicutes

Members of the *Mycoplasma* genus belonging to the class Mollicutes, are distinguished by their lack of a cell wall [1]. Species of this class are the smallest bacterial cells yet discovered (200-300 µm) and contain very small genomes - as small as 580,070bp for *Mycoplasma genitalium*. There is growing support for the hypothesis that *Mollicutes* evolved from gram positive bacteria by reductive evolution [2]. The adoption of a parasitic model allows the loss of significant genetic material. For example, obtaining core nutrients from a host permits the loss of genes that are no longer needed because they become energetically costly to maintain during reproduction [3]. The lack of a cell wall reduces the efficacy of many common antibiotics due to their target being inhibition of cell wall synthesis. This makes infections caused by species of this class more significant and therefore there is an urgent need for development of vaccines against them.

1.1.2 Association of *Mycoplasmas* with humans and animals

Mycoplasmas are a genus of bacteria known to infect animals as well as plants. *Mycoplasma* was first discovered/mentioned in the 19th century by Louis Pasteur when he was studying the causative agent of the 'Bovine pleuropneumonia'. Pasteur proposed the disease was caused by a microbe, named as Pleuropneumonia like organisms (PPLO); however, it was not possible at the time to observe or culture it. This remained the case until 1898 when E. Nocard and E. R. Roux [4] isolated and cultured *Mycoplasma mycoides* subsp. *mycoides*, now recognised as the causal organism. *Mycoplasma* has over 100 species associated with the genus, infecting a range of hosts although each species is usually specific to a single primary host.

1.1.3 Humans

Humans are currently considered to be the primary host for 14 mycoplasma species. The main mycoplasmas associated with human diseases are *Mycoplasma pneumoniae* and *Mycoplasma genitalium* [5]. *M. pneumoniae* was first isolated from the sputum of a patient by Eaton et al (1944) and designated *M. pneumoniae* in 1963 [6]. *M. pneumoniae* is restricted by its small genome and limited biosynthetic capabilities. This influences many of the organism's biological characteristics and complex medium supplement is required to cultivate the organism *in vitro* [7]. Cellular reproduction occurs by binary fission, temporally linked with duplication of its attachment organelle. *M. pneumoniae* is primarily an extracellular pathogen that depends on a close association with host cells to survive [7]. The attachment organelle consists of a specialised tip structure formed from a network of adhesions, interactive proteins and adherence accessory proteins [7]. Infections promote

a loss of cilia in the respiratory tract resulting in a reduction in oxygen consumption, glucose utilization, amino acid uptake, and macromolecular synthesis of affected cells [7-9].

The use of vaccination for human mycoplasmas has been largely unsuccessful. Historically vaccination attempts have resulted in limited protection, or in some cases an exacerbation of the disease. A number of tests were conducted between 1964-1976 on volunteers [10, 11] and military recruits [12-14] using formalin inactivated vaccines. However, when challenged either experimentally or naturally with virulent *M. pneumoniae*, the protective efficacy of the vaccines was limited. Attenuated strains of *M. pneumoniae* created by continuous passage *in vitro* [15, 16] or temperature-sensitive mutants, were successful in protecting hamsters but were unacceptable for human use because they either retained a significant level of virulence for humans or did not colonise and protect human volunteers against challenge [11]. Further testing with mutant avirulent *M. pneumoniae* strains such as P30 deficient *M. pneumoniae* resulted in exacerbated disease when challenged with virulent *M. pneumoniae*. A study in mice suggested this effect to be the result of prior sensitisation by vaccination or infection with *M. pneumoniae* creating an environment that conferred an advantage to the bacterium for survival [17].

1.1.4 Cattle

Contagious bovine pleuropneumonia (CBPP), a respiratory disease of cattle, is a major cause of economic loss resulting from an infection of *Mycoplasma mycoides* subspecies *mycoides* small colony (*M. mycoides* subsp. *mycoides* SC) [18]. *M. mycoides* subsp. *mycoides* SC was the first mycoplasma isolated by Nocard et al (1898) [4]. CBPP is widespread across Africa, the Middle East, and parts of Asia. *M. mycoides* subsp. *mycoides* SC is considered to be the most pathogenic of the *Mycoplasma* species with its antigenically and functionally diverse surface proteins suggested to aid in virulence [19]. As a result CBPP holds the potential for very serious and rapid spread across national borders [19].

The current method for control of CBPP employs the use of highly efficient diagnostic tests able to detect *M. mycoides* subsp. *mycoides* SC, in both symptomatic and asymptomatic carriers. These tests are used in conjunction with a live vaccine T1/44, which is derived from strain T1, developed in Tanzania in the 1950s [19]. The use of T1/44 has shown advantages; however, as a live vaccine the risk of adverse effects is high. 'Williams reactions', characterised by an oedema that appears 10 to 20 days after inoculation, have been observed after T1/44 vaccinations and abscesses may also develop at the site of vaccination [19]. The vaccination site is usually the tail, potentially dispensable if necessary [20].

Unfortunately, laboratory results from animals that had been vaccinated regularly have shown that the T1/44 vaccine strain does not always elicit a protective immune response. More concerning,

there have been reports of CBPP in closed vaccinated herds, suggesting the potential of T1/44 vaccine strains to regain virulence [19].

In addition to CBPP, cattle can be susceptible to another symptomatic disease, Chronic Pneumonia and Polyarthritis Syndrome (CPPS) caused by *Mycoplasma bovis*. Many *Mycoplasma* species can be isolated from milk of animals suffering from mastitis, with *M. bovis* considered the most common causative agent of mycoplasma mastitis [21]. First isolated from a severe case of mastitis in the early 1960's [22], it has since been associated with bovine respiratory disease. Affected animals present with clinical signs such as lameness, swelling of joints and ultimately weight loss as a consequence of impaired movement [21]. It is also associated with keratoconjunctivitis and otitis [23].

Currently, the primary methods for controlling *M. bovis* are improved animal management practices and antimicrobial treatments. However, as with all mycoplasmas, the lack of a cell wall results in a limited pool of available antibiotics and it has been reported that resistance to multiple antibiotics compounds (used to treat *M. bovis*) is increasing [24-28]. After the *M. bovis* outbreak first detected in July 2017 in New Zealand, the government proceeded with a test and slaughter approach. This was undertaken due to the previous absence of *M. bovis* in an attempt to eliminate *M. bovis* from New Zealand [29]. Research into developing a vaccine against *M. bovis* has been active for several years across many fronts. A major focus has been the development of a vaccine based around immunogenic proteins and inactivated whole *M. bovis*. There have been a number of potential avenues identified, however as yet, there has been no direct evidence that any of the currently developed vaccines based on these methods are effective under field conditions [21]. The third approach to vaccine development, the use of live attenuated organisms, is used to control disease of many mycoplasmas. Zhang et al (2014) [30] showed promising results with the use of P150 and P180 (passages 150 and 180, respectively); vaccination with either of these *M. bovis* strains resulted in significant reduction of gross lung lesions and lung lesion scores in calves with a slightly higher reduction in the group vaccinated with p150. However, as mentioned in relation to the T1/44 vaccine, the use of a live vaccine potentially poses risks of vaccine induced infections.

1.1.5 Pigs

Porcine enzootic pneumonia (PEP), is a respiratory disease induced by *Mycoplasma hyopneumoniae*. PEP is characterised by chronic coughing, poor growth rate and decreased feed conversion efficiency [31]. *M. hyopneumoniae* is also associated with porcine respiratory disease complex (PRDC), considered as a primary agent of the disease [32]. In addition to the primary disease caused by *M. hyopneumoniae*, infection facilitates respiratory tract invasion by other pathogens. Porcine reproductive and respiratory syndrome virus (PRRSV) is often found in tandem with *M. hyopneumoniae* [33]. *M. hyopneumoniae* is primarily located on the mucosal surface of the trachea, bronchi, and bronchioles [34], where it induces chronic, non-productive coughing which is the main

clinical sign. Under experimental conditions this sign appears 10 -16 days after initial infection, however this period can vary greatly under field conditions [32]. Presentation of clinical signs and lesions can lead to a tentative diagnosis, but laboratory testing is necessary for a conclusive diagnosis [35].

The three main strategies to control the disease include: therapies with antibiotics known to be active against *M. hyopneumoniae*, repopulation of infected herds with pigs free of *M. hyopneumoniae* and adaptations in management to minimise the risk for PEP [36]. Improvements in management is fundamental in the control of infections of *M. hyopneumoniae*. Reducing the opportunity for the spread of the disease will contribute to an improvement in the control of enzootic pneumonia [32]. However, additional factors different from housing and management conditions, such as strain differences, can influence the disease [37]. Antibiotic treatment for *M. hyopneumoniae* frequently uses tetracyclines and macrolides; however, other antimicrobials, including lincosamides, pleuromutilins, fluoroquinolones, florfenicol, aminoglycosides and aminocyclitols are also used [32]. Results from treatment and control of enzootic pneumonia outbreaks can be frustrating as the symptoms may reappear after cessation of the therapy [32]. The procedure of pulse medication over extended periods of time or continuous use of medication during any of the production stages should be discouraged due to the increased risk of promoting antibiotic resistant bacteria and the potential of antibiotic residues remaining in pig carcasses at slaughter [32].

Commercial inactivated, adjuvanted whole-cell vaccines, are widely applied worldwide with up to 70% of pig herds in some countries treated this way [32]. Protection against clinical pneumonia is often incomplete and the vaccine does not prevent colonisation [38]; however, the use of the vaccine may reduce the number of organisms in the respiratory tract [39] which in turn reduces the infection level within a herd [40]. However, it has been shown that vaccines based on inactivated whole cells (WC) have been unable to prevent transmission or establishment of the bacteria in the lungs [31].

1.2 Pneumonia in New Zealand

1.2.1 Sheep farming

Sheep farming is an important export contributor for the New Zealand economy. As of June 2019, there were 26,822,000 sheep put to ram in New Zealand consisting of 2,073,000 ewe hoggets and 16,845,000 breeding ewes [41]. For the 2019 year a total of 18,867,593 lambs were slaughtered with 10,327 animals condemned and 18,857,266 graded for export.

1.2.2 History of mycoplasma infections in New Zealand

Since the introduction of intensive farming in New Zealand, pneumonia has likely been a problem in sheep, with the first recorded incidence of the disease reported at the end of the 19th century [42].

Due to the nasal cavity being the primary habitat of the bacteria involved in ovine pneumonia it is likely that the bacteria were imported into the country from Europe with their hosts [43].

Fortunately, due to selective measures at the time of importation or the inability of infected animals to survive long journeys, New Zealand has been spared some of the significant virally-induced pneumonias that are prominent in the UK and Europe such as maedi-visna and pulmonary adenomatosis [43].

1.2.3 Respiratory disease in sheep

Respiratory disease is common in domesticated livestock around the world. The dominant diseases in sheep are:

1. Acute pneumonia (AP)
2. Chronic non progressive pneumonia (CNP)
3. Ovine progressive pneumonia (OPP) or maedi-visna (MV)
4. Pleurisy
5. Pulmonary adenomatosis /Jaagsiekte sheep retrovirus

Currently, the ovine respiratory diseases recognised in New Zealand are Acute pneumonia, Chronic non progressive pneumonia, and pleurisy [43].

Acute pneumonia

As with many respiratory diseases in farmed domestic animals, ovine pneumonia is a complex combination of infective agents and environmental factors. This type of pneumonia, also referred to as acute fibrinous pneumonia occurs primarily in older lambs during the late summer and autumn when heat stress and transportation, or mustering in dry hot conditions is most prevalent [43].

Clinical symptoms of AP include depression, weight loss, fever, inappetence, increased respiratory rate, and nasal and ocular discharge. Affected animals often become separated from the rest of the flock and a frothy fluid drooling from the mouth is usually present in the terminal stages [44]. The disease has rapid onset and affected animals can be found dead within 12 hours [43]. During outbreaks mortalities vary between 2 and 8% [45-47]. *Mannheimia haemolytica* is considered the primary agent in acute pneumonia and the major cause of lung damage in both forms of ovine pneumonia.

Chronic non progressive pneumonia

As the majority of lambs will be affected by some level of CNP during their first autumn [48] the potential cost to the New Zealand sheep market can be significant. CNP is also referred to as atypical pneumonia, prolific interstitial pneumonia, coughing syndrome, enzootic pneumonia, or summer

pneumonia. CNP has few if any overt clinical signs [48]; however, coughing, exacerbated breathing, accelerated breathing or nasal discharge may be present.

CNP increases the energy requirement of the animal. This can be from the extra metabolic requirements for repairing lung damage caused mycoplasma infections, or from the extra load on the immune system during challenge by a pathogen that requires considerable nutritional support resulting in a less efficient conversion of available feed to body mass [49].

Typically, lesions begin with dull red vertical areas of collapse which develop into firm red-grey areas of consolidation over 2-3 weeks [50]. Lesions may continue to grey areas of consolidation with attached localized pleural adhesions [50]. CNP and AP are considered pathologically different due to the severity and extent of damage [43].

Pleurisy

Pleurisy is an inflammation of the pleura resulting in adhesions between the lung and chest wall.

Pleurisy is not considered to be a separate disease but an outcome of a pneumatic event. Acute pleural lesions are likely to contain large numbers of the bacteria that caused the associated pneumonia [51]. Chronic pleurisy may contain a few of these organisms or be absent depending on the stage of disease development [52].

In the early stages of pleurisy, contact and movement between the parietal and visceral pleurae results in pain due to the inflammation of the pleura [53]. In the second stage the ventral parts of the lungs can collapse due to the accumulation of serofibrinous inflammatory exudate in the pleural cavity. The next stage results in the fluid being resorbed and adhesions developing, restricting the movement of the lung and the chest wall, further reducing the functionality of the lungs [53]. As the adhesions stretch, the restriction of breathing is reduced; however, the pleural lesions will not resolve completely, and fibrous scars will remain on the lungs [53].

1.2.4 Prevalence

Kirton (1976) reported prevalence of CNP in New Zealand to vary from 21 to 93% [54]. An average of 60% of lambs were seen to show some level of pneumonia in a trial looking at seasonal incidence of pneumonia over a five year period [54]. Two flocks from Wallaceville, New Zealand had their pneumonia status recorded over subsequent years, resulting in a prevalence of 78% (n=27) and 57% (n=58) [55]. McGowan et al (1978) [56] observed a prevalence of 78-93%, in lambs at 3 months across two regions, Hawkes bay and Feilding respectively. The prevalence of lesions in counterparts at market slaughter weights was 35-68% [56]. These identifications were determined by the presence of lesions at slaughter which may underrepresent the total incidence of pneumonia if mild cases fail to produce lesions [56].

Black (1997) [57] estimated the prevalence across seven slaughter plants engrossing 9400 animals. Results indicated a much lower prevalence of CNP, with Northland having 13%, Bay of Plenty 6%, East Coast 4%, Whanganui 6%, Marlborough 4%, Canterbury 4%, and Southland 5%. The reduced prevalence observed in this study may be a result of the designated definition of infection, which was lesions with red or red-grey consolidation covering at least a quarter of one anteroventral lung lobe with presence of fibrinous plural exudates [57].

Conversely Goodwin (2004) [49] indicated a more prominent presence of CNP, observing 21.8%, 49.2% and 60.3% in Southland, King Country, and Northland, respectively. This increase in detection is due to the differing criteria used in this study. Separating the affected lungs into multiple categories from <5% to >20% not only allows for analysis of animals that have significant lung damage as seen in the Black (1997) [57] study but also allows the analysis of animals across the spectrum of CNP potentially allowing a more accurate detection rate of CNP in New Zealand animals.

1.3 *M. ovipneumoniae* in sheep

1.3.1 History

Mycoplasma ovipneumoniae is a species of the class Mollicutes known to infect Caprinae globally. *M. ovipneumoniae* was first described in New Zealand by Clarke et al (1974) [58] after the organism was initially proposed the name by Carmichael et al (1972) [59]. *M. ovipneumoniae* was subsequently detected in the majority (79%) of lambs with CNP but was also detected (63%) in apparently normal lungs [58]. It has to be noted that the samples had been handled by meat inspectors before testing, therefore while increases in false positives due to contamination from handling have to be taken into account, the presence of *M. ovipneumoniae* in some apparently normal lungs is certain [60].

It has been shown that multiple strains of *M. ovipneumoniae* can be detected within populations of sheep [61]. Out of 60 samples obtained from 20 farms, Ionas et al (1991) [61] obtained 58 unique isolates, indicating that there are numerous strains within New Zealand. However, it is to be noted that differences in DNA do not necessarily correlate to differences in protein expression which is more relevant to the host's immune response [60]. Immune responses can potentially be induced by any foreign protein; however, the responses promoted by surface proteins are more likely to be relevant in the context of immunity [60].

In addition to the number of different isolates detected between and within populations of sheep, it has been determined that multiple isolates can be detected with individual animals [62]. When lesions of severe CNP were compared to those with less extensive lesions by restriction endonuclease analysis, the more severe lesions contained more distinct strains with three or four strains versus the two strains found in the less severe lesions. The significance of the different

number of strains within the animals and the severity of the infection has not been elucidated; however, this increases the requirement for multiple strains to be considered when new methods to control *M. ovipneumoniae* infections are devised.

1.3.2 Economic impact

Chronic non-progressive pneumonia (CNP) is one of the most prevalent diseases of young sheep in New Zealand [60]. The major causative agent of CNP in New Zealand is *M. ovipneumoniae* [48].

There are three main ways pneumonia may result in economic loss: acute pneumonia (causing mortalities in sheep across all ages), downgrading of lamb carcasses for export markets due to the presence of pleural adhesions attached to the thoracic wall and the consequences of lower weight gain caused by CNP [48]. The majority of lambs are likely to suffer from CNP in some form during their life and this holds the potential to be a more significant cause of economic loss than mortalities from acute pneumonia [48].

The average loss between healthy and CNP infected animals could be as high as 2.19 kg over a two-month period correlating to a 1.5 kg difference in carcass weight [48]. With a potential infection rate of 70 - 80%, an average loss of 1.5 kg across a herd can have huge economic outcomes every year [63]. It was estimated that the average cost in 2008 of pneumonia and pleurisy to the New Zealand sheep market was in excess of NZ \$53 million [47]. Twenty-eight million dollars of losses were due to pneumonia affecting growth of the animals and NZ \$25 million losses due to pleurisy affected carcasses. Pleurisy reduces the value of a lamb because some or all of the ribcage needs to be removed to excise the pleural lesions, downgrading the lambs to 'cutter grades' with a loss of approximately 30% of value [47]. Also, in cases where the carcasses are unable to be trimmed, the entire carcass may have to be condemned, resulting in zero pay-out for the farmer. As deaths from acute pneumonia are sporadic and difficult to quantify, they were not included in the above estimates so total losses due to pneumonia may in fact be higher.

1.3.3 Transmission and predisposing factors

Pneumonia is transmitted between infected and susceptible sheep through contamination of the air, feed, water, and equipment, exacerbated by the inclination of sheep to crowd together [64, 65].

Numerous factors have been suggested to predispose animals to CNP including lungworm or mite infection, malnutrition, inbreeding, harsh weather conditions, or stress associated with overcrowding [64, 65]; however, the extent to which each of these factors predisposes the animals to disease is not well defined.

Introduction of stress through weather, overcrowding, or other environmental and anthropogenic means, has been suggested to progress subclinical infections into acute pneumonia, pulmonary abscessation and pleurisy [65]. As *M. ovipneumoniae* is often isolated from healthy sheep in low numbers, stress can have severe outcomes for affected animals [66]. This may be due to an increase

in endocrine responses induced by environmental stresses. This has the potential to reduce resistance to disease in affected animals [66, 67].

Environmental stressors can include harsh winters, which can stress the animal increasing energy budgets or reducing access to available food [66]. There have also been implications that precipitation levels in spring can predict the risk of pneumonia epizootics in the subsequent year. For every unit (mm/m^2) of spring precipitation above average, the relative risk was reduced 0.41 times [66].

1.3.4 Clinical signs and pathological lesions

CNP like many enzootic pneumonias in livestock, has few observable clinical signs apart from poor growth rates and decreased food conversion efficiency [60]. Infected individuals may also exhibit a chronic, persistent and irregular cough [65].

CNP is used to distinguish the disease from acute pneumonias which unlike CNP are often fatal [60]. Lesions first appear as dull red ventral areas of collapse which are accompanied by bronchiolitis in associated airways. Lesions will then progress to firm red-grey areas of consolidation over 2-3 weeks. In some occasions they may continue as grey areas of consolidation, with attached localised pleural adhesions, for several months [48, 60, 68].

1.3.5 Detection, control, and prevention of *M. ovipneumoniae*

With limited clinical signs of pneumonia and few unique to *M. ovipneumoniae* infections, laboratory diagnosis is required to confirm *M. ovipneumoniae* presence in a particular animal [65].

Immunocytochemistry using *M. ovipneumoniae* specific monoclonal or polyclonal antibodies can be used to confirm the presence within diseased tissues [65]. In addition to antibodies, the use of molecular techniques can be applied. The use of *M. ovipneumoniae* specific primers can be used for polymerase chain reaction (PCR) based detection. This requires collection from the animal to be inoculated in mycoplasma broth growth media and incubated for 7-10 days, after which samples showing mycoplasma growth can be inoculated on to mycoplasma agar plates allowing single colonies to be detected [69]. Single colonies can then be grown up in mycoplasma growth media ready for DNA extraction for use with the *M. ovipneumoniae* specific primers [69]. Recently faster diagnostics have been suggested for the detection of *M. ovipneumoniae* without the requirement of culturing [70]. Two rapid, specific, and sensitive assays have been developed: an isothermal DNA amplification using recombinase polymerase amplification and a real-time PCR for the detection of *M. ovipneumoniae*. The assays were validated using bronchoalveolar lavage fluid and nasal swab samples collected from sheep. This provides a potential testing methodology requiring only nasal swabs [70].

M. ovipneumoniae can be carried asymptotically by domestic sheep and goats. This can make control of the disease and prevention of outbreaks difficult. There is currently no successful vaccination program for the control of *M. ovipneumoniae*.

1.4 Adaptive immunity

In response to a pathogen the mammalian immune system can mount a response to reduce or remove the invader. The specialised mechanism used is dependent on the type and location of infection. The two primary mechanisms of adaptive immunity are humoral, and cell mediated [71].

1.4.1 B cells and humoral immunity

B-cell development

Hematopoietic stem cells (HSCs) are the origin of B-cells [72-75]. Originating in the bone marrow, the lymphoid lineage of the HSCs develop into common lymphoid progenitor cells, which are able to develop into all types of lymphocytes [72, 76]. B-cell receptors (BCR) on the surface of the cell control two further types of selection for the B-cells [75]. The first, positive selection, activates the antigen independent pathway involving both the pre-BCR and BCR [77, 78]. If they do not bind to their ligand, they do not receive their proper signals and cease to develop [77, 78]. Alternatively, negative selection is initiated on the binding of self-antigen to the BCR, the cell undergoes one of four fates: clonal deletion, receptor editing, anergy, or ignorance (B cell ignores signal and continues development) [77].

The BCRs are comprised of the immunoglobulin (Ig) heavy (H) and light (L) chains. The pre-BCR also have IgH however, the IgL is replaced with surrogate (SL) chains. The IgSL are composed of the invariant $\lambda 5$ and VpreB polypeptides and have homology to true IgL chains [79-82].

To complete development, immature B cells migrate from the bone marrow into the spleen as transitional B-cells. [83]. Transitional B cells of type 1 (T1) are recent immigrants from the bone marrow. They develop into the transitional B cells of type 2 (T2), which are cycling and found exclusively in the primary follicles of the spleen. Mature B cells can be generated from T1 or T2 B cells [83].

B-cell subsets

Naïve B-cells are divided into the typical subsets, B-1 B-cells, follicular B-cells, and marginal zone (MZ) B cells [75]. Peripheral B cells, in addition to their role in mediating humoral immune responses, can also secrete cytokines and have potential to present antigens to naïve thymus-derived lymphocytes (T-cells) [75]. Naïve mature follicular B-cells occupy two niches during their recirculatory wandering, once they can recirculate in the spleen or bone marrow, they can migrate repeatedly through the blood and lymph to B-cell areas of lymph node, Peyer's patches, and the spleen. The follicular niche contains naïve follicular B-cells able to present T-dependent antigens to

activated T-cells. The follicular niche becomes the major site of B-cell mediated T-dependent immune response [75]. Alternatively follicular B-cells also migrate to the bone marrow, forming discrete collections around vascular sinusoids [84, 85]. When the recirculating B-cells are residing in the bone marrow rather than in follicles they form the perisinusoidal niche [75].

Marginal zone B-cells are considered to be innate-like cells that in the absence of BCR ligation, can be induced into short-lived plasma cells. The MZ B-cells can also mediate the transport of antigens in immune complexes into splenic follicles as well as involved in T-cell dependent B-cell responses and immune responses to lipid antigens [75]. In rodents it has been observed that MZ B-cells mediate T-independent responses to antigens in blood-borne pathogens. During these pathogen driven responses, integrin adhesiveness may decrease and direct the migration of activated B-cells out of the MZ into the red pulp of the spleen where they may differentiate into short-lived plasma cells [86]. The MZ cells are also able to deliver antigens to follicular B-cells [75].

There is limited knowledge on B-1 B-cells with the majority of studies completed on rodents [87] and limited defined populations in humans [88]. The B-1 B-cells can contribute to the production of IgM to T-independent antigens [75]. Dendritic cells in the intestinal lamina propria may activate B-1 B-cells via presentation of antigens from commensal bacteria. Lipopolysaccharide and some toll-like receptor ligands can induce B-1 B-cell production as well as IgM secretory plasma cells, whereas antigen specific B-1 B-cells may be induced into IgA secretory cells [89].

B-cell activation

B-cell activation occurs in the secondary lymphoid organs (SLOs), such as the spleen or lymph nodes [90] following the recognition of antigen through the BCR and causes B-cell proliferation and differentiation [91]. Activated B-cells are able to differentiate into either plasma cells or memory cells, able to produce antibodies or protect against re-infection respectively [91]. Individuals contain an enormous population of B-cells for protection against a multitude of pathogens. This diversity; however, results in a low chance for a B-cell to encounter its cognate antigen [91]. To improve the encounter rate, the body localises these events within SLOs [91]. B-cells are contained within secondary lymphoid tissue, spending approximately 24 h searching for antigens before re-joining the circulatory system and being taken to another SLO to continue antigen searching [92]. B-Cell activation is completed via two major pathways, T-cell-dependent activation (TD) or T-cell-independent activation (TI). Antigens that require the TD pathway are usually foreign proteins, and are unable to induce a humoral response in organisms that lack T-cells [90]. B-cell responses to these antigens can require multiple days to mount the necessary response, however the antibodies generated have a higher affinity than those produced via the TI pathway. Alternatively antigens which do not require T-cells, include foreign polysaccharides and unmethylated CpG DNA [93].

1.4.2 T-cells and cellular immunity

T-cell development

T-cells are a type of lymphocyte originating from HSC which are found in the bone marrow [94]. The HSC migrate to the thymus for maturation, selection, and subsequent export to the periphery [94]. The peripheral T-cells primarily consist of naïve T-cells, memory T-cells, and regulatory T-cells (Treg). Naïve T-cells have the ability to respond to new antigens, upon interaction with a pathogen the cells rapidly divide and express molecules such as cytokine proteins that help protect the host [95]. These responding cells are designated effector T-cells, specifically cytotoxic T-cell, and are able to migrate to inflamed tissues and kill infected cells [96]. Following removal of the pathogen, the majority of effector cells die; however, a small amount of long lived memory cells remain that are poised to respond on the re-emergence of the pathogen [96]. The memory T-cells persist as heterogeneous subsets based on migration, tissue localisation and self-renewal capacities [94]. Each memory subset can participate in maintaining long-term immunity and recall protective responses [94]. The Treg cells play a critical role in keeping the immune responses in check, creating tolerances to innocuous and ubiquitous antigens [94].

T-cell activation

T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) become activated when they recognise with their T cell receptor specific peptide antigens presented by MHC class II and class I molecules, respectively. This provides a clear distinction between activation of $CD4^+$ and $CD8^+$ T cells. Class I MHC molecules are found on all nucleated cells and their function is to present endogenous antigens to $CD8^+$ T cells, while Class II MHC molecules are found on the professional antigen-presenting cells (APCs): dendritic cells, macrophages, and B-cells. Their function is to present exogenous antigens to $CD4^+$ T-cells [71].

T-cell effector subsets

The $CD4^+$, $CD8^+$, and Treg T-cells are groups of cells that actively respond to a stimulus, collectively known as effector T-cells. The cells expressing CD4 glycoprotein on their surface are also referred to as T helper cells (T_H) [97]. T helper cells help other leukocytes in immunological processes, including maturation of B-cells into plasma cells and memory B-cells as well as activation of cytotoxic T-cells and macrophages. Once T helper cells have been activated, they can differentiate into one of several subtypes including T_H1 , T_H2 , T_H3 , T_H17 , T_H9 , or T_{FH} , which secrete different cytokine patterns to facilitate different types of immune responses [97].

The two major subtypes of T helper cells are known as T_H1 and T_H2 cells. T_H1 helper cells lead to an increased cell-mediated immune response, typically against intracellular bacteria and protozoa [98, 99]. The main effector cells of T_H1 cells are macrophages as well as CD8 T-cells and interferon gamma ($IFN-\gamma$) CD4 T-cells [100, 101]. The interplay between macrophages and $IFN-\gamma$ is fundamental

to the host's ability to mount an effective immune response [102]. One of the first actions to occur in innate immunity is macrophage activation [102]. During the innate and the acquired immune response, macrophages respond primarily to IFN- γ as well as to a number of different cell products [102]. Initially discovered as agents that interfere with viral replication [102, 103] IFNs were classified by the secreting cell type but are now classified into type 1 and type 2 according to receptor specificity and sequence homology [102]. The most common IFN, IFN- γ , is the sole type 2 IFN and is structurally unrelated to type 1 IFNs, binding to different receptors and encoded by a separate chromosomal locus [102]. The functional IFN- γ receptor (IFNGR) is comprised of two ligand-binding IFNGR1 chains and two signal-transducing IFNGR2 chains that are associated signalling machinery [102]. The IFNGR2 chain is generally the limiting factor in IFN- γ responsiveness as IFNGR1 is usually in excess [104, 105]. Type 1 and 2 IFN up-regulate multiple functions within the antigen presentation pathway to increase the diversity of peptides presented on the surface in the context of class 1 MHC. Up-regulation of cell-surface class I MHC by IFN- γ is important for host response to intracellular pathogens, as it increases the potential for cytotoxic T cell recognition of foreign peptides and thus promotes the induction of cell-mediated immunity [102]. Of the IFNs, IFN- γ alone can efficiently up regulate the class 2 antigen presenting pathway and thus promote peptide-specific activation of CD4⁺ T-cells [106, 107]. IFN- γ is a major product of T_H1 cells, promoting further T_H1 immune responses by upregulating characteristic T_H1 effector mechanisms: innate cell-mediated immunity (via activation of natural killer cell effector functions), specific cytotoxic immunity (via T cell-APC interactions), and macrophage activation [102, 106]. IFN- γ also influences naïve CD4⁺ cell differentiation toward a T_H1 phenotype more directly through the presence of the cytokine milieu at the time of T cell receptor engagement [102].

T_H2 cells mediate host defence against extracellular parasites [98, 99]. T_H2 cells lead to the humoral response, the main effector cells are eosinophils, basophils, and mast cells as well as B-cells [98, 99]. T helper 3 cells (T_H3) are a subset of Treg T-lymphocytes with immunoregulatory and immune suppressive functions [108]. Naïve CD4⁺ can differentiate into T_H3 cells upon ingestion of a foreign antigen through the oral route [108, 109]. T_H3 cells are able to help IgA secretion, produce TGF- β production, and suppress both T_H1 and T_H2 cells [110] in an antigen nonspecific manner [108]. Since TGF- β , the primary secretion in the contact-independent mechanism, has a wide range of expression and affects the function of various cell types, T_H3 cells may have a major role in many aspects of immune modulation T-cell homeostasis [108, 111].

T helper 17 cells (T_H17) defined by their production of interleukin 17 (IL-17), are a subset of pro inflammatory T helper cells. They are related to Treg cells; however, the signals that promote T_H17 differentiation inhibit Treg differentiation [112]. T_H17 cells are important for maintaining and

clearing pathogens from mucosal surfaces [113]. In addition to their role in pathogen clearance T_H17 cells, via IL-17, are associated with autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and psoriasis [114]. T_H17 overactivation can cause type 3 hypersensitivity resulting in immune complex diseases such as rheumatoid arthritis or Arthus reaction [115, 116]. Due at least in part to its involvement in the inflammatory pathology IL-17A is one of the best-studied cytokines in immunology [117-119], entering the spotlight of immunological attention after the discovery of a development distinct $CD4^+$ T helper subset that expresses IL-17A and mediates tissue inflammation [120, 121]. IL-17A is also shown to mediate protection against extracellular pathogens [122, 123]. T helper 9 (T_H9) cells produce interleukin 9 (IL-9) and has been observed to play a role in defence against helminth infections, in allergic responses, in autoimmunity, and tumour suppression [124]. Initially IL-9 was thought to be a T_H2 specific cytokine; however, it was observed that T_H2 cells could be reprogrammed into a distinct T_H subset that primarily secreted IL-9 [125].

T-cells found within the B-cell follicles of SLOs expressing B-cell follicle homing receptor CXCR5 are recognised as Follicular B helper T-cells (T_{FH}), [126]. T_{FH} cells are considered indispensable T-cell subsets in the generation and maintenance of germinal centre responses. Therefore in the absence of T_{FH} cells, similar to B-cell activation by T-cell independent antigens, a quick burst of low affinity plasma cell production is formed but this does not lead to germinal centre induction nor permit antibody affinity maturation or the differentiation of effective memory B cells which are essential in fortifying the body against subsequent infections [126].

The $CD4^+$, $CD8^+$, and Treg T-cells are groups of cells that actively respond to a stimulus, collectively known as effector T-cells. The cells expressing CD4 glycoprotein on their surface are also referred to as T helper cells (T_H) [97]. T helper cells help other leukocytes in immunologic processes, including maturation of B-cells into plasma cells and memory B-cells as well as activate cytotoxic T-cells and macrophages. Once T helper cells have been activated, they can differentiate into one of several subtypes including T_H1 , T_H2 , T_H3 , T_H17 , T_H9 , or T_{FH} , which secrete different cytokine patterns to facilitate different types of immune responses [97].

1.5 Pathogenesis of *M. ovipneumoniae*

1.5.1 Primary atypical pneumonia

M. ovipneumoniae has been suggested to interact with the immune system of infected animals in two main ways. The first occurs when the organism attaches to host cells in the lung. Infected animals have been shown to have high levels of ciliary autoantibodies, providing a potential mechanism for the observed ciliostasis (loss of cilia in both structure and function) [127]. When *M. ovipneumoniae* infection becomes established it can cause small lesions within the lungs [60], and this can become a problem for the animal as the percentage of affected tissue increases. There was

a noticeable negative effect on weight gain when $\geq 20\%$ of the lung was affected. With up to 50% decrease in weight gain for affected animals this can have a significant effect on the time required to reach slaughter weight [49]. The second mechanism of influencing the host immune system involves the direct interaction with host alveolar macrophage plasma membranes. Attachment of *M. ovipneumoniae* to macrophages failed to induce expected phagocytic responses [128]. Infection of live *M. ovipneumoniae* significantly decreased the expression of IgG receptors on the surface of the macrophages. Exposure to live *M. ovipneumoniae* also decreased uptake of the organisms by the phagocytic cells, and decreased activity was also observed with exposure to heat inactivated *M. ovipneumoniae* but to a limited degree [128]. There was also a noted steady decline in uptake of *Staphylococcus aureus* when pre-treated with increasing concentrations of *M. ovipneumoniae* [128]. Reducing the effectiveness of macrophages may be one of the ways *M. ovipneumoniae* can predispose animals to secondary infections.

1.5.2 Predisposing infected animals to secondary infection and fatal pneumonia

M. ovipneumoniae, in addition to causing most cases of CNP in lambs, is recognised to predispose animals to more serious infections by other pathogenic organisms such as *M. haemolytica* [129, 130]. Ciliostasis caused by *M. ovipneumoniae*, prevents the host from being able to mechanically remove bacterial infections from the lungs [131]. In addition to physically being unable to remove bacteria, *M. ovipneumoniae* can result in suppression of macrophage ability to ingest *Staphylococcus aureus* providing another avenue for infection of more pathogenic bacteria [129, 130].

Predisposing livestock to other more pathogenic infections is the most serious complication following *M. ovipneumoniae* infection. Multi-bacterial infections have been shown to progress the disease from CNP to acute pneumonia with the accompanying health effects [129, 130]. In big horn sheep multi-bacterial infections have been linked to up to 75-90% mortality in affected animals [129, 132]. Not only does *M. ovipneumoniae* predispose animals to invasion by other bacteria, animals already harbouring subclinical infections of organisms such as *M. haemolytica* can result in fatal pneumonia [133] indicating that *M. ovipneumoniae* infections can also increase the pathogenicity of pre-existing infections.

1.5.3 Immune response to *M. ovipneumoniae*

The airway epithelium acts as the first line of defence, providing a physical barrier to infection. In addition to a physical protection the epithelium also produced chemokines and cytokines such as tumour necrosis factor (TNF)- α , IL-1 β , IL-4, IL-6, and IL-8, which are important mediators in both lung defence and inflammation [134]. The ability of *Mycoplasmas* to induce these cytokines has been studied in a number of different species, in particular *M. pneumoniae* [135] and *M. hyopneumoniae* [136]. *M. ovipneumoniae* was observed to induce several cytokines with the prominent cytokine, IL-8, by far in excess of the other cytokines observed [137]. The induction of IL-8 was also observed in

human airway epithelial cells at similar overwhelming levels in response to *M. pneumoniae* antigen [138-140]. These studies suggest that IL-8 may play an important role in the mucosal immune response during *M. ovipneumoniae* infections.

The immune response to *M. ovipneumoniae* infections in domestic sheep and bighorn sheep has yet to be characterised [141]. In other respiratory associated mycoplasma infections severity of disease is reportedly dependent of the type of T helper immune response mounted by the host [141]. In humans, mice, and cattle, a T_H1 response resulted in better management of infection whereas T_H2 responses were associated with increased pathology [141-144]. Goats infected with *M. capricolum* resulted in increased T_H17 responses which is linked to lung damage [145]. This has led to the hypothesis that the reported inter-species susceptibility difference between domestic and bighorn sheep to *M. ovipneumoniae* infections is associated with the different immunopathologic responses [145]. Studies using molecular high-throughput technologies confirmed that *Mycoplasma* infections are accompanied by massive changes in the gene expression profiles of hosts [146-148]. Whole blood transcriptome profiling revealed downregulation of gene expression leading to a general immune suppression [146-148]. It can be considered that this global approach reflects the sum of all local and systemic inflammatory and immune relevant host responses during the acute phase of infection [146]. Both immune stimulation and immune suppression have been reported suggesting a complex and dynamic interplay between activation and inhibition [146, 149-153]. *M. gallisepticum* infection has been demonstrated to upregulate several cytokines including IL-1 β , IL-6, IL-8, IL-12p40, CCL-20, and NOS-2 via macrophage activation [146, 154, 155]. Additionally, *M. hyopneumoniae* and *M. bovis* were shown to stimulate macrophages, elevating the release of pro-inflammatory cytokines [146, 149, 152, 153]. Other studies have shown a general down regulation of innate immune response-related genes in mammary lymph nodes or immune suppression through reduction of PMBC proliferation as well as a shift of the cytokine profile from pro-inflammatory to anti-inflammatory [146, 150-152]. *M. gallisepticum* also decreased the expression of inflammatory cytokines such as TNF α , IL-1 β , and IL-6 in embryonic chickens [146, 156].

1.5.4 History of vaccination

Several vaccination studies on pneumonia in sheep have been undertaken; however, as the major cause of acute pneumonia in sheep is due to *M. haemolytica* infection, this organism is frequently the focus when it comes to vaccine development. As *M. ovipneumoniae* has been shown to predispose animals to secondary infections, only providing vaccination against the secondary infection does not provide protection for the animals against pneumonia. This was noted by Raghavan et al (2017) [129] when a vaccine against Leukotoxin producing (lkt+) members appeared to prevent the animals from naturally acquiring the lkt+ members of *Pasteurellaceae* from carrier ewes when comingled with animals of known infection. However, introduction of *M. ovipneumoniae* to the test

animals resulted in all animals developing pneumonia symptoms eventually resulting in the death of the animals from pneumonia over the corresponding months [129]. Upon death, Ikt+ *M. haemolytica* was able to be detected within the lower respiratory tract and growth of the organism was most likely facilitated by *M. ovipneumoniae* infection. This indicates the importance of including *M. ovipneumoniae* in any vaccine program addressing pneumonia.

This was the case when the commercial vaccine Ovipast Plus© was tested in commercial farming conditions. The vaccine contained formalin killed cells expressing iron regulated antigens from *M. haemolytica* strains A1, A2, A6, A7, A9 and *P. trehalosi* strains T3, T4, T10 and T15. As was reported by Goodwin-Ray (2008) [157], there was no distinguishable improvement between vaccinated and placebo groups, with a possible increase in severe pneumonia in the vaccinated group. As the report was focusing on the efficacy of the vaccine on pneumonia, it did not address the cause of the pneumonia in the groups, so it is not possible to determine if it was the presence of *M. ovipneumoniae* or another organism that was the cause of pneumonia. This provides another example of a vaccine targeting organisms determined to be the cause of acute pneumonia whereas focusing on the organisms predisposing animals to lethal infections may provide improved protection against both the chronic infection as well as against multiple lethal infectious agents. *M. ovipneumoniae* has more recently become the target of vaccine research. Experiments using either live *M. ovipneumoniae* or killed *M. ovipneumoniae* (50 µg or 250 µg) in oil adjuvant were tested for vaccine potential [158]. Animals vaccinated with either live *M. ovipneumoniae* or 50 µg of killed *M. ovipneumoniae* did not produce a significant antibody response, however animals vaccinated with 250 µg of killed *M. ovipneumoniae* did produce a significant cELISA antibody response and serum *M. ovipneumoniae* inhibition activity [158]. This provides potential for development of a *M. ovipneumoniae* vaccine, however further testing including challenge trials will be required to confirm any protective potential of this vaccine [158]. This is in contrast to other experiments using similar vaccines [159] suggesting that bacterin based vaccines may not provide the desired protection, especially in animals with prior exposure to *M. ovipneumoniae* [158, 159]. To overcome the potential issues observed in animals previously exposed to *M. ovipneumoniae*, research into the use of subunit vaccines is also being addressed. A few antigens, such as EF-Tu and HSP70, from *M. ovipneumoniae* have been observed to induce strong immune responses in non-target animals, e.g., mice [160]. However further testing of recombinant proteins in sheep will be necessary to corroborate data observed in non-target animals.

Vaccination research has been undertaken against a number of other species of mycoplasma. Vaccination against *M. agalactiae* using an oil-emulsion adjuvant has been shown to induce full protection against the onset of clinical signs and infection [161]. This vaccine used inactivated *M.*

agalactiae in an emulsion of Montanide ISA-563, Marcol-52 and Montane-80 (ratio: 30%, 63%, 7% respectively). *M. mycoides* subsp. *mycoides* SC, the major cause of CBPP is primarily protected against by the T1/44 vaccine, a live vaccine in continuous use in Africa for over 60 years [19, 162]. This vaccine has been shown to provide protection against infection, however the limitations of the vaccine are short duration of immunity, the tendency to cause adverse reactions, and because it is only semi attenuated, it has the potential to cause outbreaks in closed herds [19, 162]. To overcome the adverse effects of the live vaccine, inactivated vaccines have been tried, including the use of whole cell mycoplasma inactivated in saponin and a recombinant sub-unit vaccine prepared using the highly immunogenic lipoprotein LppQ [162]. Despite high antibody titres, there was no evidence that the vaccination provides any protection and may have exacerbated the disease [162]. The evidence to date suggests that only live vaccines can achieve the levels of protection needed to aid eradication [162]. A Canadian-Kenyan consortium has shown potential for the use of a cocktail of 5 antigens, immunisation with some of the antigens appearing to provide protection while others increased immune-related pathology [162-164].

M. bovis is another mycoplasma pathogen of cattle. Inactivated vaccines are the most commonly used in studies to protect against infection. Protection against intratracheal challenge with *M. bovis* was observed in calves following vaccination with experimental bacterin vaccines [162, 165, 166]. The first trial reduced clinical disease, upper-respiratory tract colonisation by the bacterium and lung lesions [162, 165]. In the second trial, total clinical and lung lesion scores were reduced in the vaccinated/ challenged calves. Additionally, this vaccine showed protection against clinical arthritis which developed in only some of the challenged animals [162, 166]. A sub-unit vaccine was prepared based on previously selected *M. bovis* antigens [167, 168]. This vaccination induced strong humoral, and cell-mediated responses, however, did not provide protection against *M. bovis* challenge [167, 168]. Additionally, a combination of recombinant proteins and cell extracts were used as vaccines to control *M. bovis* infections [167, 169, 170].

The dominant mycoplasma species infecting pigs is *M. hyopneumoniae* and is the primary agent involved in porcine respiratory disease complex. Current commercial vaccines are able to provide only partial protection, they do not prevent infection and have limited effect on transmission [32, 171]. Commercial vaccines mostly consist of adjuvanted inactivated, whole-cell preparations [171]. Additionally, an attenuated vaccines have been licenced in Mexico and China [172]. The Mexican vaccine is a thermosensitive mutant of *M. hyopneumoniae* that is applied intranasally. The Chinese vaccine is attenuated by continuous alternating passage through modified Friis medium and is mostly used by intrapulmonary administration [173].

The main mycoplasmas associated with human diseases are *Mycoplasma pneumoniae* and *Mycoplasma genitalium* [5]. Various attempts have been undertaken to produce vaccines against *M. pneumoniae*, however they have been largely unsuccessful. Between 1964-1976, volunteers [10, 11] and military recruits [12-14] were tested using formalin inactivated vaccines. However, when challenged either experimentally or naturally with virulent *M. pneumoniae*, the protective efficacy of the vaccines was limited. Attenuated strains of *M. pneumoniae* created by continuous passage *in vitro* [15, 16] or temperature-sensitive mutants, were successful in protecting hamsters but were unacceptable for human use because they either retained a significant level of virulence for humans or did not colonise and protect human volunteers against challenge [11]. Alternatively, testing with mutant avirulent *M. pneumoniae* strains such as P30 deficient *M. pneumoniae* resulted in exacerbation of disease when challenged with virulent *M. pneumoniae*. With limited protection provided by bacterin vaccines, recombinant protein subunit vaccines are an alternative approach. A number of potential targets have been identified, including cytoadherence proteins (P1, P30, P116, CARDS toxin), polysaccharides, lipids and lipoproteins [174]. These virulence factors have immunogenicity and are likely to be potential candidates for vaccine antigens [174].

1.6 Genome analysis

With the advancement of genetics, it has become possible to sequence large portions or entire genomes of organisms of interest. With the addition of the advancements in bioinformatics it is easier to build and annotate the genomes providing previously un-paralleled information on the organisms at the molecular level [175].

Determining the ideal antigens to be used in a vaccine can be the most difficult and time-consuming aspect of vaccine design [176]. This often requires multiple animal trials and subsequent experimental analyses to determine the effect of the antigen on the immune response of the animal. If an animal does not exhibit a strong response to a particular antigen, a new antigen must be selected, and the process started again. To reduce the number of trials required to find useful antigens the genome and bioinformatics can be used to predict potential vaccine targets by identifying aspects of proteins that may increase the likelihood of the protein to be detected during natural infection or induce a strong immune response [176].

One aspect of proteins useful for vaccine targets is the presence of transmembrane domains. Identifying proteins with a few transmembrane domains at the start of the amino acid coding sequence can provide a pool of proteins that are likely to have some or most of the protein on the surface of the organism [177, 178]. Using proteins that are surface bound for antigen vaccines provide targets that are visible while the organism is whole and active [179, 180]. An additional approach for using bioinformatics in vaccine development is to combine bioinformatics with

traditional antigen discovery using antibodies against the whole cells of the target pathogen and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting to identify proteins that induce an immune response. Putative immunogenic proteins are confirmed by mass spectrometry. Combining this empirical approach with bioinformatics can determine the whole sequence of the immunogenic proteins including potentially fully identifying the protein as well as the presence of defining features, such as transmembrane domains.

1.7 Proteins of *M. ovipneumoniae*

Proteins exported outside the membrane are likely to be processed through the general secretory pathway [181, 182]. In gram positive bacteria, which *M. ovipneumoniae* is thought to have evolved from, exported proteins are either released to the environment or held to the membrane. The general secretory pathway involves several proteins. Several of these proteins have been named with the prefix Sec. and for this reason the pathway has also been called the Sec-dependent pathway. This pathway appears to be common to all bacterial and eukaryote cells [183].

1.7.1 General secretory pathway (GSP)

The general secretory pathway requires the presence of a signal sequence for protein exportation. The signal sequence is recognised by ribonucleoprotein complex called the signal recognition particle (SRP). SRP binds the h-region of the signal peptide by virtue of a hydrophobic groove and delivers it to the translocase machinery in the membrane [184]. The translocase has a membrane-spanning domain made up of heterotrimers of membrane proteins. This creates a channel through which the mature portion of the protein is transferred, leaving the signal sequence embedded in the membrane [182]. Upon transferring through the membrane it is cleaved by a signal peptidase, the mature protein is then released [184]. The final destination is coded in the N-terminal signal sequence of the protein [181, 184].

1.7.2 Signal sequences

Signal peptide sequences consist of 20-40 amino acids at the amino terminus which are primarily hydrophobic [185, 186]. Signal peptides analysed from various prokaryotes species have identified common features: a short (1-5 amino acids) amino terminal n-region containing at least one positively charged residue such as arginine or lysine, followed by a core h-region which is a hydrophobic stretch of 7-20 amino acids. The last section is a carboxyl c-region that is more polar and contains the cleavage site for the signal peptidase [184]. Hydrophobicity of the h-region and the positions of the positively charged residues are considered features essential for the function of the signal sequence [187-189]. The net positive charge of the n-region is thought to interact with the negatively charged surface of the inner-membrane [189] and the core h-region, which has a high propensity for α -helical conformation, is thought to facilitate interaction with the interior of the

membrane bilayer [190]. It is generally believed that gram positive and gram negative bacteria secrete proteins by similar mechanisms as the genome sequences show they share the major components [191]. This is also observed in a number of mycoplasma species [191].

1.7.3 Immunogenic proteins

In the absence of an efficacious vaccine, it is desirable to characterise immunogenic *M.*

ovipneumoniae antigens with a view to developing a novel *M. ovipneumoniae* subunit vaccine.

Detection of immunogenic proteins from a pathogen can provide potential avenues to produce vaccines and diagnostic tests for the prevention and treatment of infections, respectively. To increase the ability of antigens to produce a protective effect, selecting antigens that are present on the surface of the pathogen may provide increased protection by targeting proteins that are present while the pathogen is actively growing in the host.

The studies of *M. ovipneumoniae* to date have identified a small number of immunological proteins. The main proteins identified are heat-shock protein 70 (HSP70) [160, 192-194], elongation factor thermo unstable (EF-Tu) [160, 195-197], and pyruvate dehydrogenase [195]. HSP70 and EF-Tu have been confirmed to be membrane associated proteins [160].

Absence of a cell wall in *M. ovipneumoniae* may further facilitate its membrane and cytoskeleton proteins to actively participate during infection of hosts [160]. As a potential intracellular pathogen, adhesion mediated by membrane-associated proteins is suggested to be a major step in mycoplasma pathogenesis [160]. Previous studies have identified immunogenic proteins such as P97, P42, and MnuA of *M. hyopneumoniae*, P30 of *M. pneumonia*, and MGC3 of *M. gallisepticum* [31, 198-201]. EF-Tu and HSP70 of *M. ovipneumoniae* have been determined to produce both humoral and cellular responses in mice [160]. Moreover, there is evidence to suggest that recombinant proteins of HSP70 and EF-Tu are able to induce a stronger innate immune response, including production of the cytokines TNF- α , IFN- γ , IL-4 and IL-5, than whole cell extracts of *M. ovipneumoniae* [160]. TNF- α is the earliest inflammatory cytokine to activate neutrophils and lymphocytes, this allows the permeability of vascular endothelial cells to increase, tissue metabolism to be adjusted, and promotes the synthesis and release of cytokines [160, 202, 203].

Recombinant protein vaccines, in general, have been known to preferentially induce T_H2 responses with limited or no T_H1 responses. However, some recombinant proteins from *M. hyopneumoniae* have been shown to induce T_H1 response in mice [31, 204, 205]. HSP70 and EF-Tu from *M. ovipneumoniae* have been shown to induce high levels of IFN- γ in mice. In addition to IFN- γ , higher concentrations of IL-4 and IL-5 were detected in response to recombinant HSP70 and EF-Tu compared to whole lysate [160].

1.8 Analysis of immune responses to *M. ovipneumoniae* antigens

1.8.1 Use of serum from bacterin-vaccinated sheep for immune-screening purposes

The use of serum from infected animals is a common approach for immune-screening antigens for subunit vaccine and diagnostic development [23, 160, 194, 206]. For direct detection of immunogenic proteins against *M. ovipneumoniae* infections the use of sera from animals naturally or artificially infected would be ideal. However, the use of sera from bacterin-vaccinated animals can provide its own benefits for exploratory experiments. Bacterin-vaccinated animals can have higher titres of antibody than infected animals, and the ethics to only vaccinate animals is often easier to justify than needing to infect animals with pathogens [207, 208]. Sera from animals vaccinated with bacterins were also chosen in this project due to their availability from previous adjuvant vaccine trials. Due to the limited protective effect of bacterin vaccines against *M. ovipneumoniae* the results obtained using sera from vaccinated animals would benefit from comparison with sera from naturally infected animals.

1.8.2 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay is an antigen antibody reaction. The assay was first introduced and described by Engvall and Perlmann (1971) [209, 210] and has become a common laboratory technique used to measure the concentration of antibodies in blood and other fluids. The assay utilises a covalently coupled enzyme system to measure specific binding of antibody with antigen. The amount of antibody that binds to antigen is determined spectrophotometrically by measuring the conversion of a clear substance to a coloured product by the coupled enzyme. ELISA is typically performed in 96-well plates enabling multiple serum samples and dilution series of sera to be measured simultaneously, along with relevant positive and negative controls. Antibodies present in sera bind to antigen coated onto the solid surface of the plate. Unbound antibodies are washed away, and a secondary antibody conjugated to horse radish peroxidase or another enzyme is then added. Unbound antibodies are removed again, and a suitable substrate is added. The intensity of the yellow colour produced through action of the coupled enzyme provides a readout of the amount of antibodies present in the serum sample [211].

1.8.3 Interferon gamma release assays

Interferon gamma release assays (IGRA) are a collection of tests widely used in the diagnostic identification of latent tuberculosis infection (LTBI) [212-214] as well as other diseases [215-217]. IGRAs rely on pre-sensitised T-lymphocytes (exposed to antigens from infection or vaccination) releasing IFN- γ when re-exposed to specific antigens. This is commonly undertaken by one of two methods, enumeration of IFN- γ secreting T cells (ELISpot) [160] or by measurement of IFN- γ

concentration (ELISA) [216]. Using Interferon gamma release assays in addition to traditional ELISA can help determine the kind of immune response produced from the infection. IFN- γ assays are able to identify the level of cellular immune response to *Mycoplasma* antigens [160] and can aid in understanding the effect of vaccination on the host immune system.

1.8.4 IL-17A

Interleukin-17A can be measured in a similar way as IFN- γ for determining the function of antigens on the production of T_H17 with T-lymphocytes releasing IL-17A when exposed to specific antigens. [218]. This can be completed using either the ELISA method or commonly flow cytometry [218-220]. Whereas measurement of IFN- γ aims to determine the effect of antigens on the upregulation of cellular responses, measurement of IL-17A can help determine the effect of particular antigens on the upregulation of the inflammation response. Measuring the IL-17A response to antigens can be useful as upregulation of IL-17A can result in a protective effect against extracellular pathogens; however, over expression of IL-17A can result in a pathogenic effect in itself [119, 145].

1.8.5 Growth inhibition analysis

Immunological assays, such as those described above are useful for detecting infection and measuring immune responses to specific pathogens but often provide limited information about the ability of the immune system to neutralise the pathogen and control infection. While identifying immunogenic antigens which produce a strong antibody response provides a pathway to finding antigens for a vaccine, this approach on its own does not guarantee the vaccine antigens will protect against the invading pathogen. This is where the use of more direct methods to determine effect of antibodies on the pathogen are useful.

Growth inhibition analysis is a technique that determines the ability of antibodies produced against a pathogen to neutralise or inhibit the growth of the pathogen in culture [160, 221-223]. This is undertaken by incubating the pathogen in media with the addition of sera over several days. Comparison of wells with sera against positive and negative controls allows determination of the effect of anti-sera on the growth of *Mycoplasma* [160].

An *in vitro* growth inhibition test using antisera of mice raised against HSP70 and EF-Tu from *M. ovipneumoniae* has provided evidence that specific antibodies against these proteins can inhibit growth of *M. ovipneumoniae* [160]. This provides a key starting point for further research into the use of recombinant proteins as potential vaccine candidates against *M. ovipneumoniae*. This will require the identification of further immunogenic *M. ovipneumoniae* proteins. Antisera from rabbits raised against any newly detected proteins will allow further *in vitro* testing for ability to inhibit growth of *M. ovipneumoniae*. It must be noted that the immune response in mice and rabbits may not extrapolate to other species, therefore these proteins along with the previously detected HSP70

and EF-Tu would benefit from further analysis using a sheep model to evaluate the ability of these proteins to induce protective immunity to *M. ovipneumoniae* in sheep.

1.8.6 Immunofluorescence microscopy

Surface located proteins may offer the best candidates for a sub-unit protein vaccine. There are several methods to determine the localisation of proteins on the surface of the cell, one of which is immunofluorescence microscopy [224-226].

Immunofluorescence microscopy uses antibodies chemically labelled with fluorescent dyes to visualise molecules under a light microscope. There are two general classes of immunofluorescence techniques: primary and secondary. Primary (direct) uses a single, primary antibody, chemically linked to a fluorophore, and secondary (indirect) uses two antibodies, an unlabelled primary antibody against the target proteins in combination with a secondary antibody with a conjugated fluorophore. If the target protein is present on the surface of the pathogen the primary antibody will attach to the target protein. Then the secondary anti-primary antibody will bind to the primary antibody. Excitation of the fluorophore by exposure to light with the required wavelength produces light at a specific wavelength allowing visualisation of the cells under a microscope [224-226].

1.8.7 Inhibition of adherence by *M. ovipneumoniae* antibodies

In response to an infection to a pathogen the immune system mounts a response to eliminate the invader. One method by which antibodies can interfere with the function of a pathogen is by preventing the pathogen from adhering to the host cells preventing colonisation of the host. To determine the ability of antibodies against a target antigen to prevent adherence, an adherence inhibition analysis can be undertaken [225, 227-229]. This involves the incubation of the pathogen with the cells they adhere to in the presence or absence of the antibodies of interest. The cells are then washed removing any unbound pathogens, the cells are subsequently released from the dish and transferred to a growth medium. If the antibodies prevent adhesion to the cells the pathogen should be removed with the washing and therefore should not grow in the medium. Comparison between samples incubated with antibodies and samples incubated without antibodies will provide an understanding if the antibodies are able to prevent adhesion and to what extent. If there is a noticeable decrease of pathogens from the sample incubated with antibodies it can be considered that there was an effect of the antibodies of the pathogen [225, 227].

1.9 Summary and aims of thesis

M. ovipneumoniae is the primary cause of CNP in sheep in New Zealand, as well as being a predisposing factor for more severe, potentially life-threatening infections. To date there are currently no commercially successful vaccines available for *M. ovipneumoniae*. Infections are difficult to treat

due to the nature of the disease producing only limited overt symptoms, and there are no simple diagnostic tests available.

There is currently limited information on immunogenic proteins of *M. ovipneumoniae*, constraining the ability of developing recombinant protein-based vaccines and antigen-based diagnostics for identifying *M. ovipneumoniae*. This study was initiated to identify novel immunogenic proteins and to determine their potential as diagnostic antigens or as antigens for a sub-unit protein vaccine. This was achieved using an empirical approach and through bioinformatic analysis of the genome of selected New Zealand isolates of *M. ovipneumoniae*.

The specific objectives of the study were as follows:

1. To build and annotate the genome of New Zealand isolates of *M. ovipneumoniae*.
2. To produce membrane fractions from selected isolates of *M. ovipneumoniae* for immunogenicity analysis.
3. To detect immunogenic proteins of *M. ovipneumoniae* by Western blot analysis with sheep antisera produced against *M. ovipneumoniae* and to confirm the identity of the immunogenic proteins using mass spectrometry.
4. To express and purify selected immunogenic proteins in *Escherichia coli* and produce protein-specific antibodies in sheep.
5. To conduct studies using the protein-specific antibodies to determine the potential of the proteins as vaccine antigens or for use as diagnostic reagents.

Chapter 2 Methods

2.1 Media preparation

2.1.1 Mycoplasma culture media

Table 2-1 Frey's complete media.

Reagent	Amount added per volume of media	
	100 ml	1 L
Mycoplasma Broth Base BBL (Frey [230]) (Fort Richard, New Zealand)	67 ml	670 ml
Bacto™ Yeast extract (Invitrogen™, Thermo Fisher Scientific™, United States)	10 ml	100 ml
Horse Serum, New Zealand origin (Gibco™ Invitrogen™, Thermo Fisher Scientific™, United States)	22 ml	220 ml
Thallium acetate (stock 2.5%) (BDH Bio-Strategy, New Zealand)	1 ml	10 ml
Penicillin G potassium salt (Sigma-Aldrich™, Germany) (stock 100,000 U/ml)	0.5 ml	5 ml
D-glucose (Sigma-Aldrich™, Germany)	0.2 ml	2 ml
Phenol Red (BDH Bio-Strategy, New Zealand)	0.2 ml	2 ml

- **Frey's broth base**[230]: 15 g of BBL Mycoplasma Broth base (Fort Richard New Zealand) was added in 670 ml of deionised water, then pH was adjusted to pH 8.0 and autoclaved at 121°C for 20 min.
- **Yeast extract**: 4.2 g of BD Bacto™ Yeast extract (Invitrogen™, Thermo Fisher Scientific™, United States) was added to 140 ml of deionised water to make 3% solution, then autoclaved at 121°C for 20 min. Store at -20°C.
- **Horse serum**: 1 L stock (Gibco™ Invitrogen™, Thermo Fisher Scientific™, United States) was heat inactivated by placing previously defrosted 1L horse serum in a pre-warmed water bath at 56°C, once temperature had stabilised serum was left for 30 min. Remove and aliquot out in 220 ml volumes into sterile containers. Froze at -20°C.
- **Thallium acetate (stock 2.5%)**: 0.250 g of Thallium acetate (BDH Bio-Strategy, New Zealand) was dissolved in 10 ml of milli-q (MQ) water.
- **Penicillin**: 2.51 g Sigma Penicillin G potassium salt (Sigma-Aldrich™, Germany) 1596U/mg - (stock 100000 U/ml) was dissolved in 40 ml to get 100149 U/ml. Froze at -20°C
- **Glucose**: 50 g of powder BDH D+Glucose (Sigma-Aldrich™, Germany) was dissolved into 50 ml MQ water and filter sterilised into a sterile bottle.
- **Phenol Red (stock 1%)**: 0.5 g of Phenol Red (BDH Bio-Strategy, New Zealand) was dissolved in 50 ml of PBS (0.01M, pH7.3) and filter sterilised. Stored at 4°C.

The medium was adjusted to a final pH of 7.8 using 1 M NaOH.

2.1.2 Luria-Bertani (LB) media

LB media was produced by combining 10 g Tryptone (Oxoid, Thermo Fisher Scientific™, United States), 10 g NaCl (Scharlau, Bio-Strategy, New Zealand), and 5 g Bacto™ Yeast extract (Invitrogen™, Thermo Fisher Scientific™, United States) to 950 mL H₂O. The working media was pH 7.0.

2.2 Reagents recipes

2.2.1 TBS 10x (concentrated Tris-buffered saline)

One litre of TBS was produced by combining 54 g Tris-HCl (Applichem, Labsupply, New Zealand), 5.6 g Tris base (Applichem, Labsupply, New Zealand), and 88 g NaCl (Scharlau Bio-Strategy, New Zealand) to H₂O. the solution was made up to one litre. The working pH was 7.6.

2.2.2 PBS (Phosphate Buffered Saline)

One litre of PBS was produced by combining 8 g NaCl (Scharlau Bio-Strategy, New Zealand), 200 mg KCl (BDH Bio-Strategy, New Zealand), 1.44 g Na₂HPO₄ (BDH Bio-Strategy, New Zealand), and 245 mg KH₂PO₄ (Sigma-Aldrich™, Germany) to H₂O. the solution was made up to one litre. The working pH was 7.4.

2.3 Selection of *M. ovipneumoniae* isolates

Prior to the commencement of this project, work had been undertaken to acquire New Zealand isolates of *M. ovipneumoniae* [231]. To study the role of *M. ovipneumoniae* in the pathogenesis of ovine pneumonia, lung lavages had been obtained at the abattoir from 128 sheep sourced randomly from 8 farms in the lower north island of New Zealand which had an history of ovine pneumonia. The lung lavages were diluted in Frey's media and incubated to grow mycoplasmas. The samples were then diluted in Frey's media and plated onto solid Frey's media to isolate single colonies. This process was repeated two more times. Identity of *M. ovipneumoniae* were then confirmed by *M. ovipneumoniae*-specific 16s rRNA PCR (2.3.1). A total of 93 isolates of *M. ovipneumoniae* were cultured from these samples. Molecular analysis using restriction fragment length polymorphism (RFLP) on the whole genome and the P113 adhesin gene [232] clustered these isolates into distinct groups (unpublished observations). In this study, 10 representative isolates (isolates 16, 23, 42, 70, 73, 83, 88, 90, 94, 103) were selected for genomic analysis. Isolates 16, 90, and 103 were selected as representatives of the three dominant groups determined by RFLP with *Eco*R1 (unpublished observations). Stocks of these and other isolates were stored in glycerol at -80°C.

2.3.1 PCR of *M. ovipneumoniae* 16s rRNA

The forward primer was LMFI (5'-TGAACGGAATATGTTAGCTT-3'), and the reverse primer was LMRI (5'-GACTTCATCCTGCACTCTGT-3'). *M. ovipneumoniae* DNA was denatured at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. Final extension was at 72°C for seven min [233, 234]. The amplified PCR product was

run on a 1% agarose gel and the presence of a PCR band at the predicted size indicated the presence of *M. ovipneumoniae*. The PCR products were not sequenced in this previous work.

2.4 Culture of *M. ovipneumoniae*

M. ovipneumoniae isolates 16, 90, and 103 were grown from frozen glycerol stocks. A 10 µl loop (LabServ, Thermo Fisher Scientific™, United States) of mycoplasma was removed from the glycerol stock and added to 3 ml of Frey's media (2.1.1) in a sterile glass bijou. The mycoplasmas were then left to grow at 37°C without shaking until a pH indication colour (phenol red) changed from red to orange. After colour change, 1 ml of the grown mycoplasma media was removed from the bijou and added to 100 ml of fresh Frey's media in a 500 ml flask and left to grow at 37°C, shaking at 150 rpm until a pH indication colour (phenol red) changed from red to orange. The culture was distributed into 30 ml aliquots and centrifugated at 3,320 x g for 20 min. The media was removed, and the cell pellet was resuspended in PBS for immediate use or stored frozen at -20°C for later use.

2.5 DNA extraction

2.5.1 Genomic DNA for high throughput sequencing (HTS)

Prior to the commencement of this project, HTS data was obtained for 10 isolates of *M.*

ovipneumoniae by Jacinda Aplin, AgResearch. The methods used are described below. Genomic DNA was extracted using a ZR fungal/bacterial DNA MiniPrep commercial kit (Zymo Research Corp., United States). The resulting genomic DNA was subsequently purified using a ZR genomic DNA clean and concentrator commercial kit.

ZR fungal/bacterial DNA MiniPrep

A quantity of 50 – 100 mg (wet weight) bacterial cells that had been resuspended in up to 200 µl isotonic buffer (e.g., PBS) was added to the ZR BashingBead™ Lysis Tube (0.1 mm and 0.5 mm). A 750 µl volume of Lysis Solution was added to the tube. The bead beater was then secured in a 2 ml tube holder assembly and processed at maximum speed for ≥ 5 min. The ZR BashingBead™ Lysis Tube was then centrifuged in a microcentrifuge at 10,000 x g for 1 min. Up to 400 µl of the supernatant was transferred to a Zymo-Spin™ IV Spin Filter (Orange Top) in a Collection Tube and centrifuged at 7,000 x g for 1 min. A volume of 1,200 µl of Fungal/Bacterial DNA Binding Buffer was added to the filtrate in the Collection Tube and 800 µl volume of the mixture from collection tube 1 was transferred to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuged at 10,000 x g for 1 min. The flow through from collection Tube 2 was discarded and the final filtrate from collection tube 1 was added to Zymo-Spin™ IIC Column and centrifuged at 10,000 x g for 1 min. A 200 µl volume of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuged at 10,000 x g for 1 min. A 500 µl volume of Fungal/Bacterial DNA Wash Buffer was added to the Zymo-Spin™ IIC Column and centrifuged at 10,000 x g for 1 min. The Zymo-Spin™ IIC

Column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl DNA Elution Buffer was added directly to the column matrix. The column was then centrifuged at 10,000 x g for 30 s to elute the DNA.

ZR genomic DNA clean and concentrator

In a 1.5 ml microcentrifuge tube, 2 volumes of ChIP DNA Binding Buffer were added to each volume of DNA sample and mixed thoroughly. The mixture was transferred to a provided Zymo-Spin™ IIC-XLR Column in a Collection Tube. The collection tube was centrifuged at 16,000 x g for 30 s and the flow-through was discarded. A 400 µl volume of DNA Wash Buffer was added to the column and centrifuged at 16,000 x g for 1 minute. The collection tube was emptied, and the wash step was repeated. The column was transferred to a 1.5 ml microcentrifuge tube. DNA Elution Buffer (≥ 50 µl) or water was added directly to the column matrix and incubated at room temperature for five minutes and centrifuged at 16,000 x g for 30 s to elute the DNA.

2.5.2 Phenol-chloroform DNA extraction

The culture (25 ml) was centrifuged at 10,000 x g for 30 min at 4°C. The pellet was washed once with Tris-EDTA (TE) buffer at 4°C. The pellet was used immediately or stored at -80°C. Cell pellets were resuspended in 500 µl of Tris-Sodium chloride-EDTA (TNE) buffer and the cells lysed by the addition of 10 µl of 10% SDS and 10 µl of 10% sarcosine. A 10 µl volume of 20 mg/ml protein kinase was added, and the sample was incubated at 37°C for 2 h. Following incubation, 100 µg/ml DNase-free RNase was added and incubated at 37°C for 30 min. The lysate was extracted once with 500 µl of phenol (Gibco™ Invitrogen™, Thermo Fisher Scientific™, United States). The emulsion was centrifuged at 13,000 x g for 10 min, and the upper aqueous phase was transferred to another tube. A volume of 500 µl of phenol:chloroform:isoamyl alcohol was added to the tube, the sample was centrifuged at 13,000 x g for 10 min. 400 µl of the upper aqueous phase was transferred to a new tube. Sodium acetate (40 µl) and 800 µl of 100% ethanol was added and mixed gently. The DNA was precipitated by incubation at -20°C for 16 h. The DNA was pelleted by centrifugation at 13,000 x g for 10 min, the supernatant was then discarded. A volume of 500 µl of 80% ethanol was added and mixed. The DNA was pelleted by centrifugation at 13,000 x g for 10 min and the supernatant was discarded. The DNA was air dried and resuspended in TE buffer.

2.6 Preparation of *M. ovipneumoniae* proteins

2.6.1 Whole cell lysates (WCL)

The cell pellet from 2.4 was resuspended in 1 ml PBS and transferred to a microcentrifuge tube. The suspension was sonicated on ice with a VCX 750 Ultrasonic Processor (Sonics & Materials, USA) to break open the cells and fragment the genomic DNA. The sample was sonicated using 40 s bursts

until lysis was complete (typically 3–4 times). The suspension was chilled on ice for 1 min between each ultrasonic treatment. Samples were stored at -20°C.

2.6.2 Preparation of membrane fractions from *M. ovipneumoniae*

Membrane protein fractions were prepared using the Bio-Rad ReadyPrep™ Protein Extraction Kit (Membrane I) [235, 236] (Bio-Rad Laboratories, United states). Buffers M1 and M2 were chilled on ice for at least 15 min before beginning the extraction and the bottle of buffer were inverted several times during the incubation period). Membrane fractions were prepared from *M. ovipneumoniae* by resuspending a cell pellet from 10 ml of culture in a microcentrifuge tube with 1 ml of buffer M1. The suspension was sonicated on ice with a VCX 750 Ultrasonic Processor (Sonics & Materials, USA) to break open the cells and fragment the genomic DNA. The sample was sonicated using 40 s bursts with 1 min intervals on ice until lysis was complete (typically 3–4 times). A 500 µl volume of chilled buffer M2 was added to the cell extract. The suspension was vortexed 4–5 times, 60 s each. The tube was kept in an ice-water bath for 30–60 s between each vortexing step. At the end of the vortexing procedure, the microcentrifuge tube was incubated in an ice-water bath for 10 min. The microcentrifuge tube was transferred to a 37°C water bath and incubated for 30 min. The suspension was mixed for 30 s periodically (3 to 4 times). The microcentrifuge tube was centrifuged at maximum speed in a microcentrifuge (16,000 x g) for 5 min at room temperature. The top layer, containing the hydrophilic proteins, was removed, and transferred to a clean microcentrifuge tube. A 500 µl volume of prechilled buffer M2 was added to the tube containing the bottom phase. The suspension was vortexed 4–5 times, 60 s each. The tube was kept in an ice-water bath for 30–60 s between each vortexing step. At the end of the vortexing procedure, the microcentrifuge tube was incubated in an ice-water bath for 10 min. The microcentrifuge tube was transferred to a 37°C water bath and incubated for 30 min. The suspension was mixed for 30 s periodically (3 to 4 times). The microcentrifuge tube was centrifuged at maximum speed in a microcentrifuge (16,000 x g) for 5 min at room temperature. Again, the top layer was removed and pooled with the top layer that was collected earlier. This tube of pooled top layers was labelled Hydrophilic Protein Fraction. The bottom phase was collected and transferred to a new microcentrifuge tube. This tube was labelled Hydrophobic Membrane Protein.

Hydrophobic and hydrophilic fractions were cleaned up using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad Laboratories, United states) according to kit instructions using ZOOM™ Carrier Ampholytes pH 3-10 Invitrogen™, Thermo Fisher Scientific™, United States. Samples were stored at -20°C. The ReadyPrep 2-D Cleanup Kit uses selective precipitation to remove ionic contaminants such as detergents, lipids, and phenolic compounds from protein samples. These contaminants may interfere with separation, particularly during isoelectric focusing (IEF) and 2-D separations. Use of the kit improves 2-D resolution and reproducibility.

A 100 µl volume of the protein preparation was transferred into a 1.5 ml microcentrifuge tube. A 300 µl volume of precipitating agent 1 was added to the protein sample, without touching the samples to avoid possible protein precipitation and mixed well by vortexing. The preparation was incubated on ice for 15 min. Precipitating agent (300 µl) was added to the mixture of protein and precipitating agent without touching the samples to avoid possible protein precipitation. The sample was mixed well by vortexing. The microcentrifuge tubes were centrifuged at maximum speed (16,000 x g) for 5 min to form a tight pellet. Without disturbing the pellet, the supernatant was removed and discarded using a pipette. The microcentrifuge tubes were centrifuged for 15-30 s to collect any residual liquid at the bottom of the microcentrifuge tube and the remaining supernatant was removed. A 40 µl volume of wash reagent 1 was added on the top of the pellet. The tube was centrifuged at maximum speed (16,000 x g) for 5 min and the wash reagent was removed using a pipette. Ultrapure water (25 µl) was added on top of the pellet and the tube was vortexed for 10-20 s. A 1 ml volume of wash reagent 2 (prechilled at -20°C for at least 1 hr) and 5 µl of wash 2 additive was added and the microcentrifuge tube was vortexed for 1 min. The microcentrifuge tube was incubated at -20°C for 30 min, vortexing for 30 s every 10 min during the incubation period. After the incubation period, the tube was centrifuged at 16,000 x g for 5 min to form a tight pellet. The supernatant was removed, and the tube was further centrifuged for 15-30 s and any remaining wash reagent was removed. The pellet would appear white at this stage. The pellet was air-dried at room temperature for no more than 5 min and until the pellet appeared translucent. Each pellet was resuspended by adding 100-150 µl of 2-D rehydration/sample buffer to the pellet. The supernatant can be used directly for IEF in IPG strips or in IEF gels. The prepared samples were stored at -20°C for short term or -80°C for long term storage. Any unused or remaining protein sample was stored in a clean tube at -80°C for later analysis.

2.6.3 Protein determination

Protein concentration in the *M. ovipneumoniae* fractions was determined using a Pierce™ BCA Protein Assay Kit, (Thermo Fisher Scientific™, United States) [237-240]. The working reagent (WR) was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A: B). A 25 µL quantity of standard or sample (3 replicates) was pipetted into a microplate well (Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate, Thermo Fisher Scientific™, United States). A 200 µL volume of the WR was added to each well and the plate was mixed thoroughly on a plate shaker for 30 s. The plate was covered and incubated at 37°C for 30 min. The plate was cooled to room temperature and the absorbance measured at or near 562 nm on a VersaMax™ ELISA Microplate Reader (Bio-Strategy, New Zealand).

2.7 Generation of antisera in sheep and rabbits

2.7.1 Inactivation of mycoplasma cells with hydrogen peroxide

Prior to using *M. ovipneumoniae* for vaccinating sheep in the various animal trials, the cells were inactivated using hydrogen peroxide (H₂O₂) (30 % W/W (Bio-Strategy, New Zealand)). H₂O₂ was chosen due to its successful use in other vaccine studies undertaken by AgResearch, to inactivate bacteria without denaturing surface proteins. *M. ovipneumoniae* isolates were grown in 200 – 600 ml volumes of Frey's media until media colour changed to orange (2.4). A 300 ml culture of each isolate was centrifuged at 10,000 x g for 20 min, the supernatant was removed, and the cell pellet re-suspended in 300 ml of PBS solution. A 49 ml quantity of H₂O₂ (30 % W/W (Bio-Strategy, New Zealand)), was added for a final concentration of 5%. The cells were gently shaken (150 rpm) for two hours at room temperature. The cells were recovered by centrifugation at 10,000 x g for 10 min and the supernatant was removed. The pellet was resuspended in 40 ml PBS and transferred to 50 ml falcon tubes. The cells were centrifuged at 3,320 x g for 10 min, the supernatant was removed, and the pellet resuspended in 40 ml PBS. The washing step was repeated, and the cells resuspended in PBS at a protein concentration of 9 mg/ml (2.6.3).

An experiment was conducted to determine the minimum concentration of H₂O₂ (30% w/w) needed to inactivate *M. ovipneumoniae* by conducting the above protocol with a range (0.1% to 10%) of concentrations. Successful inactivation of *M. ovipneumoniae* was determined by incubating aliquots of the treated cells in Frey's media for 14 days and observing any change in colour. A concentration of 5% was the lowest concentration of H₂O₂ that inhibited growth of *M. ovipneumoniae*.

2.7.2 Pre-existing sera from sheep vaccinated with *M. ovipneumoniae* and *M. haemolytica*

A trial (conducted under Grassland's Animal Ethics approval 13567) had been conducted previously at AgResearch to determine optimal adjuvants for stimulating strong serum antibody responses to *M. ovipneumoniae* and *M. haemolytica* (manuscript in preparation). The sera from this trial were made available at the start of the PhD and were used for the initial Western blotting analysis. Groups of 20 sheep were vaccinated with a mixture of *M. ovipneumoniae* (isolates 16, 90, and 103) and *M. haemolytica* WCL combined with three different adjuvants, Alum, Montanide ISA61 or Quil-A (Table 2.2). Control groups of sheep (n=7) were given adjuvant alone. Sheep were vaccinated (subcutaneously) twice at a 4-week interval with *M. haemolytica* (5 x 10⁸ cells) and *M. ovipneumoniae* (0.2 mg from each of the three isolates). Serum samples were obtained from animals before vaccination (pre-immune sera) and at 2 weeks after the second vaccination. Antibody responses to *M. ovipneumoniae* WCL antigens were measured by ELISA to identify animals that had developed a strong immune response to *M. ovipneumoniae*. The antisera from three animals, vaccinated with WCL formulated with Montanide ISA61 (Seppic, France), an oil-in-water adjuvant

were selected for Western blotting studies as these animals had high antibody titres as determined by ELISA. The sera from the three sheep were pooled and stored in aliquots at -20°C.

Table 2-2 Groups of animals vaccinated with a mixture of *M. ovipneumoniae* and *M. haemolytica* WC combined with three different adjuvants, Alum, Montanide ISA61 or Quil-A.

Group	Number of animals	Adjuvant	Antigen
1	7	Alum	Nil
2	7	Quil-A	Nil
3	7	Montanide ISA61	Nil
4	20	Alum	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i>
5	20	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i>
6	20	Montanide ISA61	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i>

2.7.3 Sera from sheep vaccinated only with *M. ovipneumoniae*

Antisera were obtained from sheep vaccinated with *M. ovipneumoniae* for Western blotting studies to compare results obtained using antisera against a mixture of *M. ovipneumoniae* and *M. haemolytica*. WCL of *M. ovipneumoniae* isolates 16, 90 and 103 were grown as described in 2.4. Two sheep were vaccinated twice by the subcutaneously route at a 4-week interval with combination of 0.6 mg the three isolates formulated with Montanide ISA61 (Seppic, France) adjuvant (64% v/v). The trial was performed with Grassland's Animal Ethics approval (AE 14263). Sera were collected pre-vaccination (pre-immune sera) and 2 weeks after the second vaccination.

2.7.4 Rabbit antisera

Three rabbits (New Zealand white) were vaccinated with WC of *M. ovipneumoniae* isolates 16, 90 and 103. Each rabbit was vaccinated four times at 2-week intervals with WCL from a single isolate emulsified Montanide ISA61 (Seppic, France) (0.6 mg for first two vaccinations and then 0.3 mg for the 3rd and 4th vaccinations). Vaccination of rabbits and collection of pre-immune sera and antisera were performed at AgResearch Ruakura under Animal Ethics approval (AE14012, 12445).

2.7.5 Sheep trials for producing antisera for studies and measurement of immune responses

Three sheep trials were conducted to collect pre-immune sera, antisera, and blood for studies and measurement of cell-mediated immune responses. All, or a selection of the animals used in these trials were used for studies in this thesis. Each of the trials are described below followed by a table indicating which animals were collected from and what was collected. Animals in these trials were vaccinated and bled by Dr Sandeep Gupta and Tania Wilson, AgResearch.

2.7.5.1 Trial 1: Antisera production (recombinant proteins from *M. ovipneumoniae* antigens)

A trial (referred to as recombinant protein trial or Antisera production trial 1) using 24 female Romney crosses approximately 6 months of age, was conducted to produce antisera against the in-house EF-Tu and the five proteins produced by GenScript (EF-Tu, GAPDH, HP3, P146, and an epitope of HP1). Antisera were also produced against WC and hydrophobic protein fractions (HPF) prepared from the three isolates (16, 90, 103) of *M. ovipneumoniae* (described in Chapter 2.6). Twelve groups of sheep (detailed in Table 2.3 n=2 per group) were vaccinated with the various antigens (Table 2.3) formulated with Montanide ISA61 (Seppic, France) adjuvant twice by the intramuscular route at a 4-week interval. This trial was conducted at the AgResearch Aorangi farm, Palmerston North under AgResearch (Grasslands) Animals Ethics Committee's approval (AE 14595).

Sera samples were collected at week 0 (pre-vaccination), week 4 (at the time of second vaccination), and week 6 for measurement of antibody responses. Heparinised blood samples were also collected at week 7 for measurement of antigen-specific IFN- γ and IL-17A responses.

Table 2-3 Groups of animals vaccinated with whole cell (WC) or hydrophobic protein fractions (HPF) of *M. ovipneumoniae* or individual recombinant proteins.

Group (n=2)	Dose (mg)	Antigen
1	0.1	<i>M. ovipneumoniae</i> recombinant protein EF-Tu (GenScript)
2	0.1	<i>M. ovipneumoniae</i> recombinant protein GAPDH (GenScript)
3	0.1	<i>M. ovipneumoniae</i> recombinant protein HP3 (GenScript)
4	0.1	<i>M. ovipneumoniae</i> recombinant protein HP1 (GenScript)
5	0.1	<i>M. ovipneumoniae</i> recombinant protein P146 (GenScript)
6	0.1	<i>M. ovipneumoniae</i> recombinant protein EF-Tu (In-house)
7	0.6	WC of <i>M. ovipneumoniae</i> isolate 16
8	0.6	WC of <i>M. ovipneumoniae</i> isolate 90
9	0.6	WC of <i>M. ovipneumoniae</i> isolate 103
10	0.5	HPF from isolate 16
11	0.5	HPF from isolate 90
12	0.5	HPF from isolate 103

2.7.5.2 Trial 2: Antisera production (WC of *M. ovipneumoniae* and *Methanobrevibacter ruminantium* M1)

A second antisera production trial (Antisera production trial 2) was conducted. Due to the loss of 8 animals due to facial eczema in Antisera production trial 1, (including animals vaccinated with WC), additional animals (1-year old castrated male Romney crosses) were vaccinated subcutaneously with

M. ovipneumoniae WC. One animal was vaccinated with WC from isolate 90 and two animals were vaccinated with WC from isolate 103. In addition, three animals were vaccinated with an unrelated organism *Methanobrevibacter ruminantium* M1. Three animals were given adjuvant alone. Vaccine antigens (*M. ovipneumoniae* 0.6 mg protein or *M. ruminantium* 5 mg wet weight) were formulated with Montanide ISA61 adjuvant (Seppic, France) and animals were vaccinated twice by the intramuscular route at a 3-week interval. This trial was conducted at the AgResearch Aorangi farm, Palmerston North under AgResearch (Grasslands) Animals Ethics Committee's approval (AE 14718). Sera were collected at three time points, week 0 (pre-vaccination), week 3 (at the time of second vaccination) and week 5. In this trial, heparinised blood samples were also collected at week 5 for measurement of antigen-specific IFN- γ and IL-17A responses.

Table 2-4 summarises the animals from Antisera production trial 2 used in this thesis.

Table 2-4 Groups of animals vaccinated with *M. ovipneumoniae* or methanogen WC.

Group	Antigen
1	Control (Adjuvant alone) (n=3)
10	Methanogen WC (M1) (n=3)
11	WC <i>M. ovipneumoniae</i> isolate 90 (n=1)
12	WC <i>M. ovipneumoniae</i> isolate 103 (n=2)

2.7.5.3 Trial 3: Adjuvant trial

A trial using 24 sheep was conducted by the Animal Health team (AgResearch, Palmerston North, New Zealand) to compare the efficacy of five different adjuvants at promoting immune response to *M. ovipneumoniae* and *M. haemolytica* antigens. Groups (n=12) of mixed sex Romney crosses approximately 6 months of age, were vaccinated twice subcutaneously with a mixture of WC from *M. ovipneumoniae* isolates 16, 90, 103 (0.6 mg protein from each of the three isolates, 1.8 mg total) and *M. haemolytica* (Approximately 10^8 inactivated cells per dose, as recommended by MSD Animal Health, New Zealand). Group 1 was comprised of antigens alone, and Group 2, had antigens formulated with Quil-A®. These animals were part of a larger trial of 72 animals. Aspects of the immune responses by animals in Groups 1 and 2 are reported in this thesis. Responses in animals in Groups 3-6 are outside the scope of the thesis. This trial was conducted at the AgResearch Aorangi farm, Palmerston North under AgResearch (Grassland) Animals Ethics Committee's approval (AE 14592).

Sera samples were collected at week 0 (pre-vaccination), week 4 (at the time of second vaccination), while sera and heparinised blood were collected at week 6 for measurement of antibody and T-cell

responses respectively. Raw IFN- γ and IL-17A data was collected by Tania Wilson, AgResearch for this trial.

Table 2-5 Groups of animals vaccinated with *M. ovipneumoniae* and *M. haemolytica* WC antigens formulated with and without adjuvant.

Group (n=12)	Antigen
1	Control (Antigens alone)
2	Antigens + Quil-A adjuvant

2.7.5.4 Trial 4 Challenge trial

A trial using 25 mixed sex Romney crosses approximately 4 months of age was conducted by the Animal Health team (AgResearch, Palmerston North, New Zealand) to determine the efficacy of a subcutaneous vaccine comprised of *M. haemolytica* (approximately 10^8 inactivated cells per dose, as recommended by MSD Animal Health, New Zealand) formulated with Quil-A to protect sheep against experimental challenge with pneumonia. Sera obtained at weeks 0 and 7 from 12 of the animals administered with *M. haemolytica* formulated with Quil-A were used to study the cross-reactivity of responses in sheep vaccinated (in Antisera production trial 1) with the various *M. ovipneumoniae* recombinant proteins. These animals were part of a larger trial of 75 animals. The other animals in this trial were vaccinated with a mixture of *M. haemolytica* (10^8 inactivated cells per dose, as recommended by MSD Animal Health, New Zealand) and *M. ovipneumoniae* (0.6 mg protein from each of the three isolates, 1.8 mg total) (n=25) formulated with Quil-A to protect sheep against experimental challenge with pneumonia. A control group (n=25) were given adjuvant alone. This trial was conducted at the AgResearch Aorangi farm, Palmerston North under AgResearch (Grassland) Animals Ethics Committee's approval (AE 14870). Other results from the trial are outside the scope of this thesis and are not reported here.

Table 2-6 Summary of sera and blood collected from the four trials and used in this thesis.

Trial	Animal No.	Antisera	Blood	Adjuvant	Antigen
1	1.1	Week 6	Week 7	Montanide ISA61	Recombinant EF-Tu
1	1.2	Week 6	Week 7	Montanide ISA61	Recombinant EF-Tu
1	1.3	NA	NA	Montanide ISA61	Recombinant GAPDH
1	1.4	Week 6	Week 7	Montanide ISA61	Recombinant GAPDH
1	1.5	Week 6	Week 7	Montanide ISA61	Recombinant HP3
1	1.6	NA	NA	Montanide ISA61	Recombinant HP3
1	1.7	Week 6	Week 7	Montanide ISA61	Recombinant HP1
1	1.8	Week 6	Week 7	Montanide ISA61	Recombinant HP1
1	1.9	Week 6	Week 7	Montanide ISA61	Recombinant P146

1	1.10	Week 6	Week 7	Montanide ISA61	Recombinant P146
1	1.11	NA	NA	Montanide ISA61	Recombinant EF-Tu (In-house)
1	1.12	Week 6	Week 7	Montanide ISA61	Recombinant EF-Tu (In-house)
1	1.13	Week 6	Week 7	Montanide ISA61	WC16
1	1.14	Week 6	Week 7	Montanide ISA61	WC16
1	1.15	Week 6	Week 7	Montanide ISA61	WC90
1	1.16	NA	NA	Montanide ISA61	WC90
1	1.17	NA	NA	Montanide ISA61	WC103
1	1.18	NA	NA	Montanide ISA61	WC103
1	1.19	NA	NA	Montanide ISA61	HPF16
1	1.20	NA	NA	Montanide ISA61	HPF16
1	1.21	Week 6	Week 7	Montanide ISA61	HPF90
1	1.22	Week 6	Week 7	Montanide ISA61	HPF90
1	1.23	Week 6	Week 7	Montanide ISA61	HPF103
1	1.24	Week 6	Week 7	Montanide ISA61	HPF103
2	2.1	Week 5	Week 5	Montanide ISA61	N/A
2	2.2	Week 5	Week 5	Montanide ISA61	N/A
2	2.3	Week 5	Week 5	Montanide ISA61	N/A
2	2.4	Week 5	Week 5	Montanide ISA61	Methanogen WC (M1)
2	2.5	Week 5	Week 5	Montanide ISA61	Methanogen WC (M1)
2	2.6	Week 5	Week 5	Montanide ISA61	Methanogen WC (M1)
2	2.7	Week 5	Week 5	Montanide ISA61	WC90
2	2.8	Week 5	Week 5	Montanide ISA61	WC103
2	2.9	Week 5	Week 5	Montanide ISA61	WC103
3	3.1	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.2	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.3	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.4	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.5	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.6	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.7	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.8	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.9	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.10	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.11	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.12	Week 6	Week 6	N/A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.13	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.14	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.15	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.16	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.17	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.18	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.19	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.20	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.21	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC

3	3.22	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.23	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
3	3.24	Week 6	Week 6	Quil-A	<i>M. ovipneumoniae</i> and <i>M. haemolytica</i> WC
4	4.1	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC
4	4.2	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC
4	4.3	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC
4	4.4	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC
4	4.5	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC
4	4.6	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC
4	4.7	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC
4	4.8	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC
4	4.9	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC
4	4.10	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC
4	4.11	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC
4	4.12	Week 7	Week 7	Quil-A	<i>M. haemolytica</i> WC

2.8 Production of recombinant proteins

2.8.1 Expression and purification of EF-Tu in *E. coli*

The gene encoding EF-TU was isolated by PCR using specific primers as described by Jiang et al (2016) [160] (Table 2.7). Restriction sites, NcoI and XhoI, were introduced at the 5- and 3'-ends of the EF-Tu gene respectively in the flanking primers.

The *M. ovipneumoniae* EF-Tu carries an UGA codon. This codes for tryptophan in mycoplasmas but codes for a stop codon in *E. coli*. Overlap PCR was used to change the A in the UGA codon to a G to avoid producing a truncated protein. The primers used for cloning and nucleotide replacement are shown in Table 2.7. PCR master mix composition and thermocycling conditions are detailed in Tables 2.8 and 2.9, respectively. The gene final product was sequenced at Massey Genome Service (MSG) in Palmerston North to confirm the codon change.

Table 2-7 Primers used for amplification of EF-Tu by overlap extension PCR.

Restriction sites are highlighted in yellow and mutation sites are indicated by underlining.

Primer No.	Primer name	Primer sequence	Description
1	MoEFTu-NcoI-A	5'-CATG CCATGG CAGTTGTAAACTGG-3'	Forward primer containing a NcoI site for cloning into pET 22b (+)
2	MoEFTu576-b	5'-GTTCAAGAACTTAGCTTCC <u>CA</u> TTCAGGTTTTCCTCAAG-3'	Reverse primer for site-directed mutagenesis, the mutation site is at genome position 576bp

3	MoEFTu576-C	5'-CTTGAAGGAAAACCTGAAT TGG GAAGCTAAAGTTCTTGAAC-3'	Forward primer for site-directed mutagenesis, the mutation site is at genome position 576bp
4	MoEFTu-XhoI-D	5'-CCG CTCGAG TTTAATAATTTCACTTACTG-3'	Reverse primer containing a XhoI site for cloning into pET 22b (+)

Table 2-8 PCR master mix (5X).

Master Mix	20µl
H ₂ O	61
BUFFER	10
dNTP	10
Forward	42
Reverse	4
Polymerase	1
Total	90

Table 2-9 Thermocycler conditions.

PCR conditions		
°C	Sec	cycles
95	120	
95	30	x30
48	30	
72	60	
72	300	
4	∞	

Electrophoresis of DNA:

DNA marker (5 µl 1 Kb Plus DNA Ladder (Invitrogen™, Thermo Fisher Scientific™, United States) was loaded to the first well of a 1% agarose gel. A 20 µl volume of PCR product was loaded into remaining wells. Following electrophoresis for 45 min at 100 V, the gel was kept in plastic frame and imaged in the ultraviolet (UV) transilluminator (Gel Doc Bio-Rad).

DNA purification:

The full-length PCR product with the mutated EF-Tu was extracted using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific™, United States). The gel in the plastic frame was placed on a UV transilluminator (Gel Doc Bio-Rad, United States). The gel slice containing the DNA fragment was excised using a clean scalpel, cutting as close to the DNA as possible to minimise the gel volume. The gel slice was placed into a pre-weighed 1.5 mL microfuge tube and weighed. Exposure to UV light was kept to a few seconds to avoid causing damage to the DNA. A volume of 100 µL of Binding Buffer was used for every 100 mg of agarose gel. The gel mixture was incubated at 60°C for 10 min until the gel slice was completely dissolved. The tube was mixed by inversion every few minutes to

facilitate the melting process. The gel mixture was vortexed briefly before loading on the column. Up to 800 µL of the solubilised gel solution was transferred to the GeneJET purification column and centrifuged for 1 min at 20,000 x g. The flow-through was discarded and the column was placed back into the same collection tube. A 700 µL volume of Wash Buffer was added to the GeneJET purification column and centrifuged for 1 min at 16,000 x g. The flow-through was discarded and the column was placed back into the same collection tube. The empty GeneJET purification column was centrifuged at 16,000 x g for an additional 1 min to completely remove residual wash buffer. The GeneJET purification column was transferred into a clean 1.5 mL microcentrifuge tube. Elution Buffer (50 µL) was added to the centre of the purification column membrane and centrifuged for 1 min. The purified DNA was stored at -20°C.

[Restriction endonuclease digestion and cloning](#)

Purified samples were double digested by the restriction enzymes NcoI and XhoI (Thermo Fisher Scientific™, United States) in 2X Tango buffer (Thermo Fisher Scientific™, United States). Samples were incubated at 37°C for six hours before inactivation by heating at 80°C for 20 min.

The digested fragments were combined with T4 DNA Ligase (Invitrogen™, Thermo Fisher Scientific™, United States) and digested pET22b (+) prokaryotic expression vector (Novagen, Merck Group, Germany) then incubated at room temperature for 1 h to construct the pET22b (+)-EF-Tu plasmid.

[Transformation of *E. coli*](#)

The completed plasmid was transformed into DH5 alpha *E. coli* (Invitrogen™, Thermo Fisher Scientific™, United States). A 50 µl quantity of DH5 alpha *E. coli* competent cells (Invitrogen™, Thermo Fisher Scientific™, United States), were added to two clean microcentrifuge tubes. A volume of 1 µl (10 ng confirmed by nanodrop) pET22b (+)-EF-Tu plasmid was added to one tube and a volume of 1 µl H₂O was added to the second tube (negative control). Samples were incubated on ice for 30-45 min then heat shocked in 42°C water bath for 1 min. A 500 µl amount of LB broth was added to each tube. The tubes were incubated vigorously on a shaker at 150 rpm for 1h at 37°C. A 100 µl aliquot was transferred to a LB plate containing 100 µg/ml of ampicillin (Roche, Sigma-Aldrich™, Germany). The tubes were centrifuged at 16,000 x g, 300 µl of the supernatant removed, and the cells re-suspended in the remaining approximately 100 µl, then plated onto a LB plate. The plates were incubated overnight at 37°C to produce colonies.

[Purification of plasmid](#)

A single colony was inoculated into 10 ml LB containing 100 µg/ml Ampicillin in a 50 ml Falcon tube and incubated overnight on a shaker at 200 rpm at 37°C. Plasmids were purified using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen™, Thermo Fisher Scientific™, United States). A 5 ml volume of the overnight LB-culture was transferred to a 15 ml Falcon tube, centrifuged at 3,320 x g and all medium removed. The cell pellet was resuspended in 1 ml milli-Q water. This was transferred to a

new 1.5 ml microcentrifuge tube, centrifuged at 12,000 × g and all water removed. Resuspension Buffer (R3) with RNase A (250 µl) was added to the cell pellet and the pellet resuspended until homogeneous. Lysis Buffer (L7) (250 µl) was added, and the sample mixed gently by inverting the capped tube until the mixture was homogeneous. The sample was incubated in the tube at room temperature for 5 min. Precipitation Buffer (N4) (350 µl) was added and the sample was mixed immediately by inverting the tube. The sample containing the lysate was centrifuged at 16,000 × g for 10 min. The supernatant was transferred onto a spin column in a 2 mL wash tube. The column was centrifuged at 12,000 × g for 1 min and the flow-through discarded and the column placed back into the wash tube. Wash Buffer (W9) (700 µl) containing ethanol was added to the column. The column was centrifuged at 12,000 × g for 1 min, the flow through discarded and the column placed into the wash tube. The column was centrifuged at 12,000 × g for 1 min. The wash tube was discarded with the flow through. The Spin Column was placed in a clean 1.5 mL elution tube. A 75 µl volume of preheated TE Buffer (TE) (65-75°C) was added to the centre of the column. The column was incubated for 1 min at room temperature. The column was centrifuged at 12,000 × g for 2 min. The elution tube contained the purified plasmid DNA. The plasmid DNA was quantified using a NanoDrop® ND-1000 (Thermo Fisher Scientific™, United States) to confirm the concentration of the plasmid. The presence of EF-Tu within the plasmid was confirmed via PCR using Primers A and D (Table 2.4). The plasmid DNA was stored at 4°C while being used then moved to -20°C for long term storage.

Production and purification of recombinant protein

The pET22b (+)-EF-Tu plasmid was transformed into Rosetta™ (DE3) competent cells (Sigma-Aldrich™, Germany), to express the recombinant protein. A 50 µl aliquot of Rosetta™ (DE3) competent cells (Sigma-Aldrich™, Germany), were added to two clean microcentrifuge tubes. A quantify of 1 µl (10 ng confirmed by nanodrop) pET22b (+)-EF-Tu plasmid was added to one tube and H₂O (1 µl) was added to the second tube (negative control). Samples were incubated on ice for 30-45 min, then heat shocked in 42°C water bath for 1 min. A 500 µl amount of LB broth was added to each tube and incubated vigorously on a shaker at 150 rpm for 1h at 37°C. A 100 µl aliquot was transferred to a LB plate containing 100 µg/ml of ampicillin (Roche, Sigma-Aldrich™, Germany). The tubes were centrifuged at 16,000 × g, 300 µl of the supernatant removed, and the cells re-suspended in the remaining approximately 100 µl, then plated onto a LB plate. The plates were incubated overnight at 37°C to produce single colonies. A single colony was inoculated into 10 ml LB containing 100 µg/ml ampicillin (Roche, Sigma-Aldrich™, Germany) in a 50 ml Falcon tube and incubated overnight on a shaker at 200 rpm at 37°C.

The competent cells carrying pET22b (+)-EF-Tu were grown in sterile Luria-Bertani (LB) media containing ampicillin (100 µg/ml). Cells were incubated for 3 h before addition of 1 mM isopropyl β-

D-thiogalactoside (IPTG, Sigma-Aldrich™, Germany) to induce protein production. Incubation of the culture was continued at 37°C for 3 h.

The cells were harvested by centrifugation at 10,000 x g for 20 min and disrupted by sonication, VCX 750 Ultrasonic Processor, (Sonics & Materials, USA) and recombinant proteins purified by His60 Ni Gravity Column Purification Kit (Takara Bio United States). A 2 ml volume of His60 Ni xTractor buffer was added per 100 mg of cell pellet. Sample was gently pipetted up and down until the cell pellet was fully resuspended. To the resuspended pellet, 1 µl DNase I was added for every 2 ml of extract and mixed gently. The sample was incubated on ice, with intermittent mixing for 15 min. The sample was centrifuged for 20 min at 10,000 x g at 4°C. The clear supernatant was collected.

[Batch/gravity-flow column purification using a His60 Ni Gravity Column](#)

The His60 Ni Gravity Column (1 ml) and all buffers were equilibrated to room temperature before use. Prior to opening the column, the matrix was fully resuspended to prevent loss of resin that may have settled near the top cap. The column was washed with 5 column volumes of His60 Ni Equilibration Buffer. The bottom stopper on the outlet of the column was put back. The clarified sample was added to the column and the top stopper carefully connected to the top of the column. The target protein was allowed to bind by slowly inverting the column for 1 h at 4°C. The column was installed in a vertical position and the resin settled at the bottom of the column. A clean empty tube was put under the outlet of the column. The top and bottom stopper was removed, and 1 ml fractions were collected. The column was washed with 10 column volumes of His60 Ni Equilibration Buffer followed by 10 column volumes of His60 Ni Wash Buffer. The target protein was eluted with approximately 10 column volumes of Elution Buffer and 1 ml fractions were collected. The collected fractions were analysed by SDS-PAGE. Recombinant protein was concentrated using Pierce™ Protein Concentrators PES, 10K MWCO, 2-6 ml (Thermo Fisher Scientific™, United States). Protein Concentrators PES are disposable ultrafiltration centrifugal devices with a polyethersulfone (PES) membrane for concentration, desalting, and buffer exchange of biological samples.

[2.8.2 Production of recombinant proteins by GenScript](#)

In addition to EF-Tu produced above, five proteins were produced for the studies by GenScript Biotech Corporation (www.genscript.com). These were EF-Tu, GAPDH, Hypothetical protein 3 (HP3), P146, and an epitope of Hypothetical protein 1 (HP1). For each protein, the nucleotide sequences were edited for *E. coli* codon preference from the peptide sequence provided. The following is a summary of the cloning and expression steps provided by GenScript.

Target DNA sequence was optimised and synthesised. The synthesised sequence was cloned into vector pET30a(+) (Novagen Sigma-Aldrich™, Germany) with His tag (EF-Tu, GAPDH, HP3, and P146) or vector pGEX4T 1 (GE Healthcare, United States) with GST tag (HP1) for protein expression in *E. coli*. *E. coli* strain BL21 star (DE3) was transformed with recombinant plasmid. A single colony was

inoculated into 5052 auto induced medium (EF-Tu, GAPDH, and HP3) or TB medium (HP1 and P146) containing related antibiotic; culture was incubated in 37°C at 200 rpm and then induced with IPTG. SDS PAGE was used to monitor the expression. Recombinant BL21 star (DE3) stored in glycerol was inoculated into 5052 auto induced medium (EF-Tu, GAPDH, and HP3) or TB medium (HP1 and P146) containing related antibiotic and cultured at 37°C. When the OD₆₀₀ reached approximately 3.0 (for EF-Tu, GAPDH, and HP3) or 1.2 (for HP1 and P146), the cell culture was induced with IPTG at 15°C for 16 h. Cells were harvested by centrifugation. Cell pellets were resuspended with lysis buffer followed by sonication. The supernatant after centrifugation was kept for future purification. Target protein was obtained by one step purification using a Ni column (HP3 and P146) or GST column (HP1). The target protein was sterilised by 0.22 µm filter before storage in aliquots. The concentration was determined by Bradford protein assay with bovine serum albumin (BSA) as standard. The protein purity and molecular weight (Mw) were determined by standard SDS PAGE along with Western blot confirmation using anti-His or anti-GST antibodies.

2.9 SDS-PAGE

Sodium dodecyl sulphate (SDS) with a denaturing polyacrylamide gel system has been widely used to separate protein molecules based on size since first described by Laemmli 1970 [241]. SDS-PAGE can be used to monitor protein purifications, check the purity of samples, and to estimate molecular weights for unknown proteins [242].

2.9.1 1D SDS-PAGE

SDS-PAGE gels were run in the iBlot® Gel Transfer Device Invitrogen™, Thermo Fisher Scientific™, United States. using 10% or 4-12% NuPAGE™ Invitrogen™, Thermo Fisher Scientific™, United States and Bolt™ pre-cast gels Invitrogen™, Thermo Fisher Scientific™, United States. The ladder used was SeeBlue™ Plus2 Pre-stained Protein Standard Invitrogen™, Thermo Fisher Scientific™, United States. Gels were run as described below.

A 13 µl aliquot of samples was combined with 5 µl NuPAGE™ LDS Sample Buffer (4X), (Invitrogen™, Thermo Fisher Scientific™, United States) and 2 µl NuPAGE™ Sample Reducing Agent (10X) (Cat: NP0009 Invitrogen™, Thermo Fisher Scientific™, United States). The samples were incubated for 10 min at 70°C in a heating block. A volume of 5 µl Ladder was loaded into the wells and samples (20 µl) were loaded into the other available wells. The gel was run at 200 V for 50 min. The gel was removed and placed in a container and covered with deionised water. The gel was microwaved for 1 min, shaken gently and water was removed. The water was replaced, and the procedure was repeated 3 times. SimplyBlue™ SafeStain (20 ml) (Invitrogen™, Thermo Fisher Scientific™, United States) was added to the gel. The gel was microwaved for 30 s, container was shaken gently and microwaved again for 30 s. The container was placed on rocker for 10 min. The stain was removed, and water

was added to a depth of 1 cm. The container was placed back on the shaker for 30 min. The water was removed, NaCl (5 M) was added to a depth of 1 cm and the gels stored at room temperature overnight. Gels were imaged next day.

2.9.2 2D SDS-PAGE

A 155 µl volume of hydrophobic fraction sample prepared for 2D electrophoresis (section 2.6.2) was placed into a strip cassette (Invitrogen™, Thermo Fisher Scientific™, United States), taking care not to introduce bubbles. The ReadyStrip™ IPG Strips pH 3-10 (Bio-Rad Laboratories, United States) were removed from the packet (ensuring the plastic back is removed) and inserted into the cassette (writing side down). The supplied tape was put over the wells and the cassette incubated at 4°C overnight. The tape and plastic tabs were removed, the supplied paper was wet with RO water and put over wells. The cassette was run at 200 V for 20 min followed by: 400 V for 15 min, 750 V for 15 min, and 2000 V for 60 min. While running at the highest voltage, the equipment was observed closely due to some risk of catching fire. The water was removed and cassette dried. Plastic backing was removed, and the plastic cover attached. The cassette was filled with 10 ml (1 x NuPAGE™ LDS Sample Buffer Invitrogen™, Thermo Fisher Scientific™, United States and 1 x NuPAGE™ Sample Reducing Agent (Invitrogen™, Thermo Fisher Scientific™, United States). The cassette was incubated at room temperature for 15 min. The liquid was removed and 10 ml solution containing (1 x NuPAGE™ LDS Sample Buffer, 1 x NuPAGE™ Sample Reducing Agent and 0.232 g iodoacetamide Sigma-Aldrich™, Germany) was added. The cassette was incubated at room temperature for 15-30 min. The plastic cover and strips were removed. The strips were put into NuPAGE™ 4 to 12%, Bis-Tris ZOOM™ (Invitrogen™, Thermo Fisher Scientific™, United States) SDS-PAGE gels and covered with 0.5% agarose. Once set the gel was run at 200 V for 50 min. The gel was removed and placed in a container and covered with deionised water. The gel was microwaved for 1 min, shaken gently and water removed. The water was replaced, and the procedure was repeated 3 times. SimplyBlue™ SafeStain (20 ml) (Invitrogen™, Thermo Fisher Scientific™, United States) was added to the gel. The gel was microwaved for 30 s, container was shaken gently and microwaved again for 30 s. The container was placed on rocker for 10 min. The stain was removed, and water added to a depth of 1 cm. The container was placed back on the shaker for 30 min. The water was removed, NaCl (5 M) added to a depth of 1 cm and the gels stored at room temperature overnight. Gels were imaged next day.

2.10 Western blot analysis

SDS-PAGE was run as above until removal of gel. Western blot analysis was performed using iBlot™ polyvinylidene difluoride (PVDF) Transfer Stack (Invitrogen™, Thermo Fisher Scientific™, United States). Blotting was performed using 1-Step™ 3,3',5,5'-Tetramethylbenzidine (TMB)-Blotting

Substrate Solution (Invitrogen™, Thermo Fisher Scientific™, United States) or Clarity™ Western enhanced chemiluminescence (ECL) Substrate, (Bio-Rad Laboratories, United states).

A gel was produced either by 1D SDS-PAGE (2.9.1) or 2D SDS-PAGE (2.9.2). The gel was transferred to a PVDF Transfer Stack, in an IBLOT transfer machine (Invitrogen™, Thermo Fisher Scientific™, United States) for 7 min. The PVDF membrane was removed from the IBLOT and transferred to a container. The PVDF membrane was blocked with 5% skimmed milk (bovine) in 1x TBS at 4°C overnight. The PVDF membrane was rinsed twice with MQ water and 10 ml of a 5% skimmed milk solution containing 24-25 µl of serum was added for a final dilution of 1/400. The PVDF membrane was incubated on a rock shaker for 3 h. The PVDF membrane was rinsed 5 times with 1X TBS + 0.01% Tween-20 and 10 ml of a 5% skimmed milk solution containing 10 µl rabbit anti-sheep secondary antibodies conjugated with horseradish peroxidase (HRP) (Invitrogen™, Thermo Fisher Scientific™, United States) was added. The PVDF membrane was rocked again for 2 h then the PVDF membrane was rinsed 5 times with TBS + 1% Tween-20. The PVDF membrane was washed with MQ water and 10 ml 1-Step™ or 7 ml Clarity™ blotting substrate solution was added to the membrane.

2.11 Recording gel and Western blot images with Gel Doc

SDS-Page and Western blots were imaged using ChemiDoc MP (Bio-Rad Laboratories, United states). SDS-PAGE gels were imaged using the inbuilt Coomassie blue setting with the provided white tray. Western Blots were imaged using the colorimetric setting on the provided black tray for 1 Step stained gels. Chemilucifer stained Western blots were imaged using the inbuilt chemilucifer option in the rapid auto setting.

2.12 Liquid chromatography–mass spectrometry (LC-MS)

Protein spots were extracted from the 2D SDS-PAGE gels and sent to AgResearch Lincoln for mass spectrometry analysis using their Liquid Chromatography - Tandem Mass Spectrometry (LC-MS-MS) system. This system is a powerful analytical technique that combines the separating power of liquid chromatography with the highly sensitive and selective mass analysis capability of triple quadrupole mass spectrometry. The methods used by AgResearch Lincoln are described below.

2.12.1 Sample preparation

The excised spots from the 2D SDS-PAGE gels were transferred into a 0.5 ml Eppendorf tube. The spots were de-stained by adding 200 µl of 200 mM NH_4HCO_3 in 50% CAN and incubated at 37°C for 1 h. The solution was discarded, and the sample was reduced with 200 µl 50 mM TCEP in 100 mM NH_4HCO_3 and incubated at 56°C for 45 min. The solution was discarded, and the sample was washed once with 100 mM NH_4HCO_3 solution. The sample was alkylated by vortexing for 30 min at room temperature in the dark with 100 µL of 150 mM Iodoacetamide in 100 mM NH_4HCO_3 solution.

The solution was discarded and washed once with 50 mM NH_4HCO_3 solution. Trypsin stock solution was prepared (20 μg trypsin/200 μl 50 mM acetic acid) then diluted 2-fold to working stock by adding 50 mM NH_4HCO_3 solution. The working solution (0.1 μg) was added to the gel pieces followed by 60 μl 10% ACN in 50 mM NH_4HCO_3 solution, vortexed briefly and then incubated at 37°C for 16-18 hours. A 1 mm x 1 mm piece of Empore disk was cut out and wet with 100% ACN for 1 min, then conditioned with 100% methanol for 1 min. The discs were equilibrated by immersing in 100 μl 0.1% formic acid. The conditioned Empore disks were immersed directly into the peptide extract and incubated for 3 hr at room temperature with light vortexing. The Empore disks were taken out and washed with 200 μl of 0.1% FA, vortexed briefly, centrifuged and the solution discarded. The Empore disks were incubated with 15 μl of 75% ACN/0.1% FA for 30 min. The eluant was transferred to a clean vial and dried down on the centrifugal concentrator and stored at -20°C until analysis.

2.12.2 LC-MS analysis

The Samples were resuspended in 50 μL of 0.1% formic acid prior to LC-MS analysis. Samples were analysed using a nanoAdvance UPLC coupled to a Bruker amaZon speed ion ETD trap mass spectrometer equipped with a CaptiveSpray ion source operated at 1900 V. Five μl of sample was loaded on a C18 nano trap column (Bruker, 75 μm x 2 cm, C18AQ, 3 μm particles, 200 Å pore size). The trap column was then switched in line with the analytical column (Bruker Magic C18AQ, 100 μm x 15 cm C18AQ, 3 μm particles, 200 Å pore size). The column oven temperature was 50 °C. Elution was with a multipart linear gradient from 2 to 45% B in 70 min at a flow rate of 800 nL/min. Total run time including column re-equilibration was 90 min. Solvent A was water with 0.1% formic acid (FA) and 2% acetonitrile (ACN); solvent B was ACN with 0.1% FA and 2% water. Auto MS/MS data acquisition was performed with 10 precursors selected for fragmentation in the m/z 350-1200 range during each MS cycle.

2.12.3 Protein identification

The PEAKS Studio v8.5 data analysis software package (Bio informatic Solutions Inc) was used to analyse the LC-MS/MS data. The raw data were refined by a built-in algorithm. The proteins/peptides were identified with the following parameters: a precursor mass tolerance of 0.3 Da and fragment mass tolerance of 0.6 Da were allowed, the Uniprot *Mycoplasma ovipneumoniae* database (v2018.12) was used, semi-trypsin was specified as digestive enzyme and up to 2 missed cleavages were allowed. Both oxidation (M) and deamidation (NQ) are chosen as variable modifications, carbamidomethylation (C) is defined as fixed modification and a maximum of 3 post translational modifications per sample were allowed. False discovery rate (FDR) estimation was made based on decoy-fusion and FDR was set to 1% at the peptide spectrum level.

2.13 Antibody ELISA

2.13.1 Checkerboard ELISA

To determine the ideal concentration of recombinant proteins to be used in the ELISAs each of the recombinant proteins were diluted as described in Table 2.10. To determine the ideal dilution of sera to be used, sera from an animal vaccinated with WC of isolate 16 (Animal 13 2.7.5.1) was diluted as laid out in Table 2.10.

Nunc MaxiSorp™ flat-bottom 96 well plates (Thermo Fisher Scientific™, United States) were used and 100 µl of antigens, diluted in 0.1 M bicarbonate coating buffer pH 9.6 (0.06 M NaHCO₃, 0.04 M Na₂CO₃), were added to each well and the plates were left at 4°C overnight. The following morning the plates were washed 4 times with PBS (pH 7.4) containing 0.05% (v/v) Tween 20, to remove the unbound antigens. Non-specific sites were blocked by incubation at room temperature for 1 hour with 5% skimmed milk in PBS (200 µl per well). The plates were washed 4 times with PBS (pH 7.4) containing 0.05% (v/v) Tween 20 and 100 µl of serum in blocking buffer was added to each well as per the template and incubated at room temperature for 1 hour. The plates were washed 4 times with PBS (pH 7.4) containing 0.05% (v/v) Tween 20 and 100 µl of HRP labelled anti-sheep IgG (Sigma-Aldrich™, Germany) in blocking buffer (1:15,000) was added and incubated at room temperature for 1 hour. The plates were washed 4 times with PBS (pH 7.4) containing 0.05% (v/v) Tween 20. Pierce™ TMB Substrate Kit (Thermo Fisher Scientific™, United States) working solution was added @ 200 µl / well and the plates were incubated in dark at room temperature for 30 min. The resultant blue colour reaction was stopped by addition of 50 µl of 2M sulphuric acid to each well. The optical density (OD) values were read at 450 nm using VersaMax™ ELISA Microplate Reader (Bio-Strategy, New Zealand).

2.13.2 Measurement of serum antibodies to *M. ovipneumoniae* WC antigens, fractions, and recombinant proteins

Nunc MaxiSorp™ flat-bottom 96 well plates were used and 100 µl of antigens (500 ng of each of the proteins EF-Tu and GAPDH, 125ng of each of the proteins HP1, HP3, and P146), diluted in 0.1 M bicarbonate coating buffer pH 9.6 (0.06 M NaHCO₃, 0.04 M Na₂CO₃), were added to each well and the plates were left at 4°C overnight. The following morning the plates were washed 4 times with PBS (pH 7.4) containing 0.05% (v/v) Tween 20, to remove the unbound antigens. Non-specific sites were blocked by incubation at room temperature for 1 h with 5% skimmed milk in PBS (200 µl per well). The plates were washed 4 times with PBS (pH 7.4) containing 0.05% (v/v) Tween 20 and 100 µl of serum in blocking buffer (1:50 for the proteins EF-Tu and GAPDH, 1:100 for the proteins HP1, HP3, and P146) was added to each well and incubated at room temperature for 1 h.

Table 2-10 Layout of antigen quantity (in ng, shown in green) and serum dilution (shown in red).

	1	2	3	4	5	6	7	8	9	10	11	12
A	1000.0 1:50	500.0 1:50	250.0 1:50	125.0 1:50	62.5 1:50	31.3 1:50	15.6 1:50	7.8 1:50	3.9 1:50	2.0 1:50	1.0 1:50	Buffer 1:50
B	1000.0 1:100	500.0 1:100	250.0 1:100	125.0 1:100	62.5 1:100	31.3 1:100	15.6 1:100	7.8 1:100	3.9 1:100	2.0 1:100	1.0 1:100	Buffer 1:100
C	1000.0 1:150	500.0 1:150	250.0 1:150	125.0 1:150	62.5 1:150	31.3 1:150	15.6 1:150	7.8 1:150	3.9 1:150	2.0 1:150	1.0 1:150	Buffer 1:150
D	1000.0 1:200	500.0 1:200	250.0 1:200	125.0 1:200	62.5 1:200	31.3 1:200	15.6 1:200	7.8 1:200	3.9 1:200	2.0 1:200	1.0 1:200	Buffer 1:200
E	1000.0 1:250	500.0 1:250	250.0 1:250	125.0 1:250	62.5 1:250	31.3 1:250	15.6 1:250	7.8 1:250	3.9 1:250	2.0 1:250	1.0 1:250	Buffer 1:250
F	1000.0 1:300	500.0 1:300	250.0 1:300	125.0 1:300	62.5 1:300	31.3 1:300	15.6 1:300	7.8 1:300	3.9 1:300	2.0 1:300	1.0 1:300	Buffer 1:300
G	1000.0 1:350	500.0 1:350	250.0 1:350	125.0 1:350	62.5 1:350	31.3 1:350	15.6 1:350	7.8 1:350	3.9 1:350	2.0 1:350	1.0 1:350	Buffer 1:350
H	1000.0 1:400	500.0 1:400	250.0 1:400	125.0 1:400	62.5 1:400	31.3 1:400	15.6 1:400	7.8 1:400	3.9 1:400	2.0 1:400	1.0 1:400	Buffer 1:400

The plates were washed 4 times with PBS (pH 7.4) containing 0.05% (v/v) Tween 20 and 100 µl of HRP labelled anti-sheep IgG (Cat A3415, Sigma-Aldrich™, Germany) in blocking buffer (1:15,000) was added and incubated at room temperature for 1 h. The plates were washed 4 times with PBS (pH 7.4) containing 0.05% (v/v) Tween 20. Pierce™ TMB Substrate Kit (Thermo Fisher Scientific™, United States) working solution was added @ 200 µl / well and the plates were incubated in dark at room temperature for 30 min. The resultant blue colour reaction was stopped with the addition of 50 µl / well of 2 M sulphuric acid. The OD values were read at 450 nm using VersaMax™ ELISA Microplate Reader (Bio-Strategy, New Zealand).

2.14 Measurement of cell-mediated responses to mycoplasma antigens

2.14.1 Blood cultures

Heparinised blood was obtained from sheep vaccinated with WC, HPF, or recombinant proteins from *M. ovipneumoniae*. The blood was diluted 1:1 with RPMI 1640 medium (Invitrogen™, Thermo Fisher Scientific™, United States) containing 5% foetal bovine serum (FBS) (Cat 10091148, Invitrogen™, Thermo Fisher Scientific™, United States) and antibiotic and antimycotic solution (Invitrogen™, Thermo Fisher Scientific™, United States) and aliquoted (1 mL per well) to 24-well cell culture plate (Greiner-Bio One, Austria) [243].

Pokeweed mitogen (20 µg/well; positive control), phosphate buffered saline (PBS; blank), recombinant EF-Tu, GAPDH, HP3, HP1, and P146 (10 µg/well), were added to wells and the plates incubated at 37°C in 5% CO₂ for 40 h. An example of a setup template for a 24 well plate is shown in Table 2.11.

Table 2-11 Example setup of 24 well plate for blood cultures.

Animal 1		Animal 2		Animal 3	
WCL 16	HP3	WCL 16	HP3	WCL 16	HP3
EF-Tu	P146	EF-Tu	Pokeweed	EF-Tu	P146
GAPDH	Pokeweed	GAPDH	Pokeweed	GAPDH	Pokeweed
HP1	PBS	HP1	PBS	HP1	PBS

Following incubation, the plates were centrifuged at 784 x g for 15 min. The supernatants (plasmas) were carefully removed using a pipette and stored in Micro Titer tubes (Quality Scientific Plastics, Thermo Fisher Scientific™, United States) at 4°C until IFN- γ concentrations were measured using ELISA then stored at -20°C.

2.14.2 Measurement of IFN- γ using ELISA

IFN- γ responses in blood cultures to different antigens were determined using a BOVIGAM ELISA kit (Thermo Fisher Scientific™, United States). ELISA plates were equilibrated to room temperature for at least 30 min before removing them from their packaging. The plates were labelled, and the individual column strips were labelled 1 to 12. The Green diluent was brought to room temperature and mixed thoroughly and 50 μ l was added to the required wells using an 8 channel, multi-dispenser pipette. The plasmas were brought to room temperature. A 50 μ l volume of samples were added to the appropriate wells containing the Green diluent. Each plate was mixed thoroughly by using a microplate shaker, shaking for one min. Each plate was covered with a lid and incubated at room temperature ($22 \pm 5^\circ\text{C}$) for 60 ± 5 min. Each plate's contents were shaken out and washed 6 times at room temperature. Each plate had wells filled with wash buffer using the Flow Laboratories Titertek Microplate Washer 120 and the wash buffer was left in the wells for a minimum of one minute. The wash buffer was shaken out and the process repeated five more times. After the sixth wash, the contents were shaken out of the well and then tapped them face down on a towel to remove as much as remaining buffer as possible. The Blue diluent was brought to room temperature and mixed thoroughly. The conjugate was prepared by diluting the 100x conjugate in Blue diluent. The working strength conjugate was prepared and used within 5 min. A 100 μ l volume of freshly prepared, diluted conjugate reagent was added to the wells. Each plate was covered with a lid and incubated at room temperature (22°C) for 60 min. The contents were shaken out. Using a hose with a spray gun, the plates were washed thoroughly from the bottom, paying attention to the gap between the strips of wells and the strips and frame. The plate was washed once with the Flow Laboratories Titertek Microplate Washer 120 and immediately emptied. Each plate had wells filled with wash buffer using the Flow Laboratories Titertek Microplate Washer 120 and the wash buffer was left in

the wells for a minimum of one minute. The wash buffer was shaken out and the process repeated five more times. After the sixth wash, the contents were shaken out of the well and then tapped them face down on a towel to remove as much as remaining buffer as possible. The enzyme substrate was brought up to room temperature. The enzyme substrate solution was prepared just prior to use by adding chromogen to the enzyme substrate at a dilution of 1/100. The enzyme substrate solution was thoroughly mixed, and the colour checked. The enzyme substrate solution should be colourless and was discarded if turned blue. A 100 µl volume of freshly prepared enzyme substrate solution was added to each well. Each plate was covered with a lid and incubated in the dark at room temperature (22°C) for 30 min. A 50 µL volume of enzyme stopping solution was added to each well. The absorbance of each well in the plates was read at 450 nm with VersaMax™ ELISA Microplate Reader (Bio-Strategy, New Zealand) within approximately five minutes of terminating the reaction. The original IFN-γ analyses were undertaken to determine if there was any detectable response, therefore ODs were determined to be adequate. Trial 3: Adjuvant trial (2.7.5.3) was analysed separately and an ovine IFN-γ standard (Kingfisher Biotech, St. Paul, USA) was titrated and the concentration of IFN-γ was calculated from the standard curve.

2.14.3 Measurement of IL-17A

IL-17A was measured using a Bovine IL17A Kingfisher DIY kit (Kingfisher biotech Inc, United States). The kit includes capture antibody (PB0274B-100), standard and detection antibody (PBB0277B-050). The plate was coated with 50 µl capture antibody at 1/8,000, diluted in PBS and Incubated in the fridge overnight. The plates were washed 3 times with PBS + 0.1% tween wash buffer and 100 µl of 0.5% BSA in PBS (10 mM) blocking buffer was added to each well. The plates were incubated for 1 hour at room temp. The plates were washed 3 times with PBS + 0.1% tween wash buffer. Undiluted blood culture supernatant samples were added to the plate (50 µl/well). The plates were incubated at room temp for 1 hour, then washed 6 times with PBS + 0.1% tween wash buffer. A 50 µl volume of conjugate (biotinylated anti bovine IL-17A polyclonal antibody, diluted 1/16,000 in 0.5% BSA buffer) was added to each well. The plate was incubated for 1 h at room temp then the plates were washed 6 times with PBS + 0.1% tween wash buffer. A 50 µl volume of Streptavidin HRP conjugate diluted 1/1,000 in 0.5% BSA buffer was added to each well and the plate incubated at room temp for 30 min. The plates were washed 6 times with PBS + 0.1% tween wash buffer. A 50 µl volume of TMB substrate was added to each well. The plates were incubated at room temp in the dark for 20 min then 50 µl of stop solution was added to each well. The plate was read at 450nm using a VersaMax™ ELISA Microplate Reader (Bio-Strategy, New Zealand). The original IL-17A analyses were undertaken to determine if there was any detectable response, therefore ODs were determined to be adequate.

Trial 3: Adjuvant trial (2.7.5.3) was analysed separately and therefore a standard curve was included in the analysis.

2.15 Colour Changing Unit (CCU) assay

To determine the concentration of *M. ovipneumoniae* in a culture, a CCU assay was undertaken. This determined the number of units of *M. ovipneumoniae* in a culture able to change the colour of mycoplasma media from red to yellow, signifying the growth of mycoplasma. The mycoplasma was serially diluted to determine the highest dilution at which there were enough mycoplasma cells to change the colour of the media.

A 180 µl volume of pre-warmed Frey's medium was added to the 96-well microtiter tissue culture plates with lids (Greiner-Bio One, Austria) according to the plate layout shown below. A 20 µl volume of the *M. ovipneumoniae* culture was added to 180 µl Frey's medium in the column 2 of a 96-well microtiter plate (Greiner-Bio One, Austria). From this column, using a multi-channel pipettor a further ten-fold serial dilution with 20 µl volumes was performed up to column 11 in Frey's medium to a final dilution of 10^{11} (Table 2.12). Plates were subsequently sealed with Parafilm M® (Cole-Parmer, United States) and incubated at 37°C for at least 2 weeks. Plates were inspected every 2 days to observe any change in colour. Wells were inspected visually to determine the highest dilution that colour changed to yellow.

Table 2-12 Colour Changing Units (CCU) plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12	
A													Blank
B		10^3	10^2	10^1	10^4	10^3	10^6	10^7	10^8	10^5	10^{10}	10^{11}	Sample 1
C		10^3	10^2	10^1	10^4	10^3	10^6	10^7	10^8	10^5	10^{10}	10^{11}	Sample 2
D		10^3	10^2	10^1	10^4	10^3	10^6	10^7	10^8	10^5	10^{10}	10^{11}	
E		10^3	10^2	10^1	10^4	10^3	10^6	10^7	10^8	10^5	10^{10}	10^{11}	
F		10^3	10^2	10^1	10^4	10^3	10^6	10^7	10^8	10^5	10^{10}	10^{11}	
G		10^3	10^2	10^1	10^4	10^3	10^6	10^7	10^8	10^5	10^{10}	10^{11}	
H													

2.16 Determination of adherence of mycoplasmas to epithelial cells

Cell adherence analysis was based on work by Hegde et al (2018) [227] and Svenstrup et al (2002) [225].

M. ovipneumoniae isolate 16 was grown in Frey's media until the pH indicator changed to orange as described in 2.4. The cells in 4 ml of culture were recovered by centrifugation at 3,320 x g, washed once with PBS and resuspended in 4 ml of RPMI + 10% FBS + 1000 U/ml penicillin.

To quantify cell adhesion, bovine endometrial epithelial cells (obtained from Professor Michel A. Fortier, Université Laval, Québec) were infected with *M. ovipneumoniae*. Unbound mycoplasma cells were washed away, and the epithelial cells were transferred to Frey's media to determine the presence of mycoplasma bound to the epithelial cells.

Determination of adherence:

Total epithelial cell count was determined using TC20™ automated cell counter (Bio-Rad Laboratories, United states) and the calculation below:

Count cells: Cells/ml (____%) = ____ x 10^x x 2 (Dilution factor) = ____ x 10

Desired cells = 5 x 10⁶ = 1000 / (____ x 10^x / 1 x 10⁶)

1000 / ____

____ µl of cells /well

Top up to 750 µl

Epithelial cells at 1 x 10⁶ were added to the wells in a 24-well plate (example template shown in Table 2.11) and topped up with 750 µl RPMI media and incubated overnight to reform a monolayer.

The following day, 50 µl *M. ovipneumoniae* at corresponding concentrations of 10⁸ CCU/ml, 10⁹ CCU/ml, and two lots of 10¹⁰ CCU/ml was added to the RPMI. As a control, *M. ovipneumoniae* cells are added to culture medium in parallel wells to quantify the adhesion to wells (Table 2.13). The plates were incubated at 37°C with 5% CO₂ for 4 h. Following incubation, unbound *M.*

ovipneumoniae cells were removed by washing the adherent epithelial cells three times with 1 ml PBS. Liquid was removed using suction between washing. The epithelial cells were detached using 300 µl TrypLE™ Express Enzyme (Gibco™ Invitrogen™, Thermo Fisher Scientific™, United States) for 30 s, and excess TrypLE™ Express Enzyme (Gibco™ Invitrogen™, Thermo Fisher Scientific™, United States) was removed. The cells were incubated at 37°C for 10 min then 250 µl of cell culture media was added to each well. A 20 µl aliquot of the cell culture media was added to 180 µl Frey's medium in the column 2 of a 96-well microtiter plate (Greiner-Bio One, Austria). From this column, using a multi-channel pipettor a further ten-fold serial dilutions were performed with 20 µl volumes up to column 11 in Frey's medium to a final dilution of 10¹⁰. This was repeated for wells containing *M. ovipneumoniae* only and for wells containing epithelial cells only. Plates were subsequently sealed and incubated at 37°C for at least 2 weeks. Plates were inspected every day to observe any change in colour. Adherence was determined by observing a difference in colour change from wells containing epithelial cells and *M. ovipneumoniae*.

Table 2-13 Incubation plate layout for adhesion experiment.

	1	2	3	4	5	6	
A	10^8						Epithelial cells + <i>M. ovipneumoniae</i>
B	10^9						<i>M. ovipneumoniae</i> only
C	10^{10}						Epithelial cells only
D	10^{10}						

2.16.1 Determination of inhibition of adherence by antibodies

An adherence inhibition assay was performed to determine the effect of antisera on binding of *M. ovipneumoniae* to the epithelial cells. The mycoplasma suspension was pre-incubated with antisera against the recombinant protein or WC16, or pre-immune serum. Following preincubation, the mycoplasma-antibody suspension was then added to the cell monolayer together with 700 μ l of cell media (example template shown in Table 2.14). The impact of antisera on adherence of *M. ovipneumoniae* to the cells was assessed by comparing CCU between the pre-immune and antisera. Incubation of antibodies was completed by mixing a 100 μ l sample of *M. ovipneumoniae* isolate 16 culture at 10^8 with the diluted recombinant protein pre-immune or antisera (10:1 v/v) in a microcentrifuge tube and incubated with agitation for 1 h at 37°C.

Table 2-14 Incubation plate layout for adhesion inhibition experiment

	1	2	3	4	5	6	
A	H1 10^9						Pre-immune
B	H3 10^9						Antisera
C	P146 10^9						No antibodies
D	WC 10^9						

2.17 Methods used to determine impact of antisera on growth of *M. ovipneumoniae*

A growth inhibition assay, based on methods described by Jiang et al (2016) [160] was used to determine whether antibodies produced against protein reduced growth of *M. ovipneumoniae* in culture. Sheep sera were serially diluted 1:1:128 in Frey's media without thallium acetate and added into wells in triplicate (Table 2.15). Wells with added pre-immune sera wells are indicated in blue and wells containing antisera are indicated in green below. The growth of *M. ovipneumoniae* in the absence of serum served as a positive control (red). As the no antiserum control, culture medium alone was in the wells (yellow). Absorbance was recorded at 560 nm after incubation at 37°C with 5% CO₂ for 7 days. The percentage growth inhibition was determined as follows:

$$\frac{\text{Absorbance of sample} - \text{absorbance of positive control}}{\text{Absorbance of no antiserum control} - \text{absorbance positive control}} \times 100$$

To remove the effect of drying in the wells during the incubation period, the outer wells (Orange) were filled with 200 µl media.

Dilution of sera:

Sera from the Antisera production trials (section 2.7.5.1) were used for the inhibition experiments. Pre-immune and antisera from sheep (no. 1) vaccinated with recombinant GenScript EF-Tu and pre-immune and antisera from sheep (no. 13) vaccinated with WC antigens from *M. ovipneumoniae* isolate 16 were used. Animal sera were 2-fold serially diluted 1:20–1:2,560 in Frey's media without thallium acetate. Sera (40µl) is added to 760 µl Frey's media to produce 1:20 dilution. A 400 µl amount of 1:20 diluted sera is added to 400 µl Frey' media to produce 1:40 dilution. This is repeated to produce serial dilution to 1:2560 dilution. Cells of *M. ovipneumoniae* isolate 16 are dispensed into column 2-10 rows B-G (100 µl per well). For each well column 2-9 rows B-G, 100 µl of sheep sera were added in triplicate. Column 10 had 100 µl media added to the *M. ovipneumoniae*. Column 11 was filled with 200 µl media only. To remove the effect of drying in the wells the outer wells (shown in orange) are filled with 200 µl media. The plates were incubated at 37°C with 5% CO₂ for 7 days. Absorbance was recorded at 560 nm using VersaMax™ ELISA Microplate Reader (Bio-Strategy, New Zealand) each day (i.e., at 24 h intervals). The percentage growth inhibition was determined as follows: [absorbance of sample – absorbance of positive control]/[absorbance of no antiserum control – absorbance positive control]×100.

Table 2-15 Layout of 96-well plates used to determine antibody inhibition of growth.

1/A	2	3	4	5	6	7	8	9	10	11	12	Blank
B	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	+	-		Pre-immune
C	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	+	-		Anti-sera
D	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	+	-		Positive control
E	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	+	-		Negative control
F	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	+	-		
G	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	+	-		
H												

2.18 Immunofluorescence microscopy

The binding of antibodies to *M. ovipneumoniae* surface antigens was visualised using immunofluorescence microscopy. A 10 ml culture of 10¹⁰ CCU *M. ovipneumoniae* isolate 16, grown in Frey's media was centrifugated and washed 3 times with PBS. A 2.1 µl volume of a 14.3 mM 4',6-diamidino-2-phenylindole (DAPI) solution was added to 100 µl PBS to make a 300 µM DAPI stock

solution and stored at -20°C. When required the stock solution was diluted 1:500 in PBS to produce a 600 nM DAPI stain solution. The cells were stained with 100 µl DAPI stain solution for 4 min, followed by centrifugation at 14,800 x g for 5 min. Following removal of the supernatant, the cells were washed with 1 ml PBS, centrifuged for 5 min @ 14,800 x g and the supernatant removed using a pipette. To the cells 99 µl 5% Donkey serum + 1 µl primary antibody (1/100 dilution) (pre-immune or antisera produced against WC or selected *M. ovipneumoniae* recombinant proteins) was added to the cells. The cells were incubated at room temperature for 2 h, centrifuged for 5 min at 14,800 x g and the supernatant removed. The cells were washed with 1 ml PBS and centrifuged for 5 min at 14,800 x g. A 100 µl volume of 5% Donkey serum and 0.25 µl (1/400 dilution) of a secondary Donkey Anti-Sheep IgG H&L (FITC) (Cat ab97124, Abcam United Kingdom) were added to the cells. The cells were incubated for 30 min then washed with 1 ml PBS and centrifuged for 5 min at 14,800 x g. The supernatant was removed, and the cells resuspended in 50 µl VECTASHIELD Antifade Mounting Medium (CatH-1000-10). A 10 µl volume of cells in VECTASHIELD Antifade Mounting Medium was placed on to a microscope slide in the dark and left for 10 min to allow cells to settle. The cells were observed using a fluorescence microscope (Olympus BX61, Olympus Corporation Japan) at 1,000x magnification using the DAPI and FITC mirror unit. Images were captured with camera at 100 ms for fluorescence and 20 ms for DAPI.

Chapter 3 Bioinformatic and genomic analysis of New Zealand isolates of *M. ovipneumoniae*

Part of this chapter has been published in the journal cited below.

Bridgeman B. J., Gupta S. K., Murray A., Dukkipati V. S. R., Altermann E., Wedlock D. N. 2020. Draft genome sequence of a New Zealand isolate of *Mycoplasma ovipneumoniae*. Microbiol Resour Announc 9:e01375-19. <https://doi.org/10.1128/MRA.01375-19>.

3.0 Abstract

Whole genome analysis of pathogenic organisms can provide insight into the molecular mechanisms of disease. Understanding the molecular functioning of a pathogen can provide avenues into preventing or early disease diagnosis. Three draft genomes of New Zealand isolates have been created and annotated. The New Zealand isolates of *M. ovipneumoniae* had similar number of genes compared to other isolates of *M. ovipneumoniae*. *De novo* amino acid and nucleotide synthesis, as with other mycoplasmas, has been lost in the genome reduction for *M. ovipneumoniae*. Annotation of three New Zealand isolates of *M. ovipneumoniae* identified more than 200 genes containing transmembrane domains, providing a large number of potential proteins that could be investigated for vaccine or diagnostic purposes. Restriction fragment length polymorphism and phylogenetic analysis have provided support for three distinct groups of *M. ovipneumoniae* isolates within New Zealand. This grouping was supported with phylogenetic analysis of the P113 fragment. Phylogenetic analysis was undertaken using four genetic loci suggested as a multi-locus sequence typing (MLST). This indicated that New Zealand Isolates form a single clade separate from USA isolates and Australia isolates are the closest sister isolates. Within the New Zealand clade there was two subclades observed suggesting the potential for multiple invasion events of *M. ovipneumoniae*. Further sequencing of New Zealand and Australia isolates may provide further support for either separate invasion events or divergent evolution within New Zealand isolates.

3.1 Introduction

The introduction of next generation sequencing has provided an immense amount of data on the genomes of pathogenic organisms. By analysing the genomes of disease inducing organisms it is possible to better understand the methods by which these organisms induce disease providing potential avenues by which infection may be detected, treated, or prevented.

Considered the smallest independently self-replicating organisms, mycoplasmas are wall-less gram-positive bacteria characterised by small physical dimensions and genome sizes [244]. The reduced genome has aided in mollicutes being the best represented phylogenetic class of organisms to have complete genomic sequences [245]. Suggested to have gone through reductive evolution from the *Bacillus/Clostridium* branch of the gram-positive eubacteria, mycoplasmas generally have less than 1000 genes, perform little *de novo* synthesis of amino acids and nucleotides, and are obligate commensals or parasites [246, 247].

M. ovipneumoniae is a pathogenic bacterium of caprinae hosts, inducing chronic non progressive pneumonia [58, 63]. Despite its prevalence within domestic [50, 58, 60, 248] and wild animals [133, 234, 249, 250], there is limited knowledge around the genetic makeup of these organisms [130, 251], and no information on New Zealand isolates is available. To better understand the positioning of New Zealand isolates within the global distribution of *M. ovipneumoniae*, lung lavages had been obtained previously at the abattoir from 128 sheep sourced from 8 farms in New Zealand which had history of ovine pneumonia. A total of 93 isolates of *M. ovipneumoniae* were cultured from these samples. Molecular analysis using RFLP on the whole genome and the P113 adhesin gene [232] clustered these isolates into 3 distinct groups [231] and 10 representative isolates were selected for genomic analysis (Chapter 2.3). Of the isolates selected, 3 representative isolates, 16, 90, and 103, of New Zealand *M. ovipneumoniae* had their sequences aligned to produce a draft genome and have also had their draft genome annotated. These three isolates are representatives for the three groups.

To confirm the three groupings, the previously identified gene P113 [232] was extracted from the High Throughput Sequencing (HTS) of the ten New Zealand isolates. This gene has previously been described as a useful target for detection and quantification of *M. ovipneumoniae* [232]. To further distinguish between the New Zealand isolates this gene was then digested *in silico* with four restriction enzymes and the grouping of the 10 isolates was determined by looking at the fragmentation. To compare the restriction patterning of New Zealand isolates, a reference strain provided by Institute of Environmental Science and Research (ESR) was included in the HTS and P113 restriction digestions. These fragmentation patterns were also compared to the phylogenetic analysis of this gene.

This study was undertaken with the following aims:

- To produce draft genomes of the three representative isolates of New Zealand *M. ovipneumoniae*.
- Confirm the three New Zealand groups using P113 RFLP patterns.
- Annotate and compare genomes of New Zealand isolate to other *M. ovipneumoniae* isolates species.
- Complete phylogenetic analysis using sequences of four MLST loci.
- Complete genetic analysis to provide support for other work completed in this thesis.

3.2 Methods

3.2.1 Culture of *M. ovipneumoniae* isolates, DNA extraction and sequencing

HTS of isolates 16, 23, 42, 70, 73, 83, 88, 90, 94, 103, and ESR strains were produced before the start of this PhD. The generalised methods used to obtain the DNA and sequences are described below.

Isolate 90 was cultured for sequencing in Frey's medium [230] at 37°C until a phenol red pH indicator indicated growth of the mycoplasma (chapter 2.4). Genomic DNA was extracted using a ZR fungal/bacterial DNA MiniPrep commercial kit (Zymo Research Corp., United states). The resulting genomic DNA was subsequently purified using a ZR genomic DNA clean and concentrator commercial kit (Chapter 2.5.1). DNA was sequenced on an Illumina MiSeq system (New Zealand Genomics Limited, New Zealand), producing 1,401,512 paired end (PE) reads. The raw reads were trimmed against a UniVec database (Geneious R10.0.9) [252], and short-length reads (<25 nucleotides) were removed. After trimming (Geneious R10.0.9) [252], 1,214,927 PE reads with an average length of 171 bases per read for a 200X coverage were used for genome assembly.

3.2.2 Isolate 90 genome building

Genome assembly

Default parameters were used for all software except where otherwise noted. The in-house Geneious (Geneious R10.09) [252] and Velvet 1.2.10 [253] de novo assemblers were used to produce the initial assemblies. The initial contigs were improved by remapping the sequence reads to the contigs using the Geneious map-to-reference function (Geneious R10.0.9) [252] and then using the in-house Geneious *de novo* assembler to combine the contigs into larger contigs. Single nucleotide polymorphisms (SNPs) between the combined contigs were resolved by making a final map-to-reference run using the raw reads and taking the most common nucleotide.

Annotation

The genome sequence was annotated using GAMOLA2 2.0.0.14 [254], and the gene model was built using Prodigal 2.6.3 [255]. Gapped Blastp was carried out with the NCBI nonredundant amino acid database [256]. Clusters of orthologous groups (COGs) were determined using the latest COG database (2014) [257]. The COGs database is an attempt on phylogenetic classification of the proteins encoded in complete genomes. Each COGs includes proteins that are inferred to be

orthologs (direct evolutionary counterparts). The Conserved Domains Database (CDD) (09/2018) [258], protein families (Pfam) database (release 32) [259], and TIGRFAM (a database of protein families designed to support manual and automated genome annotation) (release 15.0) [260, 261] databases were used to identify the respective domains. The annotation was visualised and analysed using a modified version of Artemis 16 [254, 262, 263].

3.2.3 P113 RFLP comparison

As described by Yang et al (2014) [232], the putative adhesin gene p113 has been shown to be specific and sensitive for the detection of *M. ovipneumoniae*. To compare the New Zealand isolates the sequence for this gene was obtained for 10 of the New Zealand isolates. The gene p113 was identified and extracted from the genome of isolate 90. The extracted nucleotide sequence was used as the reference sequence for the other isolates. Using the Geneious [252] map to reference sequence function the HTS data from *M. ovipneumoniae* isolates 16, 23, 42, 70, 73, 83, 88, 94, 103, and ESR strains were mapped against the Isolate 90 reference sequence to produce the p113 gene sequence.

The sequences then had the restriction site for each of AluI, DraI, HhaI, and MboI enzymes identified in Geneious using the inbuilt restriction analysis (Geneious R10.0.9) [252], and the corresponding *in silico* gel image was produced showing the digested fragments (Fig. 3.1-3.4). Each of the isolates were compared to determine the separation into the three groups previously determined. This was undertaken by comparing the fragmentation produced by the enzymes with the isolates containing bands at the same molecular weight were put into the same group. To further analyse the grouping of the New Zealand isolates the p113 gene was analysed using the MrBayes method [264] to produce a phylogenetic tree (Fig. 3.5).

3.2.4 Building of genomes for isolates 16 and 103

Using the draft genome (isolate 90) built in 3.2 as the reference sequence, the raw reads of isolate 16 and isolate 103 were mapped to the reference sequence to produce matching contigs. Each of the contigs produced were used as the reference sequence for the Geneious map to read function. The HTS reads were mapped to each of the contigs to remove any single nucleotide polymorphisms (SNPs) and close any gaps within the contigs produced in the first run.

3.2.5 Phylogenetic analysis

Four genetic loci suggested as a multi-locus sequence typing (MLST) [265] were used to phylogenetically place the isolates from New Zealand within other *M. ovipneumoniae* isolates. The four loci used were 16S-23S ribosomal RNA intergenic spacer (IGS), 16S ribosomal RNA gene (16s), DNA gyrase subunit B (gyrB), and RNA polymerase beta subunit (rpoB) gene. These loci were identified in the draft genomes of isolate 16, 90, and 103. For the other isolates the sequence from isolate 90 was used as the reference sequence and the HTS data was mapped using the Geneious

map to read function (Geneious R10.0.9) [252]. The four loci were concatenated into a single sequence and compared against the type strain from ESR and 15 isolates from the Kamath et al (2019) paper [265]. The isolates used were 2013-12928-34, 2013-12928-35, 2013-12928-36, 2013-12928-38, 2013-12928-39, 2013-12928-41, 2013-12928-43, 2013-12928-46, 2013-13860-11, 2013-13860-13, 2013-13860-15, 2013-13860-16, 16271, Mooney, and Cillian. *M. hyopneumoniae* was selected as an outgroup; however, only three of the MLST loci (IGS, 16s, and rpoB) were available from the National Center for Biotechnology Information (NCBI) for this organism. To account for the missing gyrB loci a 400-nucleotide gap was inserted into the concatenated sequence. Each of the concatenated sequences were aligned using the Geneious [252] multiple alignment feature using the default parameters (Alignment type: Global alignment with free end gaps, Cost Matrix: 65% similarity (5.0/-4.0). The alignment was then used to produce a phylogenetic tree using the MrBayes tree builder [264, 266] method (Fig. 3.6).

3.3 Results

3.3.1 Genome properties of isolate 90

In-house Geneious (Geneious RI 0.09) [252] and Velvet 1.2.10 [253] *de novo* assemblers were used to produce the initial assemblies, which had N50 values of 62 (kilo base pairs) kbp and 38 kbp, respectively. The N50 value is defined as the length of the shortest contig in the set of largest contigs that together constitute at least half of the total assembly size. In general, a high N50 value signifies a high-quality draft assembly. Combining the in-house Geneious (Geneious RI 0.0.9) [252] and Velvet 1.2.10 [253] reads produced 14 contigs. The draft genome sequence was 1,031,345 bp long with a GC content of 29% and an N50 value of 85 kbp. A total of 758 genes were predicted, 711 of which were protein coding representing 93.8%. A putative function was assigned to 463 of the protein-coding genes, with the remainder annotated as hypothetical proteins. Of these proteins, 413 could be placed into clusters of orthologous groups (COGs) via homology search [257]. The properties and statistics of the genome are summarised in Tables 3.1 and 3.2. A paper on this work has been published in Microbiology Resource Announcements [267] (Appendix 1).

3.3.2 Genomes of isolates 16 and 103

Using isolate 90 genome as the initial reference, 14 contigs were produced for isolate 16 and isolate 103. Repeating the map to reference function on the contigs of isolates 16 and 103 improved the sequences and reduced the number of gaps; however, there were several gaps not resolved, resulting in increasing the number of contigs in isolate 16 to a total of 16 contigs. The draft genome is 1,347,255 bp with a GC content of 29.1% and N50 value of 101 kbp. Isolate 103 had two gaps in contig 1 that were not resolved with additional map to reference attempts against the contigs. This

resulted in a draft genome of 1,181,876 across 15 contigs and a GC content of 28.8% and a N50 of 87 kbp. The properties and statistics of the genome are summarised in tables 3.1 and 3.2.

3.3.3 tRNA

Analysis of the tRNAs detected confirmed 46, 44, and 46 tRNAs in isolates 16, 90, and 103 respectively. This incidence was higher than the 30 tRNA observed in genomes NM2010 [251] and SC01 [130]. New Zealand isolates detected specificity for all the amino acids except for tryptophan (Trp) in isolates 16 and 90.

3.3.4 Prediction of transmembrane domains

Proteins were analysed for the presence of transmembrane domains. Isolates 16, 90, and 103 predicted 286, 215, and 253 proteins containing transmembrane domains respectively (Table 3.1).

3.3.5 Functional analysis and metabolism

A functional analysis of the proteins encoded by the genes present in the three *M. ovipneumoniae* isolates are shown in Table 3.2, with an emphasis on transport of compounds and protein synthesis. The genome encodes numerous transporters with a wide range of substrates. Both ATP Binding Cassettes (ABC) and phosphoenolpyruvate-dependent System (PTS) transporters are represented. It appears that *M. ovipneumoniae* should be able to transport and metabolise glucose, sucrose, maltose/maltodextrin, trehalose, and xylose as an energy source. Glycerol should also be used as an energy source. As is common with other mycoplasmas, *M. ovipneumoniae* should be able to both produce and utilise glycogen and starches [268].

M. ovipneumoniae contains a single copy of each of the 16S–23S–5S ribosomal RNAs. The 5s rRNA is not located within the 16S–23S rRNA operon. The 5s rRNA is located 42kb from the two other rRNAs. The conserved gene order rpmH → dnaA → dnaN is present as is observed in *M. mobile* and several other mycoplasmas [268].

3.3.6 Comparison with other sequenced mollicute genomes

The *M. ovipneumoniae* genome is larger than many of the sequenced genomes from mollicutes. in the genome with 711-971 protein coding genes, this is larger than *M. genitalium* [269], *M. pneumoniae* [270], *M. mobile* [268] and *U. urealyticum* [271] with 483, 688, 635, and 613 protein coding genes respectively.

Table 3-1 Nucleotide content and gene details of the *M. ovipneumoniae* genome of three New Zealand *M. ovipneumoniae* isolates.

Two overseas isolates of *M. ovipneumoniae*, SC01 [130] and NM2010 [251] were included for comparison.

	Isolate 16		Isolate 90		Isolate 103		SC01		NM2010	
Attribute	Value	% of total	Value	% of total	Value	% of total	Value	% of total	Value	% of total
Size (bp)	1,347,255	100	1,031,345	100	1,181,876	100	1,020,601	100	1084159	
G + C content (bp)	404,176	30	266,315	29	351,372	29.73	294,443	28.85	315,924	29.14
Coding region (bp)	1,147,861	85.2	894,249	86.7	999,876	84.6	821,380	80.48	-	-
Total genes	1020	100	758	100	908	100	-	-	-	-
RNA genes	3	.29	9	1.19	3	.33	-	-	-	-
Protein-coding genes	971	95.19	711	93.8	859	94.6	864	100	888	100
Genes assigned to COGs*	640	62.75	413	54.49	549	60.46	864	100	888	100
Average gene length (bp)	1182		1257		1164		950		973	
Genes with transmembrane helices	286	28.04	215	28.36	253	27.86	-	-	-	-

* COGs = Clusters of orthologous groups

Table 3-2 Clusters of orthologous grouping of genes in the genomes of three New Zealand *M. ovipneumoniae* isolates.

	Isolate 16		Isolate 90		Isolate 103		
Code	Value*	% of total	Value	% of total	Value	% of total	COG description
J	153	15.76	88	12.38	104	12.11	Translation
A	-	-	-	-	-	-	RNA processing and modification
K	30	3.09	15	2.11	23	2.68	Transcription
L	52	5.36	41	5.77	60	6.98	Replication, recombination, and repair
B	-	-	-	-	-	-	Chromatin structure and dynamics
D	7	0.72	3	0.42	4	0.47	Cell cycle control, mitosis, and meiosis
Y	-	-	-	-	-	-	Nuclear structure
V	29	2.99	17	2.39	25	2.91	Defence mechanisms
T	1	0.10	-	-	-	-	Signal transduction mechanisms
M	8	0.82	8	1.13	13	1.51	Cell wall/membrane biogenesis
N	1	0.10	1	0.14	1	0.12	Cell motility
Z	-	-	-	-	-	-	Cytoskeleton
W	-	-	-	-	-	-	Extracellular structures
U	8	0.82	7	0.98	7	0.81	Intracellular trafficking and secretion
O	27	2.78	17	2.39	23	2.68	Posttranslational modification, protein turnover, chaperones
C	27	2.78	20	2.81	29	3.38	Energy production and conversion
G	76	7.83	48	6.75	66	7.68	Carbohydrate transport and metabolism
E	33	3.40	21	2.95	26	3.03	Amino acid transport and metabolism
F	30	3.09	22	3.09	25	2.91	Nucleotide transport and metabolism
H	14	1.44	11	1.55	14	1.63	Coenzyme transport and metabolism
I	6	0.62	5	0.70	7	0.81	Lipid transport and metabolism
P	13	1.34	6	0.84	12	1.40	Inorganic ion transport and metabolism
Q	-	-	-	-	-	-	Secondary metabolites biosynthesis, transport, and catabolism
R	36	3.71	23	3.23	24	2.79	General function prediction only
S	14	1.44	6	0.84	15	1.75	Function unknown
	228	23.48	54	7.59	71	8.27	Associated with multiple COGS
-	331	34.09	298	41.91	310	36.09	Not in COGs

* Value refers to number of genes in each COG

3.3.7 P113 RFLP comparison

The P113 gene sequence was successfully derived from the HTS for each of the isolates. The predicted number of restriction fragments of the gene for Alul, DraI, HhaI, and MboI enzymes, for each isolate, are shown in Table 3.3. The *in silico* gel for each restriction enzyme grouped each of the isolates into one of the previously identified three main groups represented by Isolates 16, 90, and 103 (Fig. 3.1-3.4). Based on the similarity of RFLP patterns, isolates 42 and 16 were considered group 1, isolates 23 and 90 group 2, and isolates 70, 73, 83, 88 and isolate 103 group 3. Isolate 94 had similarities with both group 1 and group 3. The RFLP pattern of the reference strain from ESR did not appear to associate with any of the three groups. It contained several of unique bands from isolate 90 digested with Alul, whereas it also contained bands unique to isolate 16 digested with DraI, HhaI and MboI. However, it only contained some of the bands but was missing many of the other unique bands making it difficult to place it in any of the New Zealand groups (Table 3.3).

Comparing the results from the RFLP with the phylogenetic analysis (Fig. 3.5) showed congruence with the RFLP results. Isolate 94 was located with group 3 helping to distinguish between grouping isolate 94 into group 1 or 3. ESR was also located outside the three groups from New Zealand as was observed from the RFLP data.

Table 3-3 *In silico* restriction enzyme analysis of the P113 gene in different New Zealand isolates of *M. ovipneumoniae*, showing the number of restriction sites for each isolate. Phylogenetic analysis of the P113 was included for comparison.

<i>M. ovipneumoniae</i> Isolate	Predicted gene length (bp)	Restriction enzyme				Grouping RFLP	Grouping Phylogenetic
		Alul	DraI	HhaI	MboI		
16	3,239	18	6	5	7	1	1
90	3,240	21	7	12	5	2	2
103	3,259	19	8	3	7	3	3
42	3,221	18	8	6	7	1	1
70	3,236	24	9	2	9	3	3
23	3,227	18	9	10	3	2	2
73	3,233	16	7	3	8	3	3
83	3,236	18	8	3	9	3	3
88	3,236	18	9	2	9	3	3
94	3,226	17	7	3	9	1,3	3
ESR	3,237	15	6	8	8	-	-

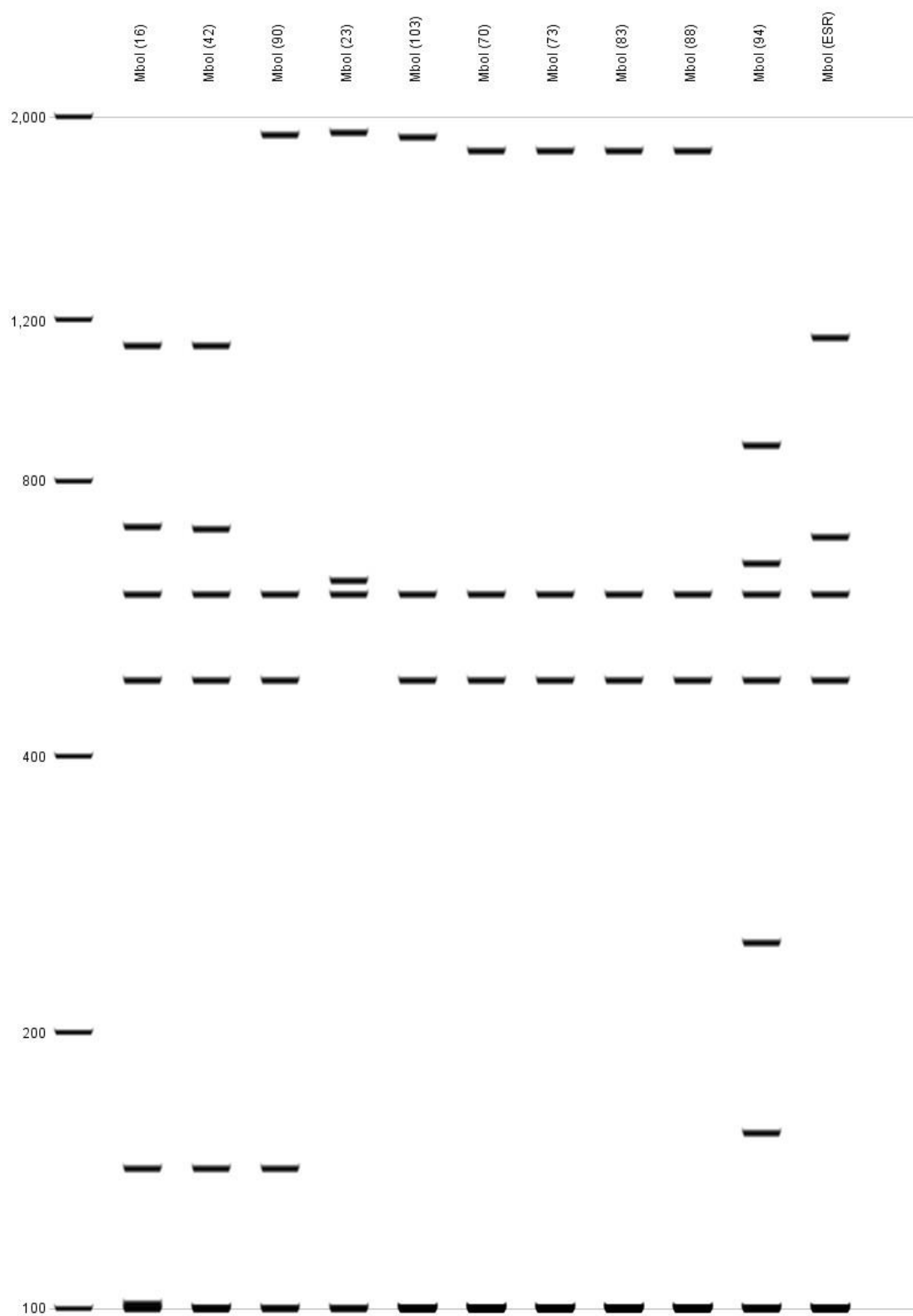


Figure 3-1 *In silico* gel electrophoresis pattern from *in silico* digestion of P113 fragment with Mbo1 restriction enzyme.

Ladder is Low DNA mass ladder.

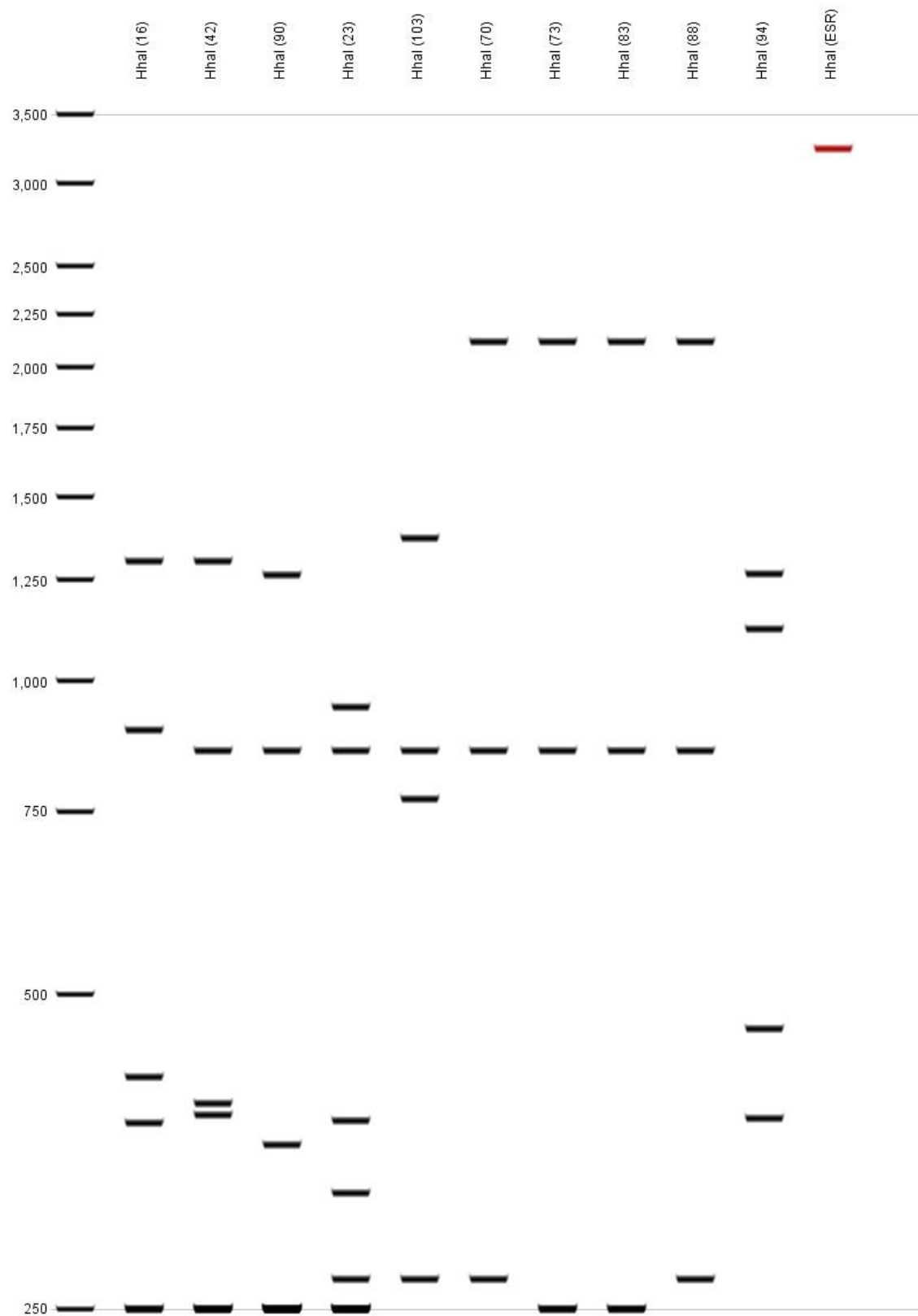


Figure 3-2 *In silico* gel electrophoresis pattern from *in silico* digestion of P113 fragment with Hha1 restriction enzyme.

Ladder is 250 bp DNA ladder.

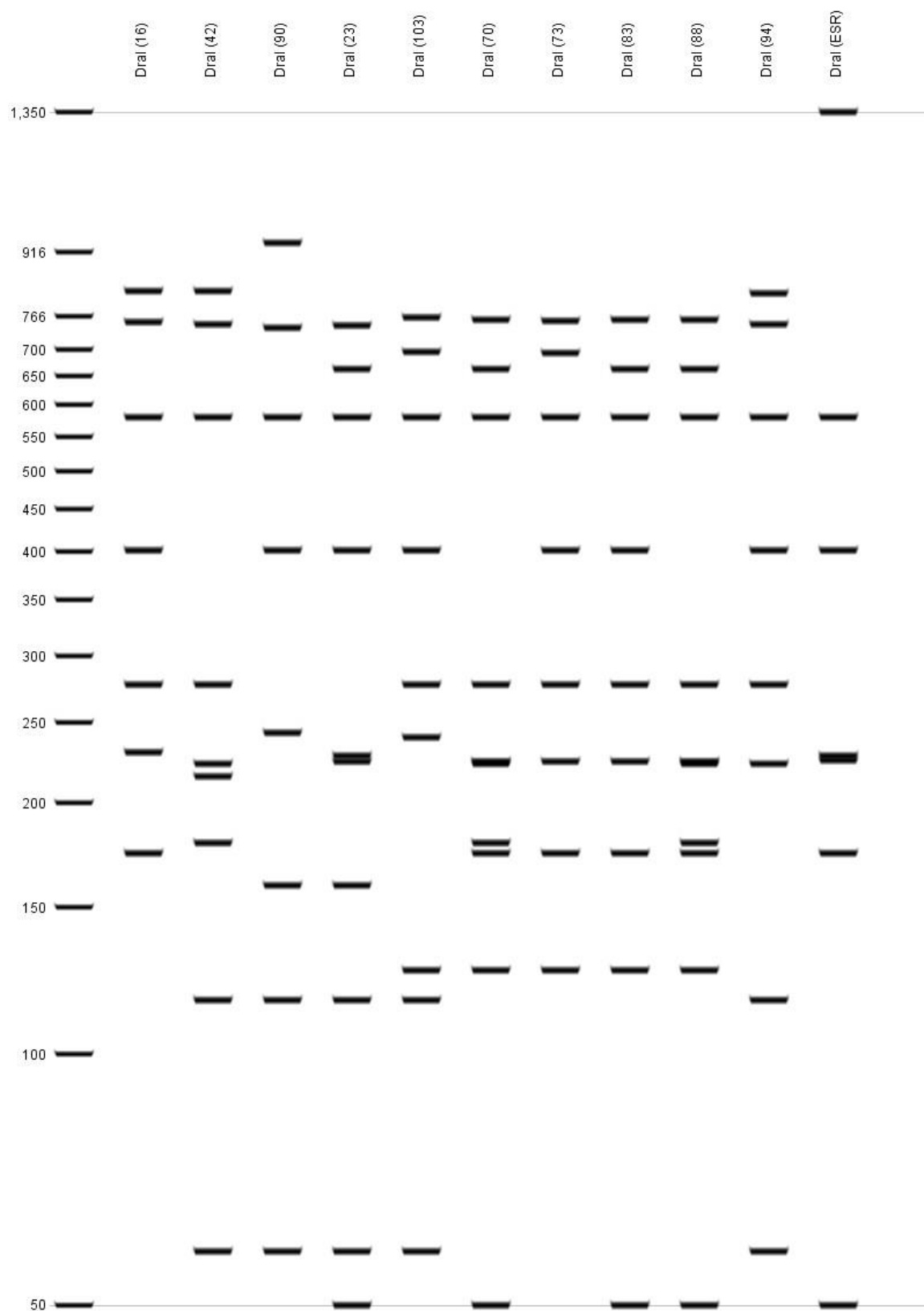


Figure 3-3 *In silico* gel electrophoresis pattern from *in silico* digestion of P113 fragment with Dra1 restriction enzyme.

Ladder is 50 bp (NEB)

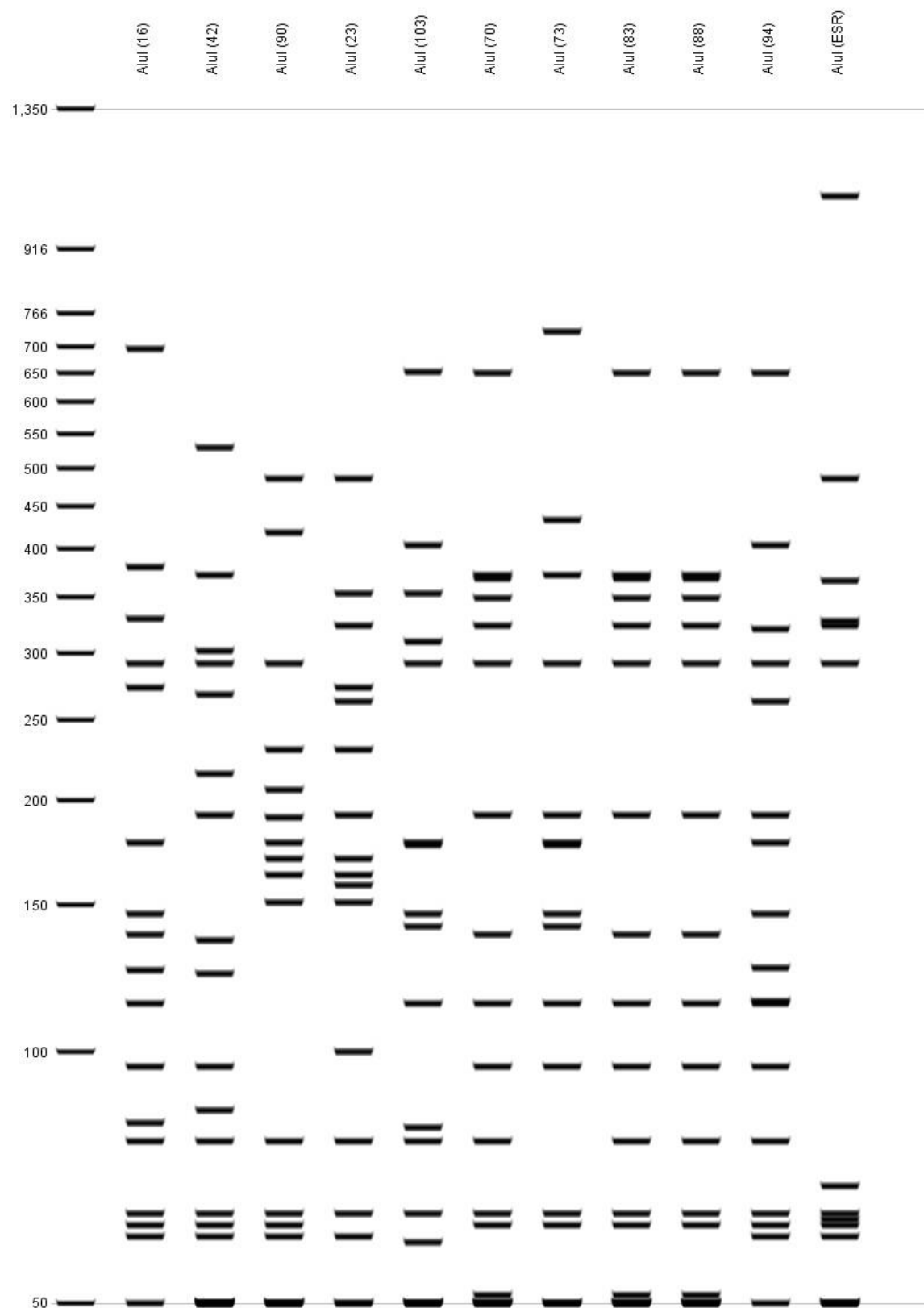


Figure 3-4 *In silico* gel electrophoresis pattern from *in silico* digestion of P113 fragment with AluI restriction enzyme.

Ladder is 50 bp (NEB).

3.3.8 Phylogenetic analysis

Sequences for the four loci from the MLST approach were obtained from the genome libraries for each of the 10 New Zealand isolates and the ESR type strain. The four sequences, for each of the isolates, were then concatenated into a single sequence in the order IGS→16s→gyrB→rpoB. This was repeated with the 15 sequences from Kamath et al (2019) [265]. Phylogenetic analysis grouped the New Zealand isolates into a single clade with the isolates from the Kamath et al paper [265] being separated into their own clade (Fig. 3.6). The type strain from ESR was located outside of the New Zealand clade but was more closely related to the New Zealand isolates than to the other isolates. Within the New Zealand clade there are two potential sub-clades, the first containing Isolates 23, 90, and 94, and the second containing isolates 16, 42, 70, 73, 83, 88, and 103 (Fig. 3.6).

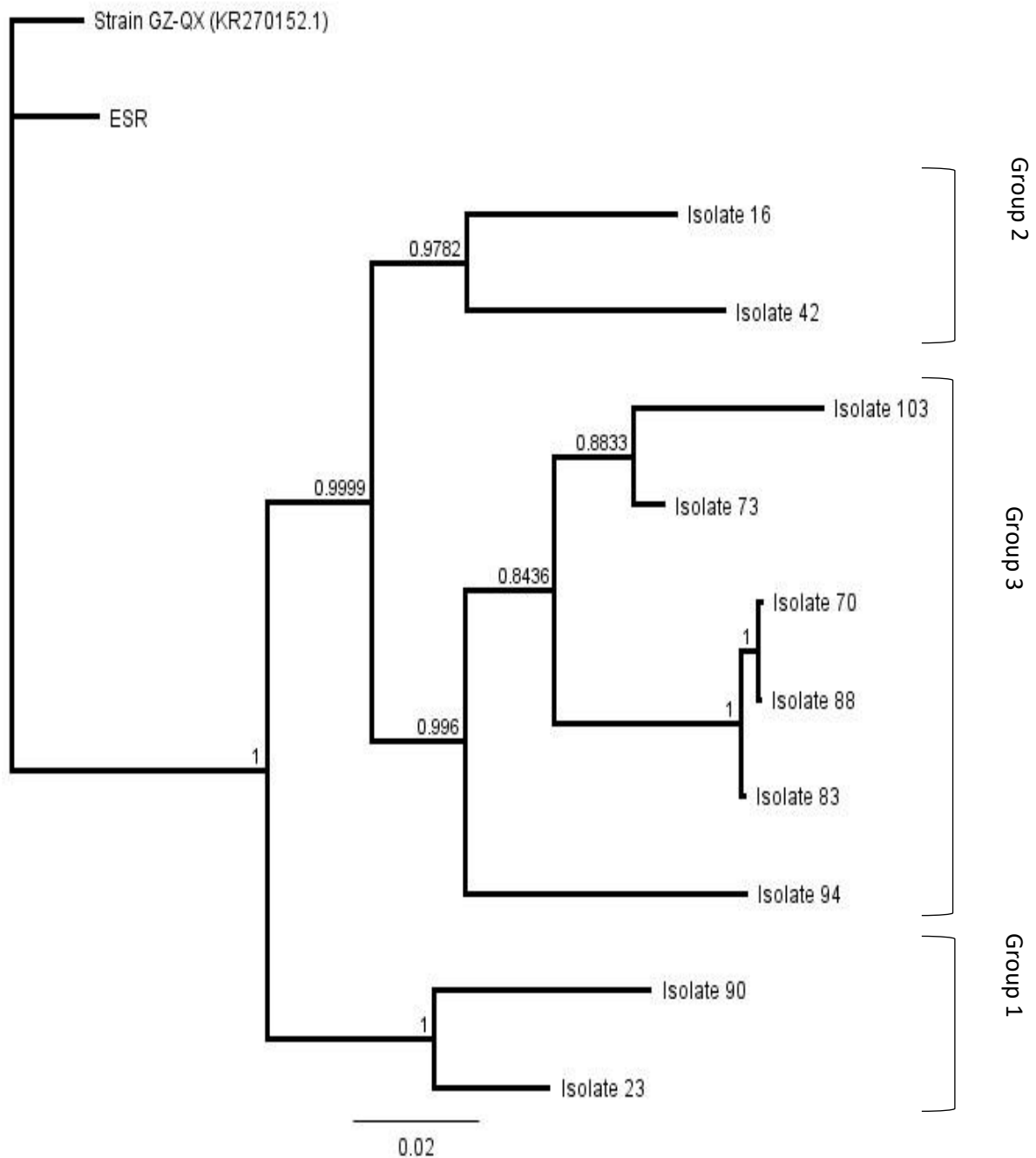


Figure 3-5 Phylogenetic tree of the P113 gene showing the distribution of New Zealand *M. ovipneumoniae* isolates into three groups.

The sequence of the type strain (ESR) and strain GZ-QX were used as the out groups establishing the root of the tree. Although strain GZ-Qx is unpublished, it appears in GenBank under the accession number KR270152.1. The tree was constructed using MrBayes [262] method.

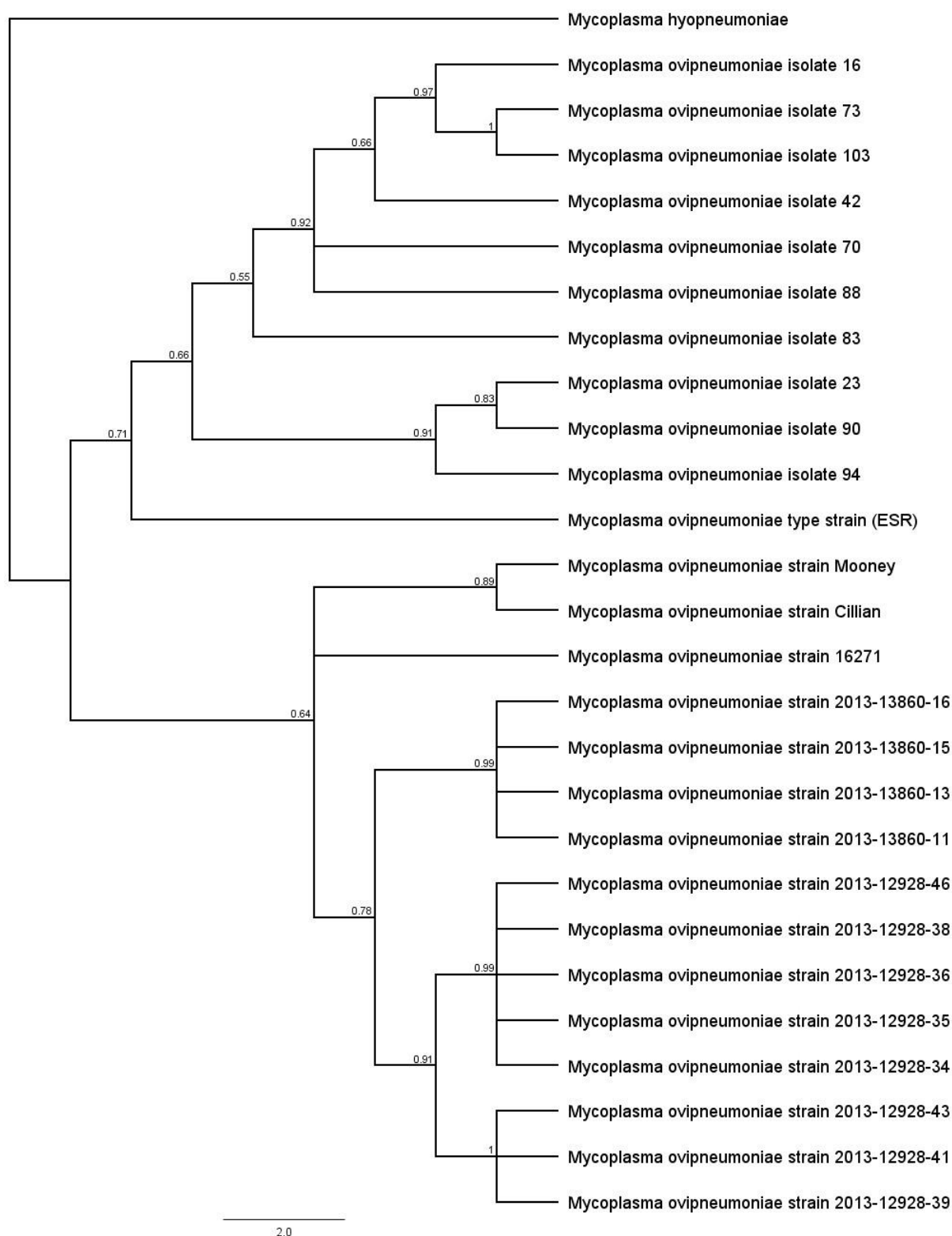


Figure 3-6 Phylogenetic tree of the MLST loci showing the position of New Zealand *M. ovipneumoniae* isolates.

The sequence for *M. hyopneumoniae* was used as the out group establishing the root of the tree. The other sequences included that were not a direct part of this study were produced by Kamath et al (2019) [263]. The tree was constructed using MrBayes [29] method.

3.4 Discussion

3.4.1 Genome properties of New Zealand isolates

To increase the genetic information available for New Zealand *M. ovipneumoniae*, three of the isolates obtained from sheep in New Zealand farms, the genomes of isolates 16, 90, and 103 were sequenced by HTS. Using the HTS shotgun sequences a draft genome was produced and annotated for isolates 16, 90, and 103.

Comparing New Zealand isolates with two other genomes available SC01 [130] and NM2010 [251] indicated similar genome lengths between isolates 90 and 103 with the genomes of SC01 and NM2010. Isolate 16 is currently the largest of the genomes being compared here at 1.3 mbp, approximately 263 kbp larger than NM2010. The difference in genome size may represent one or multiple gene duplication events [272, 273]. Comparing each of the genes identified in isolates 16 with those in isolates 90 and 103 might determine where the duplication events may have occurred. Errors in the building of the genomes are always a potential area for increase or decrease in the total length of the genome [274]. Using additional data such as using longer read length sequences such as those produced by PacBio [275] for each of the genomes can help increase the accuracy of the built genomes and can help cover gaps between contigs as well as ensuring the contigs produced have not had extra sequences introduced into them.

Despite the differences in genome size the percentage of the genome that was coding stayed similar (80-90%). The New Zealand isolates had a slightly higher coding percentage (84.6-86.7%) than that of strain SC01 (80.5%). As the genome size increased the number of genes detected increased, as would be expected since the coding region percentage stayed the same. The length of the proteins; however, did change outside of what was expected with all the New Zealand isolates having larger average gene lengths compared to that in SC01 and NM2010. The current data suggests that the average gene length of the New Zealand isolates was longer at 1,164-1,257 bases compared to 950 and 973 in SC01 and NM2010 respectively. This may provide some explanation into why New Zealand isolates appear to have larger genomes than other strains of *M. ovipneumoniae*.

In congruence with other mycoplasma species, including other *M. ovipneumoniae* strains, the genomes of the New Zealand isolates contained a single 16s-23s rRNA operon. The 5s rRNA was located away from the other two rRNAs which appears to be a common characteristic among mycoplasmas [276]. However, the level of gap between them appears to differ between species with the New Zealand isolates having a gap of 42 kb whereas *M. pulmonis* [276] was shown to have a gap of 320 kb. This suggests that there may be evolutionary pressure on the conservation of the 16s-23s operon but limited pressure on the location of the 5s rRNA. The separation of the 5s from the other rRNA operons may provide information on the phylogenetics of the mycoplasma branch by comparing the distance of separation and the genes present in the gap. *De novo* amino acid and

nucleotide synthesis, as with other mycoplasmas, has been lost during genome reduction of *M. ovipneumoniae* [268]. Amino acids are presumed to be obtained by ABC-type oligopeptide transporters followed by proteolytic degradation of the peptides. Extensive nucleotide salvage pathways, including the conversion of ribonucleotides to deoxyribonucleotides by *nrdA*, are also present [268].

Analysis of the COGs for each of the isolates 16, 90, and 103 predicted 640, 413, and 549 COGs respectively assigned to 54.49-62.75% of the total genes. Of the genes that had COG functions assigned not all genes were able to be assigned to a single COG. Isolate 16 had 228, isolate 90 had 54, and isolate 103 had 71 genes with multiple COG assignments. Undertaking further analysis of the genes assigned to multiple COGs may allow for proper assignment of COGs. Determining the protein function of the genes with multiple COG assignments will be useful in determining the correct assignment, and as more *M. ovipneumoniae* isolates are sequenced and annotated the accuracy of the annotation pipeline will continue to increase, reducing the incidence of multiple COG assignments.

3.4.2 tRNA

New Zealand isolates genomes contained specificity for all the amino acids except for tryptophan (Trp) in the genomes of isolates 16 and 90. The genome contained genes for tryptophan ligase and tryptophan is present in the sequences of proteins, therefore the absence of tryptophan tRNA is more likely to be due to the draft genome not overlapping the entire genome rather than loss of the genes from *M. ovipneumoniae*. Tryptophan is a unique amino acid in mycoplasmas as UGA and UGG codons, which are normally read as universal stop codons, are read as tryptophan instead [277, 278]. Therefore, it would be unlikely that no tryptophan tRNAs would be present in these isolates. One other potential reason for the lack of presence of tryptophan tRNAs may be the result of the annotation pipeline not correctly identifying tRNA_{UCA} or tRNA_{CCA} genes. Deeper analysis of these genomes will allow for the determination of the presence of the extra tRNA genes.

3.4.3 Functional analysis and metabolism

M. ovipneumoniae is an obligate parasite of caprine hosts, requiring nutrients and amino acids directly from their host. This is represented in the functional analysis of proteins (Table 3.2), indicating an emphasis on transport of compounds and protein synthesis. Closer inspection indicates that *M. ovipneumoniae* should be able to transport and metabolise a range of sugar compounds, including glucose, sucrose, maltose/maltodextrin, trehalose, and xylose as an energy source. In addition to using sugars as an energy source, glycerol is suspected to be metabolised and is a known preferred carbon source in *M. pneumoniae* [279]. Glycerol and glycerol-containing compounds such as lipids belong to the most abundant organic compounds that may serve as nutrients for many bacteria. These small molecules can easily be imported and used for energy production. Since

glycerol is usually found as a component of phospholipids, these lipids must be broken down to release glycerol. The use of glycerol by mycoplasmas as an energy source is only one of the suspected uses. Degradation of glycerol is also suspected to be important for the cytotoxicity of mycoplasma [279, 280]. The major virulence factor that causes host damage in mollicutes is hydrogen peroxide as a by-product of glycerol metabolism. Many mycoplasmas do not encode common virulence factors, in contrast to the severe virulence factor exhibited by *M. mycoides subsp. mycoides* [281]. This highlights the use of fundamental functions of metabolism, such as glycerol metabolism, to ensure their survival in the host tissue and cause disease [282-284].

Ciliostasis is a major pathogenic outcome from *M. ovipneumoniae* infections, which has been correlated to hydrogen peroxide production [285]. This provides potential avenues to protect infected animals if a method to prevent hydrogen peroxide production in *M. ovipneumoniae* can be discovered or there is a mechanism for degradation of hydrogen peroxide before it gets to the levels to cause disease.

3.4.4 Prediction of transmembrane proteins

The identification of suitable antigens is pivotal in the production of vaccines and diagnostics. The localisation of a protein on the surface of the pathogen may improve the likelihood of that protein being useful for vaccines and diagnostics. With an annotated genome, one of the methods to determine the localisation of a protein is the presence of transmembrane domains [177, 178].

Transmembrane domains are regions of the protein that are predicted to be located or embedded within the cell membrane and therefore the presence of these domains indicates that at least part of the protein is located as an extracellular domain outside of the cell. In isolate 16, 90 and 103 there were 286, 215, and 253 proteins with predicted transmembrane domains, respectively, providing a large pool of potential antigens for further analysis. Reducing this pool down to proteins with single or few transmembrane domains may help produce a more manageable pool of antigens for targeted analysis. This is because proteins with multiple transmembrane domains can be difficult to be produced in recombinant form. Comparing the proteins detected as potential antigens across isolate 16, 90, and 103 may assist in further reducing the number of antigens common across all three isolates.

3.4.5 Phylogenetics

In a previous unpublished analysis of New Zealand isolates of *M. ovipneumoniae*, three genetic groups were identified (chapter 2.3). An attempt was undertaken to regrow the isolates however after extended period with limited number of successful cultures the decision was made to proceed *in silico*. To re-confirm this grouping, the putative adhesin gene p113 sequence was obtained for 10 of the New Zealand isolates and the ESR reference strain. Comparison of the *in silico* restriction patterns separated the three isolates 16, 90, and 103 into unique groups as was suggested in the

previous study. The patterns of the other 7 New Zealand isolates produced restriction fragment patterns similar to one of the three groups; group 1 contained isolates 16, and 42, group 2 had isolates 90 and 23, and group 3 had isolates 103, 70, 73, 83, and 88 (Table 3.3). Isolate 94 was difficult to distinguish between group 1 and group 3. Comparing the sequence data for p113 showed isolate 94 was more closely related to group 3 than group 1 (Fig. 3.5). The other isolates in group 3 however, were closer to each other than to isolate 94 which may account for the difficulty in distinguishing between the two groups (Fig. 3.5). The ESR reference strain did not produce a restriction fragment pattern that could be associated with any of the groups identified in the New Zealand isolates (Table 3.3). This supports the use of this gene as a typing tool for grouping isolates of *M. ovipneumoniae*, not only in New Zealand but useful in following the distribution of different isolates as they move between domestic animals and wild animals such as big horn sheep [249]. Phylogenetic analysis grouped the New Zealand isolates into a single clade separate from the isolates described by Kamath et al (2019) [265] and the *M. hyopneumoniae* outgroup (Fig. 3.6). This is expected considering the geographical separation between New Zealand and the United States. The Type strain (ESR) was positioned as a sister isolate to the New Zealand isolates with a posterior probability of 0.71. This suggests Australia, the location of the type strain, is the source of New Zealand *M. ovipneumoniae* (Fig. 3.6). With both countries being located close together it is likely that mycoplasmas could be transported from one country, especially in the early stages of the budding sheep industry in New Zealand and before the identification of the presence of *M. ovipneumoniae* [59, 248] and strict biosecurity measures were implemented in both countries [286]. Further analysis of Australian *M. ovipneumoniae* could determine whether there is support for movement of *M. ovipneumoniae* between the two countries or if there has been predominately one-way movement from Australia to New Zealand.

Within the New Zealand clade there is support for the presence of two separate groups of New Zealand *M. ovipneumoniae* isolates (Fig. 3.6). Except for isolate 94, the split was between group 2 and, groups 1 and 3. Isolate 94 was originally located within group 3 along with isolate 103 according to its P113 sequence; however, using the MLST sequence, isolate 94 was more closely related to isolates 23 and 90 in group 2. With the posterior probability of 0.66, the separation between the two groups is not as strong as some of the other clades within the New Zealand isolates. This may be due to the grouping of isolate 94 with isolates 23 and 90, suggesting that isolate 94 may have undergone genetic shift between the two groups. This could be undertaken via horizontal gene transfer [287] when different isolates on *M. ovipneumoniae* mix within a host. This could allow movement of either P113 or MLST genes between groups resulting in disparity between the two phylogenies. Another potential would be convergent evolution of one or more of the genes used in the phylogenetic

analysis. The primary differences between the two clades is two insertions in the IGS of 3 and 5 nucleotides within the isolates 23, 90, and 94.

The separation of *M. ovipneumoniae* into two clades may support the concept of multiple invasions of this organism into New Zealand from Australia. To confirm this, further analysis of New Zealand isolates would be required to increase the number of isolates associated with the clade containing isolate 90 to confirm the separation of the clades. The second analysis would require further samples of Australian isolates to determine if there are isolates closer related to each group. This would determine whether the two groups are a result of multiple invasion events or due to divergent evolution within the New Zealand isolates.

3.4.6 Comparison with other mycoplasmas

Members of the mollicutes are known for their small genome size and reduced number of genes with most members containing less than 1,000 genes and many genomes under 1,000 kb [268, 288]. *M. ovipneumoniae* have a similar gene count of 711-971 protein coding genes; however, the genome is over 1,000 kb. The *M. ovipneumoniae* genome has been shown to be over 1,000 kb in several strains, with strain SC01 having a genome size of 1020 kb [130] and strain NM2010 having a genome size of 1,084 kb [251]. The GC content across the five genomes (isolate 16, 90, 103, SC01, and NM2010) of *M. ovipneumoniae* was consistent at around 29-30%. This was higher than that detected in *M. mobile* at 24.9% [268] and *M. pulmonis* at 26.6%. Comparatively *M. pneumoniae* has a GC content of 40%. The larger genomes of *M. ovipneumoniae* compared to other mycoplasma species may provide advantages to promote distribution across a number of domestic and wild species as well as allowing *M. ovipneumoniae* to evade detection and/or reduce the immune response to infection, increasing its ability to take hold in the host animals. Further comparisons between mycoplasma species may reveal the different/additional genes present in *M. ovipneumoniae* and whether they provide advantages to *M. ovipneumoniae*. It would also be of interest to determine if the extra genes present in *M. ovipneumoniae* are a result of gene duplication from a smaller genome ancestor or a remnant from a larger genome ancestor. It would also be of interest to understand the role of selection pressure on these events.

3.5 Conclusion

Three isolates of New Zealand isolates of *M. ovipneumoniae* have had a draft genome created and annotated. This provides a new avenue of research into the molecular understanding of this pathogen. The isolates had a genome size of 1.03-1.34 mb and a genomic GC content of 29-30% similar to the genomes of isolates SC01 and NM2010. Annotation identified 971, 711, and 859 protein coding genes in isolates 16, 90, 103 respectively with over 200 genes containing transmembrane domains, providing a large number of potential proteins that could be investigated

for further vaccine or diagnostic use. Comparison between New Zealand isolates and other *M. ovipneumoniae* genomes has shown similar range of genes, with the two external *M. ovipneumoniae* isolates gene count sitting between the three New Zealand isolates. RFLP and phylogenetic analysis of P113 has provided support to the establishment of three groups of *M. ovipneumoniae* in New Zealand. Phylogenetic analysis of the three New Zealand isolates (16, 90 and 103) grouped all three isolates in a single clade with the ESR reference strain as the closest related strain. Within the New Zealand clade, two further subclades were observed, which may indicate two separate invasion events of *M. ovipneumoniae* from Australia. Further analysis of New Zealand and Australia isolates of *M. ovipneumoniae* will provide support for either separate invasion events or divergent evolution within New Zealand isolates.

3.6 Data availability

This whole-genome shotgun project for isolate 90 has been deposited at DDBJ/ENA/GenBank under the accession number VZDPOOOOOOOO. The version described in this paper is version VZDPOIOOOOOO, and the BioProject number is PRJNA573593. The sequence reads have been deposited at the SRA under the accession number SRR10959050.

Chapter 4 Identification of *Mycoplasma ovipneumoniae* immunogenic proteins by two-dimensional SDS-PAGE, Western blotting, and mass spectrometry analysis

4.0 Abstract

As part of research aimed at developing a vaccine against *M. ovipneumoniae* or a diagnostic test to detect disease caused by this bacteria, hydrophobic membrane protein fractions were produced and immunoblotted with sheep antisera generated against three New Zealand isolates of *M. ovipneumoniae*. Proteins in these fractions were separated by 2D gel electrophoresis and immunogenic proteins were detected by Western blotting using antisera against *M. ovipneumoniae*. The identity of immunogenic proteins was determined by mass spectrometry with reference to the genome of *M. ovipneumoniae* produced in Chapter 3. Eight proteins were successfully identified across the three isolates including two previously known immunogenic proteins of *M. ovipneumoniae*, HSP70 and EF-Tu. Each of the eight proteins were analysed for B lymphocyte epitope prediction, transmembrane helices prediction, and hydrophobicity prediction. This resulted in four proteins (EF-Tu, GAPDH, P146, and HP3) and one epitope of HP1 being selected for further study.

4.1 Introduction

Throughout the history of the biological arms race between pathogens and their hosts, pathogens have developed mechanisms to inhibit, or hide from, the host immune system whereas the host has developed mechanisms to better detect and eliminate the invading pathogen [90]. Since the emergence of vertebrates and the development of their adaptive immune system, the host has been able to produce a stronger immune response due to the ability to gain an immunological memory where pathogens are remembered by select antigens [289]. The adaptive immune response is antigen-specific and uses the process of antigen presentation to recognise non-self-antigens [77, 78]. The specificity of the adaptive immune system allows for the generation of tailored responses to specific pathogens and pathogen infected cells. This process is managed by 'memory cells', which, in response to repeated detection of the same pathogen elicit an appropriate immune response to quickly eliminate it [91].

The ability of the adaptive immune system to 'remember' antigens from pathogens allows the development of preventative measures against known pathogens. Through the introduction of antigens of a specific pathogen, it is possible to prime the immune system, reducing the time it takes from the initial detection of the pathogen to mounting a strong immune response against the invading organism [290]. This 'priming' of the immune system is the basis for using vaccines to protect against disease. However, a key component of developing a vaccine is to identify proteins within the agent that trigger a strong immune reaction [290]. Identification of such immunogenic proteins is an important step in vaccine development. Of the proteins determined to be immunogenic, the proteins presented on the surface of the cell will provide the best chance for the host to be protected against the organism, as they are more likely to be detected by the immune system while the organism is still alive and pathogenic [291, 292]. This mainly relates to antibody-based vaccines and extracellular pathogens, which *M. ovipneumoniae* appears to be. Alternatively, T-cells recognise processed peptides which don't require the antigens to be cell surface located. Limited exposure to a pathogen reduces the ability of the adaptive immune system to protect the animal as much of the damage is caused before the immune response can mount the required response. Therefore, priming the immune system through vaccination can protect against or reduce the effect of the related infection.

This is often the case in production animals where animals, such as lambs, will be kept for a limited period of time for growing and may be slaughtered before they are able to remove the infection. *M. ovipneumoniae* is an organism that causes sub-lethal infection of the lungs of lambs. This reduces the wellbeing of the animals and increases the energy load on the animal, reducing the feed conversion rate. This results in the animals being smaller and lighter at time of slaughter or requiring longer time to reach the required weight [60].

Vaccination can provide the potential to prevent infection from occurring or establishing in the host. However, in the absence of a viable vaccine, treatment of the animal if infection occurs will be required. Two difficulties of treating *M. ovipneumoniae* infections are the limited clinical symptoms of infection [60, 248], and the inefficacy of common antibiotics on *M. ovipneumoniae* due to the lack of a cell wall [50]. Therefore, the development of quick and accurate identification techniques for *M. ovipneumoniae* infections is required for prompt treatment using antibiotics effective against mycoplasmas.

Hence, this study was undertaken with the following aims:

- Detect immunogenic proteins in New Zealand isolates of *M. ovipneumoniae*
- Identify the immunogenic proteins in terms of amino acid and corresponding nucleotide sequences
- Analyse the proteins for potential use in a vaccine or for diagnostic use

Previous work (unpublished) has suggested three groups of *M. ovipneumoniae* isolates within New Zealand. This grouping was based on RFLP analysis of the P113 gene in genomic DNA (chapter 2.3). The restriction digestion pattern of P113 gene was further tested *in silico* in chapter 3. Three isolates, 16, 90, and 103, were chosen as representatives of the three groups identified for the studies in this chapter.

To detect potential immunogenic proteins, Western blotting was performed on hydrophobic protein fractions (HPF) prepared from the three isolates using sera collected from animals vaccinated with *M. ovipneumoniae* whole cell antigens. The proteins in the hydrophobic fractions were separated on one-dimensional (1D) and two-dimensional (2D) SDS-PAGE gels. The first experiments used sera collected from sheep vaccinated with a mixture of *M. ovipneumoniae* and *M. haemolytica*. This sera were used as it was readily available at the start of the study. During the studies, further sera were obtained from animals that had only been vaccinated against only *M. ovipneumoniae* in both sheep and rabbits to examine *M. ovipneumoniae*-specific immunoreactions. The Western blotting experiments identified a number of potential proteins for further studies. These proteins were extracted from SDS-PAGE gels for identification by mass spectrometry. To identify the immunogenic proteins of interest and obtain the complete protein and nucleotide sequences, the peptide sequences from mass spectrometry of the extracted proteins were compared with sequences from the built and annotated genome of *M. ovipneumoniae* (Chapter 3).

4.2 Methods

4.2.1 Extraction of proteins

Three New Zealand isolates (16, 90, and 103) of *M. ovipneumoniae* were cultured as described in Chapter 2 (Section 2.4). Whole cell lysates (WCL) of *M. ovipneumoniae* were obtained via sonication

(Section 2.6.1), and hydrophilic and hydrophobic membrane proteins fractions were extracted using Bio-Rad ReadyPrep™ Protein Extraction Kit (Membrane I) [235, 236] (Section 2.6.2) (Bio-Rad Laboratories, United states). Protein concentrations were determined using the Pierce protein determination kit (Section 2.6.3).

4.2.2 Production of sera

Sera were collected from sheep vaccinated with the three New Zealand isolates of *M.*

ovipneumoniae (isolates 16, 90, and 103) and *M. haemolytica* as described in Chapter 2 (chapter 2.7.2). Sera were also produced in sheep against only *M. ovipneumoniae* (isolates 16, 90, and 103) (chapter 2.7.3). This serum was to determine the effect *M. haemolytica* antibodies might have on the downstream experiments. To test the effect of using a host other than sheep on the immune response to *M. ovipneumoniae*, sera were also collected from rabbits vaccinated with *M. ovipneumoniae* (isolates 16, 90, and 103) (chapter 2.7.4).

4.2.3 SDS-PAGE and Western blotting

WCL or membrane proteins were separated by molecular weight using 1D and 2D SDS-PAGE as described in Chapter 2 (chapter 2.9). The gels were either stained with Coomassie blue for detection of all proteins present or transferred to a PVDF transfer stack. For Western blotting, the PVDF membrane was incubated with primary antibodies (chapter 2.10) from sheep sera (chapter 2.7.2 and 2.7.3) or rabbit sera (chapter 2.7.4). The membrane was then incubated with rabbit anti-sheep or goat anti-rabbit secondary antibodies respectively. For colorimetric analysis 1-Step™ TMB-Blotting Substrate Solution (Invitrogen™, Thermo Fisher Scientific™, United States), was used or for Chemiluminescent analysis, Clarity™ Western ECL Substrate, (Bio-Rad Laboratories, United states) was used.

4.2.4 Protein identification by mass spectrometry

Protein spots identified as immunogenic by Western blotting were extracted from the 2D SDS-PAGE gel, sent to AgResearch Lincoln and analysed via their LC-MS/MS platform as described in Chapter 2 (chapter 2.12). The mass spectrometer produces a number of small peptide sequences (~5-7 amino acids) for each of the proteins provided. The small peptide fragments provided were then aligned to the annotated genome produced in chapter 3 to determine the associated protein. The peptide sequences that were unique to single protein were recorded and if multiple proteins were detected. proteins of the correct molecular weight were selected as the most likely protein of interest. This allowed the full nucleotide and amino acid sequence to be obtained for further protein analysis.

4.2.5 Protein sequence analysis

The full-length peptide and nucleotide sequence for each of the immunogenic proteins were extracted from the genome of isolate 90 (chapter 3). These sequences were used to analyse the predicted structure of each of the proteins. The first analysis conducted was B lymphocyte epitope

prediction. B lymphocyte epitopes were calculated by prediction of linear epitopes from a protein sequence (<http://tools.iedb.org/bcell/>). The calculation method used was the Bepipred-1.0 Linear Epitope Prediction [293]. It must be noted that B cell epitope predictions are not optimised for ruminants. The second analysis predicted transmembrane domains. Transmembrane domains were predicted using the Technical University of Denmark's TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) Predicting transmembrane helices in proteins [294, 295]. The final analysis looked at predicting the hydrophilic and hydrophobic regions of the proteins. For this, Kyte/Doolittle analysis was completed using the ExPASy SIB Bioinformatics Resource Portal (<https://www.expasy.org/>) implementing the Kyte/Doolittle method for displaying the hydropathic nature of a protein [296].

4.2.6 Comparison of immunogenic proteins

The sequence data for each of the detected immunogenic proteins, HSP70, EF-Tu, GAPDH, MATE, HP1, HP2, HP3, and P146 were extracted from the genome of isolate 90. As described in Chapter 3 (chapter 3.2.2) the nucleotide sequences were used as the reference sequences for the map to reference function. HTS data from isolates 16, 23, 42, 70, 73, 83, 88, 94, and 103 were mapped against the reference sequence from isolate 90 to produce the gene sequence of those isolates. For each of the proteins, nucleotide and protein sequence data were compared between each of the New Zealand isolates of *M. ovipneumoniae*. The sequences were then compared to sequences from other mycoplasmas.

4.3 Results

4.3.1 Protein analysis of *M. ovipneumoniae*

Analysis of proteins in whole cell lysates

Proteins in cell lysates prepared from *M. ovipneumoniae* isolates 16, 90, and 103, were separated by SDS-PAGE. Different protein amounts were used to observe the banding patterns of each of the isolates without the effect of saturation from abundant proteins. The majority of proteins had molecular masses between 28 and 97 kDa (Fig. 4.1). One-dimensional Western blotting analysis of WCL from the three isolates was conducted with pooled sera (pre-immune and antisera) obtained from sheep vaccinated with both *M. ovipneumoniae* and *M. haemolytica* (chapter 2.7.2), detected a large repertoire of immunogenic proteins unique to the antisera sera (Fig. 4.2). These immunogenic proteins ranged in molecular size from less than 19 kDa to greater than 97 kDa.

There were differences between each of the three isolates in their pattern of reactivity to both the pre-immune sera and antisera sera with several bands either present or absent in the different isolates. This indicates that there may be some significant differences in the immunogenicity of proteins between the three isolates.

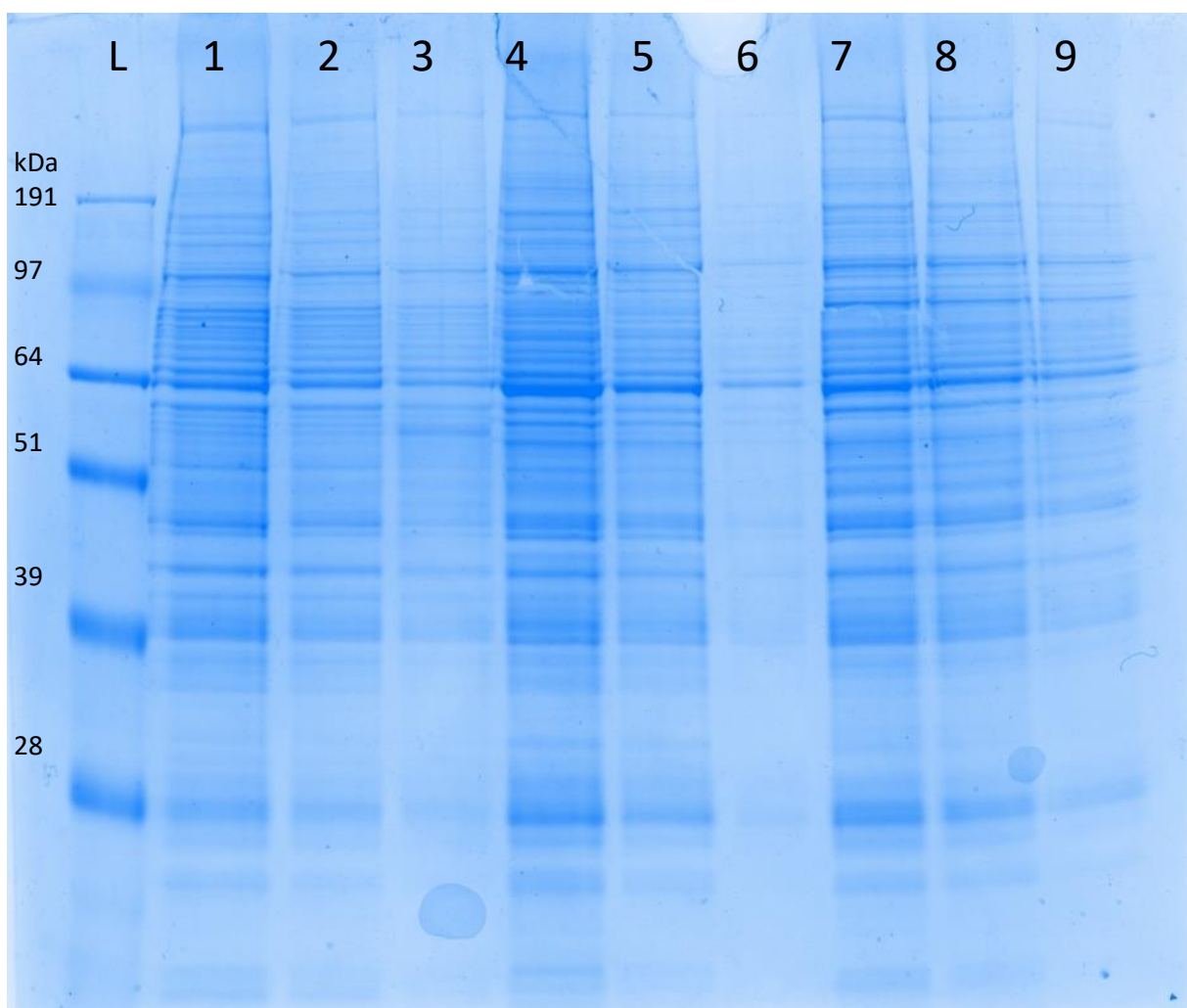


Figure 4-1 SDS-PAGE of proteins from whole cell lysates of *M. ovipneumoniae*.

Lane L: Ladder SeeBlue™ Plus2 Pre-stained Protein Standard; Lane 1: isolate 16, 6.5 µg; Lane 2: isolate 16, 3.2 µg; Lane 3: isolate 16, 1.6 µg; Lane 4: isolate 90, 6.5 µg; Lane 5: isolate 90, 3.2 µg; Lane 6: isolate 90, 1.6 µg; Lane 7: isolate 103, 6.5 µg; Lane 8: isolate 103, 3.2 µg; Lane 9: isolate 103, 1.6 µg. Different protein concentrations were used to observe the banding patterns of each of the isolates without the effect of saturation from abundant proteins.

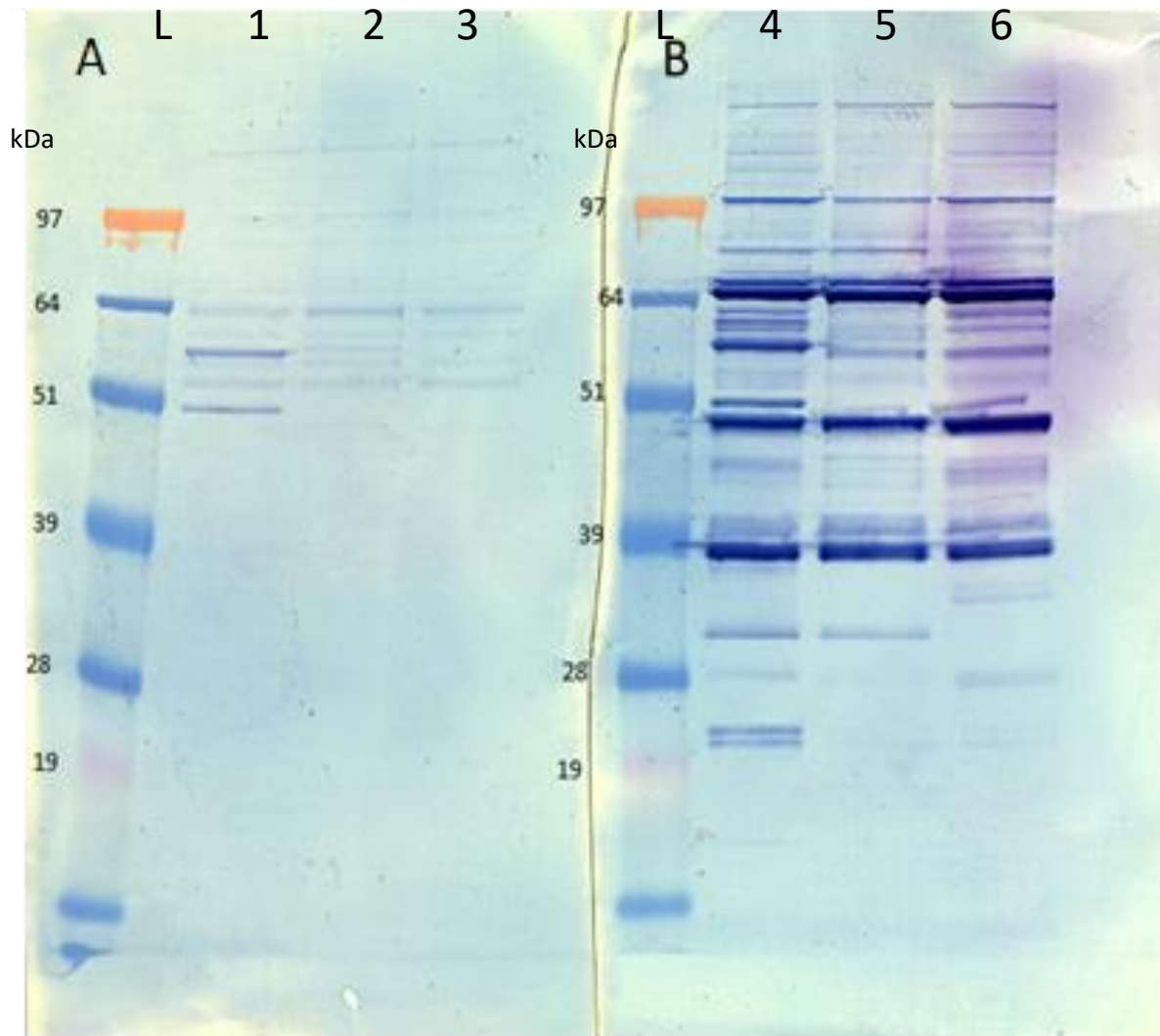


Figure 4-2 1D Western blot showing immunogenic proteins in whole cell lysates prepared from *M. ovipneumoniae*.

Proteins were separated by SDS-PAGE and probed using pooled sera from three sheep vaccinated with *M. ovipneumoniae* (isolates 16, 90, and 103) and *M. haemolytica*. A) Western blot using pre-immune sera. B) Western blot using antisera. Lanes L: Ladder SeeBlue™ Plus2 Pre-stained Protein Standard, Lanes 1, 2, and 3: whole cell lysates from isolates 16, 90 and 103, respectively; Lanes 4, 5, and 6: whole cell lysates from isolates 16, 90 and 103, respectively. 1 step colorimetric blotting was used as the detecting agent.

Analysis of proteins in membrane fractions using sera from sheep vaccinated with *M. ovipneumoniae* (isolates 16, 90, and 103) and *M. haemolytica*

Separation of the proteins in the hydrophobic fractions showed the presence of many proteins, with the majority having a molecular size between 39 and 97 kDa (Fig. 4.3). Western blotting showed of the presence of immunogenic protein bands (Fig. 4.4). The hydrophobic fraction prepared from isolate 16 contained approximately 13 immunogenic protein bands, isolate 90 approximately 14 bands and isolate 103 approximately 15 bands. The immunogenic proteins in these bands had molecular sizes ranging between 28 and 100 kDa. Many of the immunogenic protein bands were common across all isolates, with prominent antigens corresponding to molecular masses of 38, 40,

49, 66, 69, and 97 kDa being detected in all 3 isolates. Notably, the immunogenic protein bands at 71 kDa and 86 kDa, visible in isolates 16 and 103, were not detected in the hydrophobic fraction from isolate 90.

The hydrophilic protein fraction contained many immunogenic protein bands (Fig. 4.4). The hydrophilic band pattern was similar to that of the hydrophobic fraction, with prominent bands around 38, 40, 49, 66, 69, and 97 kDa in size. In addition to the bands common between hydrophobic and hydrophilic fractions, a number of bands were apparent between approximately 57 and 61 kDa only within the hydrophilic fraction.

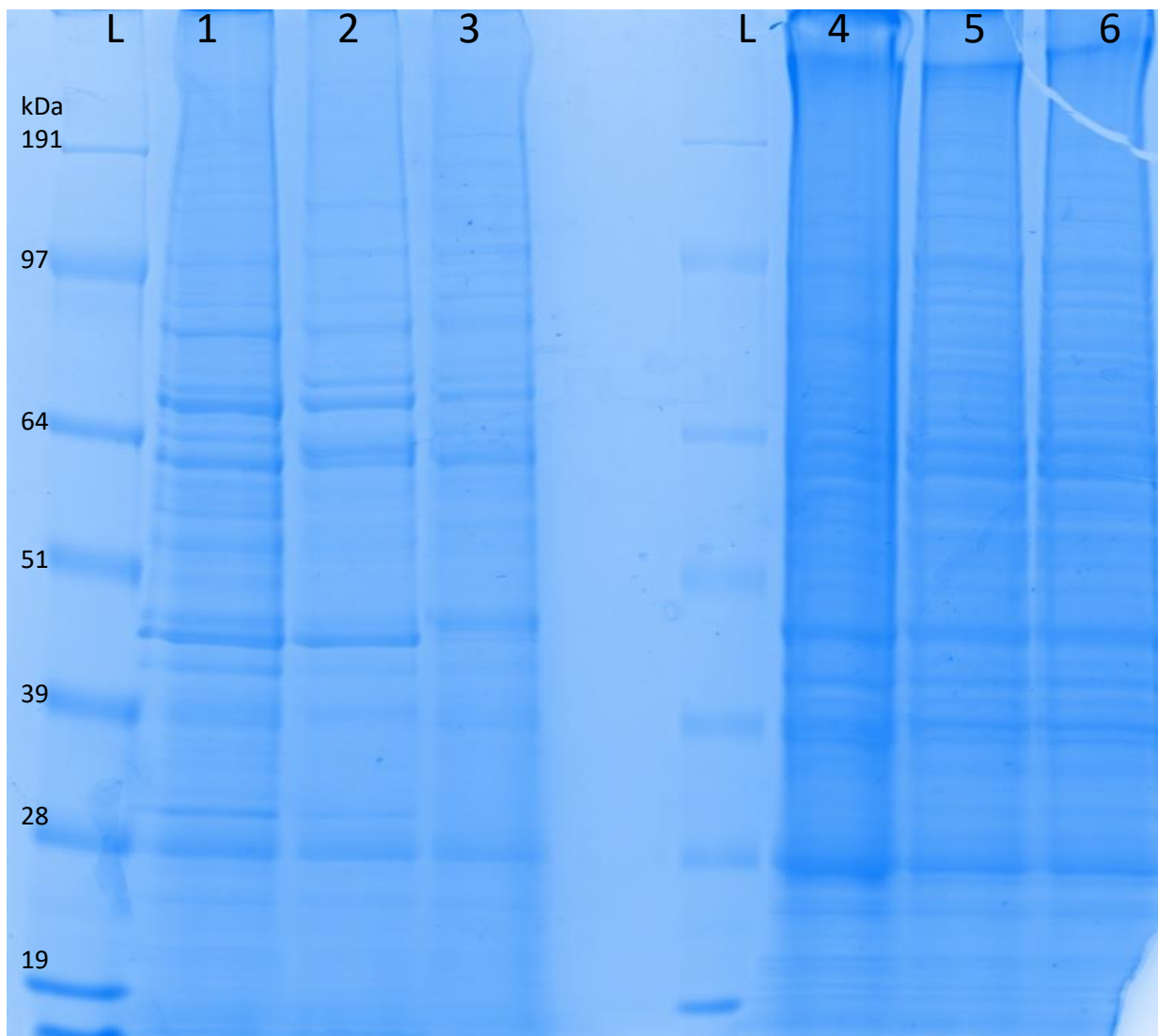


Figure 4-3 SDS-PAGE separation of proteins in hydrophobic and hydrophilic fractions prepared from *M. ovipneumoniae*.

Lanes L: Ladder SeeBlue™ Plus2 Pre-stained Protein Standard; Lanes 1, 2, and 3: hydrophobic fractions from isolates 16, 90 and 103, respectively; Lanes 4, 5, and 6 hydrophilic fractions from isolates 16, 90 and 103, respectively.

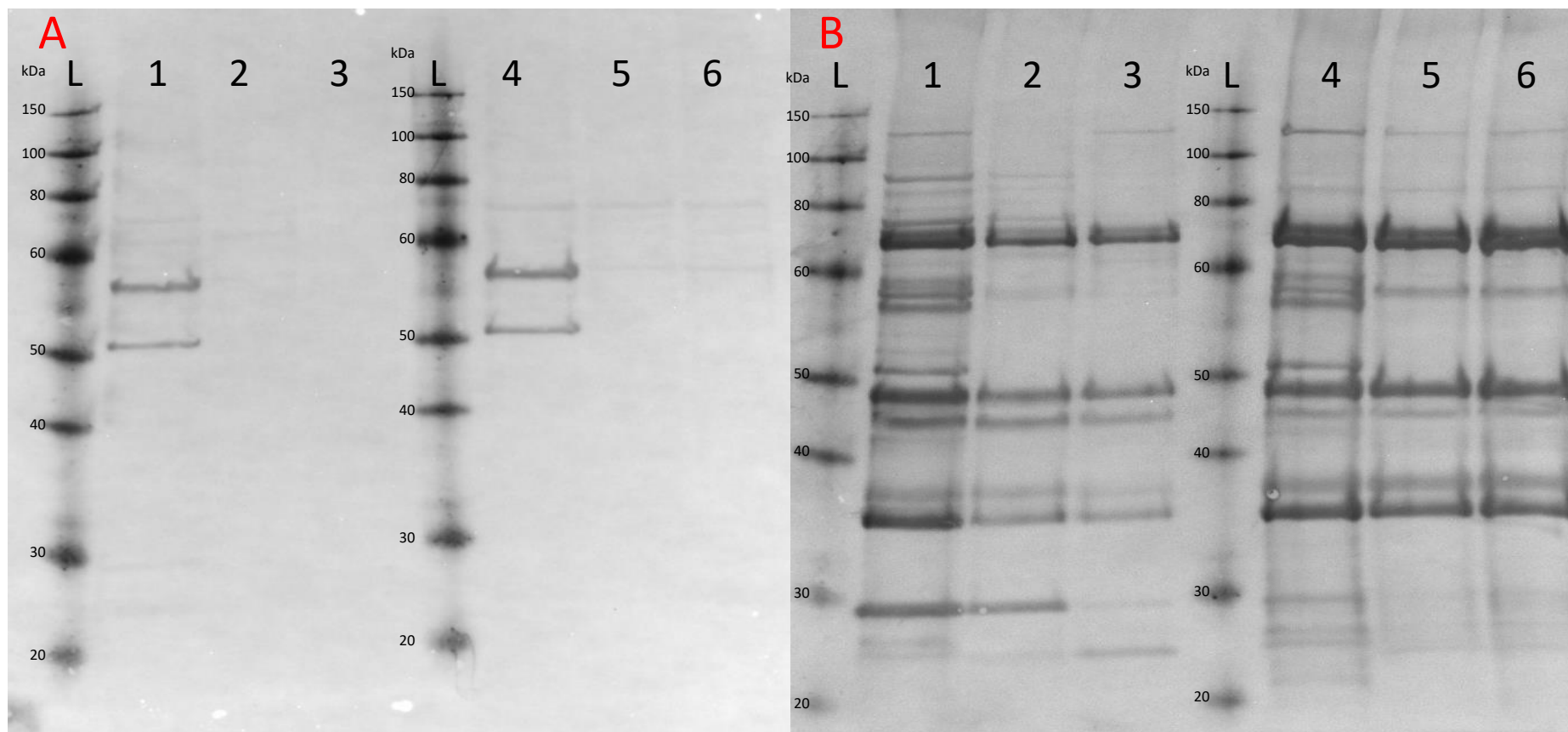


Figure 4-4 1D Western blots showing immunogenic proteins from hydrophobic and hydrophilic protein fractions of *M. ovipneumoniae*.

Proteins were separated by 1D SDS-PAGE and probed using sheep sera from sheep vaccinated with *M. ovipneumoniae* (isolates 16, 90, and 103) and *M. haemolytica*. A) Western blot using pooled pre-immune sera. B) Western blot using pooled antisera sera. In each blot, Lanes L: Ladder SuperSignal™ Molecular Weight Protein Ladder; Lanes 1, 2, and 3: hydrophobic fractions from isolates 16, 90 and 103, respectively, and Lanes 4, 5, and 6: hydrophilic fractions from isolates 16, 90 and 103, respectively.

Rabbit antibody response to *M. ovipneumoniae* antigens

Whole cell, and hydrophilic membrane protein fractions from the three *M. ovipneumoniae* isolates blotted against the rabbit sera (vaccinated with *M. ovipneumoniae* isolates 16, 90, and 103) (chapter 2.7.4) showed similar banding (Fig. 4.5, 4.6) to those observed using the pooled sheep sera. This suggested that *M. ovipneumoniae* is producing a similar immunogenic response in rabbits when blotted against WCL and hydrophilic membrane proteins. However, Western blotting on the HPF showed less immunoreactivity to the hydrophobic proteins in the middle of the molecular weight range, with most of the proteins detected having molecular sizes around 64 kDa or 28kDa.

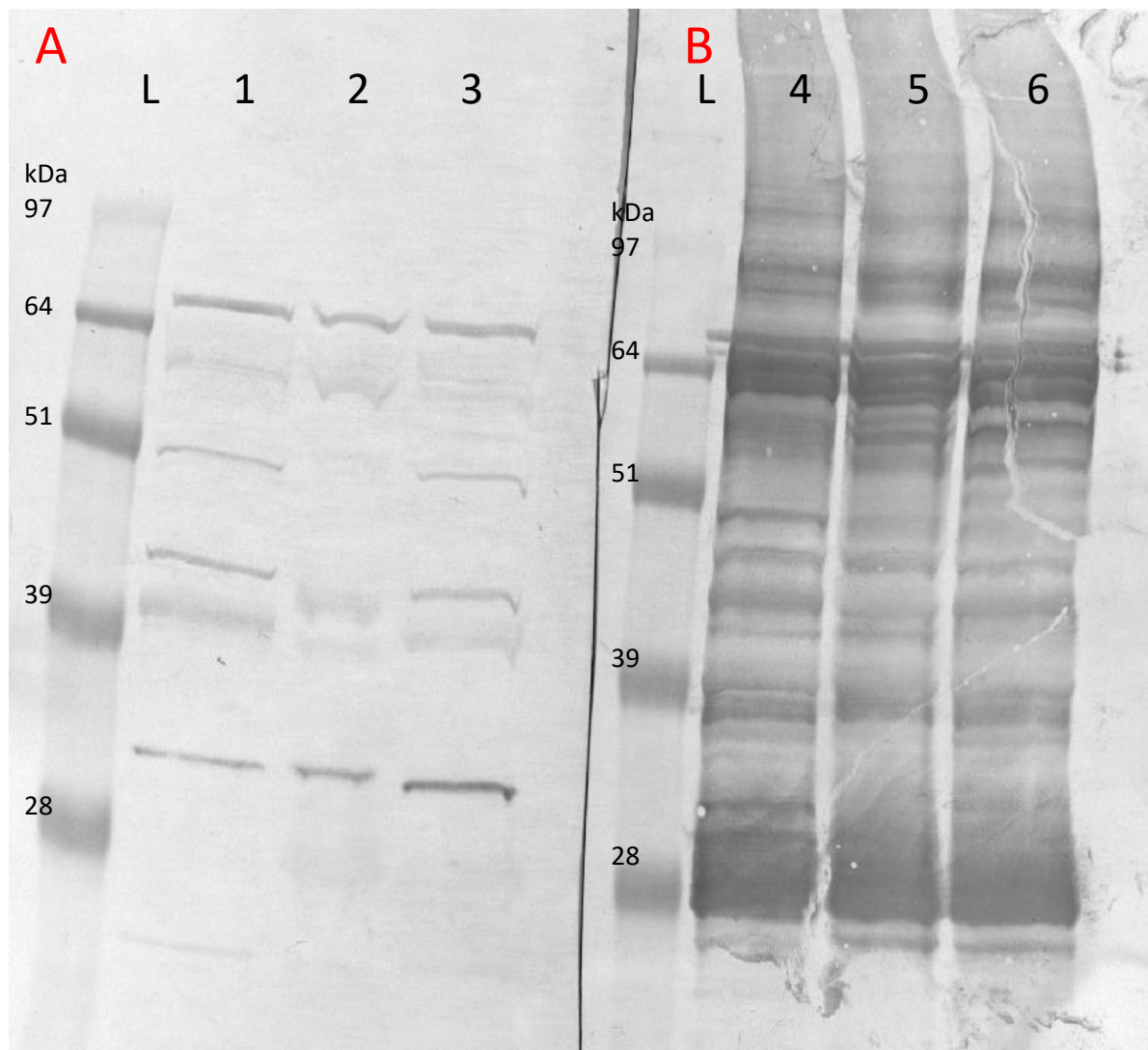


Figure 4-5 1D Western blots showing immunogenic proteins in whole cell lysates prepared from *M. ovipneumoniae*.

Proteins were separated by 1D SDS-PAGE and probed using rabbit sera from rabbits vaccinated with *M. ovipneumoniae* (isolates 16, 90, and 103). A) Western blot using pre-immune sera. B) Western blot using anti sera. Lanes L Ladder SeeBlue™ Plus2 Pre-stained Protein Standard, Lanes 1, 2, and 3 isolates 16, 90 and 103, respectively. Lanes 4, 5, and 6 isolates 16, 90 and 103, respectively.

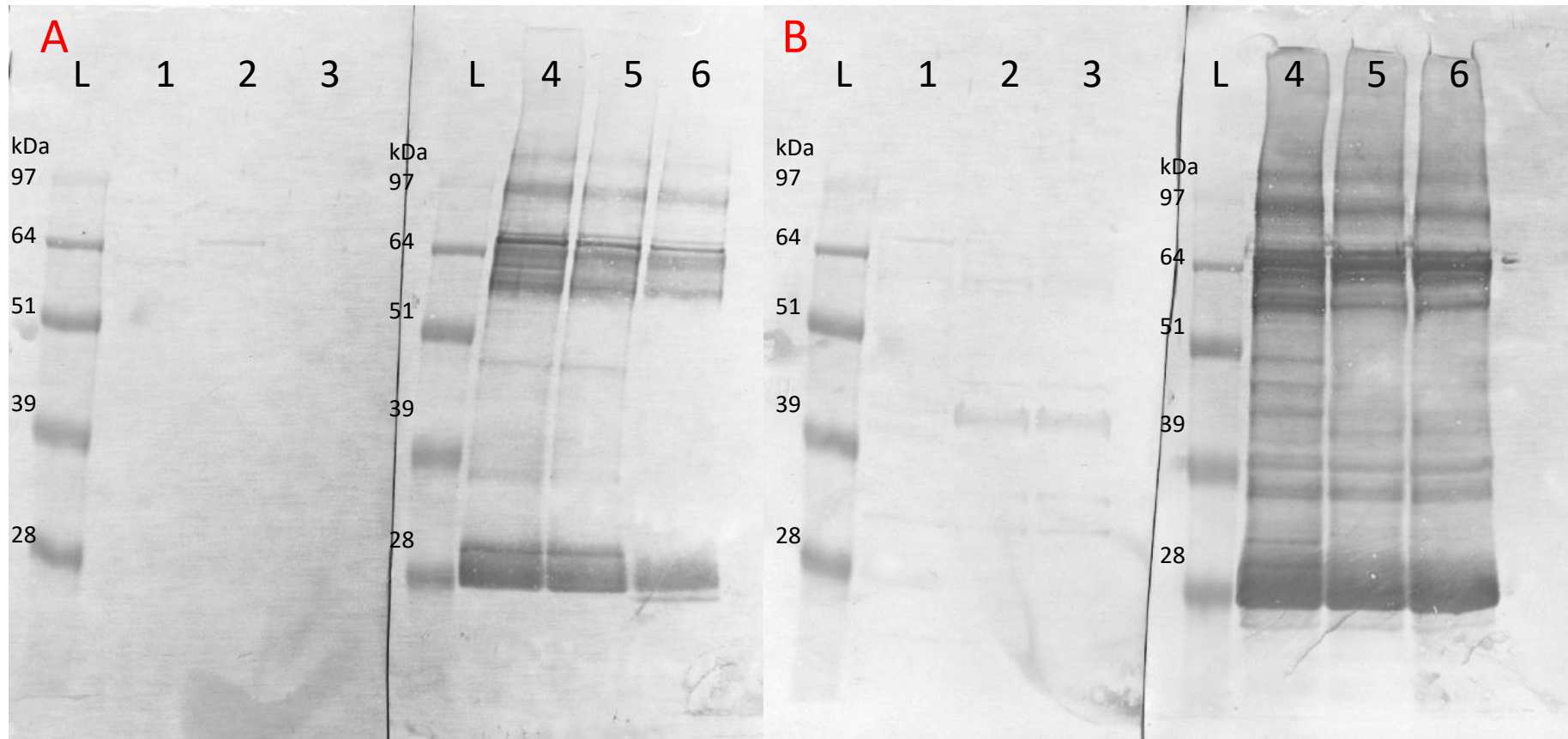


Figure 4-6 1D Western blots showing immunogenic proteins present in hydrophobic and hydrophilic fractions prepared from *M. ovipneumoniae*.

Proteins were separated by 1D SDS-PAGE and probed using rabbit sera from rabbits vaccinated with *M. ovipneumoniae* isolates 16, 90, and 103. A) Western blot against hydrophobic fractions. B) Western blot against hydrophilic fractions. Lanes L Ladder SeeBlue™ Plus2 Pre-stained Protein Standard, Lanes 1, 2, and 3 pre-immune sera against isolates 16, 90 and 103, respectively. Lanes 4, 5, and 6 antisera against isolates 16, 90 and 103, respectively.

Sheep antibody response to *M. ovipneumoniae* antigens without *M. haemolytica* antigens

Western blots of whole cell lysates using pre-immune sera and antisera from sheep vaccinated against the three isolates of *M. ovipneumoniae* (isolates 16, 90, and 103) (chapter 2.4.3) identified a similar size range of immunogenic proteins bands to that detected using the sera from sheep vaccinated with a mixture of *M. ovipneumoniae* and *M. haemolytica* (Fig. 4.7). Comparing the hydrophobic and the hydrophilic membrane fraction Western blots (Fig. 4.7) to the hydrophobic and the hydrophilic membrane fraction Western blots produced using the pooled sera (Fig. 4.4) identified a similar protein banding pattern in the middle of the molecular range (100-50 kDa). However, a larger number of high molecular weight proteins were detected in both the hydrophobic and hydrophilic membrane protein fraction *M. ovipneumoniae* Western blots and conversely there were fewer lower molecular weight proteins detected.

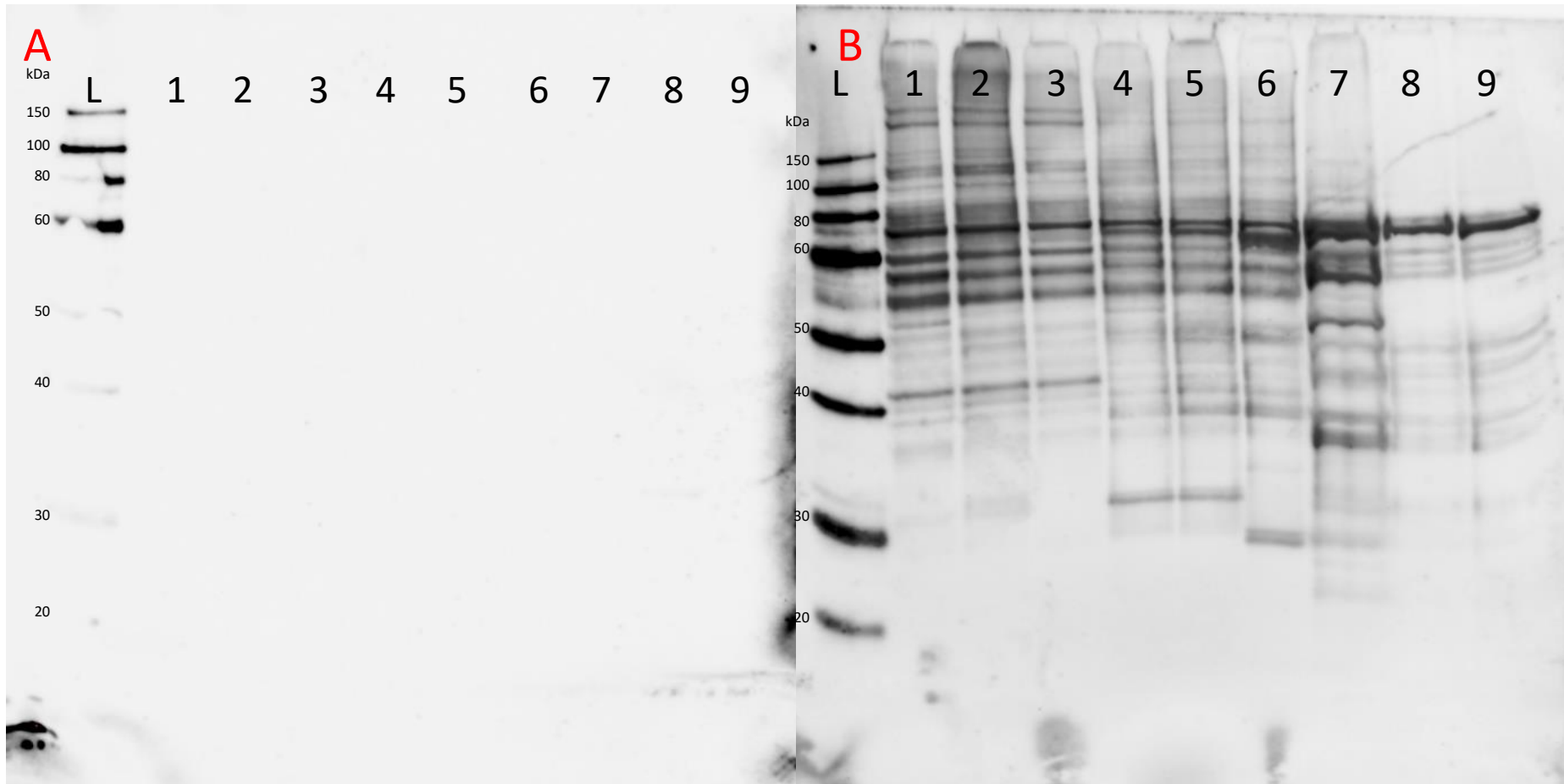


Figure 4-7 1D Western blots showing immunogenic proteins in whole cell lysates, hydrophobic, and hydrophilic fractions prepared from *M. ovipneumoniae*.

Proteins were separated by SDS-PAGE and probed using sheep sera from sheep vaccinated with *M. ovipneumoniae* (isolates 16, 90, and 103). A) Western blot using pre-immune sera. B) Western blot using anti sera. Lanes L SuperSignal™ Molecular Weight Protein Ladder, Lanes 1, 2, and 3 whole cell of isolates 16, 90 and 103, respectively. Lanes 4, 5, and 6 HPF of isolates 16, 90 and 103, respectively, Lanes 7, 8, and 9 hydrophilic membrane fraction of isolates 16, 90 and 103, respectively.

2D Western blotting

Two-dimensional western blotting of the HPF from *M. ovipneumoniae* isolate 16 with the pooled antisera (2.7.2) (against *M. ovipneumoniae* isolates 16, 90, and 103 and *M. haemolytica*) showed four intense immuno-reactive protein spots and a larger number of less intensive areas of immuno-reactivity (Fig. 4.8). These spots were then located in the 2D gel (Fig. 4.9) by aligning the Western blot (Fig. 4.8) with the gel and the corresponding protein spots excised for mass spectrometry. This identified eight protein spots of interest. Comparison of the peptide sequences obtained from the mass spectrometry analysis to an annotated genome of *M. ovipneumoniae* identified four proteins (Table 4.1). These proteins were identified as the known immunogenic proteins HSP70 and EF-Tu, as well as two new potential immunogenic proteins Multidrug and Toxic Compound Extrusion (MATE) efflux transporter protein, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The full amino acid sequence of the MATE and GAPDH proteins were obtained from the annotated genome (chapter 3).

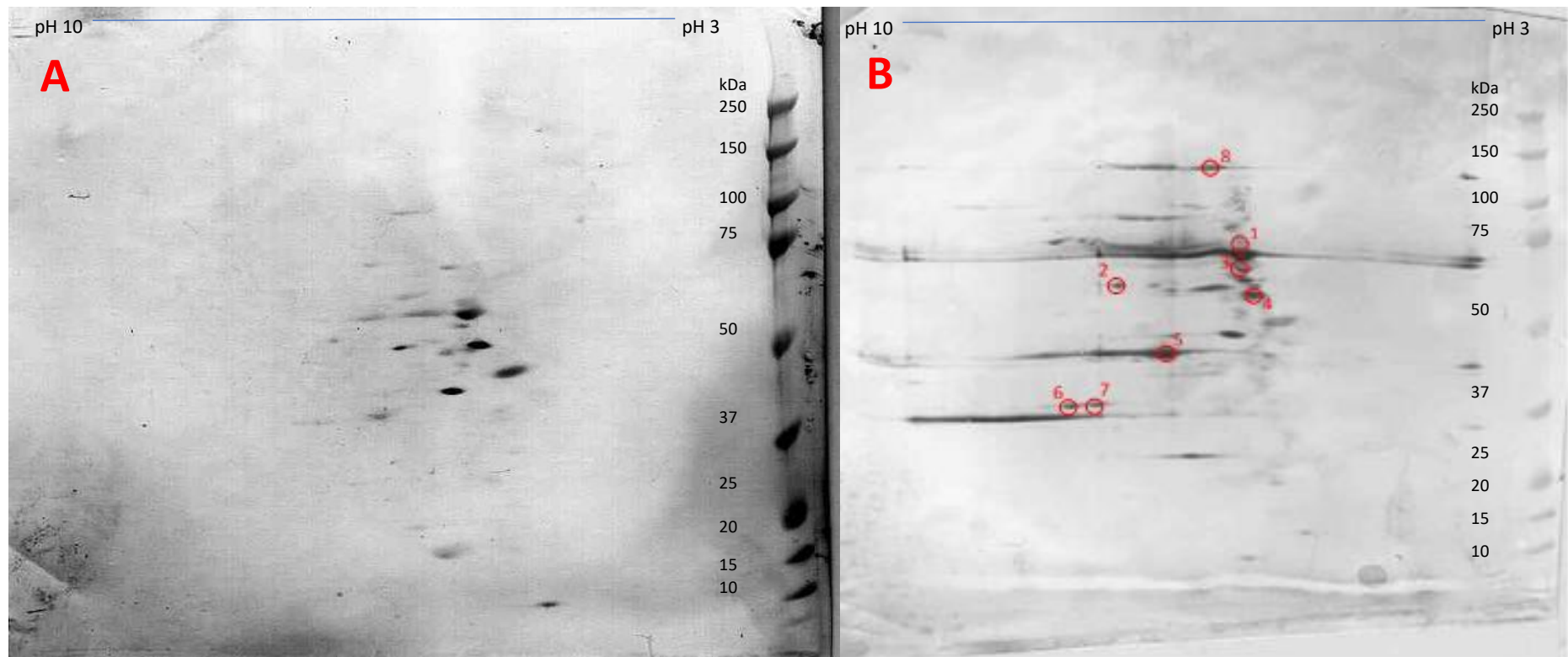


Figure 4-8 2D Western blots showing reactivity of proteins in the hydrophobic protein fraction prepared from *M. ovipneumoniae* isolate 16 and probed using sheep sera from sheep vaccinated with *M. ovipneumoniae* (isolates 16, 90, and 103) and *M. haemolytica*.

A) Western blot using pooled pre-immune sera. B) Western blot using pooled antisera produced against mycoplasmas. Proteins sent for mass spectrometry are indicated by red circles. Ladder Precision Plus Protein Western C Standards.

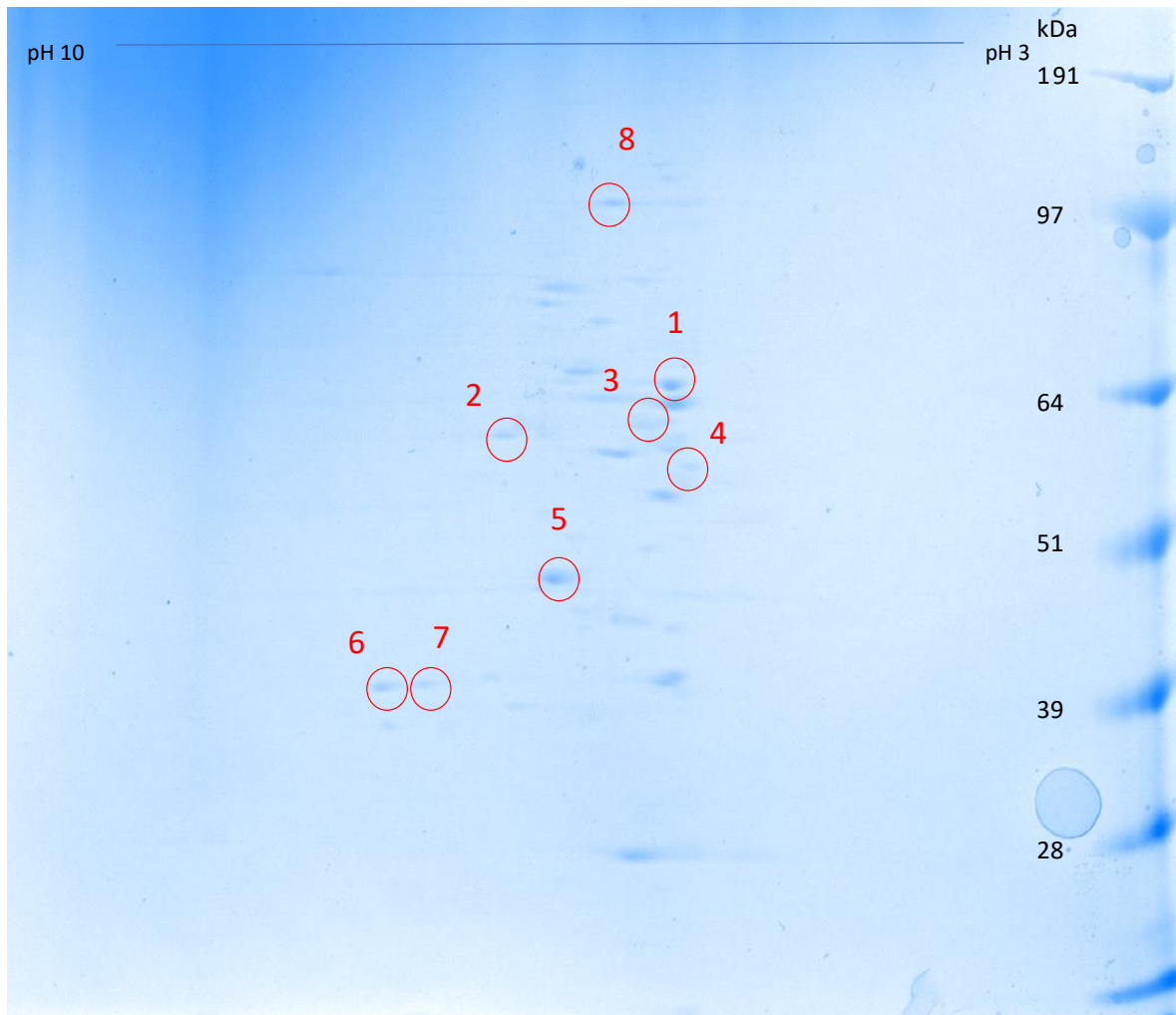


Figure 4-9 2D gel electrophoresis separation of proteins in hydrophobic protein fraction prepared from *M. ovipneumoniae* isolate 16.

Proteins sent for mass spectrometry analysis are indicated by red circles. Ladder SeeBlue™ Plus2 Pre-stained protein standard.

Two-dimensional gel electrophoresis of proteins in the HPF prepared from *M. ovipneumoniae* isolate 90 showed four spots of immunoreactivity when analysed with the pooled pre-immune sera (against *M. ovipneumoniae* isolates 16, 90, and 103 and *M. haemolytica*) (Fig. 4.10). Immunoblotting with the antisera showed 10 spots of immunoreactivity. These spots were located on a 2D gel (Fig. 4.11) and the 10 potential protein bands were excised. Mass spectrometry analysis identified six proteins. In addition to EF-Tu and GAPDH which were also detected in *M. ovipneumoniae* isolate 16, three hypothetical proteins and Mycoplasma adhesin protein P146 were detected (Table 4.1). P146 was detected at two positions within the 2D gel, running between 80 and 100 kDa molecular size and between 50 and 60 kDa.

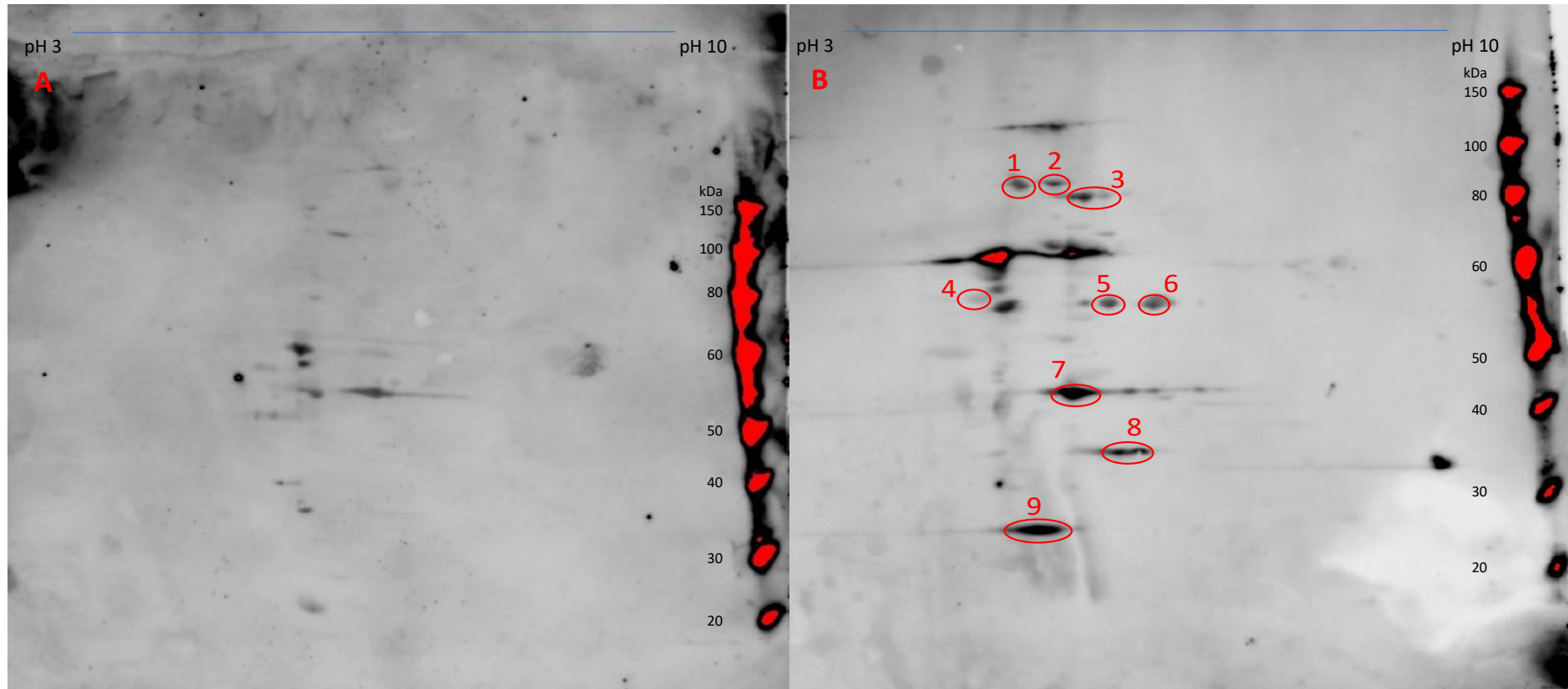


Figure 4-10 2D Western blots showing reactivity of proteins in the hydrophobic protein fraction prepared from *M. ovipneumoniae* isolate 90 and probed using sheep sera from sheep vaccinated with *M. ovipneumoniae* (isolates 16, 90, and 103) and *M. haemolytica*.

A) Western blot using pooled pre-immune sera. B) Western blot using pooled antisera produced against mycoplasmas. Proteins excised and sent for mass spectrometry analysis are indicated by red circles. Ladder SuperSignal™ Molecular Weight Protein Ladder. The red signal is saturated reading of the chemiluminescence.

pH 3 ————— pH 10

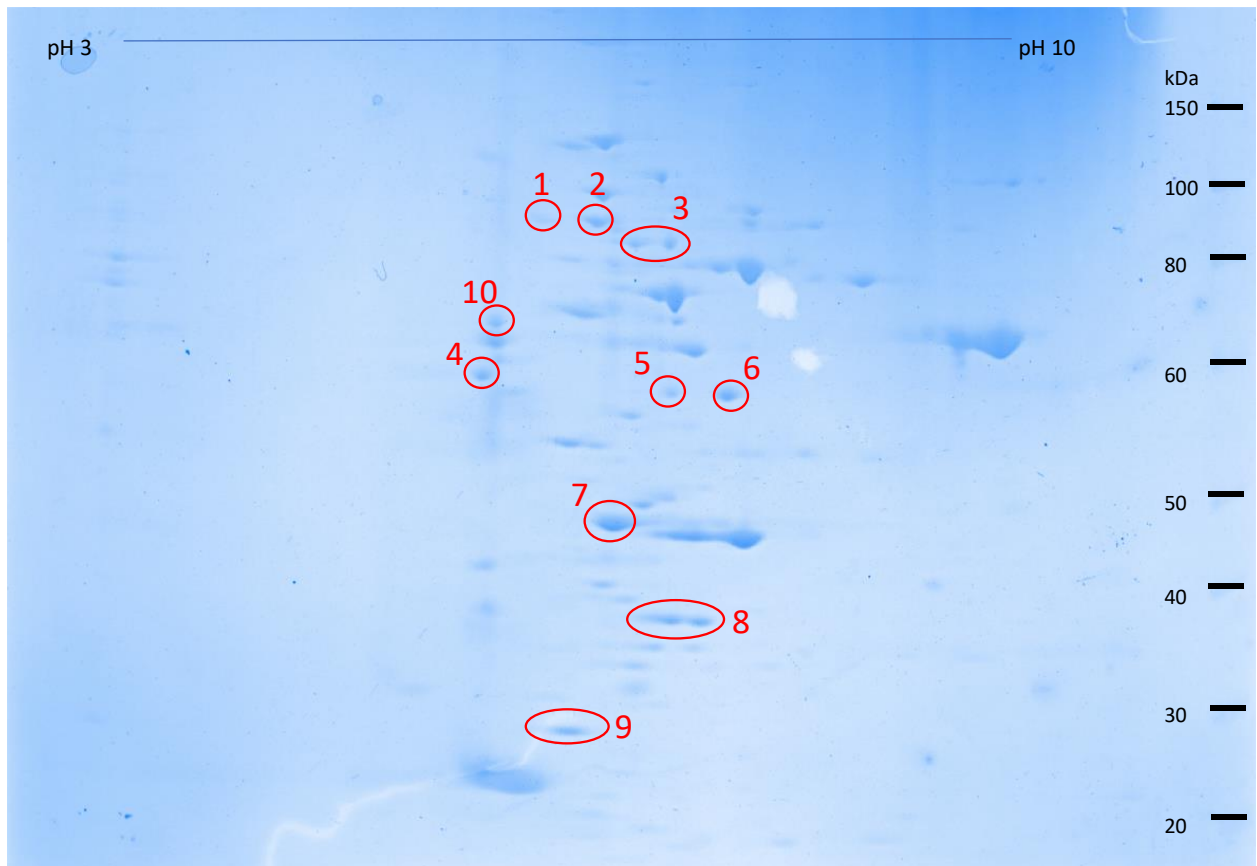


Figure 4-11 2D gel electrophoresis separation of proteins in hydrophobic protein fraction prepared from *M. ovipneumoniae* isolate 90.

Proteins sent for mass spectrometry analysis are indicated by red circles. Ladder SuperSignal™ Molecular Weight Protein Ladder.

Western blotting of proteins in the HPF prepared from *M. ovipneumoniae* isolate 103 using pre-immune sera (against *M. ovipneumoniae* isolates 16, 90, and 103 and *M. haemolytica*) identified four strong spots and two lighter spots (Fig. 4.12). Not including these spots in the antisera Western left six spots of interest remaining (Fig. 4.12). These spots were located on a 2D gel and the spots were excised and sent for mass spectrometry analysis (Fig. 4.13). Three of the six proteins were successfully identified by mass spectrometry analysis, these were EF-Tu, GAPDH, and P146 (Table 4.1). These three proteins have been identified as immunogenic across all three *M. ovipneumoniae* isolates.

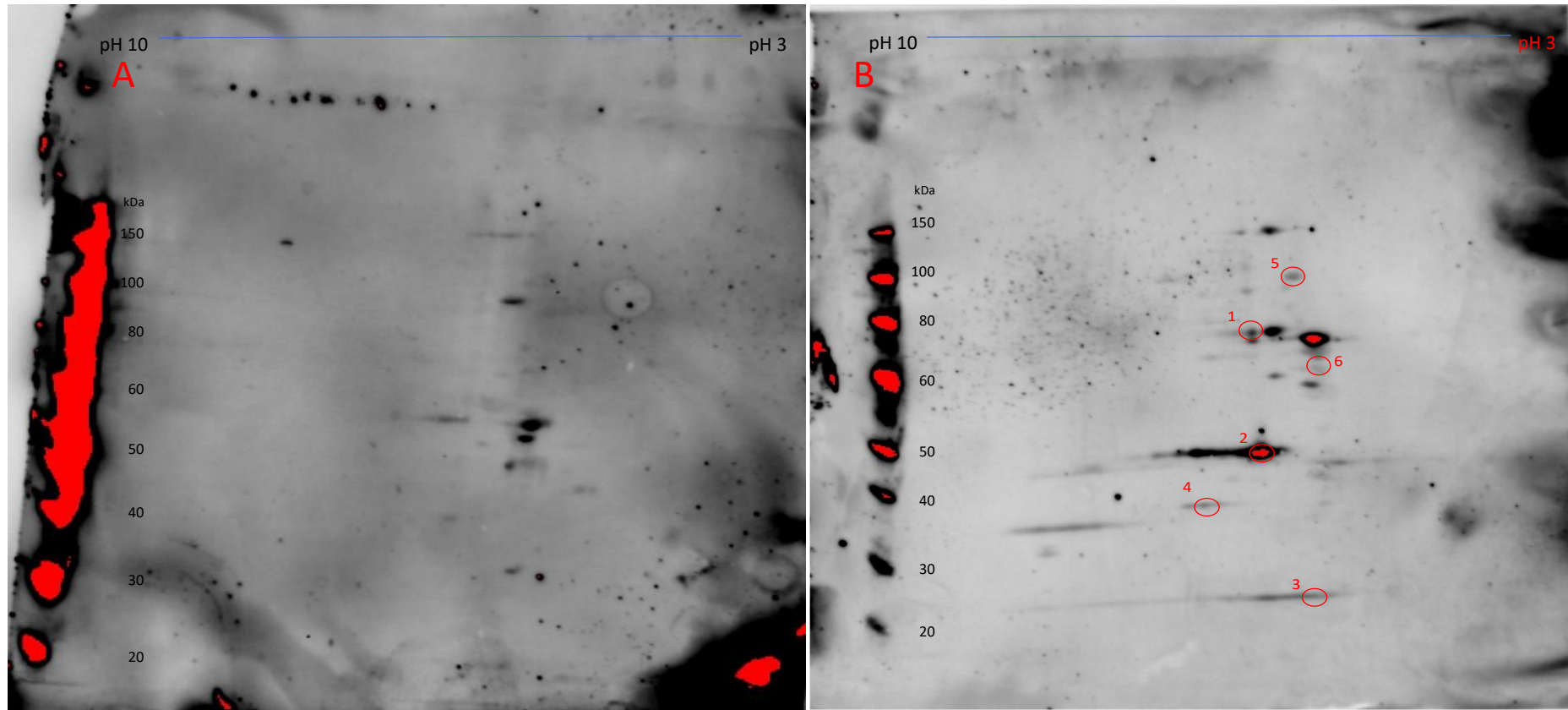


Figure 4-12 2D Western blots showing reactivity of proteins in the hydrophobic protein fraction prepared from *M. ovipneumoniae* isolate 103 and probed using sheep sera from sheep vaccinated with *M. ovipneumoniae* (isolates 16, 90, and 103) and *M. haemolytica*.

A) Western blot using pooled pre-immune sera. B) Western blot using pooled anti-sera produced against *M. ovipneumoniae*. Proteins sent for mass spectrometry are indicated by red circles. Ladder SuperSignal™ Molecular Weight Protein Ladder. The red signal is saturated reading of the chemiluminescence.

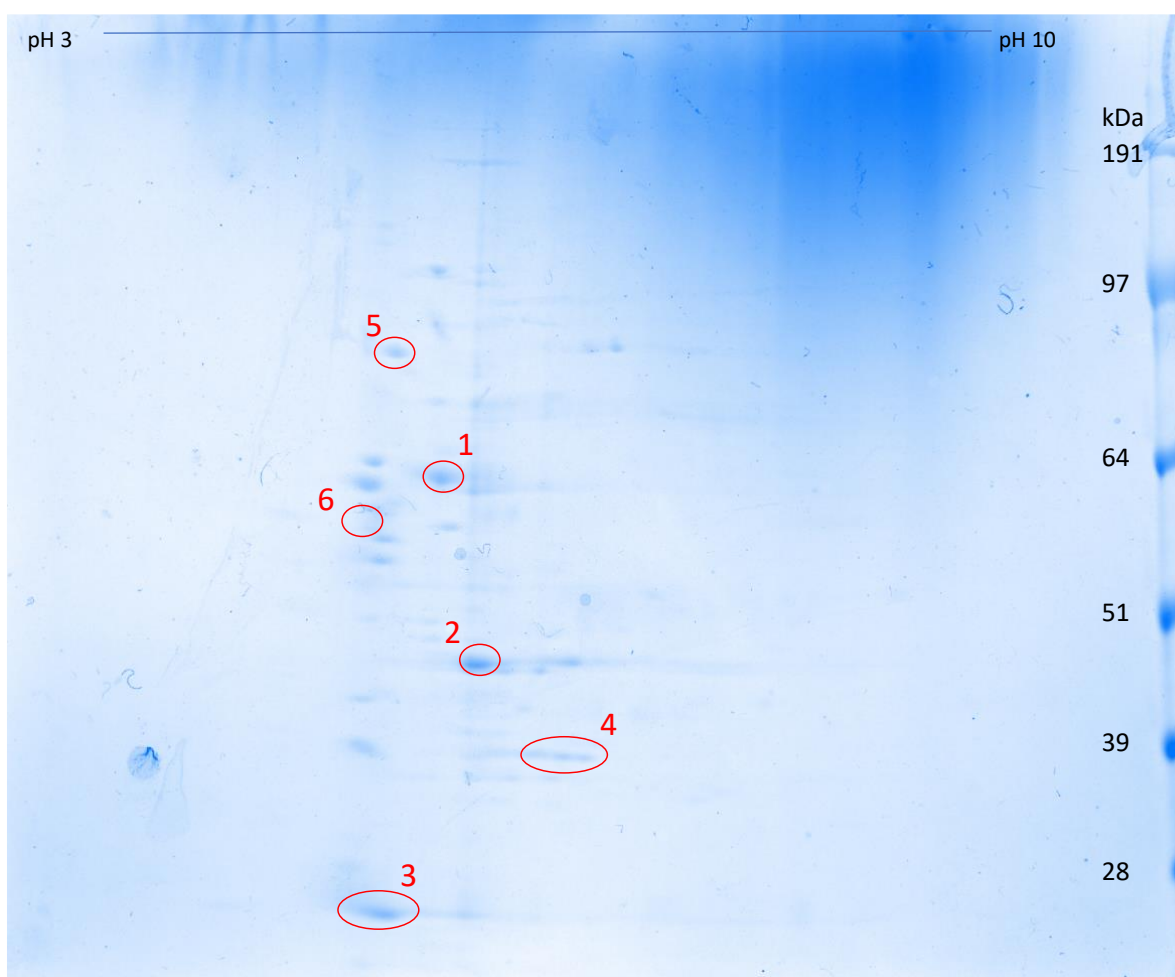


Figure 4-13 2D gel electrophoresis separation of proteins in hydrophobic protein fraction prepared from *M. ovipneumoniae* isolate 103.

Proteins sent for mass spectrometry analysis are indicated by red circles. Ladder SeeBlue™ Plus2 Pre-stained Protein Standard.

4.3.2 Sequence analysis of identified proteins

Each of the proteins identified by mass spectrometry analysis had their full peptide sequence extracted from the whole genome of *M. ovipneumoniae* isolate 90, (described in chapter 3) (Appendix 2-9). A B-cell epitope map was calculated using Bepipred-1.0 Linear Epitope Prediction [293] (Appendix 10-17). A hydrophilicity plot was also produced to complement the B-cell epitope map indicating which areas are hydrophobic and which are hydrophilic (Appendix 18-25). For each protein, transmembrane domain analysis was done to determine the probability of the protein being membrane associated or located on the cell surface (Appendix 26-34). In addition to the sequence analysis completed on isolate 90 sequences, nucleotide sequences for each of the immunogenic proteins in each of the isolates 16, 23, 42, 70, 73, 83, 88, 94, and 103 was successfully derived and translated into the corresponding peptide sequence.

Table 4-1 Summary of protein spots excised from 2D gel and corresponding proteins identified via mass spectrometry analysis

<i>M. ovipneumoniae</i>	Spot number	Approximate Mw size on the gel	Protein detected by MS
16	1	70	HSP70
	2	60	Not Identified
	3	62	Not Identified
	4	55	MATE
	5	44	EF-Tu
	6	37	GAPDH
	7	37	Not Identified
	8	110	Not Identified
90	1	90	P146
	2	95	Hypothetical Protein 1 (HP1)
	3	85	Hypothetical Protein 2 (HP2)
	4	58	Not Identified
	5	56	Not Identified
	6	56	P146
	7	44	EF-Tu
	8	35	GAPDH
	9	24	Hypothetical Protein 3 (HP3)
	10	70	HSP70
103	1	63	Not Identified
	2	45	EF-Tu
	3	24	Not Identified
	4	37	GAPDH
	5	84	P146
	6	59	Not Identified

HSP70 had a protein coding sequence of 1,797-1,800 bp, across the 10 isolates with 1,715 identical sites (95.35%). Translated peptide sequences produced a sequence length of 598-599 amino acids (aa) with a sequence identity of 594 (99.2%) and a pairwise identity of 99.8%. The differences in sequence length were due to an in-frame addition of TCA in isolate 16 at site 1,758 resulting in the

addition of a glutamine residue at site 587. The predicted molecular weight was 65 kDa. No transmembrane domains were detected in this protein, with the entire protein being predicted to be located on the outside of the cell membrane (Appendix 26). B-cell epitope analysis indicated 20 potential immunogenic epitopes of 4 amino acids or larger of which 17 were hydrophilic (Appendix 34). HSP70 had an intra-species nucleotide sequence identity of 95.35%, and comparison to *M. hyopneumoniae*, *M. conjunctivae*, and *M. bovis* produced a sequence identity of 86.3%, 80%, and 67.2% respectively.

EF-Tu had a protein coding sequence of 1,209bp, in all the isolates, with 1,176 (97.3%) identical sites. Translated peptide sequences produced a sequence length of 402 aa with 401 (99.8%) identical sites and pairwise % identity of 99.9%. All the Isolates had the same peptide sequence except for isolates 103 and 73 which had a change from Alanine to Valine at position 319. The predicted molecular weight was 44 kDa. As with HSP70 no transmembrane domains were detected (Appendix 27), and all the protein is predicted to lie outside the cell membrane. B-cell epitope analysis detected 17 potential immunogenic epitopes of 4 amino acids or larger, 13 were determined to be hydrophilic (Appendix 35). The EF-Tu sequence from *M. ovipneumoniae* was compared to closely related species, *M. hyopneumoniae*, *M. conjunctivae*, and *Mycoplasma bovis*, this revealed a sequence identity of 84.9%, 83.1%, and 74.8% respectively.

GAPDH had a protein coding sequence of 1008 bp with 974 (96.6%) identical sites. Translated peptide sequences produced a sequence length of 336 aa with 318 (94.6%) identical sites and pairwise % identity of 97.8%. Isolates 16, 23, 73, and 83 have an internal stop codon reducing the length to 332 aa. The predicted molecular weight was 36.9 kDa in size. There were no predicted transmembrane domains (Appendix 28). B-cell epitope analysis showed 14 potential epitopes of which 8 are predicted to be hydrophilic (Appendix 36). Comparing it to the equivalent protein in *M. hyopneumoniae*, *M. conjunctivae*, and *M. bovis* showed a similarity of 77.3%, 72.2%, and 63.8% respectively.

MATE produced a protein coding sequence of 1563bp with 1,448 (92.6%) identical sites, translated peptide sequence showed a sequence length of 521 with 502 (96.4%) identical sites and pairwise % identity of 98.6%, resulting in a 58 kDa sized protein. This protein has been predicted to contain 12 transmembrane domains, seven intracellular and six extracellular (Appendix 29). B-cell epitope analysis indicated the presence of 12 potential immunogenic epitopes of which 4 are predicted to be hydrophilic (Appendix 37). Comparison to *M. hyopneumoniae* revealed a sequence similarity of 71.2%.

P146 is a large 129 kDa protein with a nucleotide sequence length of 3507-3630 bp with 2747 (74.3%) identical sites. Translated peptide sequences produced a sequence length of 1157-1170 aa

with 407 (33.0%) identical sites and pairwise identity of 71.7%. It contains one potential transmembrane domain at the start of the protein, with the majority of the protein sitting outside of the cell (Appendix 30). B-cell epitope analysis indicates 32 potential sites of B-Cell immunogenicity of which 29 are predicted to be hydrophilic (Appendix 38).

Three different hypothetical proteins, HP1, HP2, and HP3, were identified. HP1 had a sequence length of 3613-3844 bp with 2,669 (69.4%) identical sites. Translated peptide sequences produced a sequence length of 1204-1231 aa, with 318 (25.3%) identical sites and a pairwise % identity of 56.2%. Predicted protein was 133 kDa in size. There was a single transmembrane domain at the N-terminus of the protein (Appendix 31). B-cell epitope analysis identified 40 potential epitopes, with two large epitopes of 129 and 73 amino acids respectively. 39 of the 40 epitopes are predicted to be hydrophilic (Appendix 39).

HP2 had a sequence length of 2886-2908 bp with 2,587 (89.0%) identical sites. Translated peptide sequences produced a sequence length of 910-968 aa with 789 (81.4%) identical sites and pairwise identity of 92.6%. Internal stop codons are the cause for the smaller sequences. A protein of 111 kDa was predicted. There was a single transmembrane domain detected at the start of the protein (Appendix 32). B-cell epitope analysis indicated 28 potential epitopes of which 26 are predicted to be hydrophilic (Appendix 40).

HP3 had a sequence length of 756-757 bp with 648 (85.6%) identical sites. Translated peptide sequences produced a sequence length of 251-252 aa with 166 (64.1%) identical sites and pairwise identity of 90.5%. The predicted molecular weight was 28 kDa. This protein has one transmembrane domain predicted near the start of the protein and occurring after a short intracellular domain (Appendix 33). The majority of the protein is predicted to lie outside the cell membrane. B-cell epitope analysis indicated in the presence of eight potential immunogenic epitopes all of which were predicted to be hydrophilic (Appendix 41).

Sequence similarity of the *M. ovipneumoniae* proteins identified is summarised in Table 4.2.

Sequence alignments of the 10 New Zealand isolates are presented in Appendix 42-49.

Table 4-2 Summary of sequence similarity of the identified proteins.

The nucleotide sequence for each of the proteins was compared within the 10 New Zealand isolates. Additionally, the nucleotide sequence from isolate 90 was compared to sequences from *M. hyopneumoniae*, *M. conjunctivae*, and *Mycoplasma bovis* where available. Results represented as percentage of sequence similarity.

	New Zealand Isolates	<i>M. hyopneumoniae</i>	<i>M. conjunctivae</i>	<i>M. bovis</i>
HSP70	95.35	86.3	80	67.2
EF-Tu	97.3	84.9	83.1	74.8
GAPDH	96.6	77.3	72.2	63.8
MATE	92.6	71.2	-	-
P146	74.3	57.9	-	-
HP1	69.4	-	-	-
HP2	92.6	-	-	-
HP3	90.5	-	-	-

4.4 Discussion

Having an annotated draft genome enabled the determination of proteins from the sequences produced by mass spectrometry analysis. Of the 24 spots sent for mass spectrometry analysis, eight proteins were detected from the sequences produced. Of these eight proteins, five were identified to be named proteins. These were HSP70, EF-Tu, GAPDH, MATE, and P146. The other three proteins were only identified as hypothetical proteins. Of the eight proteins identified, two have been previously identified as immunogenic proteins, HSP70 and EF-Tu [160, 297]. Identification of these known proteins helped validate the methodology used in this chapter.

Identification of the role of the proteins that were detected may assist our understanding of how inhibition of the proteins of *M. ovipneumoniae* may protect the animal from disease. This could inform rational experimental design for testing the ability of antibodies to protect the animal. The ideal outcome for a vaccine is to produce an immune response that facilitates killing of the invading pathogen. However, that is not always possible, but in this case inhibiting certain functions, such as the adhesion ability of the pathogens could potentially reduce the effect of the infection limiting the damage to the host. In this example, testing the antibodies in an adhesion experiment may provide more useful information than relying on challenge studies. Understanding the sequence can also provide information on the location of the protein of interest. This can help confirm the location of the protein on the surface of the cell. This is important for the host's ability to detect the antigen while the pathogen is active, increasing the ability of the immune system to detect and act upon the invading pathogen.

4.4.1 Western blotting

Western blotting of *M. ovipneumoniae* proteins was undertaken using pre-immune sera and anti-sera produced against *M. ovipneumoniae* in both sheep and rabbits. Sera and antisera collected from a *M. ovipneumoniae* and *M. haemolytica* adjuvant trial was the primary sera used in the identification of immunogenic *M. ovipneumoniae* proteins. The three pooled sera used were selected because of their high titres against *M. ovipneumoniae* (data not shown). The pooled sera produced a number of bands on a Western blot indicating the presence of immunogenic proteins. This banding pattern was compared to the banding pattern using antisera against *M. ovipneumoniae* in rabbits. This was done to compare reactivity of the proteins to sera from different host species and to determine if the inclusion of *M. haemolytica* in the vaccine used to produce the pooled sera had an effect on the detection of the immunogenic proteins. The banding pattern observed in the Western blots using the rabbit antisera had the largest differences between any of the different antisera tested. Overall, the Western blots of WCL and hydrophilic membrane fractions with sheep antisera and rabbit antisera showed similar patterns of immunoreactivity. In the HPF; however, there was a stark difference between the two Western blots with an observed reduction in antibodies against *M. ovipneumoniae* proteins in the middle of the molecular weight range with proteins being detected around the 64 kDa marker and the 28 kDa marker with limited response between those molecular weights. This suggests that the immune response to *M. ovipneumoniae* proteins may differ between different animal models. This is an important observation because much of the published immunogenic work on *M. ovipneumoniae* has been done using non target animal models such as mice [160] as the host. If a difference in immunogenic patterning observed between the sheep sera and the rabbit sera is also observed in other animal models, it may mean that immunological studies using species other than sheep should be interpreted with caution.

Two sheep were also vaccinated only with the three isolates of *M. ovipneumoniae* without inclusion of *M. haemolytica* antigens in the vaccine (chapter 2.7.3). Western blots using the sera produced against *M. ovipneumoniae* alone produced a patterning similar to that observed in the pooled sera from sheep vaccinated with both *M. ovipneumoniae* and *M. haemolytica* and thus containing anti *M. haemolytica* antibodies. The differences between the Western blots using antisera generated against antigens from both organisms was an increase in some high molecular weight and a decrease in some low molecular weight immunogenic protein bands compared to Western blots using antisera against *M. ovipneumoniae* alone. The addition of *M. haemolytica* in the vaccine appeared to have had less of an effect on the immunogenic patterning observed, than the use of rabbits as the host.

4.4.2 Analysis of immunogenic proteins

The annotated genome of *M. ovipneumoniae* isolate 90 not only enabled the identification of the proteins but facilitated the extraction of the nucleotide and peptide sequences for the proteins. Using this sequence data, a total of 10 versions of each of the proteins was obtained across New Zealand isolates. HSP70, EF-Tu, GAPDH, and MATE all had similar sequences across all 10 isolates that were sequenced, with identical sites ranging from 92.6% for MATE to 97.3% for EF-Tu.

Elongation Factor Thermo unstable (EF-Tu)

Elongation Factor Thermo unstable (EF-Tu) is among the most abundant bacterial proteins, consisting of 10% of total protein for *M. pneumoniae* [195]. It plays a crucial role in protein synthesis, facilitating the binding and selection of an aa-tRNA to the A-site of the ribosome [195]. In addition to its primary role in protein synthesis, EF-Tu has been linked to a number of secondary functions including protein disulphide activities [298], chaperone like properties [299], initiation of Q β phage replication [300], and upregulation in the presence of high concentrations of iron [301]. Surface localised EF-Tu has also been demonstrated to mediate the binding of mycoplasma to fibronectin [195]. Fibronectin (Fn) is a high molecular-weight glycoprotein found abundantly in fibrillary form in the extracellular matrix (ECM). Fn is primarily used as a substrate for mammalian cell adhesion. However, this substrate has been hijacked by a number of microbial pathogens by binding Fn for colonisation purposes, which contribute to the infectious process [302].

B-cell epitope analysis of EF-Tu detected 17 potential immunogenic epitopes, with 15 of them consisting of 8 or more amino acids. This suggests there are many sites for interaction of the protein with the immune system. Balasubramanian et al [302] determined that Fn binding is primarily mediated by the carboxyl terminus (amino acids 192 to 394). Within this section, five B-cell epitopes were detected including an epitope at the start and end of the section. Producing antibodies against these sections may provide protection against *M. ovipneumoniae* by reducing its ability to colonise the host lung tissue. This provides another avenue for the immune system to protect the host against infection in addition to induction of cellular immunity and destruction of infected cells.

Transmembrane analysis did not detect any transmembrane domains within the peptide sequence of EF-Tu. However, EF-Tu has been shown to be surface localised [160]. Of the 17 epitopes, 13 were determined to be hydrophilic, suggesting that these epitopes are externally located on the protein. These include the two epitopes at the start of the carboxyl terminus (183-193 and 205-214) as well as an epitope at the end of the carboxyl region (382-395). These epitopes, in addition to the other five epitopes in this region, may provide protection by inhibiting the ability of EF-Tu to adhere to the host cells.

Due to the significance of EF-Tu in protein production, it is one of the most highly conserved proteins in prokaryotes [195]. Being a highly conserved protein provides both benefits and obstacles to the production of protein-based vaccines. Being highly conserved reduces the likelihood that antibodies against EF-Tu from a particular isolate of *M. ovipneumoniae* will be unable to recognise and bind to the protein from other isolates. This increases the universality of potential vaccine components against not only New Zealand isolates but strains from abroad. The downside of this high degree of similarity in amino acid sequence is potential for an overactive immune system; antibodies produced against this protein may cross-react with similar organisms. This could produce antibodies to beneficial non-target organisms. For a host species such as sheep being a ruminant, this could cause issues as production of such antibodies could be reactive against rumen and gut dwelling bacteria necessary for proper function as a ruminant animal. If the antigen is to be considered for a diagnostic target, then cross reaction between multiple organisms would reduce the specificity of the test. The sequence comparison of EF-Tu, within the New Zealand isolates, was the most conserved of the proteins identified. Comparing the EF-Tu sequence with closely related species *M. hyopneumoniae*, *M. conjunctivae*, and *Mycoplasma bovis* revealed a sequence identity of 84.9%, 83.1%, and 74.8% respectively.

Heat Shock Protein (HSP70)

Heat Shock Protein (HSP70) is a 70 kDa protein belonging to a group of proteins induced by environmental stress conditions. These proteins play an important role in stimulating both host innate and adaptive immunity [193, 303]. HSP70 functions as a molecular chaperone but has been suggested to also play an important role in the organisation of the terminal organelle, critical for the adhesion to host cells. HSP70 has been shown to be a strong antigen containing multiple B- and T-cell epitopes [160]. From the analysis of the protein sequence derived from *M. ovipneumoniae*, 20 B-cell epitopes were determined, of which 17 were hydrophilic. This increases the probability that they are located on the outside of the protein. There are no predicted transmembrane domains in this protein. This may be due to the main function of the protein occurring intracellularly. It has been demonstrated that the antigenic properties of HSP70 can be exploited to enhance and induce humoral and cellular response, as both an adjuvant and immunogen [304].

HSP70 had an intra-species nucleotide sequence identity of 95.35%, and comparison to *M. hyopneumoniae*, *M. conjunctivae*, and *M. bovis* produced a sequence identity of 86.3%, 80%, and 67.2% respectively. This showed the highest inter-species sequence similarity of the proteins so far for *M. hyopneumoniae* and *M. conjunctivae*. The higher inter-species sequence similarity suggests that this protein would not be useful for vaccine or diagnostic use. However, the reduced similarity with *M. bovis* indicates that the likelihood of the *M. ovipneumoniae* protein cross identifying with

proteins outside of the *Mycoplasma* genus is unlikely. Therefore, as a diagnostic protein for an immune based assay it may be useful for detecting the presence of *Mycoplasma* but may not be specific enough for determining the *Mycoplasma* infection at the species level.

EF-Tu and HSP70 from *M. ovipneumoniae* have been shown to produce both humoral and cellular responses in mice [160]. Moreover, there is evidence to suggest that recombinant proteins of HSP70 and EF-Tu are able to induce a stronger innate immune response, including production of the cytokines TNF- α , IFN- γ , IL-4 and IL-5, than whole cell extracts from *M. ovipneumoniae* [160]. TNF- α is the earliest inflammatory cytokine to activate neutrophils and lymphocytes; this allows the permeability of vascular endothelial cells to increase, tissue metabolism to be adjusted and enhanced synthesis and release of cytokines [160, 202, 203]. This provides an interesting avenue of research into potential recombinant proteins for a vaccine in sheep. Caution should be taken from these results since, as mentioned above, model animals and the target sheep host may not respond identically, therefore the immunogenic response to these proteins will need to be tested in sheep.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Glyceraldehyde 3-phosphate dehydrogenase was the first of the novel immunogenic proteins identified in this study by mass spectrometry to be detected across all three strains of *M. ovipneumoniae*. GAPDH is a protein that has been determined to be immunogenic in a large number of different pathogenic organisms including a number of different *Mycoplasma* species [170, 305]. The mycoplasma variant of GAPDH, like EF-Tu, has been shown to have a number of secondary roles outside of the cytoplasm [306, 307]. GAPDH has been shown to have an affinity for haemoglobin and haeme, suggesting an additional role for this protein in iron acquisition [308, 309]. *M. ovipneumoniae* is an obligate pathogen requiring all of its nutrients from the host, prevention of the function of transporter proteins may help limit the functionality of *M. ovipneumoniae*. Vaccination against GAPDH may provide protection even if the immune response is not able to kill *M. ovipneumoniae* cells.

No transmembrane domains were predicted in this protein. This is likely because the primary function of GAPDH occurs within the cytoplasm and therefore transmembrane domains are not required. However, it has been documented a number of times in various pathogens that GAPDH can be located on the cell surface and thus provides an opportunity for detection by the host [305]. B-Cell epitope analysis of GAPDH detected 14 potential immunogenic peptides with 12 of them consisting of 8 amino acids or more. Eight of the epitopes are expected to be hydrophilic, suggesting that these epitopes are likely to be on the exterior of the folded protein with an increased likelihood of detection by the host immune system.

Similar to EF-Tu, GAPDH has also been implicated in an adhesion role by interacting with the primary component in the mucosal epithelial lining, mucin (Mn) [310]. The mucosal barrier is considered a major component of the immune system, contributing to adaptive and innate immunity, and providing a physical and mechanical barrier by which pathogens are cleared [311]. With 90% of all human infections starting at the mucosal membrane, and the same likely to be true for infections in sheep, understanding how pathogens adapt and subvert this mechanism of the immune system to invade and infect the underlying tissues is of increasing interest for developing preventive measures. GAPDH from *Mycoplasma genitalium* has been shown to bind to human vaginal/cervical Mn and introduction of antisera raised against recombinant GAPDH blocked binding of mycoplasma to mucin by approximately 70% [305]. Blockage of *M. ovipneumoniae* GAPDH by inducing antibody production against this protein using vaccination may provide a preventive mechanism for reducing the colonising ability of *M. ovipneumoniae* and therefore preventing infection by this bacterium.

GAPDH was the second most conserved protein of the eight proteins. Comparing it to the equivalent protein in *M. hyopneumoniae*, *M. conjunctivae*, and *M. bovis* showed a similarity of 77.3%, 72.2%, and 63.8% respectively. This indicates that there is a higher chance of cross detection by either the immune system or a GAPDH based diagnostic when compared to EF-Tu [312, 313]. However, there is still a sufficiently large difference between the *M. ovipneumoniae* version of GAPDH and GAPDH from the others to warrant further study with this protein.

As has been the case with *M. ovipneumoniae* vaccination efforts to date, vaccinations for *Staphylococcus* species have produced limited success. Vaccination with autogenous whole cell bacterins have failed to elicit protection against new infections and the use of specific antigen fractions did not provide heterologous protection [314]. However, GAPDH has been detected on the surface of multiple *Staphylococcus* species and was determined to be able to bind transferrin (dependent on the strain) [314, 315]. Vaccination of dairy cows with recombinant GAPDH conferred protection against challenge with *Streptococcus uberis* [315]. Vaccinated animals post-challenge showed a decreased observable mastitis compared to the control group [315]. One possible explanation is the circulating GAPDH antibodies may inhibit cell surface-associated GAPDH on the *S. uberis* bacteria, resulting in a decrease in bacterial virulence [315]. This hypothesis is derived from work on *Schistosoma mansoni*, in which GAPDH was shown to be a selective target for antibodies in the sera of humans resistant to reinfections [316]. This result was corroborated by the findings of a different study, where the introduction of drugs inhibiting GAPDH resulted in decreased survival of parasites, *Trypanosoma brucei* and *Leishmania mexicana* [317]. A DNA vaccination composed of the *Onchocerca volvulus* GAPDH has recently been used to successfully reduce the number of rodent filarial *Litomosoides sigmodontis* in a BALB/c mouse model [318]. Vaccination of mice using

recombinant GAPDH from *Streptococcus pneumoniae* resulted in a 36% survival rate compared to the control group [319].

Mycoplasma bovis has significant antigenic variation between different isolates, and this is a significant obstacle in developing effective antigen-based vaccines [21, 310]. GAPDH has been suggested as a potential protein for vaccine development due to its high level of protein conservation [310]. Recombinant GAPDH has been shown to produce significant humoral immune responses in bovine species, but field studies have failed to demonstrate induction of protective immunity [21, 170]. GAPDH has been identified in a number of pathogenic species as a potential vaccine target, the detection of GAPDH as immunogenic in *M. ovipneumoniae* increases the potential for use in diagnostics and vaccine development. Developments in vaccines against other pathogens using GAPDH may be transferable to *M. ovipneumoniae*.

Multidrug And Toxin Extrusion protein (MATE)

The fourth protein identified from the mass spectrometry analysis was a MATE efflux transporter protein. This family of proteins are predicted to be involved as drug/sodium antiporters. At present there are five Multidrug efflux transporter families identified, with the MATE family being the most recently characterised [320]. While being the least well-characterised family of the drug exporters, those studied often accommodate fluoroquinolones, providing resistance [321]. Mycoplasmas lack a cell wall and is therefore resistant to antimicrobials that target the cell wall; consequently, mycoplasmas such as *M. pneumonia* are susceptible to fluoroquinolones [7]. Inhibition of the MATE proteins may provide increased sensitivity to antimicrobials extruded by the MATE complex.

B-cell epitope analysis of the MATE protein identified 12 potential antigenic areas within the peptide sequence. Eleven of these consisted of at least 8 amino acids in length and these sections may provide better sensitivity for future diagnostic experiments and increased specificity in potential vaccine or pathogenesis studies. Of the 12 predicted epitopes, 4 were determined to be hydrophilic, and therefore potentially be present on the exterior of the protein. This suggests there are a limited number of viable epitopes on this protein for antibody targeting.

MATE had a sequence similarity >90% between the New Zealand isolates. Comparison of this protein to *M. hyopneumoniae*, *M. conjunctivae*, and *M. bovis* only produced a single homolog, from *M. hyopneumoniae* with a sequence similarity of 71.2%. The high homology within *M. ovipneumoniae* isolates and limited homology with other species including the three sister Mycoplasma species suggests it could be useful for a targeted vaccine or diagnostic test. However, the MATE protein was predicted to have seven transmembrane domains, which is expected given its function is as an efflux pump designed to remove compounds from the cell. The presence of multiple transmembrane domains would make the protein difficult to produce as a recombinant protein in commonly used

host expression systems. Further research will be required to determine the feasibility of the production of this protein as an intact protein or if an epitope-based production of the surface-located as specific peptides epitopes will be a better approach. However, the limited number of external epitopes available, this suggests that this protein may not be a viable option for further development as a vaccine or diagnostic antigen.

Adhesin like protein P146

The final named protein detected in this study was the Adhesin like protein, P146. This protein is also referred to as the Multifunctional Cilium Adhesion (Mhp684) protein and has been shown to bind to cilia, various glycosaminoglycans (GAG), and plasminogens [322]. P146 in *M. hyopneumoniae* has been shown to be able to be cleaved into multiple fragments that reside on the cell surface [322]. This may also be the case in *M. ovipneumoniae* as this protein was detected in isolate 90 as different variants corresponding to multiple protein sizes (approximately 60 kDa and 90 kDa molecular size). P146 had 32 potential B-cell epitopes and 29 are expected to be hydrophilic, suggesting that the majority of epitopes detected may be on the external surface of the folded protein. Potentially due to the large size of the protein, there were also larger B-cell epitopes; the largest consisting of 84 amino acids, providing large sites for potential B-cell targeting.

Hypothetical proteins

The final three proteins detected in the study have not currently been characterised and remain hypothetical proteins. The first two proteins (HP1 and HP2) are large and may not be ideal for use in downstream applications in their current form; however, they contain several large B-cell epitopes that may be of use and will require further analysis. Large proteins can provide additional obstacles in the production of antigens for vaccines or diagnostic tests. There are likely increased costs to produce a larger protein in a recombinant pathway and technical difficulties associated with producing the entire protein. The HP1 had 40 B-cell epitopes predicted, with all but the last epitope predicted to be hydrophilic providing several immunogenic targets including two large epitopes of 129 and 73 amino acids. Since the large size of this protein makes it difficult to work with in its entirety, synthetic peptides or small recombinant proteins could be made based on the two large predicted B-cell epitopes providing a more viable pathway for using this protein in further immunogenic analysis. This protein had a single predicted transmembrane domain at the start of the protein suggesting that the protein will be membrane located with the majority of the protein being located outside the cell membrane.

The second hypothetical protein (HP2) had 28 predicted B-cell epitopes, 26 of them being hydrophilic. This, in combination with the single transmembrane domain at the start of the protein provides a similar structure to the first hypothetical protein. Unlike the first hypothetical protein,

this protein does not have large predicted epitopes, making it more challenging to further investigate its immunogenicity. Due to its large size, the protein may be difficult to produce as a recombinant protein and therefore is a less attractive target for further immunological analysis.

The third hypothetical protein (HP3) detected was identified in *M. ovipneumoniae* isolate 90. This protein contains a transmembrane domain at the start of the protein indicating the potential surface localisation of the protein and increasing its potential as an immune target. B-cell epitope analysis detected eight potential immunogenic epitopes, and all of these were predicted to be hydrophilic, suggesting that all epitopes are present on the exterior of the folded protein.

The presence of an immunogenic protein located on the surface of the cell might provide an opportunity for the immune system to directly target and remove the invading pathogen.

Conversely, these proteins may also have the added role of acting as surface adhesion proteins. This provides a secondary method by which the immune system can act upon the *Mycoplasmas*.

Antibodies binding to the protein may prevent the pathogen from adhering to host cells such as epithelial cell and impede the ability of the *Mycoplasmas* to establish, thereby potentially preventing the development or reducing the severity of the disease. Increasing the number of potential mechanisms by which a vaccine could prevent *M. ovipneumoniae* propagating and causing disease could increase the likelihood of success of the vaccine.

Moving from the theoretical to the practical scenario will require the production of these proteins or selected peptides based on the B-cell epitope predictions. Protein specific antisera can be produced in sheep and be used for further studies and analyses to determine the ability of each of the proteins to prevent infection of sheep with *M. ovipneumoniae*.

4.5 Conclusions and next steps

Eight proteins were identified as immunogenic from conducting Western blots on hydrophobic protein fractions from *M. ovipneumoniae*. Two of the identified proteins (HSP70 and EF-Tu) were known *M. ovipneumoniae* immunogenic proteins from previous studies and three were identified as GAPDH, MATE and P146 by using the built *M. ovipneumoniae* genome (chapter 3). In addition to the five named proteins, three hypothetical proteins were also detected. Five of the proteins were produced as recombinant proteins and used in further analysis (chapter 5). The proteins chosen were EF-Tu, GAPDH, P146, and the smaller of the hypothetical proteins, HP3. In addition to the full sequences, an epitope of HP1 was also included for further studies.

Chapter 5 Production of recombinant *M. ovipneumoniae* proteins and determination of their immunogenicity in sheep

5.0 Abstract

Of the eight immunogenic proteins identified in Chapter 4, five were chosen for further study. These proteins were produced as recombinant proteins by GenScript. Additionally, one of the proteins, EF-Tu was also produced in-house. Sheep were vaccinated with these six recombinant proteins to produce antisera. Additional sheep were vaccinated with WC or HPF from *M. ovipneumoniae* isolates 16, 90 and 103. Antigen-specific antibody titres in sera were determined by ELISA. All animals produced an antibody response to the recombinant proteins used for vaccination. Antigens P146 and hypothetical protein 3 showed potential for an antigen based diagnostic tool, as observed in ELISAs using P146 or hypothetical protein 3 as the target. In combination with other antigens, they could provide a quick and efficient diagnostic tool for the identification of *M. ovipneumoniae* infections. Cell-mediated immune (CMI) responses to the recombinant proteins, WCL and HPF antigens were determined by measuring antigen-specific IFN- γ and IL-17A responses in cultured blood. Expression of IFN- γ and IL-17A in the blood cultures from sheep vaccinated with the recombinant proteins was low suggesting that the recombinant proteins induced only weak cell-mediated immune responses. Interestingly, sheep vaccinated with WC antigens showed strong antigen-specific IL-17A responses to both WC lysates antigens and recombinant GAPDH. This indicates a potential use for GAPDH as a diagnostic tool for identifying mycoplasma infections or detecting over expression of IL-17A in hypersensitive animals such as big horn sheep. Overall, the results suggest that the proteins from *M. ovipneumoniae* studied, induce predominately antibody responses and limited CMI responses similar to other reports from study of proteins in *M. ovipneumoniae*.

5.1 Introduction

Proteins that produce an immune response in a host have the potential to be used as diagnostic tools or as antigenic targets for a vaccine [323]. In Chapter 4, eight proteins (HSP70, MATE, EF-Tu, GAPDH, P146, hypothetical protein 1 (HP1), hypothetical protein 2 (HP2) and hypothetical protein 3 (HP3)) were detected from hydrophobic membrane protein fractions of three New Zealand isolates of *M. ovipneumoniae*, using Western blotting followed by mass spectrometry analysis of immuno-reactive spots. The nucleotide and peptide sequences of these proteins were determined from the whole genome of *M. ovipneumoniae* isolate 90 (described in Chapter 3 [267]). Based on the amino acid sequences, B-Cell epitope prediction, and analysis of hydrophobicity and transmembrane domains of the proteins were predicted and five proteins (EF-Tu, GAPDH, HP3, P146, and an epitope of HP1) were selected for assessment of immunogenicity.

Many of the risks associated with the use of live attenuated or killed whole organism vaccines can be avoided with a strategy that uses only specific, purified macromolecules derived from the pathogen, also known as the subunit approach [323]. Vaccination using individual antigenic proteins, or a small number of specific proteins can induce an immune response in the host against the specific desired antigens. This enables the production of protein-specific antibodies to study the functions of the proteins. Therefore, any response observed using these antibodies can be narrowed down to a single or small subset of proteins, resulting in focused research on useful proteins and limiting time spent on irrelevant proteins. These antibodies can then be used to confirm knowledge about the protein(s) of interest including but not limited to adherence, invasion, localisation, and post translational differences.

As the nucleotide and amino acid sequences are known for the selected proteins it is possible to produce each protein in a recombinant form in an *E. coli* host. This approach usually enables production of proteins in quantities needed for further studies. The addition of a histidine tag to the recombinant proteins can facilitate their purification from *E. coli* native proteins [324].

In response to an infection by a pathogen the immune system mounts a response to eliminate the invader. This can be undertaken by several different mechanisms depending on the location of the infection, whether the pathogen is intracellular or extracellular, or the size of the pathogen.

There are two main mechanisms of immunity within the adaptive immune system – humoral and cell-mediated.

Humoral immunity comprises of antibodies generated against specific foreign antigens. The extracellular spaces of the body are protected by humoral immunity [90]. Antibodies produced by the B cells will bind to pathogens leading to their inactivation or clearance by different effector mechanisms like neutralisation, agglutination, opsonisation, complement activation, antibody-dependent cell-mediated cytotoxicity and degranulation [90, 323].

Cell-mediated immunity is useful for protection against intracellular pathogens. The activated cytotoxic T lymphocytes (CTLs) then kill the target cells containing the specific pathogen [90]. T_H1 cells facilitate a cell-mediated response and are regarded as critical for immunity against intracellular microorganisms [98-100]. They are triggered by the cytokine interleukin 12 (IL-12) and produce the cytokines interferon γ (IFN- γ) and IL-2. The main effector cells of T_H1 response are macrophages as well as CTLs and IFN- γ T_H1 cells [98-100]. IFN- γ secreted by T_H1 cells can activate macrophages to phagocytose and digest intracellular bacteria and protozoa [100]. Detecting IFN- γ expressed against specific antigens can indicate the level of T_H1 cell mediated response induced by the antigen. This is useful for determining the effectiveness of an antigen in inducing cell-mediated protection.

Another cellular T_H response is T_H17 that results in the production of IL-17. T_H17 cells mediate immune responses against extracellular bacteria and fungi [100, 325]. T_H17 cells produce IL-17A, IL-17F, IL-21, and IL-22 [100]. IL-17A can induce many inflammatory cytokines, IL-6, and chemokines such as IL-8, therefore has an important role in inducing inflammatory responses [100, 326]. Both IL-17A and IL-17F recruit and activate neutrophils during immune responses against extracellular bacteria [100]. IL-17A is one of the best-studied cytokines in immunology, potentially due to its involvement in diseases caused by an excessive inflammatory response [117-119]. IL-17A has been shown to be of interest because of its involvement in both inflammation and protective antimicrobial immunity [327]. IL-17A has also been suggested to be able to exert both protective and destructive effects [328-330] with the net effect of IL-17A signalling suggested to promote disease development [116]. Observing the IL-17A response to specific antigens can be useful for determining the effectiveness of particular antigens at inducing a cellular immune protection against extracellular bacteria and fungi [100, 325]. Additionally, testing the levels of IL-17A in an animal can help in determining if an animal is over expressing IL-17A and may require treatment [116, 141].

Once the immunogenic proteins that were identified in chapter 4 has been isolated and purified, protein specific antibodies could be produced by vaccinating sheep with these proteins and collecting the antisera. In addition to the recombinant proteins, HPF from three *M. ovipneumoniae* isolates (16, 90, 103) and H₂O₂ inactivated WCL of the three isolates were also used as vaccine antigens. Antibody and CMI responses will be measured in each of the animals vaccinated with the recombinant proteins to determine the immune response induced by the recombinant proteins. To determine potential diagnostic abilities, each of the recombinant proteins was used as the target for ELISA (for measuring antibody responses), and in cellular assays (whole blood cultures) measuring protein-specific production of IFN- γ , and IL-17A.

This study was undertaken with the following aims:

- To produce recombinant proteins of the immunogenic proteins identified in Chapter 4
- Vaccinate sheep with the recombinant proteins
- Measure humoral and cellular response to the recombinant proteins

5.2 Methods

5.2.1 *M. ovipneumoniae* culture and genomic DNA extraction

Three New Zealand isolates (16, 90, 103) of *M. ovipneumoniae* were cultured for sequencing in Frey's medium [230] at 37°C until a phenol red pH indicator indicated growth of the mycoplasma (chapter 2.4). DNA was extracted using a phenol-chloroform extraction method (chapter 2.5.2) and stored at -20°C.

5.2.2 Production of recombinant proteins

Knowledge of the nucleotide sequence of the five proteins enabled production of each of the proteins in recombinant form. The five proteins were produced commercially by GenScript Biotech Corporation (www.genscript.com). In addition, EF-Tu which was the first protein identified in the studies, was produced in-house using mutational polymerase chain reaction (PCR) to edit the *M. ovipneumoniae* sequence to be read by the *E. coli* translational machinery.

In-house EF-Tu

The complete nucleotide sequence encoding the EF-Tu protein was amplified by PCR in two fragments using EF-Tu specific primers as described in Jiang et al (2016) [160] (chapter 2.8.1). Restriction sites for two enzymes, NcoI and XhoI, were introduced into the forward and reverse primers, respectively. *M. ovipneumoniae* EF-Tu carries the UGA codon which, codes for tryptophan in mycoplasma but codes for a stop codon in the *E. coli*. Therefore, overlap PCR was used to mutate the A in UGA to a G in the gene sequence to avoid a truncated protein (Fig. 5.1). The primers used are shown in Table 2.7. This involves multiple rounds of PCR with the first round sequencing the first half of the gene (Primers A & B) and the second half of the gene (primers C & D) separately. This mutates the sequence in both fragments. The second round combines the two fragments using primers A & D (Fig. 5.1)

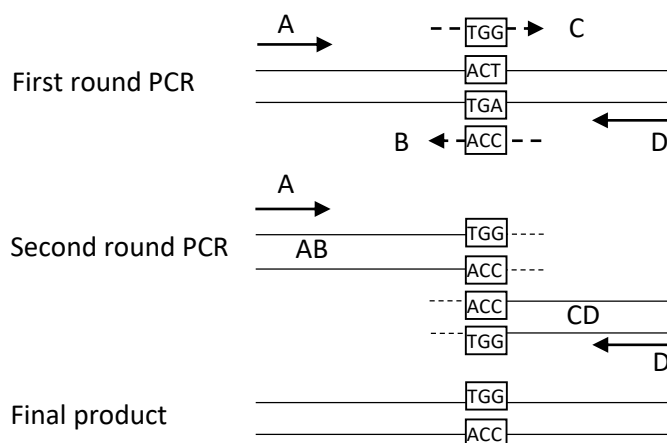


Figure 5-1 Construction of the EF-Tu mutant using site-directed mutagenesis with overlap extension.

In the first PCR round, primer pairs A-B and C-D were used to obtain two intermediate products with the TGA→TGG mutation, which were then used as templates for the second PCR round with primer pair A-D to obtain the full-length sequence encoding the EF-Tu variant. The overlapping regions of the two fragments ensured sequence hybridisation in the second round of PCR.

The full-length fragment was extracted from an agarose gel using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific™, United States) (Chapter 2.8.1). The purified fragment was sequenced to confirm proper editing (Massey Genome service, New Zealand). The fragment was then double digested by the restriction enzymes NcoI and XhoI (Thermo Fisher Scientific™, United States) (chapter 2.8.1). The purified digested fragments were then ligated into the pET22b (+) prokaryotic expression vector (Novagen, Merck, Germany) which was pre-digested with the same restriction enzymes NcoI and XhoI (Thermo Fisher Scientific™, United States) to re-construct the pET22b (+)-EF-Tu plasmid. The EF-Tu sequence including his tag is represented in Fig. 5.2.

The pET22b (+)-EF-Tu plasmid was transformed into DH5 alpha cells (Invitrogen™, Thermo Fisher Scientific™, United States) using heat shock as described in Chapter 2 (chapter 2.8.1). The *E. coli* transformant was grown in 500 µl Luria-Bertani (LB) medium (chapter 2.1.2) for one hour and plated on LB plates containing ampicillin (100 µg/ml) (Sigma-Aldrich™, Germany). The plates were left overnight at 30°C to produce single colonies. Single colonies containing the plasmid were then grown overnight in 10 ml LB medium at 30°C to amplify the plasmid. Plasmids were purified using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen™, Thermo Fisher Scientific™, United States) (chapter 2.8.1). The plasmid was sequenced (Massey Genome service, New Zealand) to confirm correct insertion of the gene. The pET22b (+)-EF-Tu plasmid with the correct gene insert was transformed into Rosetta (DE3) Chemical competent cells (Sigma-Aldrich™, Germany) to express the recombinant protein as described in Chapter 2 (chapter 2.8.1). The transformant was grown in 500 µl LB medium (chapter 2.1.2) for one hour at 30°C and plated on LB plates containing ampicillin (100

μg/ml). The plates were incubated over night at 30°C to produce single colonies. Single colonies were grown in 300 ml sterile LB medium containing ampicillin (100 μg/ml) on a shaker at 150 rpm and 30°C. The culture was incubated for 3 h before addition of 1 mM isopropyl β-D-thiogalactoside (IPTG) Sigma-Aldrich™, Germany to induce protein production. Incubation of the culture was continued at 37°C for 3 hr until the OD₆₀₀ reached 0.6.

The cells in the final culture were harvested by centrifugation at 10,000 x g for 20 min and disrupted by sonication at 30% for 40s on and 59s off, repeated five times (VCX 750 Ultrasonic Processor Sonics & Materials, USA). The recombinant protein was then purified using a His60 Ni Gravity Column Purification Kit Takara Bio USA, Inc (chapter 2.5.1). The protein was run on a SDS-PAGE gel and Western blotting was performed to check for protein production and purity. The purified EF-Tu was concentrated using Pierce™ Protein Concentrators PES, 10K MWCO, 0.5 mL Thermo Fisher Scientific™, United States by centrifuging at 3,222 x g filtering out proteins less than 10 kDa in molecular weight. Protein concentration was determined by Pierce™ BCA Protein Assay kit Thermo Fisher Scientific™, United States.

ATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCAGCCGGCGATGGCC
 M K Y L L P T A A A G L L L L A A Q P A M A
 ATGGCAGTTGTTAAAACTGGTGCAAAAAAAGATTTTGACCGTTCAAAAGAGCATATCAATATTGGG
 M A V V K T G A K K D F D R S K E H I N I G
 ACAATTGGTCATGTTGACCCAGGAAAAACCACTCTAACAGCGGCAATTTCAACTGTATTATCAAAA
 T I G H V T D H G K T T L T A A I S T V L S K
 AAAGGTCTAGCTGAAGCAAAAGATTATGCTTCTATTGACGCAGCCCTGAAGAAAAAGCGCGTGGA
 K G L A E A K D Y A S I D A A P E E K A R G
 ATTACAATCAATACAGCCACATCGAATATAGCACAGACAAGCGTCACTATGCCCATTGTTGATTGC
 I T I N T A H I E Y S T D K R H Y A H V D C
 CCTGGTCACGCCGATTATATTAATAAATATGATCACAGGAGCAGCACAAATGGATGGTGCCATTCTT
 P G H A D Y I K N M I T G A A Q M D G A I L
 GTTGTGGCCGCAACAGATGGTCCAATGCCCAAACCTCGTGAGCACATTCTTCTTTCAAAACAAGTT
 V V A A A T D G P M P Q T R E H I L L S K Q V
 GGTGTGCCAAAAATGGTTGTTTCTTAAACAAATCGACTTACTTGAAGGTGAAGAAGAAATGGTT
 G V P K M V V F L N K I D L L E G E E E M V
 GACCTTGTGAGGTTGAAATTCGTGAACCTTCTTCTTCATATGATTTTGACGGAGACAACACCCCA
 D L V E V E I R E L L S S Y D F D G D N T P
 ATAATCCGTGGTTCAGCTCGTGGTGCTCTTGAAGGAAACCTGAATGGGAAGCTAAAGTTCTTGAA
 I I R G S A R G A L E G K P E W E A K V L E
 CTAATGGATGCAGTTGACTCATACATTGACTCCCCAGTTCGTGAAATGGACAAACCATTCCTAATG
 L M D A V D S Y I D S P V R E M D K P F L M
 GCAGTTGAAGACGTCTTTACCATTACAGGTCGTGGAACCTGTTGCTACTGGTAAAGTTGAAAGAGGA
 A V E D V F T I T G R G T V A T G K V E R G
 CAAGTTAAACTAAATGAAGAGGTTGAAATTTGTCGGATATCGTCCTGAACCTAAAAAACAGTTGTA
 Q V K L N E E V E I V G Y R P E P K K T V V
 ACCGGAATTGAAATGTTTAAACAAAAACCTTCAATCTGCAATGGCTGGAGATAATGCTGGAGTTCTT
 T G I E M F N K N L Q S A M A G D N A G V L
 CTTCTGTTGGTGTGACCGTAAAGATATTGAACGTGGTCAGGTTATTGCCAAACCAAAAAACCATTT
 L R G V D R K D I E R G Q V I A K P K T I I
 CCCACACTAAATTTTAAAGCAGCAATTTACGCGCTCAAAAAAGAAGAAGGTGGAAGACATACTCCA
 P H T K F K A A I Y A L K K E E G G R H T P
 TTTTCAAAAAATTACAAACCTCAATTTTATTTCCGTACAACCGATGTTACTGGTGGAAATTGAATTT
 F F K N Y K P Q F Y F R T T D V T G G I E F
 GAAGCTGGCCGTGAAATGGTAATTCCTGGGGATAATGTTGACCTTACTGTTGAACCTTATTGCCCT
 E A G R E M V I P G D N V D L T V E L I A P
 ATCGCTGTTGAGCAAGGTACAAAATTCTCAATTCGCGAAGGTGGAAGAACTGTTGGAGCCGGAACA
 I A V E Q G T K F S I R E G G R T V G A G T
 GTAAGTGAATATTAAACTCGAGCACCACCACCACCACCTGA
 V T E I I K L E H H H H H H H *

Figure 5-2 Sequence analysis of the in-house EF-Tu.

The start codon is highlighted in orange. The pelB leader region is indicated in yellow. The His tag is indicated in purple. The stop codon is highlighted in red. The sequence is 1299 base pairs in length. The corresponding translated 432 amino acid sequence is shown as derived from the DNA sequence using Geneious.

Custom production by GenScript

In addition to EF-Tu produced above, five proteins were produced for the studies by GenScript Biotech Corporation (www.genscript.com). These were EF-Tu, GAPDH, HP3, P146, and an epitope of HP1. For each protein, the nucleotide sequences were edited for *E. coli* codon preference and changing the UGA codon to UGG to avoid producing truncated proteins. The following is a summary of the cloning and expression steps provided by GenScript.

1. Gene synthesis and subcloning: Target DNA sequence was optimised and synthesised. The synthesised sequence was cloned into the vector pET 30a(+) Novagen Sigma-Aldrich™, Germany with histidine (His tag) (EF-Tu, GAPDH, HP3, and P146) or into the vector pGEX 4T 1 (GE Healthcare, Chicago, Illinois) with Glutathione S-transferase (GST) tag (HP1) for protein expression in *E. coli*.

2. Expression evaluation: *E. coli* strain BL21 star (DE3) was transformed with recombinant plasmids. A single colony was inoculated into 5052 auto induced medium (EF-Tu, GAPDH, and HP3) or TB medium (HP1 and P146) containing related antibiotic; culture was incubated in 37°C at 200 rpm and then induced with IPTG. SDS PAGE was used to monitor the expression.
3. Scale up expression: Recombinant BL21 star (DE3) stored in glycerol was inoculated into 5052 auto induced medium (EF-Tu, GAPDH, and HP3) or terrific broth (TB) medium (HP1 and P146) containing related antibiotic and cultured at 37°C. When the OD₆₀₀ reached about 3 (EF-Tu, GAPDH, and HP3) or 1.2 (HP1 and P146), cell culture was induced with IPTG at 15 °C/16h. Cells were harvested by centrifugation.
4. Purification and Analysis: Cell pellets were resuspended with lysis buffer followed by sonication. The supernatant after centrifugation was kept for future purification. Target protein was obtained by one step purification using Ni column (HP3 and P146) or GST column (HP1). Target protein was sterilized by 0.22 µm filter before being stored in aliquots. The concentration was determined by Bradford protein assay with bovine serum albumin as standard. The protein purity and molecular weight were determined by standard SDS PAGE along with Western blot confirmation using anti-his antibodies.

The nucleotide sequences, along with corresponding amino acid sequences of the five genes are shown in appendices 50-54.

5.2.3 Production of antisera against *M. ovipneumoniae* antigens

Four different animal trials were conducted to produce antibodies and determine cellular immune responses. Each of the trials are described in detail in chapter 2.7.5. The trials are briefly described below.

Trial 1: Sera were collected from sheep vaccinated against the in-house recombinant EF-Tu and the five recombinant proteins produced by GenScript (EF-Tu, GAPDH, HP3, P146, and an epitope of HP1) (2.7.5.1). Antisera were also produced against WC and HPF prepared from the three isolates (16, 90, 103) of *M. ovipneumoniae* (described in Chapter 2.6). There were twelve groups of sheep (detailed in Table 2.3, n=2 per group) (2.7.5.1).

Trial 2: Sera were from one animal vaccinated with WC from isolate 90 and two animals with WC from isolate 103. In addition, three animals were vaccinated with an unrelated organism *M. ruminantium* M1. Three animals were given adjuvant alone (Montanide ISA61) (2.7.5.2).

Trial 3: Sera were collected from 12 sheep vaccinated with a mixture of the three New Zealand isolates of *M. ovipneumoniae* and *M. haemolytica*. Group 1 was comprised of antigens alone, and Group 2, had antigens formulated with Quil-A (2.7.5.3).

Trial 4: Sera were collected from 12 sheep administered with *M. haemolytica* formulated with Quil-A® (2.7.5.4)

5.2.4 SDS-PAGE and Western blotting

WCL, HPF, or recombinant proteins were separated by their molecular weight using SDS-PAGE as described in Chapter 2.9. The gels were either stained with Coomassie blue for detection of proteins or the separated proteins were transferred onto a polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membrane (Invitrogen™, Thermo Fisher Scientific™, United States) by Western blotting (Chapter 2.10). The PVDF membrane was incubated with primary antibodies (Section 2.10) from sheep sera (chapter 2.7.2, 2.7.3). The membrane was then incubated with rabbit anti-sheep IgG secondary antibody, HRP conjugated (Invitrogen™, Thermo Fisher Scientific™, United States). For colorimetric analysis, 1-Step™ TMB-Blotting Substrate Solution (Invitrogen™, Thermo Fisher Scientific™, United States) was used and for chemiluminescent analysis Clarity™ Western ECL Substrate (Bio-Rad Laboratories, New Zealand) was used. Details of antigens and sera used in the Western blots, together with their purpose are presented in Table 5.1.

Table 5-1 Details and purpose of the Western blots

Figure	Antigen(s)	Primary antibodies used	Purpose of study
5.3	Recombinant EF-Tu produced in-house.	Pooled sera from three animals vaccinated with a combination of three isolates of <i>M. ovipneumoniae</i> (16, 90, 103) and <i>M. haemolytica</i> .	Confirm a band at the expected size of EF-Tu and determine any effect of purification of the protein.
5.4	Five individual recombinant proteins produced by GenScript	Pooled sera from three animals vaccinated with a combination of three isolates of <i>M. ovipneumoniae</i> (16, 90, 103) and <i>M. haemolytica</i> .	Confirm proteins produced by GenScript are detected by the sera originally used to detect the proteins
5.5	Five individual recombinant proteins produced by GenScript	Pooled sera from two animals vaccinated with a combination of three isolates of <i>M. ovipneumoniae</i> (16, 90, 103)	Confirm proteins produced by GenScript are detected by the sera produced in absence of <i>M. haemolytica</i> antigens
5.6	Recombinant EF-Tu produced by GenScript	Sera from animal 1.1 vaccinated with recombinant EF-Tu produced by GenScript	Confirm detection of recombinant EF-Tu, and detection of EF-Tu in WCL
5.7	Recombinant EF-Tu produced by GenScript	Sera from animal 1.2 vaccinated with recombinant EF-Tu produced by GenScript	Confirm detection of recombinant EF-Tu, and detection of EF-Tu in WCL
5.8	Recombinant GAPDH produced by GenScript	Sera from animal 1.4 vaccinated with recombinant GAPDH produced by GenScript	Confirm detection of recombinant GAPDH, and detection of GAPDH in WCL
5.9	Recombinant HP1 produced by GenScript	Sera from animal 1.7 vaccinated with recombinant HP1 produced by GenScript	Confirm detection of recombinant HP1, and detection of HP1 in WCL
5.10	Recombinant HP1 produced by GenScript	Sera from animal 1.8 vaccinated with recombinant HP1 produced by GenScript	Confirm detection of recombinant HP1, and detection of HP1 in WCL
5.11	Recombinant HP3 produced by GenScript	Sera from animal 1.5 vaccinated with recombinant HP3 produced by GenScript	Confirm detection of recombinant HP3, and detection of HP3 in WCL
5.12	Recombinant P146 produced by GenScript	Sera from animal 1.9 vaccinated with recombinant P146 produced by GenScript	Confirm detection of recombinant P146, and detection of P146 in WCL
5.13	Recombinant P146 produced by GenScript	Sera from animal 1.10 vaccinated with recombinant P146 produced by GenScript	Confirm detection of recombinant P146, and detection of P146 in WCL
5.14	Recombinant EF-Tu produced by GenScript	Sera from animal 1.12 vaccinated with recombinant EF-Tu produced inhouse.	Confirm detection of recombinant EF-Tu, and detection of EF-Tu in WCL

5.2.5 Measurement of serum antibodies to *M. ovipneumoniae* whole cells antigens, fractions, and recombinant proteins

Antibodies in serum from animals vaccinated against the recombinant proteins, WCL and HPF were detected using in-house ELISA. Using sera collected at week 6, the optimal amount of protein and serum concentration for the antibody ELISA for each of the proteins were determined by checkerboard titrations as detailed in Chapter 2.13.1. A serum dilution of 1:50 was found optimal for EF-Tu and GAPDH, and a dilution 1:100 optimal for HP1, HP3, and P146. Similarly, 500 ng/well for EF-Tu and GAPDH and 125 ng/well for proteins; HP1, HP3, and P146 were found optimal for the ELISAs. Subsequent, ELISAs were carried out as per the protocol detailed in Chapter 2.13.2, on all sera collected from the vaccinated animals to measure antibody responses to the recombinant proteins.

5.2.6 Measurement of T cell responses to *M. ovipneumoniae* antigens

Heparinised blood obtained at week 7 (chapter 5.2.2.1) or week 5 (chapter 5.2.2.2) from sheep vaccinated with WCL, HPF or recombinant proteins was used to assess IFN- γ and IL-17A responses to different antigens.

Responses to Pokeweed mitogen (Sigma-Aldrich™, Germany) (20 μ g/well; positive control), phosphate buffered saline (PBS; blank), recombinant EF-Tu, GAPDH, HP3, HP1 and P146 (each @ 10 μ g/well), were tested in duplicate by culturing 0.5 ml blood mixed with 0.5 ml RPMI 1640 medium (Invitrogen™, Thermo Fisher Scientific™, United States) containing 5% foetal bovine serum (Invitrogen™, Thermo Fisher Scientific™, United States) and antibiotic and antimycotic solution (Invitrogen™, Thermo Fisher Scientific™, United States), in a humidified CO₂ incubator (37 °C) for 48 hours (chapter 2.14.1). After incubation, the plates were centrifuged at 784 x g for 15 min and supernatants collected for measuring IFN- γ and IL-17. Supernatants were stored at 4°C during testing then moved to -20°C for long term storage.

IFN- γ

Interferon- γ responses in blood cultures to different antigens were determined using a BOVIGAM ELISA kit (Prionics AG, Switzerland) as described in chapter 2 (2.14.2). The absorbance in each well was read at 450 nm wavelength with an VersaMax™ ELISA Microplate Reader (Bio-Strategy, New Zealand). Results were expressed as PBS-corrected (value of the unstimulated (PBS) blood cultures subtracted from the antigen-stimulated blood) optical density 450 (OD₄₅₀) values. Data in Figure 5.20 is expressed as ng/ml using an ovine IFN- γ standard (Kingfisher Biotech, St. Paul, USA).

IL-17A

IL-17A responses in whole blood culture supernatants were measured using a Bovine IL17A Do-It-Yourself kit (DIY0673B-003, Kingfisher Biotech USA). The kit includes capture antibody, standard and detection antibody and the assay protocol is described in chapter 2.14.3. The absorbance in each well was read at 450 nm wavelength with an ELISA plate reader and the concentration of IL-17A was expressed as PBS-corrected OD₄₅₀ readings.

5.2.7 Statistical analysis

Data pertaining to antibody responses using HP3 and P146 as the targets (Fig. 5.17), and IFN- γ (Fig. 5.20) and IL-17A (Fig. 5.23) responses against GAPDH (as a potential diagnostic target) were analysed using generalized linear mixed model analysis in SAS® 9.4 (SAS Institute Inc. Cary, NC, USA). The model included fixed effects of treatments, time and their interaction, and random effects of animals. A first-order autoregressive model was used to model the covariance error structure for repeated measures over time points, within animals. The OD₄₅₀ values were Blom-transformed [331] employing PROC RANK procedure in SAS® 9.4, prior to mixed model analysis. In the graphs, least square means in the usual scale were presented.

5.3 Results

5.3.1 Production of recombinant proteins

GenScript

Five *M. ovipneumoniae* proteins (EF-Tu, GAPDH, P146, HP3 and a large epitope of HP1) were custom produced by GenScript™. SDS-PAGE was used to confirm size and purity of each protein (Fig. 5.3). Each protein ran as a single band slightly higher than the expected molecular weight; EF-Tu ~45 kDa, GAPDH ~38 kDa, HP1 ~39 kDa, HP3 ~27 kDa, P146 ~127 kDa and no other proteins were visible using Coomassie blue staining. A certificate of analysis (COA) was provided by GenScript for each protein. The COA included a photograph of the protein run on SDS-PAGE and Western blotting with anti-histidine antibodies. The proteins were electrophoresed on SDS-PAGE gels and the results shown in Figure 5.3 agree with the COA for each protein.

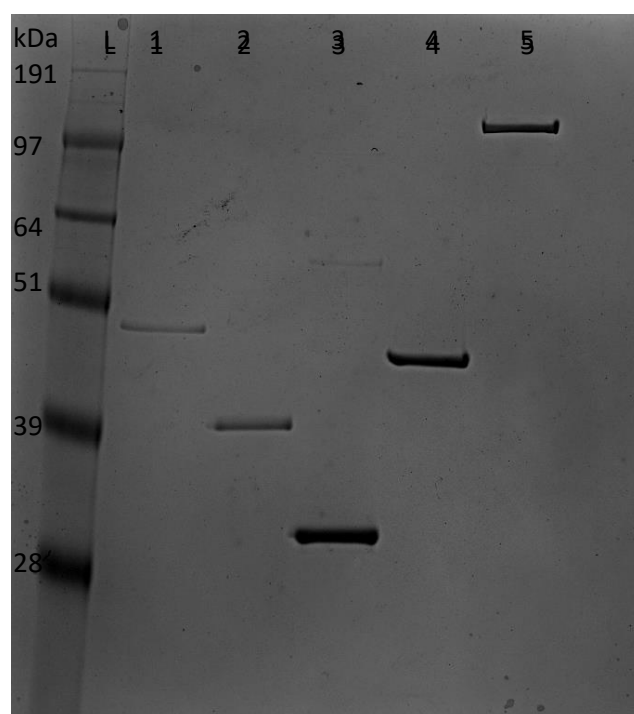


Figure 5-3 SDS-PAGE of the recombinant proteins produced by GenScript.

Lane L Ladder (SeeBlue™ Plus2 pre-stained protein standard), Lane 1 EF-Tu; Lane 2, GAPDH; Lane 3, HP3; Lane 4, HP1; lane 5, P146. Each well was loaded with 25-35 μ g of protein. The gels were stained with Coomassie blue.

In-house EF-Tu

In addition to the proteins produced by GenScript, one of the proteins, EF-Tu, was also produced in-house as part of the work conducted in this thesis. As described in chapter 2, 1 mg of recombinant EF-Tu was produced (chapter 2.8.1). Fig. 5.4 and Fig. 5.5 show analysis of the protein by SDS-PAGE and Western blotting, respectively. Western blotting confirmed a presence of a protein of the expected molecular weight (~45 kDa) for EF-Tu.

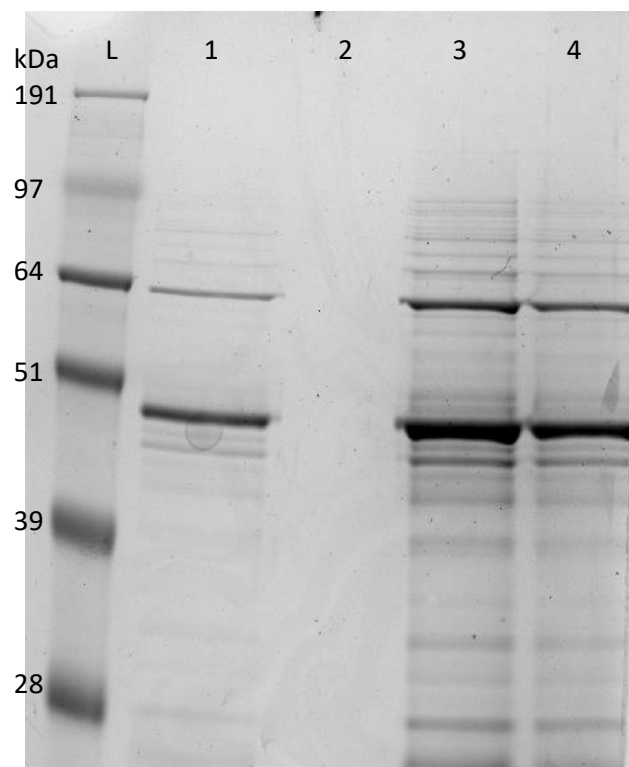


Figure 5-4 1D SDS-PAGE gel showing stages of purification of the EF-Tu produced in-house.

EF-Tu expected Mw 45 kDa. Lane L, Ladder (SeeBlue™ Plus2 pre-stained protein standard); Lane 1, final elution; Lane 2, column wash; Lane 3, un-purified sample, Lane 4 un-purified sample (1:1 dilution of sample).

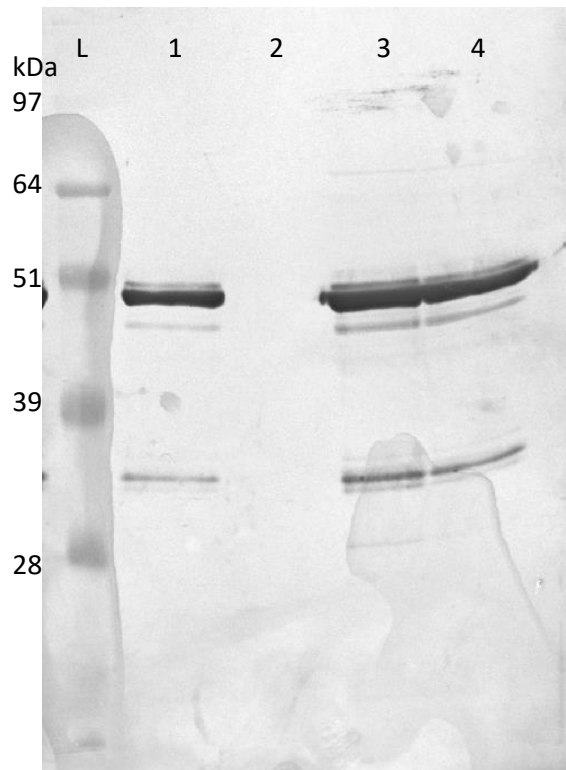


Figure 5-5 Western blot on EF-Tu produced in-house at different stages of purification.

Lane L ladder (SeeBlue™ Plus2 Pre-stained protein standard), Lane 1, final elution, Lane 2, Column wash, Lane 3, un-purified sample, Lane 4, un-purified sample 1:2 dilution. The gel was blotted with antisera produced against *M. ovipneumoniae* (pooled antisera against isolates 16, 90, and 103 from three sheep (methods 2.7.2)).

5.3.2 Western blot analysis

SDS-PAGE Western blotting was done on the GenScript recombinant proteins using both pooled sera from three sheep vaccinated with *M. ovipneumoniae* and *M. haemolytica* (chapter 2.7.2) (Fig. 5.6) and sera produced against *M. ovipneumoniae* alone (chapter 2.7.3) (Fig. 5.7). Western blotting was performed to confirm reactivity of antibodies to the recombinant proteins in the same sheep sera used originally to identify the immunogenic proteins (studies in chapter 4). With the exception of HP1, the Western blotting results indicated that the recombinant proteins reacted with antibodies in the sera. This provides support for the selection of the proteins for further analysis.

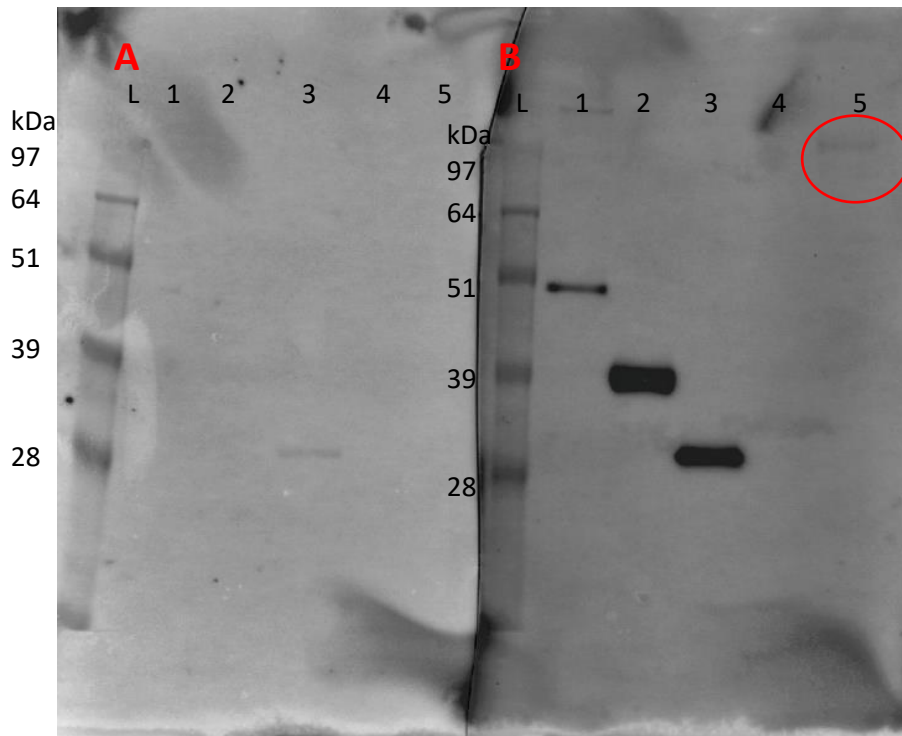


Figure 5-6 Western blotting of recombinant proteins using sera from sheep vaccinated with *M. ovipneumoniae* and *M. haemolytica*.

Proteins were separated by 1D SDS-PAGE and probed using pooled sera against isolates 16, 90, and 103 described in section 2.7.2.

A) Western blot using pre-immune sera. B) Western blot using antisera. Lanes L, Ladder (SeeBlue™ Plus2 pre-stained protein standard); Lane 1, EF-Tu; Lane 2, GAPDH; Lane 3, HP3; Lane 4, HP1; lane 5, P146. A red circle is drawn around a weakly immunogenic band of the expected molecular size of P146.

The set of pre-immune and post-vaccination sera produced against each of the six recombinant proteins was used for Western blotting analysis of the proteins in WCL prepared from *M. ovipneumoniae* (chapter 2.4). The results are shown in Fig. 5.8-5.16. In each experiment, the relevant recombinant protein was also included as a positive control and to confirm the correct identification of the target protein. Immunoreactive bands at the predicted molecular size were shown for four of the proteins, ET-Tu Fig. 5.8-9, GAPDH, Fig. 10, HP3 Fig. 5.13, and P146 Fig 5.14-15. In the case of HP1 Fig. 5.11-12, there were no clear immunoreactive bands at the expected molecular size.

For HP3 (Fig 5.13) the immunoreactive band in the cell lysate from *M. ovipneumoniae* isolate 103 was at a lower molecular weight than the immunoreactive bands seen in cell lysates from isolates 16 and 90 and recombinant HP3.

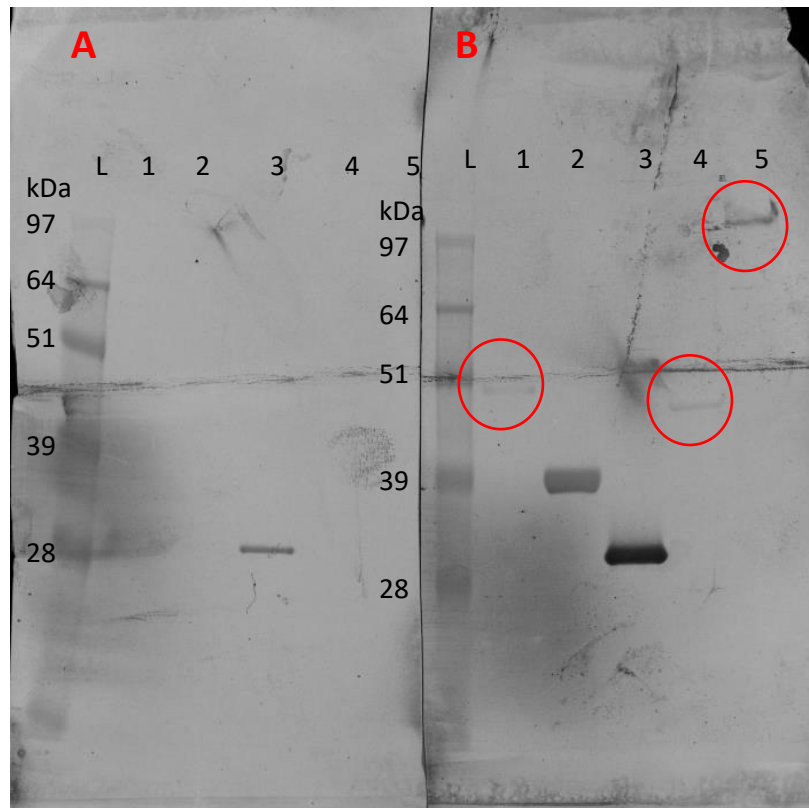


Figure 5-7 1D Western blotting of recombinant proteins using sera from sheep vaccinated against *M. ovipneumoniae*.

Proteins were separated by 1D SDS-PAGE and probed using sera from two animals vaccinated with *M. ovipneumoniae* isolates 16, 90, 103 (section 2.7.3).

A) Western blot using pre-immune sera. B) Western blot using anti sera. Lanes L, Ladder SeeBlue™ Plus2 Pre-stained Protein Standard; Lane 1, EF-Tu; Lane 2, GAPDH; Lane 3, HP3; Lane 4, HP1; lane 5, P146. A red circle is drawn around a weak immunogenic band of the expected molecular size.

Sera from several animals vaccinated with the recombinant proteins detected other protein bands in addition to the recombinant protein. The animal vaccinated with GAPDH showed several higher molecular weight proteins in the anti-sera Western blot image (Fig. 5.10). This suggests that the animal may have been exposed to mycoplasma during the trial. Both animals vaccinated with HP1 (Fig. 5.11-12) and P146 (5.14-15) had antibodies against *M. ovipneumoniae* antigens in the pre-immune sera and antisera. This suggests that the animal may have been exposed to mycoplasma before the start of the trial.

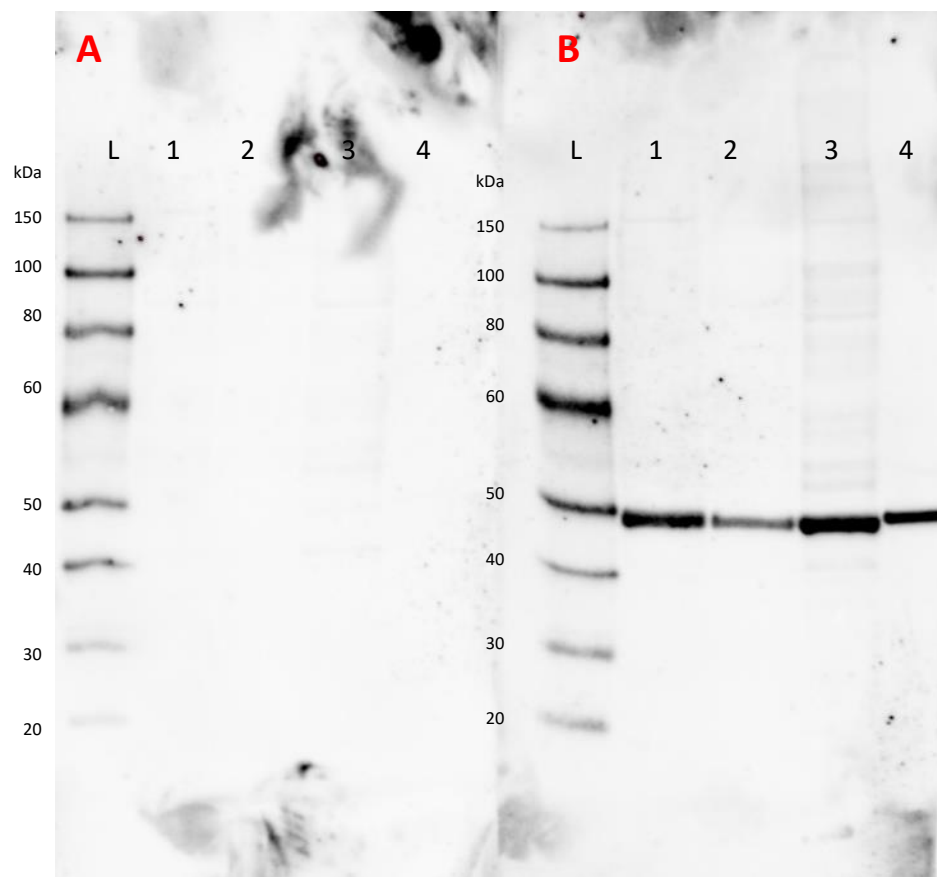


Figure 5-8 Western blotting of recombinant protein EF-Tu and whole cell lysates (WCL) of *M. ovipneumoniae* isolates using sera against EF-Tu.

Proteins were separated by 1D SDS-PAGE and probed using sera produced against EF-Tu (GenScript) (Animal 1.1 Table 2.6).

A) Western blot using pre-immune sera. B) Western blot using antisera. Lanes L: Ladder SuperSignal™ Molecular Weight Protein Ladder; Lanes 1, 2 and 3: WCLs from isolates 16, 90 and 103, respectively; Lanes 4 recombinant EF-Tu (GenScript).

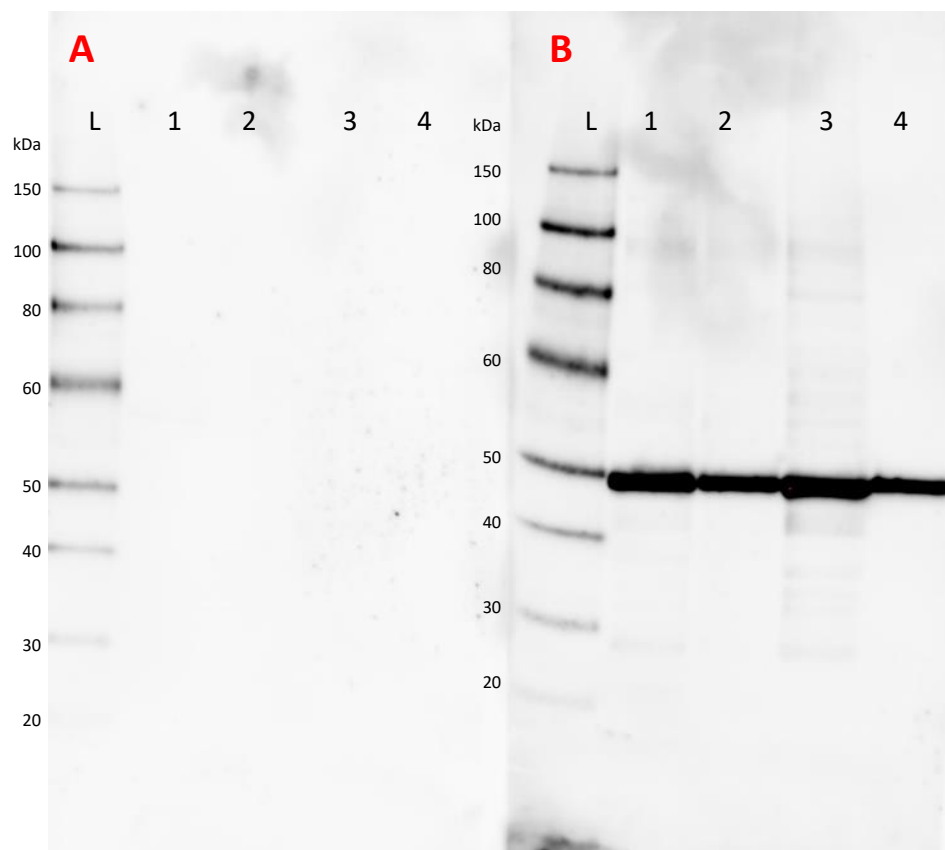


Figure 5-9 Western blotting of recombinant protein EF-Tu and whole cell lysates (WCL) of *M. ovipneumoniae* isolates using sera against EF-Tu.

Proteins were separated by 1D SDS-PAGE and probed using sera against EF-Tu (GenScript) Animal 1.2 Table 2.6.

A) Western blot using pre-immune sera. B) Western blot using anti sera. Lanes L: Ladder SuperSignal™ Molecular Weight Protein Ladder; Lanes 1, 2 and 3: WCLs from isolates 16, 90 and 103, respectively; Lanes 4 recombinant EF-Tu (GenScript).

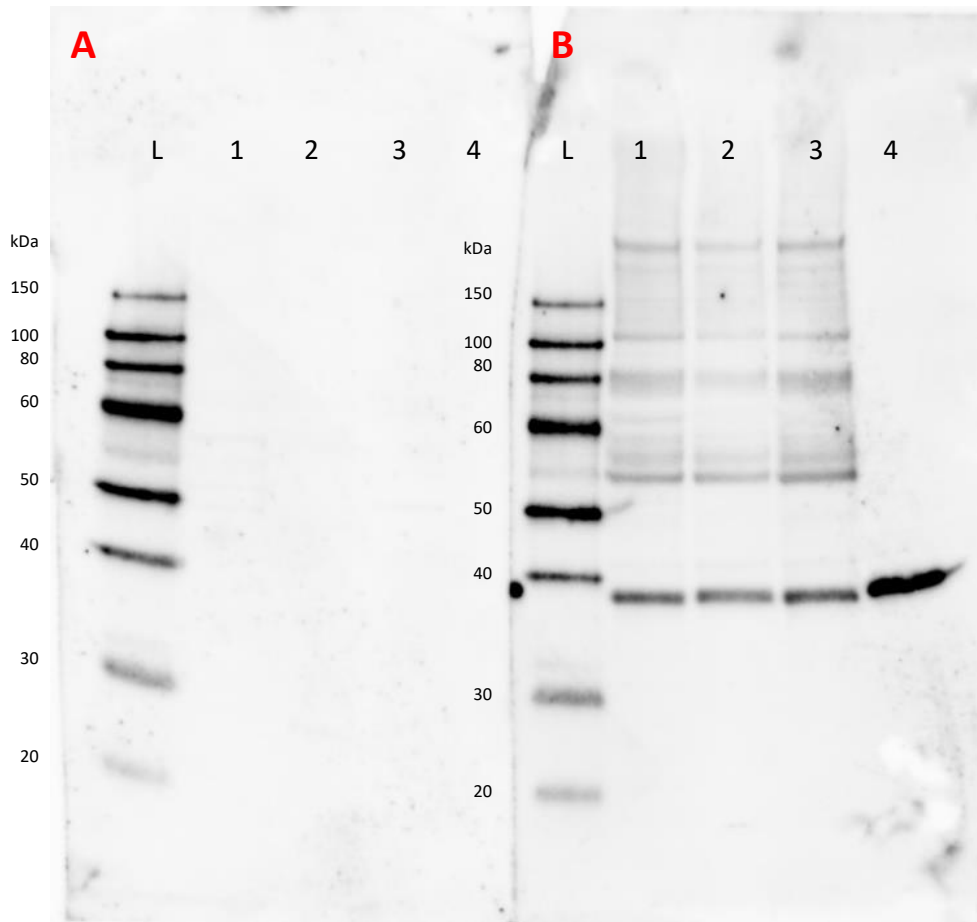


Figure 5-10 Western blotting of recombinant protein GAPDH and whole cell lysates (WCL) of *M. ovipneumoniae* isolates using sera against GAPDH.

Proteins were separated by 1D SDS-PAGE and probed using sera produced against GAPDH (GenScript) (Animal 1.4 Table 2.6).

A) Western blot using pre-immune sera. B) Western blot using anti sera. Lanes L: Ladder SuperSignal™ Molecular Weight Protein Ladder; Lanes 1, 2 and 3: WCLs from isolates 16, 90 and 103, respectively; Lanes 4 recombinant GAPDH (GenScript).

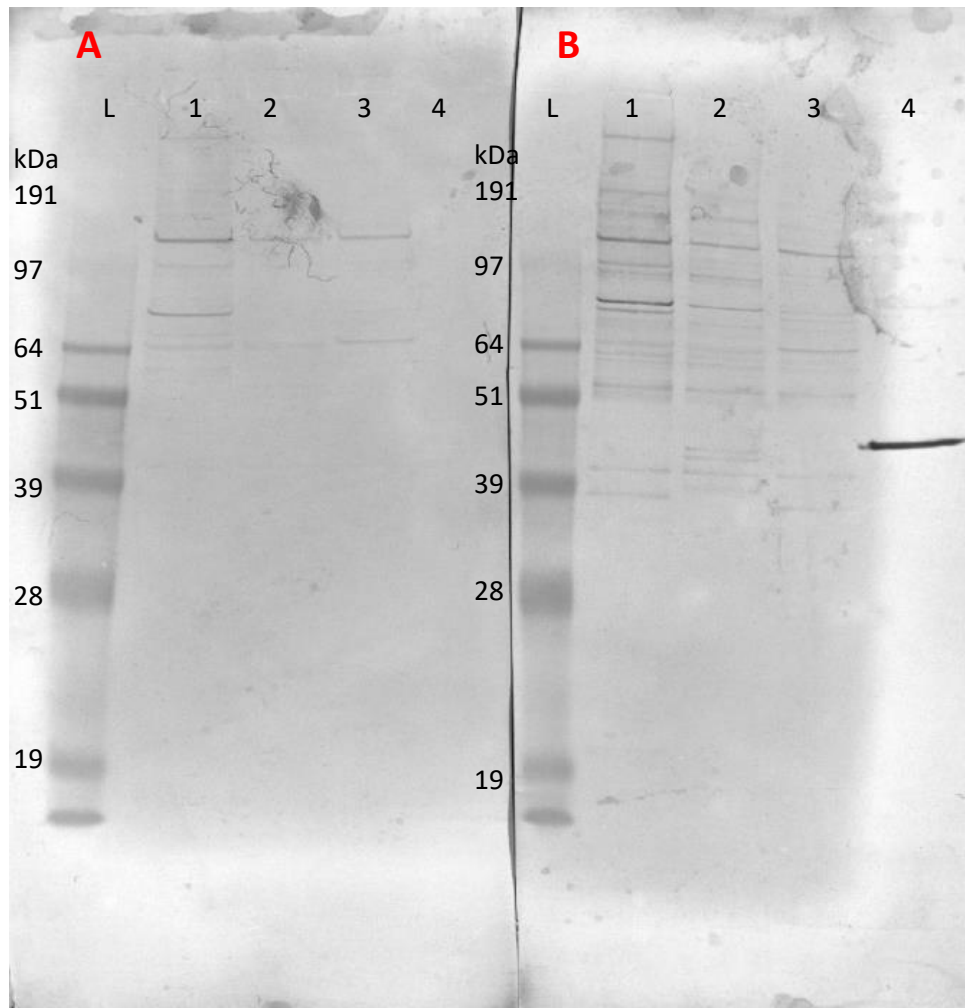


Figure 5-11 Western blotting of recombinant protein HP1 and whole cell lysates (WCL) of *M. ovipneumoniae* isolates using sera against HP1.

Proteins were separated by 1D SDS-PAGE and probed using sera against HP1 (GenScript) Animal 1.7 Table 2.6.

A) Western blot using pre-immune sera. B) Western blot using anti sera. Lanes L: Ladder SuperSignal™ Molecular Weight Protein Ladder; Lanes 1, 2 and 3: WCLs from isolates 16, 90 and 103, respectively; Lanes 4 recombinant HP1 (GenScript).

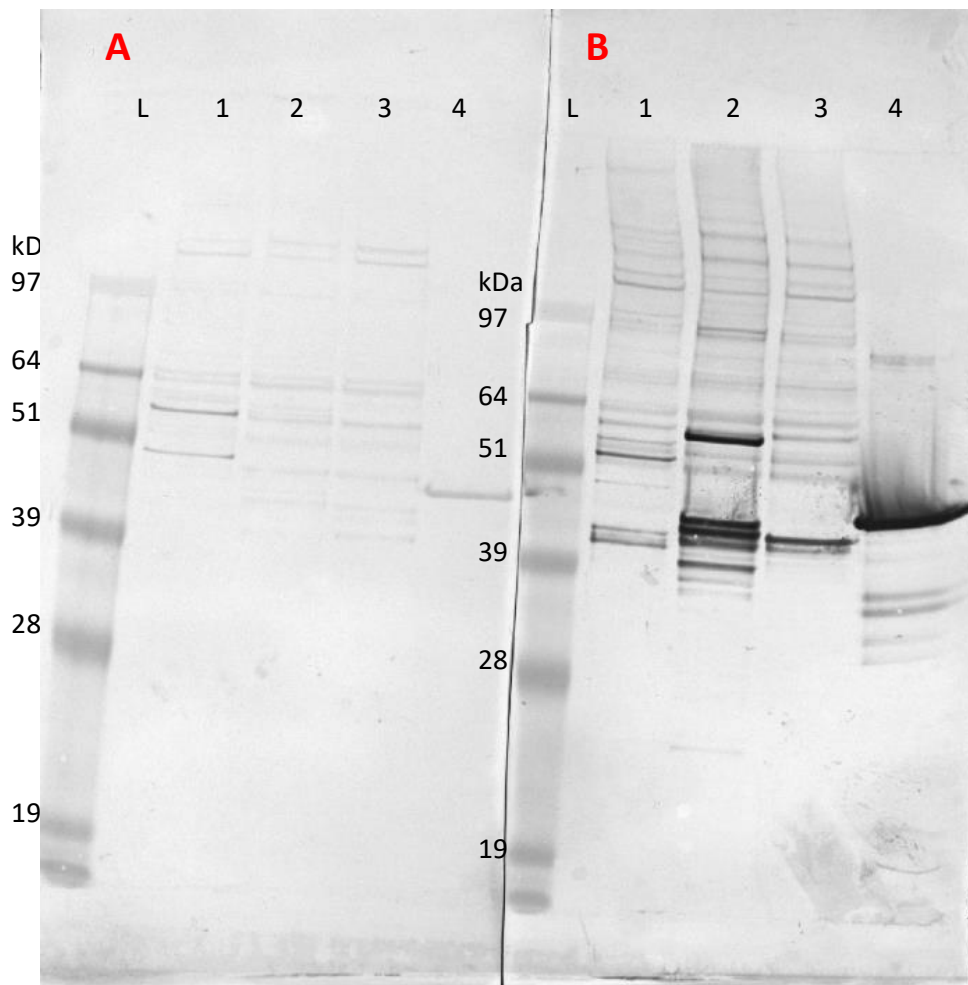


Figure 5-12 Western blotting of recombinant protein HP1 and whole cell lysates (WCL) of *M. ovipneumoniae* isolates using sera against HP1.

Proteins were separated by 1D SDS-PAGE and probed using sera against HP1 (GenScript) Animal 1.8 Table 2.6.

A) Western blot using pre-immune sera. B) Western blot using anti sera. Lanes L: Ladder SuperSignal™ Molecular Weight Protein Ladder; Lanes 1, 2 and 3: WCLs from isolates 16, 90 and 103, respectively; Lanes 4 recombinant HP1 (GenScript).

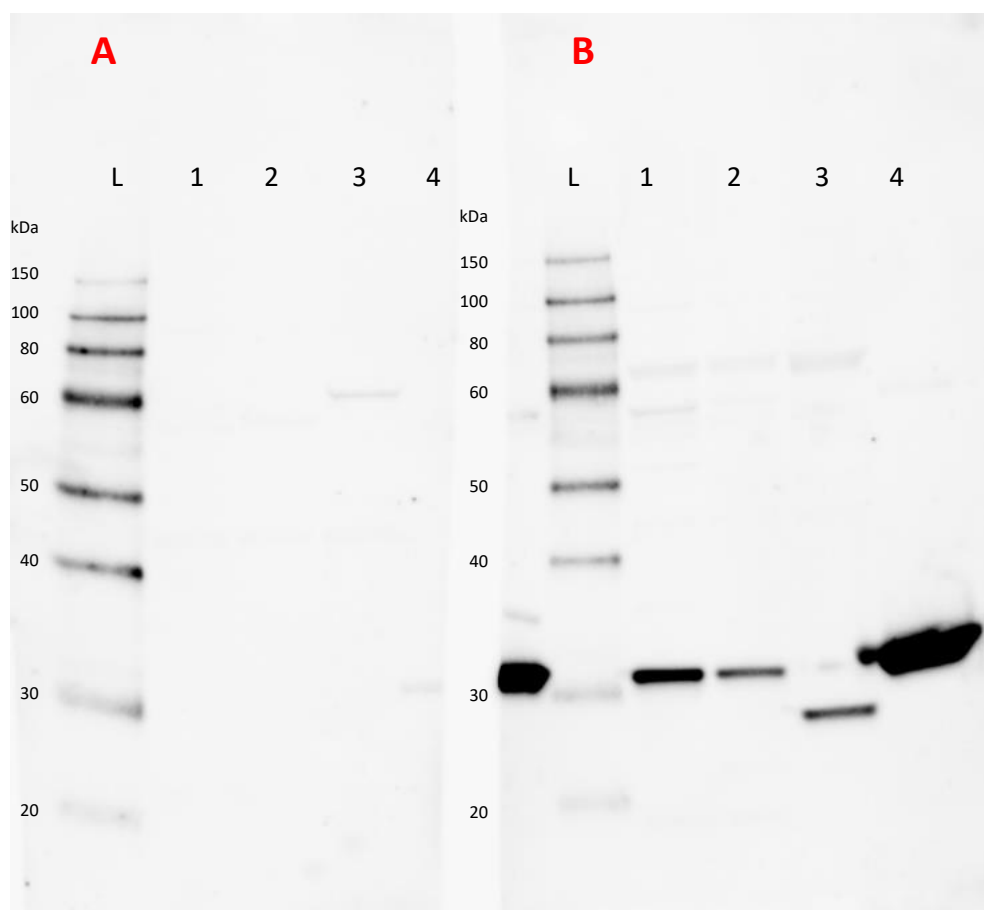


Figure 5-13 Western blotting of recombinant protein HP3 and whole cell lysates (WCL) of *M. ovipneumoniae* isolates using sera against HP3.

Proteins were separated by 1D SDS-PAGE and probed using sera against HP3 (GenScript) Animal 1.5 Table 2.6.

A) Western blot using pre-immune sera. B) Western blot using anti sera. Lanes L: Ladder SuperSignal™ Molecular Weight Protein Ladder; Lanes 1, 2 and 3: WCLs from isolates 16, 90 and 103, respectively; Lanes 4 recombinant HP3 (GenScript).

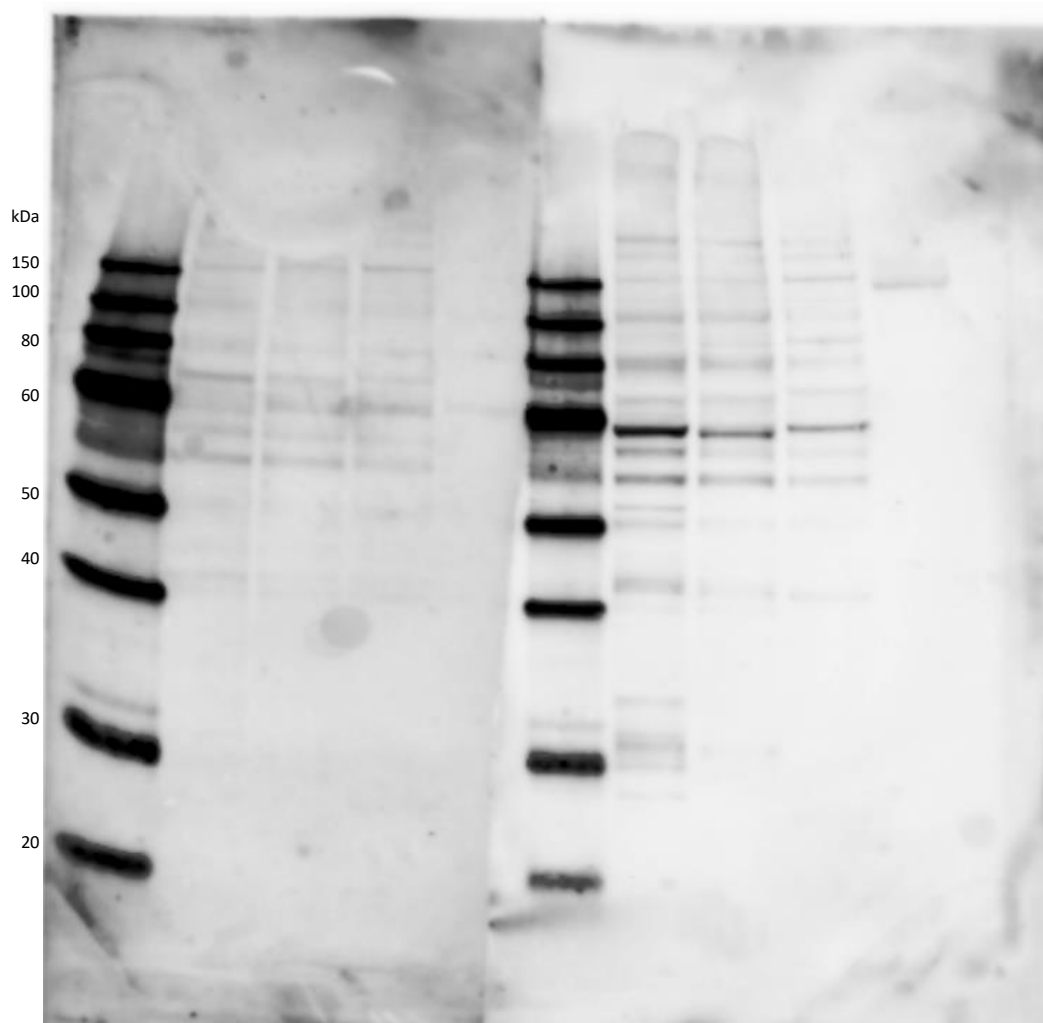


Figure 5-14 Western blotting of recombinant protein P146 and whole cell lysates (WCL) of *M. ovipneumoniae* isolates using sera against P146.

Proteins were separated by 1D SDS-PAGE and probed using sera against P146 (GenScript) Animal 1.9 Table 2.6.

A) Western blot using pre-immune sera. B) Western blot using anti sera. Lanes L: Ladder SuperSignal™ Molecular Weight Protein Ladder; Lanes 1, 2 and 3: WCLs from isolates 16, 90 and 103, respectively; Lanes 4 recombinant P146 (GenScript).

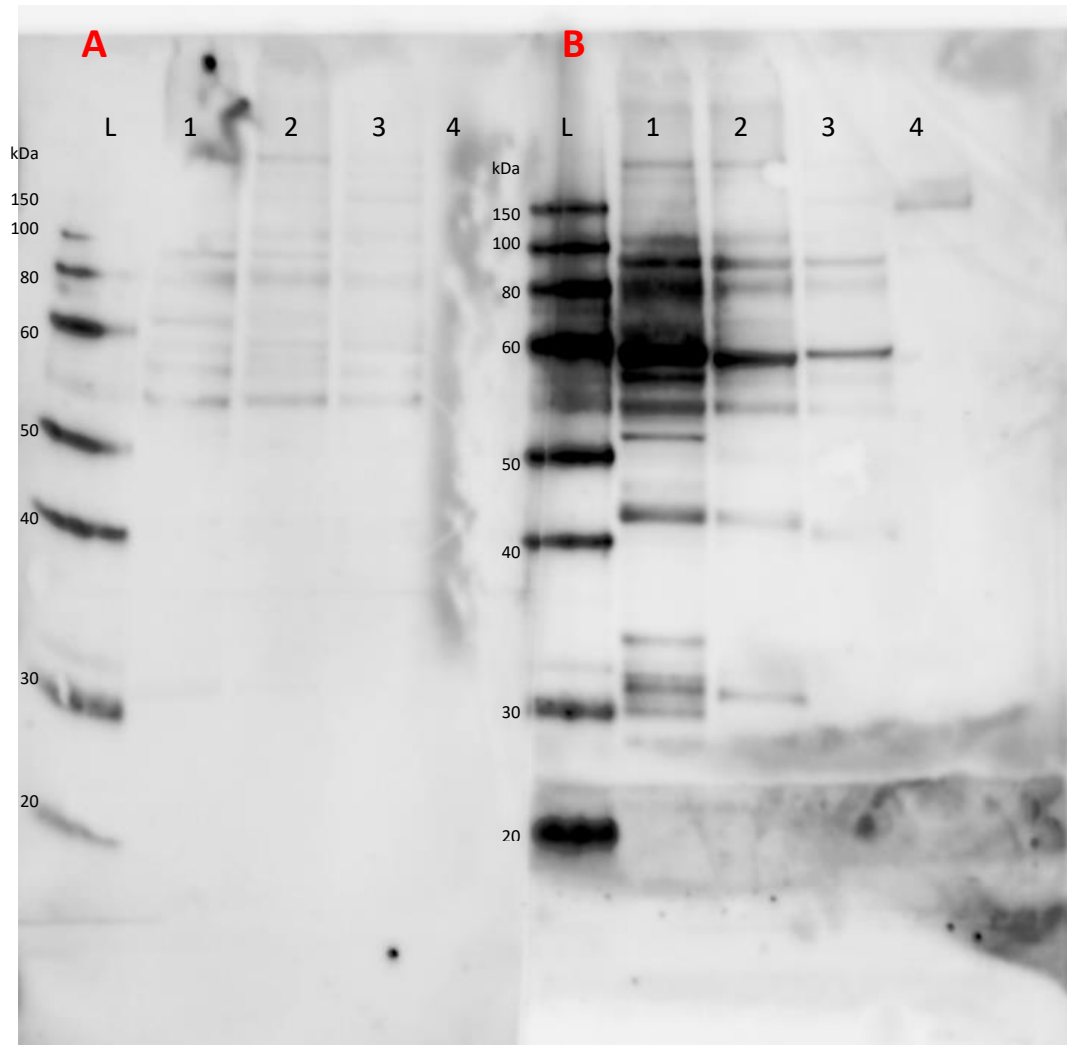


Figure 5-15 Western blotting of recombinant protein P146 and whole cell lysates (WCL) of *M. ovipneumoniae* isolates using sera against P146.

Proteins were separated by 1D SDS-PAGE and probed using sera against P146 (GenScript) Animal 1.10 Table 2.6.

A) Western blot using pre-immune sera. B) Western blot using anti sera. Lanes L: Ladder SuperSignal™ Molecular Weight Protein Ladder; Lanes 1, 2 and 3: WCLs from isolates 16, 90 and 103, respectively; Lanes 4 recombinant P146 (GenScript).

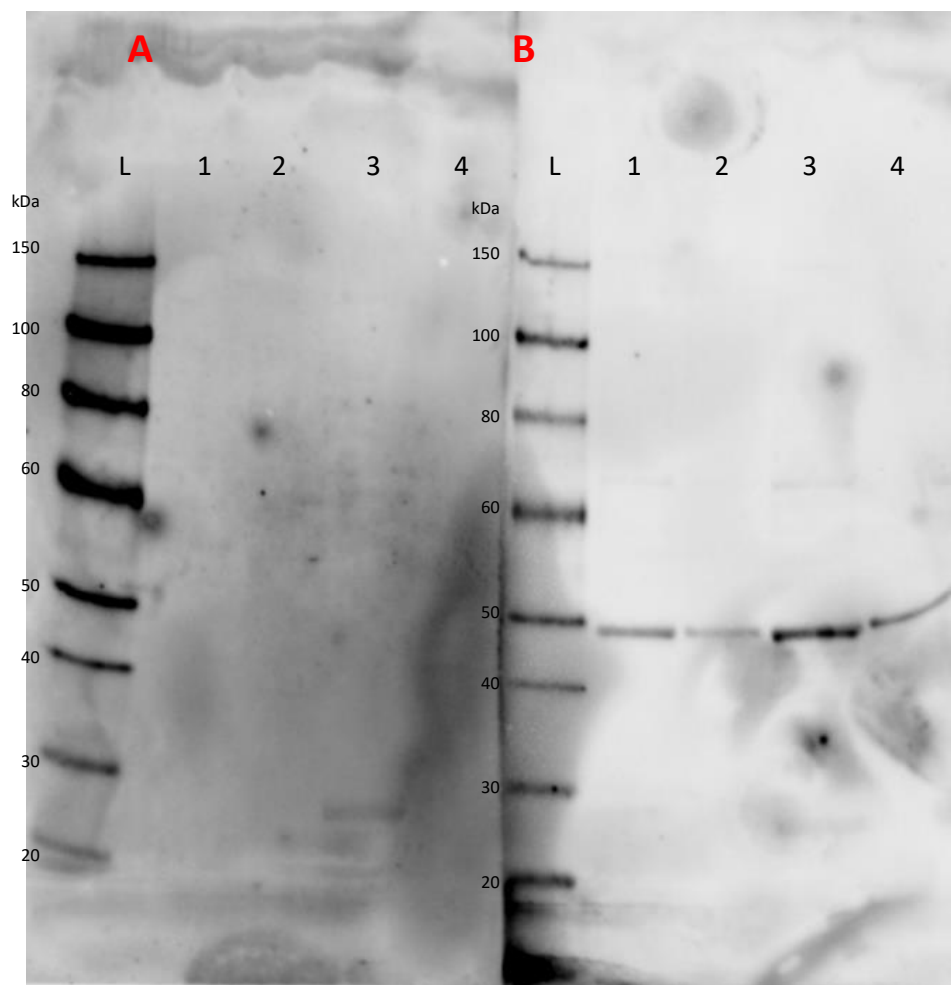


Figure 5-16 Western blotting of recombinant protein EF-Tu and whole cell lysates (WCL) of *M. ovipneumoniae* isolates using sera against in-house EF-Tu.

Proteins were separated by 1D SDS-PAGE and probed using sera against in-house EF-Tu Animal 1.12 Table 2.6.

A) Western blot using pre-immune sera. B) Western blot using anti sera. Lanes L: Ladder SuperSignal™ Molecular Weight Protein Ladder; Lanes 1, 2 and 3: WCLs from isolates 16, 90 and 103, respectively; Lanes 4 recombinant EF-Tu (GenScript).

5.3.3 Antibody responses to recombinant proteins

A standardised ELISA (chapter 2.13.2) was used to measure antibody responses in the vaccinated sheep. The results indicated that all recombinant proteins produced antigen specific antibody response following vaccination with the respective recombinant proteins at week 4 and week 6, compared to week 0 antibody levels (Table 5.2). Animals vaccinated with WCL *M. ovipneumoniae* and HPF produced inconsistent results. For some of the antigens, pre-immune antibody levels were higher than those measured at 4 weeks when the 2nd (booster) vaccination was administered or at 6 weeks (2 weeks after booster). This was the case for EF-Tu and GAPDH in animals vaccinated with WCL from isolate 16. In contrast, HP3 and P146 showed more typical responses to vaccination i.e. elevated levels of specific-antibody post-vaccination compared to levels before vaccination. This was

also the case for the other animals vaccinated with WCL from isolates 90 and 103; however, in these animals, the pre-immune response was not as strong. One animal vaccinated with HPF 90 and HPF 103 had a strong response to HP3 after the first vaccination; however, this response did not remain after the booster vaccination at the 6-week mark. HPF from isolate 103 also produced a strong response against EF-Tu. Animals vaccinated with adjuvant only or with *M. ruminantium* M1 produced weak responses to all the recombinant proteins.

Table 5-2 Mean (of duplicates) serum antibody responses to recombinant proteins in sheep vaccinated with recombinant proteins, whole cells (WC) or hydrophobic protein fractions (HPF).

The target antigen for the ELISA is represented in row 1. The antigen the animal was vaccinated with, and the animal number (Table 2.6) is represented in column 1. The time point (weeks) post vaccination the sera was collected is represented in row 3. Protein 1 & 2 represent the two animals vaccinated with the corresponding antigen target in the ELISA. Responses are expressed as PBS-corrected OD₄₅₀. Responses are highlighted in bold where there is a response over time.

	EF-Tu (GenScript)			GAPDH			HP1			HP3			P146		
	Weeks			Weeks			Weeks			Weeks			Weeks		
Antisera (Animal no.)	0	4	6	0	4	6	0	4	6	0	4	6	0	4	6
EF-Tu (in-house #1.12)	0.84	2.77	3.31												
Protein (#2)	0.39	3.16	3.03				0.00	2.45	1.95				0.09	2.72	2.16
Protein (#1)	0.92	2.78	3.15	0.40	1.96	3.42	0.20	1.38	1.55	0.00	2.61	2.53	0.00	2.99	1.85
WC 16 #1.13)	2.24	0.79	2.12	1.74	0.36	1.15	0.02	0.00	0.05	0.04	1.79	1.70	0.05	0.88	0.77
WC 16 (#1.14)	1.78	0.43	0.81	1.63	0.05	0.15	0.10	0.33	0.18	0.06	1.36	2.62	0.17	0.46	1.29
WC 90 (#1.15)	0.52	0.41	0.57	0.12	0.13	0.22	0.12	0.06	0.02	0.09	0.16	0.03	0.21	0.08	0.28
WC 90 (#2.7)	0.21	0.18	0.14	0.14	0.15	0.21	0.07	0.05	0.07	0.08	0.65	0.73	0.38	1.45	1.40
WC 103 (#2.8)	0.33	0.23	0.22	0.15	0.15	0.19	0.09	0.08	0.06	0.08	1.81	1.83	0.13	1.94	1.95
WC 103 (#2.9)	0.27	0.32	0.23	0.25	0.42	0.75	0.06	0.11	0.12	0.06	1.50	1.73	0.09	2.03	2.09
HPF 90 (#1.21)	0.46	0.41	0.56	0.21	0.16	0.14	0.00	0.55	0.02	0.01	2.09	0.14	0.03	0.32	0.40
HPF 90 (#1.22)	0.58	0.29	0.29	0.16	0.19	0.23	0.00	0.06	0.04	0.01	0.53	0.12	0.13	0.25	0.21
HPF 103 (#1.23)	0.78	1.16	2.55	0.50	0.75	0.89	0.02	0.02	0.02	0.03	2.44	0.45	0.01	0.22	0.71
HPF 103 (#1.24)	0.37	0.46	0.88	0.87	0.23	0.37	0.04	0.07	0.03	0.05	0.05	0.34	0.14	0.09	0.47
Control (#2.1)	0.54	0.17	0.13	0.20	0.10	0.19	0.08	0.09	0.08	0.11	0.08	0.09	0.18	0.14	0.17
Control (#2.2)	0.15	0.15	0.32	0.16	0.13	0.18	0.07	0.05	0.05	0.07	0.06	0.06	0.21	0.31	0.33
Control (#2.3)	0.12	0.09	0.10	0.12	0.12	0.09	0.08	0.06	0.06	0.08	0.09	0.08	0.21	0.15	0.15
Methanogen (#2.4)	0.43	0.31	0.13	0.15	0.19	0.16	0.07	0.05	0.08	0.10	0.08	0.08	0.13	0.15	0.08
Methanogen (#2.5)	0.28	0.20	0.24	0.15	0.17	0.20	0.09	0.08	0.14	0.10	0.11	0.09	0.14	0.15	0.10
Methanogen (#2.6)	0.09	0.12	0.27	0.27	0.37	0.30	0.16	0.18	0.05	0.20	0.25	0.07	0.20	0.27	0.09

Testing antibody response to P146 and HP3

Due to the good antibody response observed with P146 and HP3 these proteins were used as the target antigen in further ELISA experiments, using sera from other animal trials, to determine their functionality as a diagnostic protein (Fig. 5.17). Animals vaccinated with *M. haemolytica* were used as negative controls. these animals produced limited response to both P146 and HP3. Animals from the adjuvant trial control group (n=12) also showed a limited response to both P146 and HP3. Animals in the Quil-A group (vaccinated with *M. ovipneumoniae* and *M. haemolytica* WC) (2.7.5.3) showed a strong response (significantly elevated OD compared to week 0) to P146 and an improved response to HP3 compared to the control group. Combining both proteins in the ELISA increased the response for both the control group and the Quil-A group compared to using each of the proteins individually (Fig. 5.17).

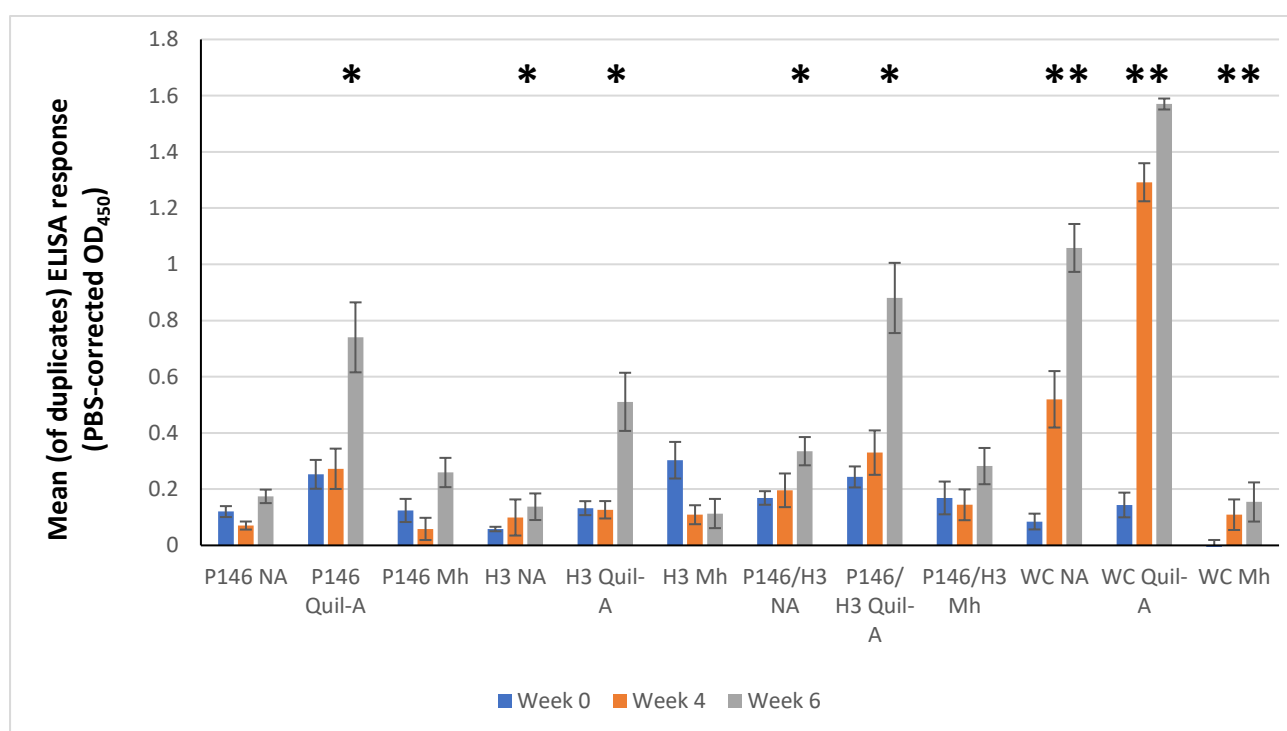


Figure 5-17 Antibody responses (Mean $\pm$ SE OD₄₅₀) to P146, HP3 and WCL antigens in animals (n=12) vaccinated with WC from *M. ovipneumoniae* isolates 16, 90, 103 and *M. haemolytica* with either no adjuvant (NA) or Quil-A as adjuvant, or vaccinated with *M. haemolytica* (Mh) (Negative control).

Responses were measured against whole cell antigens or recombinant, P146, HP3, or a combination of P146 and HP3 by ELISA Results are expressed as PBS-corrected OD₄₅₀. Sera were diluted at 1/100.

* Statistically different (P<0.05) to respective week 0 response.

5.3.4 T-cell responses to recombinant proteins

T-cell responses to the recombinant proteins were determined by measuring the release of IFN- γ and IL-17A from antigen-stimulated whole blood cultures. Whole blood was collected from sheep in trial 1 at week 7 (3 weeks after the second vaccination) as well as those in the antisera production

trial 2 at week 5 (2 weeks after the second vaccination), for determining antigen-specific IFN- γ and IL17 responses.

IFN- γ

Stimulation of blood cultures from animals vaccinated with WCL and HPF of *M. ovipneumoniae* with individual recombinant proteins or WCL revealed low IFN- γ responses (expressed as PBS-corrected OD₄₅₀) in general (mostly < 0.1), (Fig. 5.18). The two animals vaccinated with WC of *M. ovipneumoniae* isolate 16 exhibited a higher response to recombinant GAPDH. These responses of 0.86 and 0.6 were similar to the large responses measured using Pokeweed mitogen (0.91 and 0.42). Blood cultures from animals vaccinated with individual recombinant proteins, when stimulated with the various recombinant proteins, showed very low levels of IFN- γ release (Fig. 5.19). These responses were all <0.05, except for a response of 0.12 for the P146 protein in a sheep vaccinated with the same protein. Animals vaccinated with HPF also showed limited IFN- γ response to antigenic stimulation of peripheral leukocytes, except for responses of 0.17 and 0.21 respectively in two animals stimulated with WCL (Fig. 5.18).

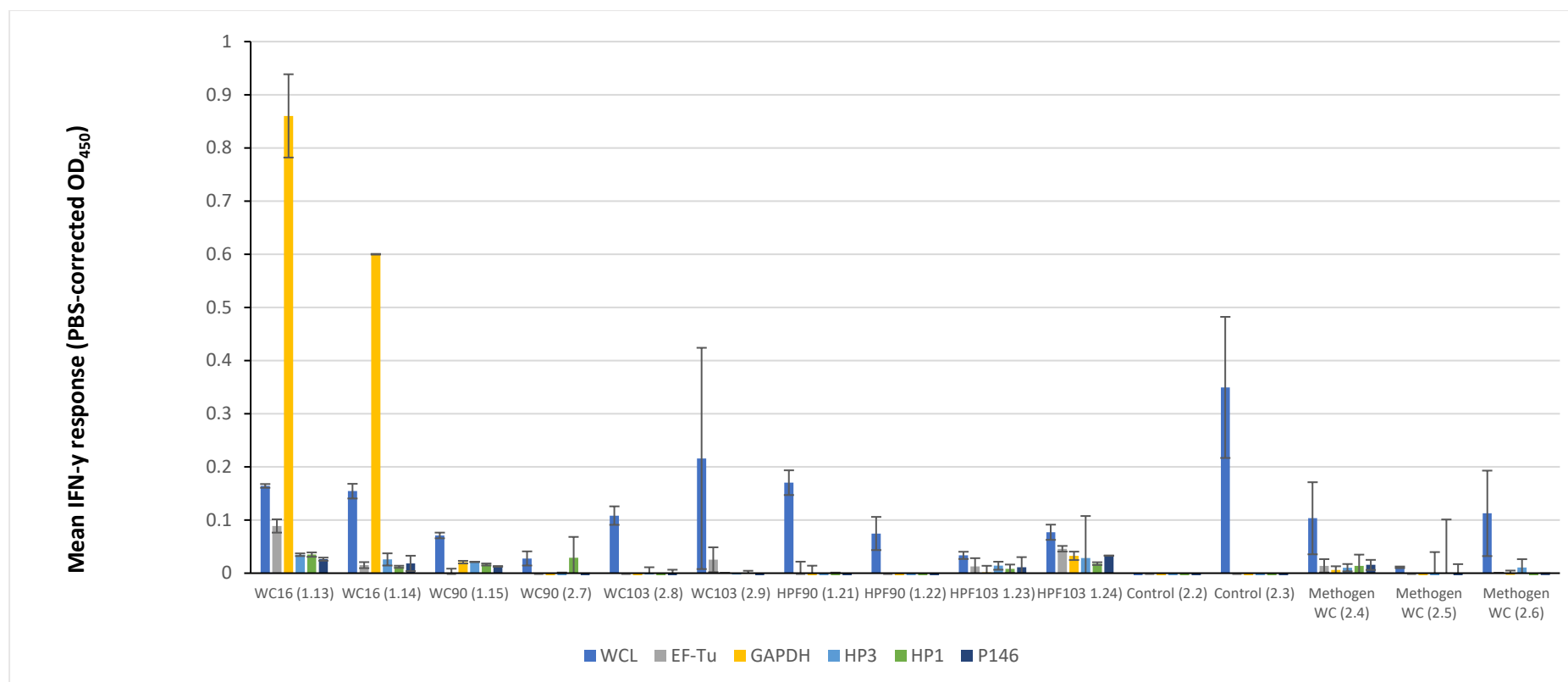


Figure 5-18 Mean ($\pm$ SE) IFN- γ responses in whole blood cultures of animals vaccinated with whole cell (WC) and hydrophobic protein fractions (HPF) of *M. ovipneumoniae* isolates 16, 90, 103, adjuvant only (negative control), and WC of *M. ruminantium* M1. All responses were measured in duplicate.

Responses were measured against WCL or recombinant EF-Tu, GAPDH, HP3, HP1, P146, or In-house EF-Tu. Responses against Pokeweed mitogen was used as a positive control but not included in the comparative analysis. Results are expressed as PBS-corrected OD₄₅₀ values.

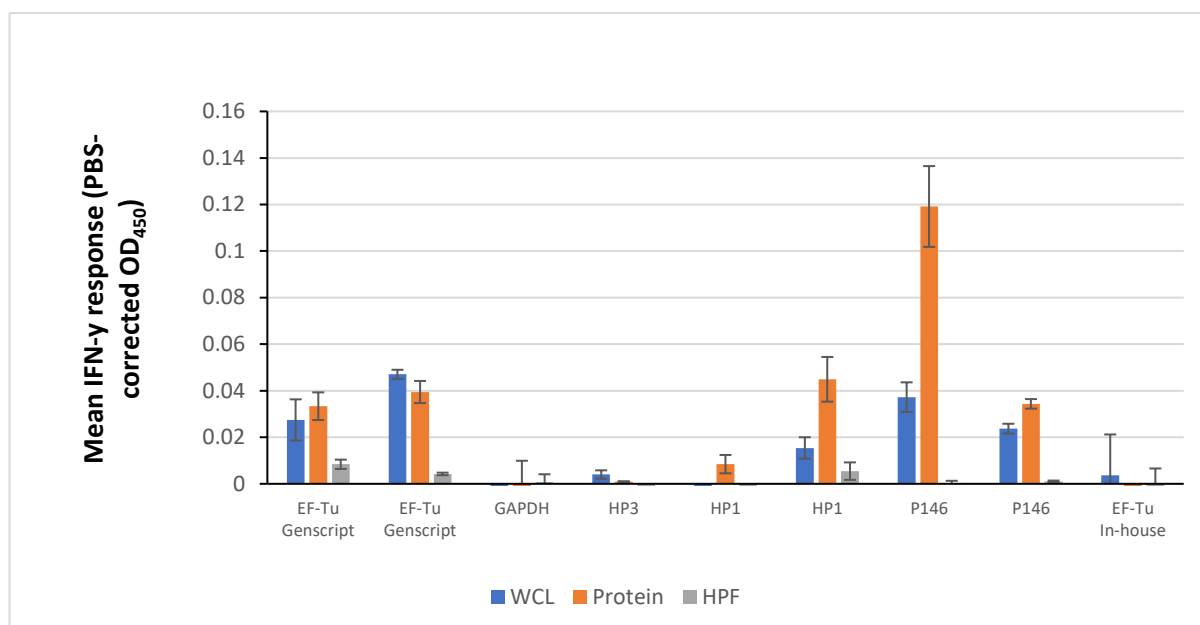


Figure 5-19 Mean ($\pm$ SE) IFN- γ responses in whole blood cultures of sheep vaccinated with recombinant proteins EF-Tu, GAPDH, HP3, HP1, P146, or In-house EF-Tu. All responses were measured in duplicate.

Responses were measured against whole cell lysate (WCL), the recombinant protein the animal was inoculated with, hydrophobic protein fraction (HPF). Responses against Pokeweed mitogen was used as a positive control but not included in the comparative analysis. Results are expressed as PBS-corrected OD₄₅₀ values.

As a control, whole blood cultures from animals vaccinated with adjuvant only or vaccinated with an unrelated microorganism, the methanogen *M. ruminantium* M1 were also tested for cellular response to WCL of *M. ovipneumoniae* and the five different recombinant proteins. While there was low IFN- γ response to the recombinant antigens, moderate responses to WCL of *M. ovipneumoniae* were observed; however, those responses were well below the responses to Pokeweed mitogen (positive control).

To investigate the potential use of GAPDH as a target antigen for cellular based diagnostics, sera were collected from a large animal trial comparing different adjuvants. Animals in both the control group (administered a mixture of WC of *M. ovipneumoniae* isolates 16, 90, 103 and *M. haemolytica*, without an adjuvant) and the Quil-A group (administered a mixture of WC of *M. ovipneumoniae* isolates 16, 90, 103 and *M. haemolytica*, together with the adjuvant Quil-A) all produced a strong response to the Pokeweed mitogen (Fig. 5.20). However, there were limited IFN- γ responses to both GAPDH and WCL.

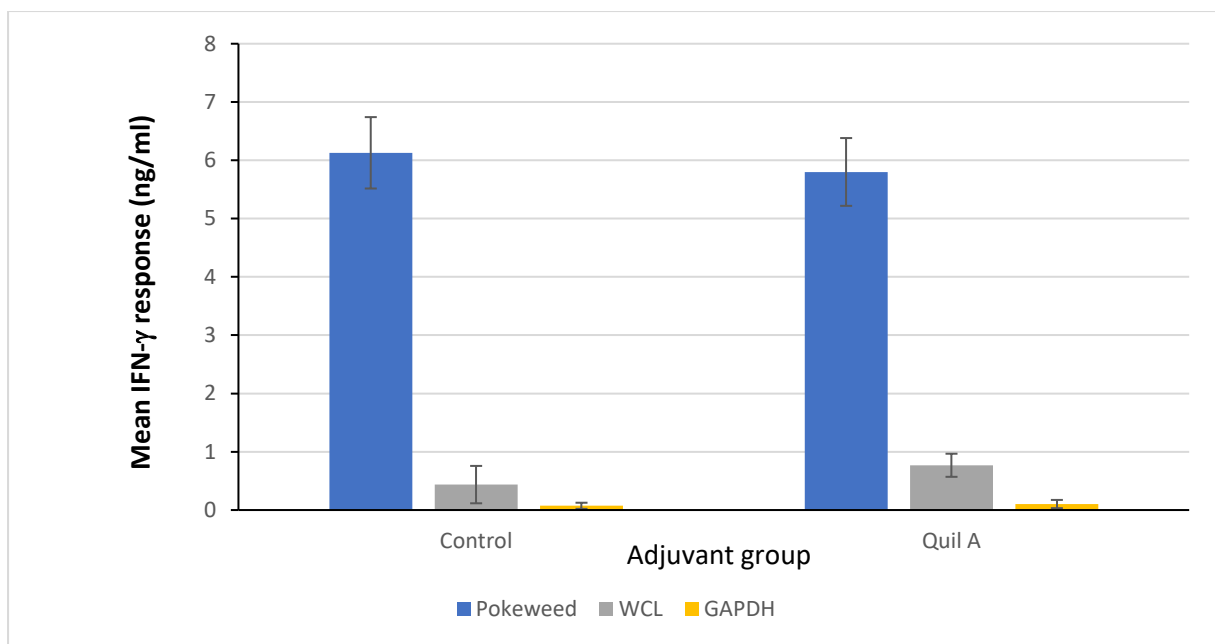


Figure 5-20 Mean ($\pm$ SE) IFN- γ responses in whole blood cultures of sheep (n=12) vaccinated with a mixture of whole cell lysate from *M. ovipneumoniae* isolates 16, 90, 103 and *M. haemolytica*, either alone (control) or in combination with an adjuvant (Quil-A).

Responses were measured against *M. ovipneumoniae* WCL or recombinant GAPDH. Responses against Pokeweed mitogen was used as positive control. Results are expressed as ng/ml.

5.3.5 IL-17A

Animals vaccinated with the individual recombinant proteins showed limited IL-17A responses (PBS-corrected OD₄₅₀ <0.5) as observed in whole blood cultures stimulated with WCL or HPF from *M. ovipneumoniae*, or the specific recombinant protein the animal was vaccinated with (Fig. 5.21). The Pokeweed mitogen elicited a strong response indicating that the lymphocytes in the cultures were able to successfully proliferate in the presence of a mitogen.

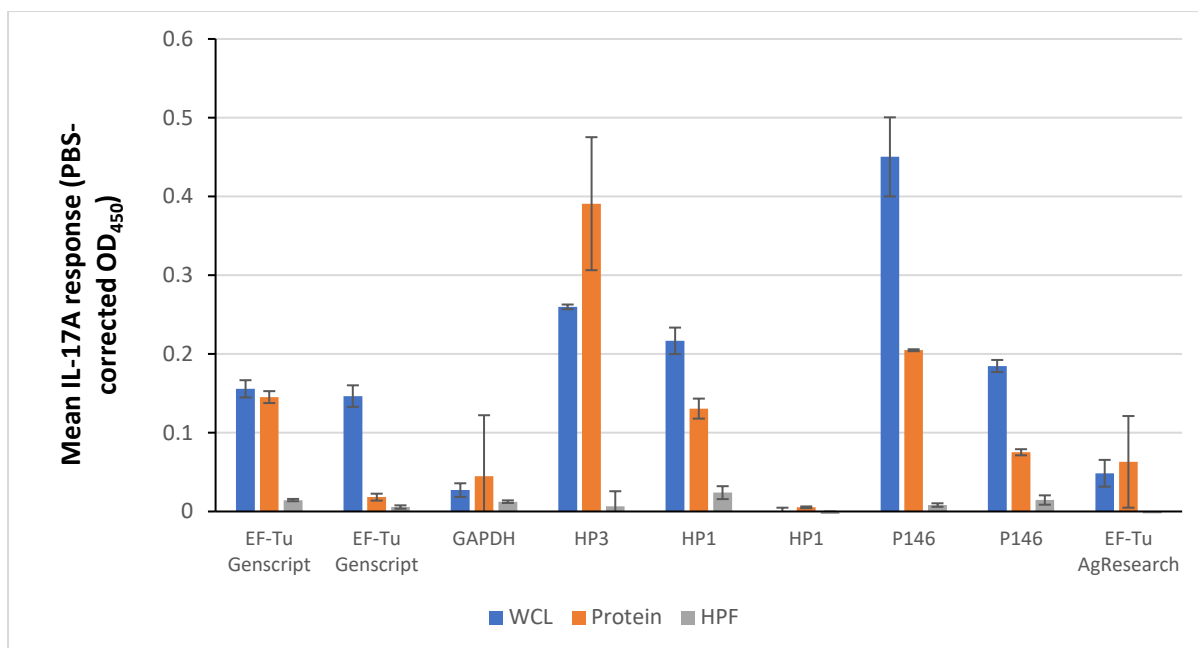


Figure 5-21 Mean ($\pm$ SE) IL-17A responses in whole blood cultures of sheep vaccinated with recombinant proteins EF-Tu, GAPDH, HP3, HP1, P146, or In-house EF-Tu. All responses were measured in duplicate.

Responses were measured against WCL, the recombinant protein the animal was vaccinated with, or HPF. Responses against Pokeweed mitogen was used as a positive control (not shown). Results are expressed as PBS-corrected OD₄₅₀ values.

Animals vaccinated with either WC or HPF of *M. ovipneumoniae* isolates 90 and 103 produced IL-17A responses similar to those observed in animals vaccinated with specific recombinant proteins. Two animals vaccinated with WC of *M. ovipneumoniae* isolate 16 produced a large response to GAPDH. WCL induced a stronger IL-17A response in animals vaccinated with WC of isolates 16, 90, and 103 than that observed for IFN- γ responses (Fig. 5.22).

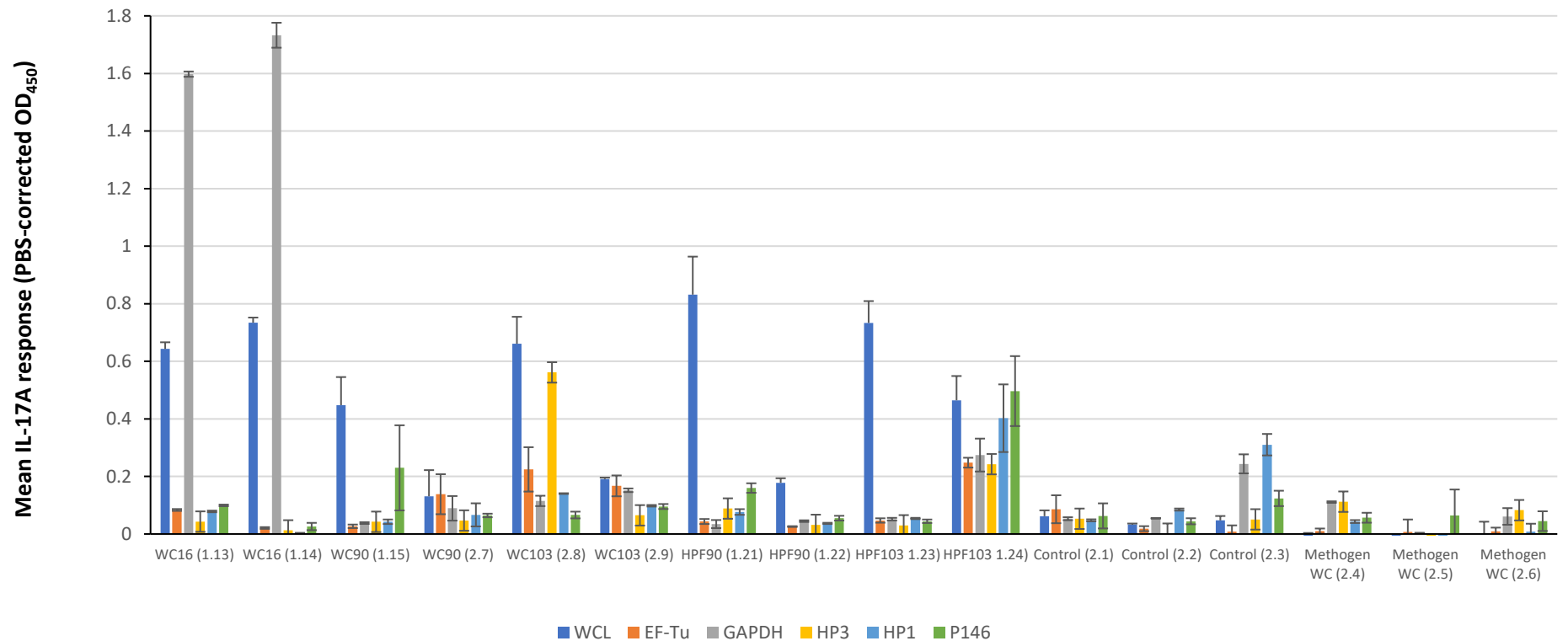


Figure 5-22 Mean ($\pm$ SE) IL-17A responses in whole blood cultures of animals vaccinated with whole cell lysates (WCL) and hydrophobic protein fractions (HPF) of *M. ovipneumoniae* isolates 16, 90 and 103, adjuvant only (negative control), and WCL of *M. ruminantium* M1. All responses were measured in duplicate.

Responses were measured against WCL or recombinant EF-Tu, GAPDH, HP3, HP1, P146, or Inhouse EF-Tu. Responses against Pokeweed mitogen was used as a positive control (not shown). Results are expressed as PBS-corrected OD₄₅₀ values.

In trial 2, three animals vaccinated with whole cell antigens from *M. ruminantium* M1 were used as an unrelated microorganism control (Fig. 5.22). All animals produced a strong response to the Pokeweed mitogen positive control (OD₄₅₀ between 2.38 and 2.5). Except for moderate responses in one of the adjuvant control animals against HP1 and GAPDH (0.31 and 0.24, respectively), there was limited IL-17A response from the other animals against *M. ovipneumoniae* antigens.

The animals from the adjuvant trial were also tested for IL-17A response to WCL from *M. ovipneumoniae* and recombinant GAPDH (Fig. 5.23). All the sheep produced strong responses to pokeweed mitogen. The group that produced the strongest IL-17A response was group 2 (animals administered a mixture of WCL of *M. ovipneumoniae* isolates 16, 90, 103 and *M. haemolytica*, together with adjuvant Quil-A), this resulted in a WCL response of 0.295 and a GAPDH response of 0.410.

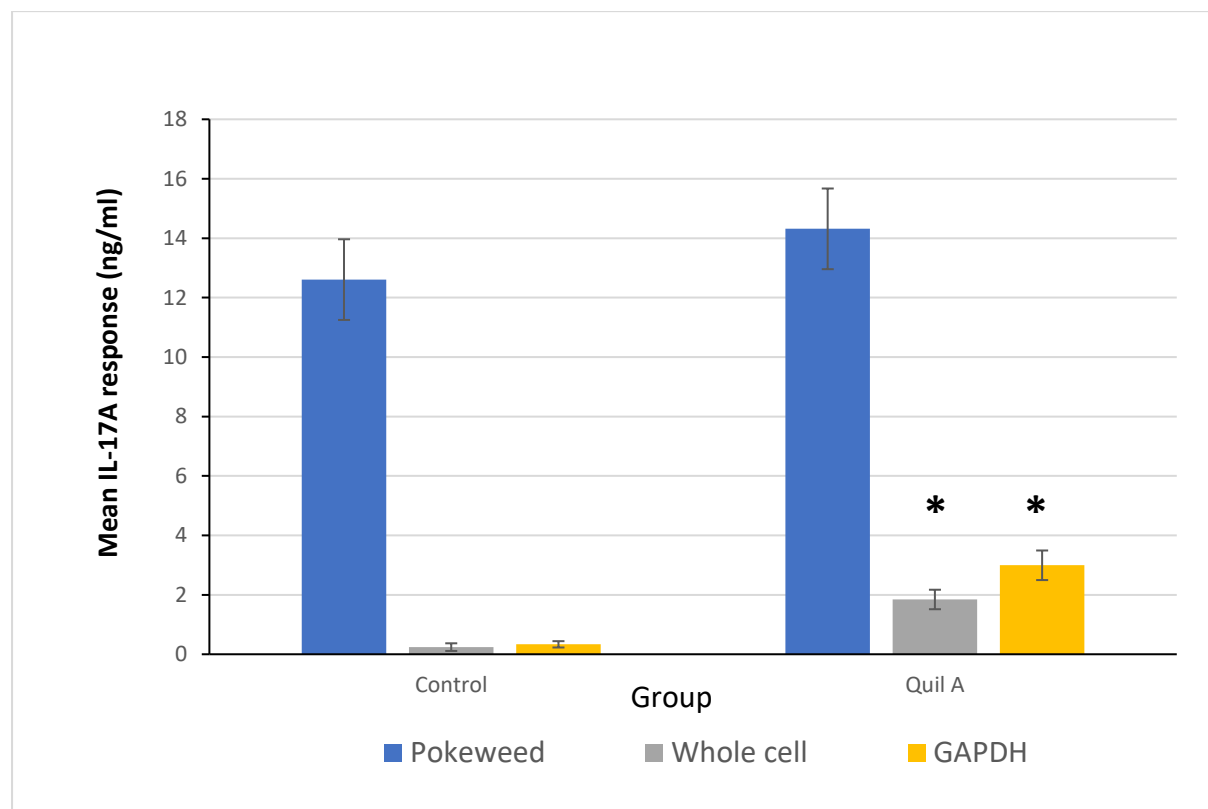


Figure 5-23 Mean ($\pm$ SE) IL-17A responses in whole blood cultures of sheep (n=12) vaccinated with a mixture of whole cell lysate (WCL) from *M. ovipneumoniae* isolates 16, 90, 103 and *M. haemolytica*, either alone (control) or in combination with an adjuvant (Quil-A).

Responses were measured against WCL or recombinant GAPDH. Responses against Pokeweed mitogen was used as a positive control. Results are expressed ng/ml. * Statistically higher compared to control (P<0.05).

5.4 Discussion

Since the use of cowpox to prevent smallpox infections, humans have been using antigens to jump start the adaptive immune system, taking advantage of the specific memory of the adaptive immune system. The use of vaccination can provide protection to animals against pathogens before encountering them, reducing the time an animal will be infected and limiting the damage caused. Determining the best antigens to use as a vaccine; however, can be a difficult process. The first step in developing a vaccine is identification of proteins or other antigens that induce strong adaptive responses. This chapter looked at the potential immunogenicity of five antigens from *M. ovipneumoniae*.

5.4.1 Recombinant protein production and Western blot analysis

The production of recombinant proteins is an invaluable tool in proteomics research. Compared to native proteins they have two main advantages: 1) more flexibility regarding which and how much recombinant protein is produced, and 2) purified recombinant proteins are often much cheaper and easier to produce than purifying native proteins. The proteins were produced using *E. coli* codon preference, to prevent the reading of the tryptophan codon as a stop codon. One of the proteins, EF-Tu was successfully produced in-house by firstly using PCR to alter a single codon recognised as a stop codon in *E. coli*. SDS-PAGE gel analysis of the EF-Tu produced in-house, and the proteins produced by GenScript confirmed that each protein had the expected molecular weight. The in-house produced EF-Tu had several additional bands separate from the expected size of EF-Tu. Western blotting (using pooled sera against *M. ovipneumoniae* isolates 16, 90, and 103) showed antibody reactivity with mainly one protein band but there were still additional bands detected by the sheep sera unrelated to the recombinant EF-Tu. One explanation is the bands are some remnant *E. coli* proteins from the purification that the sheep have previously encountered [332].

Alternatively, as most of the bands were smaller in size than EF-Tu, they could be incomplete versions or degraded forms of EF-Tu. These proteins could be produced by mechanisms such as incomplete production of the protein or proteolysis. Incomplete production is unlikely to be the cause because the his-tag used to purify the protein is located at the end of the protein, therefore an incomplete sequence is unlikely to be purified. Proteolysis; however, could affect complete proteins removing parts of the N-terminus of the protein [332]. Western blotting with anti-his antibodies would distinguish between bacterial proteins and EF-Tu derivatives. Due to the concentration difference between EF-Tu and these other proteins it is considered unlikely to affect studies using the EF-Tu.

All the recombinant proteins induced an antibody response in sheep. Western blotting using pooled sera from sheep vaccinated with a mixture of *M. ovipneumoniae* Isolate (16, 90, 103) and *M. haemolytica* and sera from individual sheep vaccinated with *M. ovipneumoniae* alone reacted both

with the recombinant proteins and against protein bands of the expected molecular size in the WC and HPF fractions. Western blotting using the sera against *M. ovipneumoniae* alone produced a much stronger response to the recombinant proteins than that of the pooled sera which had antibodies against both *M. ovipneumoniae* and *M. haemolytica*. This may be due to the addition of *M. haemolytica* whole cell antigens in the vaccine which may have reduced the *M. ovipneumoniae* specific immune responses [333]. The vaccine used to produce the pooled sera contained 0.2 mg of *M. ovipneumoniae* WCL whereas the vaccine containing *M. ovipneumoniae* alone had 0.6 mg of *M. ovipneumoniae* WC. This 3-fold higher dose may have resulted in higher titre of anti-*M. ovipneumoniae* being produced [334-336].

Western blotting detected differences between the expected band size and the observed band size for HP1 (Fig. 5.11-5.12) and HP3 (Fig. 5.13). The recombinant HP1 was produced from a 129 amino acid epitope of the whole protein and therefore would be significantly smaller (~39 kDa) than the natural protein, ~95 kDa. This fragment also had a large GST tag attached and because of the large difference between the full-size protein and the fragment there was no corresponding band in the WCL at the expected size. The reason for the band not being present is unknown. It could be due to the size of the protein i.e. a larger protein not being easily detected or transferred to the Western blot membrane. Another explanation could be that the epitope used for vaccinating the sheep did not produce antibodies able to interact with the full-size protein.

In the case of HP3, WCL from isolate 103 identified a protein band of a smaller molecular weight than that detected by WCL from isolates 16 and 90. The band detected in isolate 103 was also smaller than that detected by the recombinant protein produced by GenScript. Analysis of the nucleotide and peptide sequence from Isolate 103 from the genome library produced in Chapter 3, for this protein did not indicate any internal stop codons to account for the truncated protein. As the protein function is not known it is unclear what kind of post translational modifications are likely to occur and if this may be the cause of the truncation; however, a number of protein modification pathways have been observed in mycoplasmas including protein cleavage [337-339]. The effect of the truncated protein on the efficacy of this protein to be used in a potential vaccine or as a diagnostic protein may not be a problem because it was detected in a Western blot with antisera raised against the full length-protein. This suggests that the truncated protein is sufficiently similar to the full-length protein and has not had significant frame shifts within the protein.

Western blotting was completed using antisera from the animal vaccinated with the in-house EF-Tu against *M. ovipneumoniae* WCL and the recombinant EF-Tu produced by GenScript (Fig. 5.16). This detected a protein band in three WCL (isolates 16, 90, and 103) and EF-Tu (GenScript) lanes,

corresponding to the expected molecular weight (~45 kDa) for EF-Tu. This provides support for the correct editing and production of the inhouse EF-Tu protein.

5.4.2 Antibody responses to recombinant proteins

It has been suggested that infections of *M. ovipneumoniae* induce limited immune responses in sheep [158, 159]. It has also been previously shown that there is a dramatically reduced induction of antibodies in sheep that were previously exposed to *M. ovipneumoniae* compared to naïve animals, when vaccinated against this bacteria [158, 159]. It has been demonstrated in the current study that the five studied proteins of *M. ovipneumoniae* are immunogenic in sheep. This is an important finding in the continuing research into preventing and treating infections of *M. ovipneumoniae*. By detecting specific proteins that can elicit an immune response, it may be possible to provide a directed immune response against *M. ovipneumoniae* overcoming the hindrance of pre-existing anti-mycoplasma immune responses [158, 159] by focussing immune responses to the novel targets. ELISA were performed on sera from each of the animals vaccinated with the recombinant proteins (Table 5.2). All animals that were vaccinated with the recombinant proteins showed higher antigen-specific responses (compared to those in pre-immune sera) at boost (week 3) as well as post-boost (week 6), confirming the humoral immunogenicity of all five proteins. Comparison of the ELISA and Western blots for EF-Tu confirmed the strong antibody response to EF-Tu. The absence of any other significant immunoreactive bands in the Western blots indicates that the response to EF-Tu is due to the vaccination with the recombinant protein and not due to a previous or current mycoplasma exposure. EF-Tu in conjunction with HSP70 from *M. ovipneumoniae* has been previously shown to be immunogenic in mice [160]. *M. ovipneumoniae* HSP70 has also been shown to be immunogenic in sheep [194]. This is the first report of *M. ovipneumoniae* EF-Tu being immunogenic in sheep further supporting the findings in mice presented by Jiang et al (2016) [160].

HP1 antigen was produced as a 129 amino acid long epitope determined from the B-cell epitope analysis performed in chapter 4. This epitope was able to produce an immune response in the two vaccinated animals; however, the response was more variable than that observed for the other antigens. As this antigen was a fragment of the whole protein, either a different epitope of the protein or a larger fragment containing the selected epitope may induce a stronger and more consistent immune response. The Western blotting clearly indicated a response to the recombinant protein but did not distinctly identify the native protein in WCL. There was, however, a detection of high molecular weight mycoplasma proteins suggesting that the animals vaccinated with HP1 had previous exposure to mycoplasmas.

HP3 had a similar immunogenic response to that observed with EF-Tu with an increase in antibody response after the first vaccination but no further increase after the boost. However, as with GAPDH this observation is based on results from a single animal and further analysis of this protein will be

required to determine if the response observed is indicative of the immunoreactivity of this antigen. Further analysis will also be required to determine the long-term immune response from this antigen. Comparison with the Western blot images suggest a strong specific response to HP3 due to vaccination.

Protein P146 produced a good response from the initial vaccination. However, at week 6 (2 weeks after second vaccination) both animals showed a decrease in antibody response. The use of this protein as a potential vaccine antigen will require long term studies using a larger group of animals to determine if the decline in immune responses continues. Western blot analysis suggests that the animals vaccinated with P146 were previously exposed to mycoplasma, with animal 1.10 showing quite a strong response to the other mycoplasma proteins. Despite the strong ELISA response, the Western blots did not have a strong response to P146 in either the recombinant protein or in the WCL lanes. Due to the large size of the protein, there may be a transfer limitation of native protein to the PDVF accounting for the apparent limited response.

The animals that were vaccinated with WC from isolate 16, 90, and 103 and HPF antigens from isolate 90 and 103 were tested against each of the recombinant proteins. The two animals vaccinated with WC of isolate 16 showed a strong response to EF-Tu and GAPDH in the pre-immune sera. This may suggest that these animals could have been exposed previously to *M. ovipneumoniae* or other mycoplasma and there were pre-existing antibodies. This high response did not remain after the first vaccination and decreased to less than half. This result was different for HP3 and P146, where the response in pre-immune sera were minimal, but substantially after vaccination. This response to HP3 and P146 was also replicated in one of the animals vaccinated with isolate 90 and in both animals vaccinated with isolate 103. Animals vaccinated with HPF of isolate 90 showed limited increase in response to each of the recombinant proteins after vaccination except for HP3 which had a strong response after the first vaccination. Animals vaccinated with HPF from isolate 103 did produce a response to EF-Tu, this response was also observed for one of the animals vaccinated with HPF (from isolate 103) to GAPDH. The lack of increasing response to the recombinant proteins from animals vaccinated with HPF does not suggest that HPF will provide protection against *M. ovipneumoniae* infection.

The strong antibody responses to P146 and HP3 provide a potential avenue to use these proteins for a standardised diagnostic test. The Western blot of HP3, indicates that isolate 103 has a different protein size for HP3, this in combination with the ELISA data for animals vaccinated with isolate 103 suggests that this recombinant protein may be useful across different strains even if there is slight genetic difference between strains. Interestingly comparing the pre-immune and the antisera Western blots for P146 did not detect the recombinant protein in the pre immune Western blot

despite other *M. ovipneumoniae* antigens being detected. If the antigens detected are due to cross reactivity from exposure to other mycoplasma species, such as *Mycoplasma arginini* [248, 340-344], the lack of P146 detection in the pre-immune sera provides evidence for the use of this protein for diagnostic detection of *M. ovipneumoniae*. This was also observed in the ELISA data, despite the Western blots detecting *M. ovipneumoniae* antigens. ELISA against P146 did not detect a response to this protein in pre-immune sera. Collectively these two observations provide evidence for the specificity of P146 for the detection of *M. ovipneumoniae* in sheep. P146 has recently been suggested as a PCR target for detection of *M. hyopneumoniae* in pigs and it did not detect many other bacteria present within pigs including *M. arginini* [345].

To determine the ability of the recombinant proteins, HP3 and P146 to detect mycoplasma infections using antibody ELISA, sera from the 12 animals from the adjuvant trial were tested against each of the proteins. The animals that were vaccinated with antigen in the absence of adjuvant produced a limited antibody response against both HP3 and P146 (Fig. 5.17). This result can be explained by the lack of adjuvant in the vaccination resulting in a lower antibody response overall to *M. ovipneumoniae*, with fewer antibodies produced against HP3 and P146. The animals that had the adjuvant Quil-A® in the vaccine produced a stronger response when tested against P146 and HP3. This provides support of the use of the recombinant proteins being investigated further to ascertain the practicability of using these proteins as a diagnostic target. ELISAs using WCL as the targets were conducted as a comparison to the recombinant proteins. ELISAs using the WCL showed a more consistent detection of *M. ovipneumoniae*. This is likely due to the number of potential targets available when using whole cell as the target antigen. Additionally, animals vaccinated with only *M. haemolytica* were used to test each of the recombinant proteins for specificity against *M. ovipneumoniae*. As expected, animals vaccinated with only *M. haemolytica* showed a minimal antibody response to each of the *M. ovipneumoniae* recombinant proteins. This lack of cross-reactivity supports the use of the recombinant proteins for diagnosis of *M. ovipneumoniae*.

One of the major obstacles in treating mycoplasma disease is having an efficient and practicable test for determining the infection status of animals. There are multiple approaches for trying to determine the infection status of *M. ovipneumoniae*; however, many of the established methods are expensive and/or time-consuming requiring specialised lab equipment or expertise [69, 158, 160, 232]. The main methods are pathogen diagnosis/culturing, ELISA, and PCR [69, 158, 160, 232]. While these methods can provide high sensitivity to mycoplasma infections, they are not very practicable for on farm analysis or wildlife management. Studies are being completed to provide alternative methods, such as LAMP-LFD [346]. This method combines loop mediated isothermal amplification (LAMP) which produces small PCR products at 60°C in an hour, and lateral flow dipstick (LFD), which

allows visual identification of the PCR product without any hazardous chemicals or specialised equipment. While this method provides potential increased sensitivity and quicker turnaround time for results, its major limitation is the current need for DNA extraction which does require specialised kits and equipment that may be common to many labs but are less likely to be available to farmers and wildlife management. This limitation appears to be common to this technology [347-350].

Alternatively, given the observed ELISA responses to P146 and HP3, there may be an opportunity to produce an antibody-based test with. Compared to LFD, which detects an analyte such as antigen or DNA, a Flow through assay (FTA) can be used to detect antibodies in a similar manner using a specific antigen bound to a membrane [351, 352]. Because the test uses antibodies from the animal it may be less sensitive than LAMP-LFD; however, because it can test antibodies directly it does not require a pre-step such as DNA extraction providing the potential for a contained on-site test requiring no equipment and the complete test can be completed within 10 min. One aspect of this test that will require optimisation is the prevention of false positives by reducing the background response in the test. The use of recombinant proteins as the target can work to reduce the level of background by specifying the antibodies being detected. This was observed in comparing the ELISAs using WCL as the targets. The PBS-corrected OD₄₅₀ values for the WC were 0.402 on average, compared to 0.103 and 0.078 for P146 and HP3 respectively, therefore showing much lower nonspecific binding to the recombinant proteins. In ELISAs the values can be PBS-corrected to account for the background values however, this could not be possible in an FTA as the test is a binary test. To reduce the background of the WCL ELISA the antigen concentration could be reduced; however, this would also reduce the response at the high end potentially reducing the sensitivity of the test. Another limitation of using WCL as the antigen is the specificity of the antigens present. The ELISAs performed here used the same strains as was used in the vaccinations, this increases the likelihood that the ELISA will detect antibodies in these trials, but it may not correlate as well to natural infections with mixed strains or use in different countries with different strains. Also as observed with the Western blots and ELISAs on the animals vaccinated with P146. Western blots showed a response to *M. ovipneumoniae* proteins in the pre-immune sera whereas this response was not detected in the ELISA data. If this difference is due to the cross reactivity of antibodies against another mycoplasma species to *M. ovipneumoniae* proteins, then the use WCL proteins of *M. ovipneumoniae* as a point of care diagnostic tool may result in increased false positives due to exposure to other mycoplasma species. Further testing would need to be completed to determine the full extent of cross reactivity of antibodies between mycoplasma species.

This is where the use of specific proteins conserved among all strains could provide better widespread detection of *M. ovipneumoniae*. A better method to increase the response to the

recombinant test could be to use a combination of recombinant proteins. This would allow the test to keep the consistency of accurate concentration of proteins and the improved response by having multiple targets. This was tested by combining the P146 and HP3 proteins into a single ELISA. There was a reduction in response to P146 in animals vaccinated using Quil-A in the week 6 sera; however, animals vaccinated in the absence of an adjuvant showed an improvement when tested against both antigens compared to being tested individually. Finding further *M. ovipneumoniae* proteins that elicit a strong immune response will be the key to developing a robust diagnostic tool as well as further development work with combinations of P146 and HP3 (different ratios etc) and different ELISA methods such as using titration ELISAs.

The potential of these proteins to be useful in an FTA test will be dependent on the ability to collect antibodies from the animal. Antibodies are normally collected from an animal by preparing serum from blood, this requires drawing blood, allowing the blood to clot and then a centrifugation step to separate the serum from the blood cells. This pre step, like in the LFD makes it difficult to do on site and adds specialised equipment required for the test; however, antibodies can also be collected at lower levels from other orifices. If it can be determined that there are enough antibodies in the nasal cavity to be collected from a nasal swab there is potential for a direct FTA using nasal swabs. Saliva also contains antibodies to pathogens as demonstrated by the Carla assay developed by AgResearch for the detection of protective immunity to ingested parasite larvae [353]. Additionally, antibodies can be collected from tear fluid [354-356]. If antibodies against *M. ovipneumoniae* can be detected in tear fluid and tear fluid is easily and consistently collectable from sheep it could be another avenue for developing a direct FTA. This would allow for a test for the presence of mycoplasma in sheep to be completed on farm within a matter of minutes for each animal.

This functionality may be most useful in the management of wild bighorn sheep, as they appear to be the species most severely affected by *M. ovipneumoniae*, resulting in population limiting disease. Recent research into bighorn sheep suggests that the removal of chronic sufferers of *M. ovipneumoniae* in the populations could help prevent the loss of the population [250]. Having a test that can be completed quickly in the field could allow wildlife managers to immediately treat or remove infective animals from the population while limiting the time interacting with the animals. Confirmation of the immunogenicity of each of the recombinant proteins in sheep provides support for the methodology and techniques undertaken in chapter 4. The use of antisera against *M. ovipneumoniae* for the detection of immunogenic proteins has been shown to be successful, producing five proteins able to induce an immune response in sheep. Additionally, the recombinant form of the proteins were able to be detected by antisera against *M. ovipneumoniae* (Fig. 5.3). Vaccination with the recombinant proteins EF-Tu, GAPDH, and HP3 was also able to detect the

native protein from WCL in Western blots providing support for the use of recombinant proteins to induce immune responses to native proteins.

5.4.3 T-cell responses to recombinant proteins

IFN- γ

In response to intracellular pathogens T_H1 cells begin to proliferate and release targeted cytokines. IFN- γ released from T_H1 cells upregulates macrophage activation resulting in phagocytosis and digestion of intracellular bacteria and protozoa [98-100]. IFN- γ levels can be used as a measure for cellular response to intracellular microorganisms. IFN- γ analysis was done using whole blood from the final bleed (week 6). Animals vaccinated against the recombinant proteins produced limited release of IFN- γ in response to stimulation with WCL of *M. ovipneumoniae*, or the same recombinant protein the animal was vaccinated with. The highest response detected was observed with blood from one of the two sheep vaccinated with P146 protein, following stimulation with P146 protein. Overall, the results suggests that the proteins in this study are unlikely to induce significant cellular immunity in sheep.

There was limited IFN- γ response in animals vaccinated with WC of *M. ovipneumoniae*, except for two animals, vaccinated with WC from isolate 16, gave high responses to GAPDH. Interpretation of this result should be done with caution because of the limited number of animals producing this response. To further elucidate the potential of GAPDH to be used as a cellular based diagnostic antigen for detection of mycoplasma infections, GAPDH was included as a recall antigen for stimulation of blood collected from the 24 animals in the adjuvant trial (5.7.5.3). Minimal responses were observed to both WC and GAPDH at week 0 (data not shown). GAPDH did not produce the level of antigen-specific IFN- γ expression observed in the two in the earlier trial. The GAPDH-specific IFN- γ responses were lower than the corresponding responses from incubating with *M.*

ovipneumoniae WCL antigens. These results suggest that using GAPDH to measure IFN- γ responses would not be useful as a diagnostic tool to identify infection with *M. ovipneumoniae*.

Six animals in Antisera trial 1 were vaccinated with HPF of *M. ovipneumoniae* isolates 16, 90, and 103. Of the four animals that survived to week 7 and had their IFN- γ responses measured, all produced minimal IFN- γ responses, with only the WCL antigens producing moderate response. This suggests that the HPF did not stimulate significant cellular immunity.

To determine the specificity of the recombinant proteins to mycoplasma infections, three sheep were vaccinated with adjuvant only while another three animals were vaccinated with an unrelated bacterium *M. ruminantium* M1. All animals failed to produce an IFN- γ response. This is the expected result and suggests that the various recombinant proteins will not have an issue with cross-reactivity or producing false positive results. There were a few animals (Control 2.3, Methanogen WC 2.4, and

Methanogen WC 2.6, Table 2.6) that responded slightly to WCL of *M. ovipneumoniae* suggesting that they may have had previous exposure with *M. ovipneumoniae* or other mycoplasma.

IL-17

Another response from the cellular immune system is the T_H17 inflammatory response. Where T_H1 responds to intracellular pathogens, T_H17 responds to extracellular bacteria and fungi [325, 357]. The release of IL-17A from the T_H17 cells can induce many inflammatory cytokines IL-1 β , TNF- α , IL-6, and IL-23 [358]. Having IL-17A as the key inducer allows for direct measurement of T_H17 response by measuring IL-17A levels.

Animals vaccinated with the recombinant proteins induced limited antigen-specific IL-17A responses. The animal vaccinated with HP3 (animal 1.5) and P146 (animal 1.9) produced an elevated response compared to the other proteins. However, with a single animal vaccinated with HP3 and the other animal vaccinated with P146 not producing the same IL-17A response it is difficult to conclude anything about the ability of recombinant proteins to recruit T_H17 cells. Animals vaccinated with WC of *M. ovipneumoniae* showed an IL-17A response to WCL antigens however they showed limited response to the recombinant proteins. This may suggest that *M. ovipneumoniae* WC antigens are able to elicit an IL-17A response, however the proteins studied in this thesis do not. The one exception is the recombinant protein GAPDH, which when used as the recall antigen appeared to produce a strong response in animals vaccinated with WC16. This response was not observed in other animals vaccinated with the other isolates; therefore, the results must be taken with caution. To further assess the potential of GAPDH stimulated release of IL-17A in cultured blood as a cellular diagnostic, WCL and GAPDH-specific IL-17A production was measured in the twenty-four animals from the adjuvant trial. Minimal responses were observed to both WC and GAPDH at week 0 (data not shown). Animals vaccinated with *M. ovipneumoniae* and *M. haemolytica* with the adjuvant Quil-A[®] produced a significant response to both WCL *M. ovipneumoniae* and GAPDH which was not observed in the IFN- γ assay. While IFN- γ does not appear to be a good readout for a recombinant protein based diagnostic test for *M. ovipneumoniae* infections, a test that measures IL-17A may have potential. Measurement of this cytokine may be useful for bighorn sheep as it has been suggested that they have significant increase in IL-17A in bronchus-associated lymphoid tissue compared to domestic sheep in response to *M. ovipneumoniae* infection. In the bronchial/bronchiolar epithelium, domestic sheep saw a significant decrease in IL-17A with infection whereas bighorn sheep saw levels similar to uninfected domestic sheep [141]. This response confirms that big horn sheep have consistently higher IL-17A cytokine concentrations in response to *M. ovipneumoniae* infections compared to domestic sheep. An exacerbation of IL-17A in bighorn sheep resulting in heightened neutrophil recruitment may in part explain the increased morbidity and mortality of this breed of sheep [116, 141]. Measurement of GAPDH-specific IL-17A release in blood cultures of sheep,

particularly bighorn sheep naturally infected or experimentally challenged with *M. ovipneumoniae* will help determine the utility of this cytokine-based assay as a diagnostic tool for infection.

Determining the infection status as well as the levels of IL-17A present in bighorn sheep may allow for efficient treatment and reduction of IL-17A. This may prevent further chronic effects of *M. ovipneumoniae* infections and reducing population limiting disease [116, 141].

Two sets of controls were used to determine the baseline of IL-17A responses from animals not vaccinated against antigens and animals vaccinated against unrelated pathogens. All animals except for one (control 2.3) produced the expected minimal IL-17A response. Control 2.3 (Fig. 5.22) had an elevated response to HP1 and GAPDH. This may be due to a natural interaction with *M. ovipneumoniae* in this animal. The limited IL-17A response to animals vaccinated with methanogen antigens does suggest any responses to these proteins is due to interactions with *M. ovipneumoniae* rather than against other bacteria.

Two of the proteins, P146 and HP3, have shown potential for the detection of *M. ovipneumoniae*. When combined into a single ELISA they showed improved response to anti-*M. ovipneumoniae* antibodies from animals vaccinated with and without adjuvants. This provides support for the use of recombinant proteins as a diagnostic tool. The addition of more recombinant proteins in the ELISA may increase the sensitivity of the test.

5.4.4 Significance of the five proteins (EF-Tu, GAPDH, HP3, P146, and an epitope of HP1)

To provide protection for an animal to a particular infection a vaccine must be able to induce a strong, long lasting immune response. All five proteins used produced a strong antibody response confirming these proteins are immunogenic in sheep. *M. ovipneumoniae* EF-Tu has been shown to be immunogenic in mice [160]; however, this is the first report, that EF-Tu is immunogenic in sheep. The five proteins were also assessed for their ability to induce cellular immune responses by measuring release of the cytokines IFN- γ and IL-17A in antigen-stimulated blood cultures. None of the recombinant proteins induced an IFN- γ response, suggesting that when used in a vaccine these proteins would not induce significant cell-mediated protective immunity to *M. ovipneumoniae* infection. There is limited evidence that *M. ovipneumoniae* is an intracellular pathogen [159] and therefore the lack of IFN- γ response may not be of concern when determining the significance of these proteins as vaccine candidates. There was also limited IL-17A response generated against the recombinant proteins. While this may seem like a disadvantage for the use of these proteins, it has been suggested that bighorn sheep who are the most severely affected by *M. ovipneumoniae* [141], appear to be susceptible to over expression of IL-17A. Therefore, a vaccine target that does not induce IL-17A may have some advantages for broad use across domestic sheep and bighorn sheep. As a potential diagnostic tool, GAPDH-specific IL-17A showed some promising results in animals vaccinated with *M. ovipneumoniae* and *M. haemolytica* WC. This may be useful in observing the IL-

17A response to *M. ovipneumoniae* infections in Bighorn sheep, potentially detecting elevated IL-17A levels before chronic inflammation has occurred.

5.4.5 Vaccine methodology, limitations, and further research

In the current experiments it has been shown that all five proteins are immunogenic in sheep; however, their immunogenicity was limited to stimulating humoral immune responses. This response may be modified with the use of different adjuvants or through the use of immunomodulators such as CpG. When used as vaccine adjuvants, CpG oligodeoxynucleotides (ODN) improve the function of professional antigen-presenting cells and boost the generation of humoral and cellular vaccine-specific immune responses [359]. Determining the best adjuvant to use in addition to an antigen is an ongoing task, as emphasised by the need for undertaking the adjuvant trial mentioned in this chapter. Montanide was used in combination with the recombinant proteins due to the positive results of previous adjuvant trials using *M. ovipneumoniae* and *M. haemolytica* (unpublished data). This; however, was determined using WCL of *M. ovipneumoniae* and may not necessarily extrapolate to recombinant proteins. Because of this, repeating the recombinant antigen trial with other adjuvants such as squalene-based immunologic adjuvants [360-362], emulsigen [363], polydi(sodium carboxylatoethylphenoxy)phosphazene (PCEP) [363, 364], or trehalose diamide glycolipids [365] may provide increased immunogenic response to the recombinant proteins and potentially increased protection to disease. Additionally, the concentration of antigen within the vaccine can have an effect on the immune response. Low antigen doses may be insufficient to induce an immune response, whereas too high a dose can result in hypersensitivity reactions [366]. Other recombinant protein vaccines in sheep have used a range of 50-200 µg per dose [367-370]. This study used 100 µg which is within the range of the other trials; however, there is room for further increased protein concentration. Further trials may be needed at varying concentrations to determine the ideal protein dose for vaccination. Vaccinations in this thesis were completed subcutaneously; however, other methods of administration are available, such as intramuscularly, or orally [371-374]. Determining the ideal mode of administration may improve the response to vaccination.

The number of animals and group size used in the antisera production trial was one of the major limitations in analysing the results as there were only two animals vaccinated with each of the recombinant proteins, and due to the losses caused by the animals contracting facial eczema, some of the recombinant proteins only had a single surviving animal. With sera from two (or one) animals it is difficult to separate the effect of the vaccination from the individual animal responses. The purpose of the trial was to collect sera against each of the recombinant proteins and therefore having multiple animals was not necessary for the trial. Increasing the number of animals in a larger study would help confirm a pattern of response observed in the original trial. Another limitation is

the lack of knowledge on the long-term effects of any of the vaccinations. The final collection of sera was done two weeks after the booster vaccination, at a time expected to be the maximum response. However, this does not provide insight into the long-term response to the vaccination and the corresponding protection, if any. Further trials allowing for longer term studies of immune responses in the animals could provide better understanding of the longer-term response to each of the recombinant proteins. Combining longer term study with challenge trials will provide insight in the protective effect, if any of the recombinant proteins.

As, discussed earlier, IFN- γ expression can decrease in response to mycoplasma infections [141] in domestic sheep. Collection of blood from animals at multiple time points, in longer-term trials aiming to study the kinetics of cytokine expression will provide further information on IFN- γ and also IL-17A responses to the recombinant proteins.

5.5 Conclusion

The five recombinant *M. ovipneumoniae* proteins (EF-Tu, GAPDH, HP3, P146, and an epitope of HP1) produced strong antibody responses in vaccinated sheep. The responses to in-house produced EF-Tu recombinant protein were similar to those produced by the commercially produced EF-Tu. Animals vaccinated with individual recombinant proteins produced minimal IFN- γ and IL-17A responses. Moderate to high IL-17A responses to GAPDH and WCL were evident in animals vaccinated with WC and HPF. This might provide an avenue for a cellular based diagnostic test for *M. ovipneumoniae*; however, further studies will be required to determine the ability of this test to detect an immune response during natural infections as well as determining if a test based on measurement of IL-17A is able to distinguish between active and past infection with *M. ovipneumoniae*. An IL-17A test may be more valuable in bighorn sheep rather than domestic sheep due to the relatively higher IL-17A responses in bighorn sheep and their observed sensitivity to elevated levels of IL-17A. Testing each of the proteins against animals vaccinated with WC of *M. ovipneumoniae* suggested two proteins, HP3 and P146 may have potential for use in an antibody based diagnostic test.

Chapter 6 *In vitro* studies with antibodies produced against recombinant *M. ovipneumoniae* proteins

6.0 Abstract

In Chapter 4, eight immunogenic proteins in New Zealand isolates were identified and chapter 5 described the vaccination of animals with 5 of these proteins produced as recombinant proteins. Blood and sera were collected from these animals and used to test the ability of the recombinant proteins to induce humoral and cellular immune responses in sheep. All these proteins were able to induce humoral responses; however, they induced limited cellular responses. To gain additional information on the antibodies produced against the recombinant proteins, their ability to inhibit growth and inhibit adherence of *M. ovipneumoniae* was investigated. Additionally, immunofluorescence was used to determine whether the proteins are located on the surface of the cell. There was evidence for EF-Tu and GAPDH being located on the surface of the cell. However hypothetical proteins 1 and 3 were not able to be detected by the corresponding antisera. Due to the high background in the mycoplasma cells probed with the pre-immune and antisera against P146, the location of the protein could not be determined. An endometrial epithelial cell line was used to study adherence of *M. ovipneumoniae* to epithelial cells. Antisera against WC of isolate 16 was able to inhibit adherence; however, antisera against the recombinant proteins were not able to inhibit adherence. Further optimisation of the inhibition assay will be needed to determine the role of antibodies against the recombinant proteins in inhibiting *M. ovipneumoniae*.

6.1 Introduction

Chapter 4 described the identification of immunogenic proteins from *M. ovipneumoniae* that have potential for use as vaccine antigens or for diagnosis of infection. Chapter 5 described the production of these proteins as recombinant proteins in *E. coli* and investigated immunological responses in sheep vaccinated with the proteins. These studies provided initial information about their utility as diagnostic proteins or vaccine antigens. The aim of the study reported in this chapter is to provide further understanding of the function of these proteins. This was undertaken by testing the ability of the protein-specific antibodies to prevent adherence of *M. ovipneumoniae* to bovine endometrial epithelial cells, used as a surrogate for sheep lung epithelial cells. The bovine endometrial epithelial cells were produced by Professor Michel A. Fortier (Université Laval, Québec). As no sheep lung model was available and the bovine endometrial epithelial cells were available in the lab they were considered as an alternative. Bovine cells are not the natural host for *M. ovipneumoniae*; therefore, they were initially tested for their ability to allow *M. ovipneumoniae* cells to adhere. Following successful demonstration of adherence, the experiments were expanded to test the effect of antibodies on the adherence ability of *M. ovipneumoniae* based on previous adherence studies on *M. genitalium* and *M. pneumonia* [225, 227].

Preventing infections from a pathogen is the optimal outcome when it comes to vaccination; however, that is not always possible because of lack of understanding of the pathogen, the immune response to the pathogen, or the pathogen's ability to avoid the host immune system [375]. Minimising the ability of a pathogen to colonise a host can be a desired outcome. The adherence of *M. ovipneumoniae* to the host cells is a major aspect in the pathogen's ability to cause disease, therefore the ability to disrupt the adherence of *M. ovipneumoniae* could help reduce the ability of *M. ovipneumoniae* to propagate [376, 377]. The adherence analysis tests the ability of antibodies produced against the recombinant proteins to prevent or reduce adherence of mycoplasma cells to an epithelial cell line. The antisera produced against each of the recombinant proteins and whole cell lysate of *M. ovipneumoniae* isolate 16 were tested in an adherence assay based on methods described by Hegde et al (2018) [227] and Svenstrup et al (2002) [225].

To confirm the expected localisation of each of the immunogenic proteins on the surface of the cell, indirect immunofluorescence microscopy [224-226] was undertaken using the antisera produced against each of the recombinant proteins. Comparing sera from before and after vaccination should allow the determination of the location of the recombinant protein as the antisera should contain antibodies against the recombinant proteins whereas the pre immune sera should not. Antibodies in the antisera that bind to the relevant antigen on the microbial cell surface can be detected by fluorescence under ultraviolet light, using a fluorescent labelled secondary antibody. Confirmation of surface localisation is important for proteins considered as antigens for vaccines. A surface located

protein, could be more targetable by antibodies and thus neutralising antibodies may be able to reduce the growth of pathogens or interfere with the adherence to host cells. This chapter describes a number of pilot studies designed and undertaken to test the ability of antibodies to protect against *M. ovipneumoniae* infections.

6.2 Methods

6.2.1 Determination of Colour Changing Units (CCU) to quantify *M. ovipneumoniae* growth

To determine the concentration of *M. ovipneumoniae* in culture, a Colour Changing Units (CCU) assay based on methodologies described by Calus et al (2010) [378] and Garcia-Morante et al (2018) [379] was conducted (details are included in chapter 2.15). *M. ovipneumoniae* creates an acidic environment as it grows and inclusion of a pH indicator such as phenol red in the growth media can indicate growth of mycoplasma via a colour change, from red to yellow.

6.2.2 Adhesion of *M. ovipneumoniae* to endometrial epithelial cells

The method was described in Chapter 2.16. Briefly *M. ovipneumoniae* isolate 16 was grown in Frey's media until pH indicator changed to orange (chapter 2.4). Cells from a 10 ml *M. ovipneumoniae* culture (CCU 10⁸) were centrifuged and washed with phosphate buffered saline (PBS) and resuspended in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% foetal bovine serum (FBS) (heat inactivated).

To quantify cell adhesion, epithelial cell monolayers growing in RPMI with 10% FBS and 5% penicillin (750 µl media/well) in a 24-well plate, were infected with the mycoplasma cell RPMI-suspension (50 µl/well) and incubated at 37°C with 5% CO₂ for 4 h. As a control, the mycoplasma cell suspension was incubated alone in cell culture medium in parallel wells to determine any adhesion of mycoplasma cells to the wells. To determine the effect of the endometrial epithelial cells on the CCU test, endometrial epithelial cells were incubated alone in wells. Following incubation, plates were washed three times with PBS to remove unbound mycoplasma cells. The endometrial epithelial cells were detached with TrypLE™ Express Enzyme (Gibco™ Invitrogen™, Thermo Fisher Scientific™, United States) and transferred to a CCU assay (chapter 2.15). Adhesion was confirmed by colour change in the CCU from wells with both endometrial epithelial cells and *M. ovipneumoniae* and absence of colour change in CCU from wells with only one of the cells.

For adhesion inhibition assays, the mycoplasma suspension (100 µl) was pre-incubated with diluted sheep pre-immune sera (week 0) or antisera (week 6) produced against the recombinant proteins (EF-Tu, GAPDH, HP1, HP3, and P146) at a ratio of 10:1, v/v for 1 h at 37°C. In parallel, dilutions of *M. ovipneumoniae* culture was also pre-incubated with *M. ovipneumoniae* whole cell-specific sheep antisera (10:1, v/v ratio). After preincubation, the mycoplasma-antibody suspension (100 µl) was

then added to the cell monolayer together with 700 µl of RPMI with 5% FBS and penicillin. The adhesion assay was carried out as described above. Each serum was tested in duplicate. Any effect of the respective antisera on the viability of *M. ovipneumoniae* was assessed by comparing CCU between the pre-immune and antisera. Wells containing endometrial epithelial cells and mycoplasma in the absence of any added sera were used as positive controls. A duplicate CCU assay was completed for each test, resulting in four CCU assays per sera.

6.2.3 Immunofluorescence assay for detecting *M. ovipneumoniae* antigens

To confirm the location of the selected proteins on the surface of the mycoplasma, *M.*

ovipneumoniae cells were incubated with pre-immune sera (week 0) or antisera (week 6) of each of the recombinant proteins (EF-Tu, GAPDH, HP1, HP3, and P146) as well as whole cells from isolate 16. Reactivity of the antibodies with surface located proteins were visualised using a FITC-labelled secondary antibody and mycoplasmas were observed under a fluorescence microscope (methods described in chapter 2.18).

6.3 Results

6.3.1 Determination of CCU

The CCU of *M. ovipneumoniae* isolate 16 was determined using three separate cultures, each measured in duplicate as described in Chapter 2.4. All cultures had a final colour change dilution of between 10^9 and 10^{11} . The averaged 10^{10} CCU/ml value was used for all subsequent cultures.

6.3.2 Adherence of *M. ovipneumoniae* to bovine endometrial epithelial cells

The results indicated adherence of *M. ovipneumoniae* to bovine endometrial epithelial cells in culture. *M. ovipneumoniae* was detected in wells seeded with endometrial epithelial cells after washing as indicated by a colour change in the subsequent CCU assay. To ensure *M. ovipneumoniae* was adhering to the epithelial cells and not the plate wall *M. ovipneumoniae* was incubated alone and tested after washing. No colour change was observed from these wells indicating *M. ovipneumoniae* was not adhering to the wells. Additionally, to ensure the epithelial cells did not cause a colour change in the CCU assay, epithelial cells incubated in absence of *M. ovipneumoniae* were tested in the CCU assay. No colour change was observed from these wells indicating the epithelial cells had no effect on the CCU assay.

The antisera produced against the *M. ovipneumoniae* recombinant proteins were then tested for their ability to inhibit the ability of mycoplasma cells to adhere to the endometrial epithelial cells. No observable difference was seen between pre-immune (week 0) and antisera (week 6) produced against the recombinant proteins (EF-Tu, GAPDH, HP1, HP3, and P146) or between wells containing sera and the control wells. The addition of antisera against WC16 did reduce the ability of *M. ovipneumoniae* to bind to the epithelial cells, with no mycoplasmas detected (by determination of CCU) after washing.

Table 6-1 Summary of CCU results from the adherence assay.

Each of the incubated wells were tested using the CCU analysis in duplicate.

Well (in duplicate)	Contains mycoplasma cells	Contains epithelial cells	Antibody used	Colour change	CCU/ml observed
Test	Yes	Yes	Nil	Yes	Not recorded
Control 1	Yes	No	Nil	No	Not detected
Control 2	No	Yes	Nil	No	Not detected
EF-Tu	Yes	Yes	Pre-immune	Yes	10 ²
EF-Tu	Yes	Yes	Antisera	Yes	10 ²
GAPDH	Yes	Yes	Pre-immune	Yes	10 ²
GAPDH	Yes	Yes	Antisera	Yes	10 ²
HP1	Yes	Yes	Pre-immune	Yes	10 ²
HP1	Yes	Yes	Antisera	Yes	10 ²
HP3	Yes	Yes	Pre-immune	Yes	10 ²
HP3	Yes	Yes	Antisera	Yes	10 ²
P146	Yes	Yes	Pre-immune	Yes	10 ²
P146	Yes	Yes	Antisera	Yes	10 ²
WC16	Yes	Yes	Pre-immune	Yes	10 ²
WC16	Yes	Yes	Antisera	No	Not detected

6.3.3 Immunofluorescence assay for detecting *M. ovipneumoniae* antigens

To determine if the proteins EF-Tu, GAPDH, HP1, HP3, and P146 are located on the cell surface, immunofluorescence was performed on *M. ovipneumoniae* isolate 16 cells using pre-immune (week 0) and antisera (week 6) produced against the various recombinant proteins. Binding of antibodies to proteins located on the surface of the cell was visualised using a secondary FITC-labelled antibody and the results are shown in Fig. 6.1-6.6. The microscopy photographs are representative fields of view of labelled cells at magnification 1000.

M. ovipneumoniae cells labelled with antisera against EF-Tu (GenScript), GAPDH, P146, and *M. ovipneumoniae* WC16 had stronger fluorescence signals compared to signals with matched pre-immune serum (Fig. 6.1, 6.2, 6.5, 6.6). Microscopy clearly showed the presence of labelled cells. Weaker signals were observed with antisera against HP1 and HP3 (Fig 6.3 and 6.4 respectively).

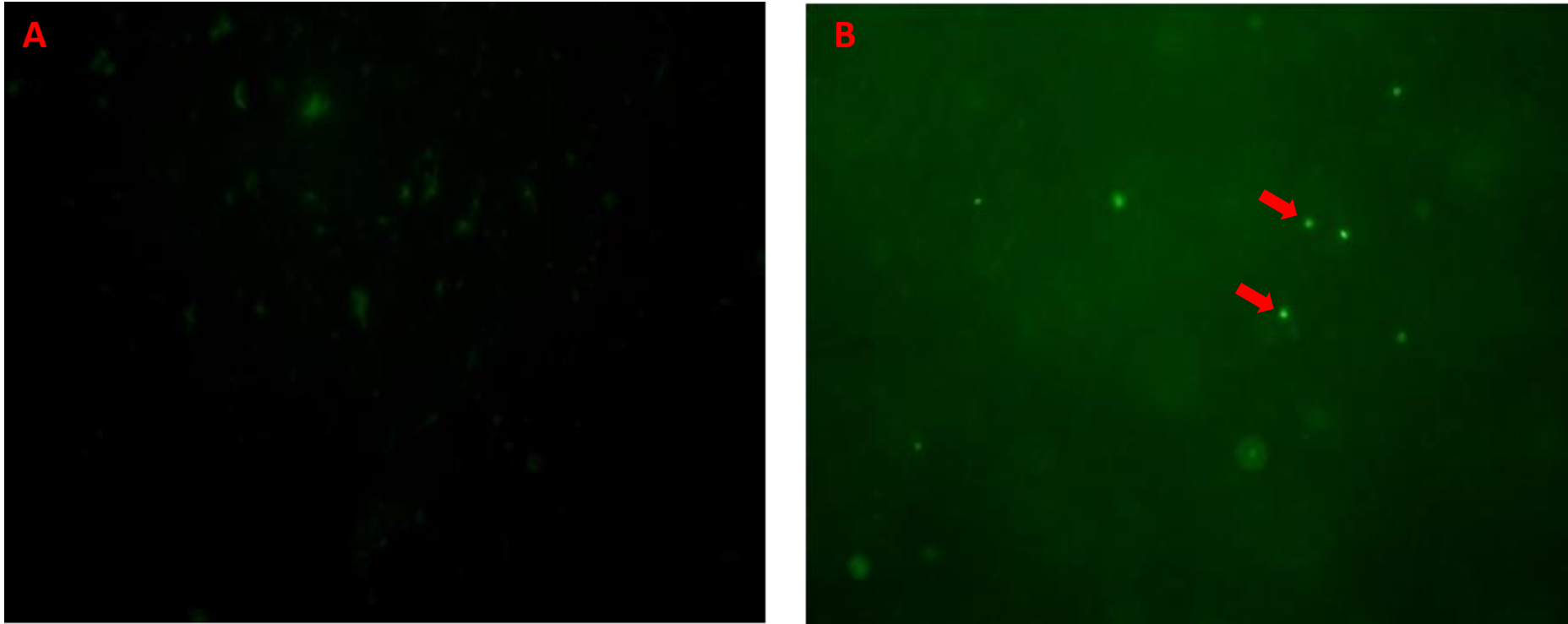


Figure 6-1 Immunofluorescence microscopy images using *M. ovipneumoniae* whole cells fixed with VECTASHIELD and probed with A) pre-immune sera or B) antisera (1:100 dilution) produced against recombinant EF-Tu and FITC-conjugated donkey anti-sheep IgG H&L (FITC) (1:400 dilution).

The excitation wavelength was 493 nm and emission wavelength 528 nm. The microscopy photographs are representative fields of view of labelled cells at magnification 1,000x and exposure of 100 ms. Red arrows indicate location of fluorescence.

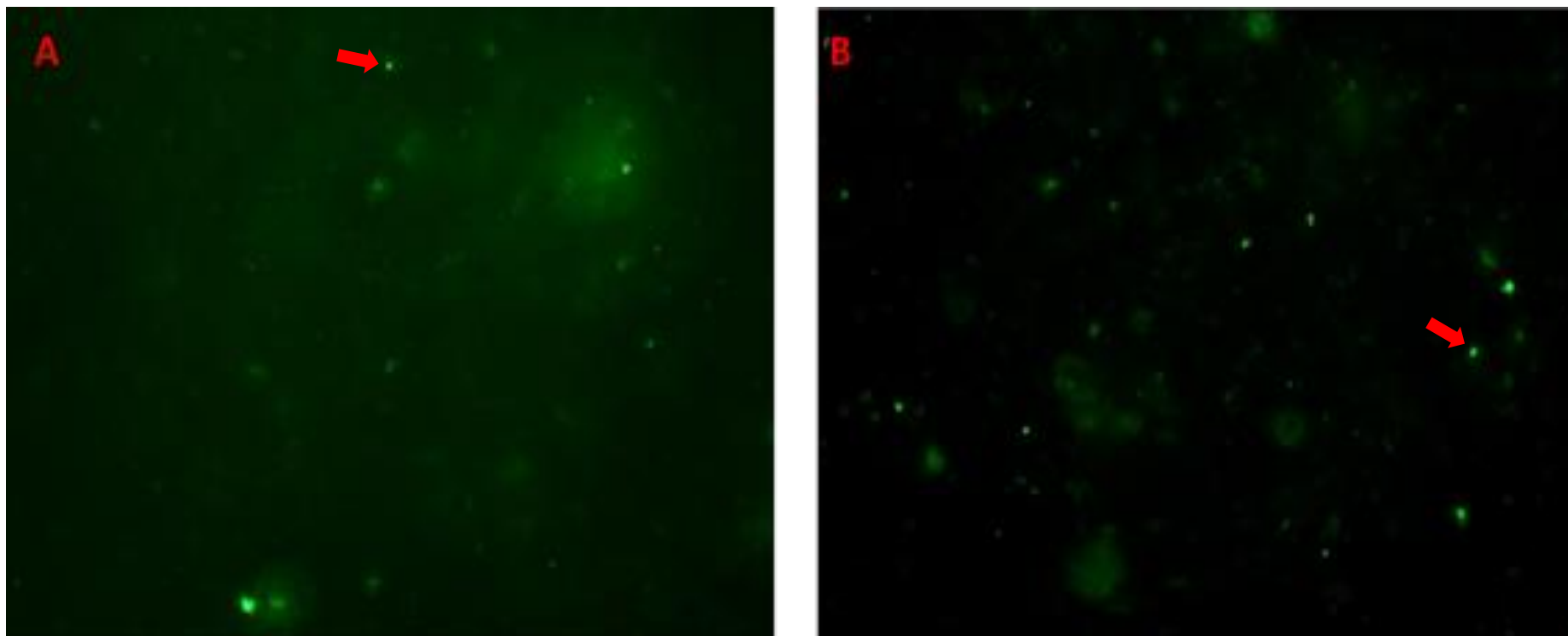


Figure 6-2 Immunofluorescence microscopy images using *M. ovipneumoniae* whole cells fixed with VECTASHIELD and probed with A) pre-immune sera or B) antisera (1:100 dilution) produced against recombinant GAPDH and FITC-conjugated donkey anti-sheep IgG H&L (FITC) (1:400 dilution).

The excitation wavelength was 493 nm and emission wavelength 528 nm. The microscopy photographs are representative fields of view of labelled cells at magnification 1,000x and exposure of 100 ms. Red arrows indicate location of fluorescence.

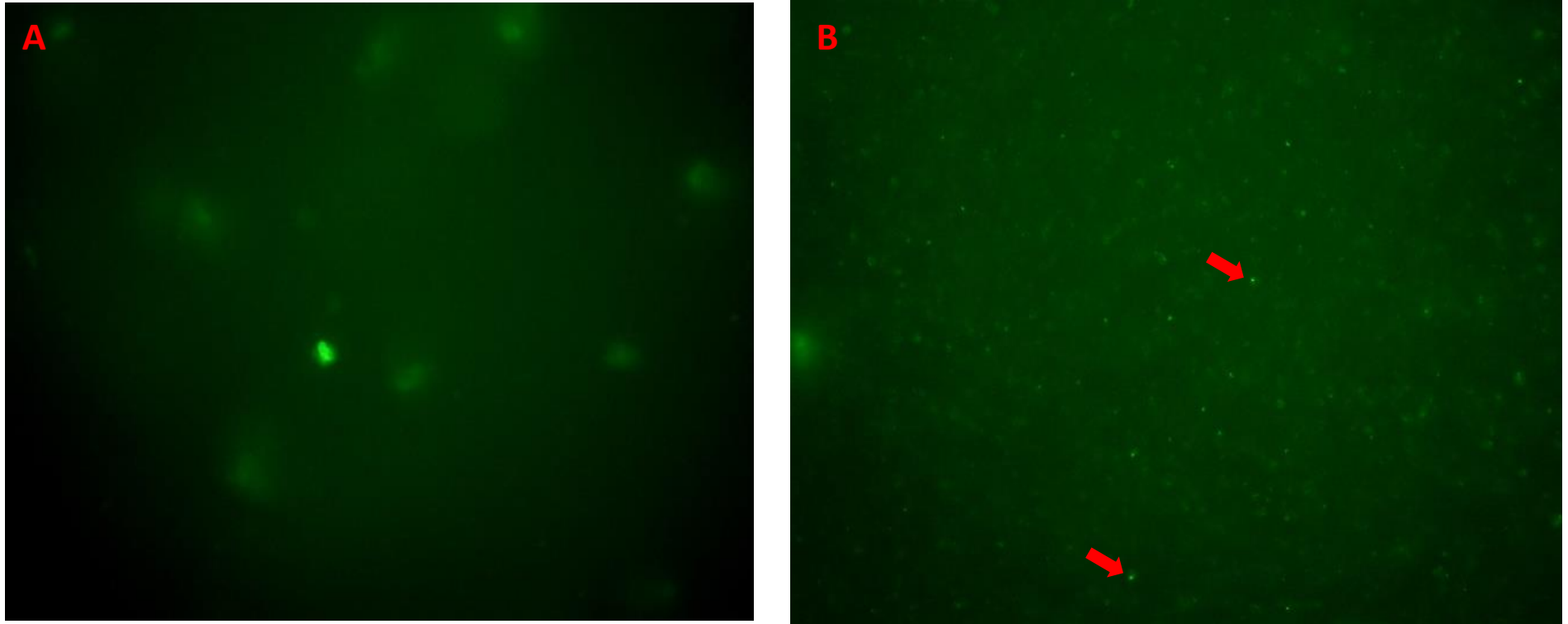


Figure 6-3 Immunofluorescence microscopy images using *M. ovipneumoniae* whole cells fixed with VECTASHIELD and probed with A) pre-immune sera or B) antisera (1: 100 dilution) produced against recombinant Hypothetical protein 1 and FITC-conjugated donkey anti-sheep IgG H&L (FITC) (1:400 dilution).

The excitation wavelength was 493 nm and emission wavelength 528 nm. The microscopy photographs are representative fields of view of labelled cells at magnification 1,000x and exposure of 100 ms. Red arrows indicate location of fluorescence.

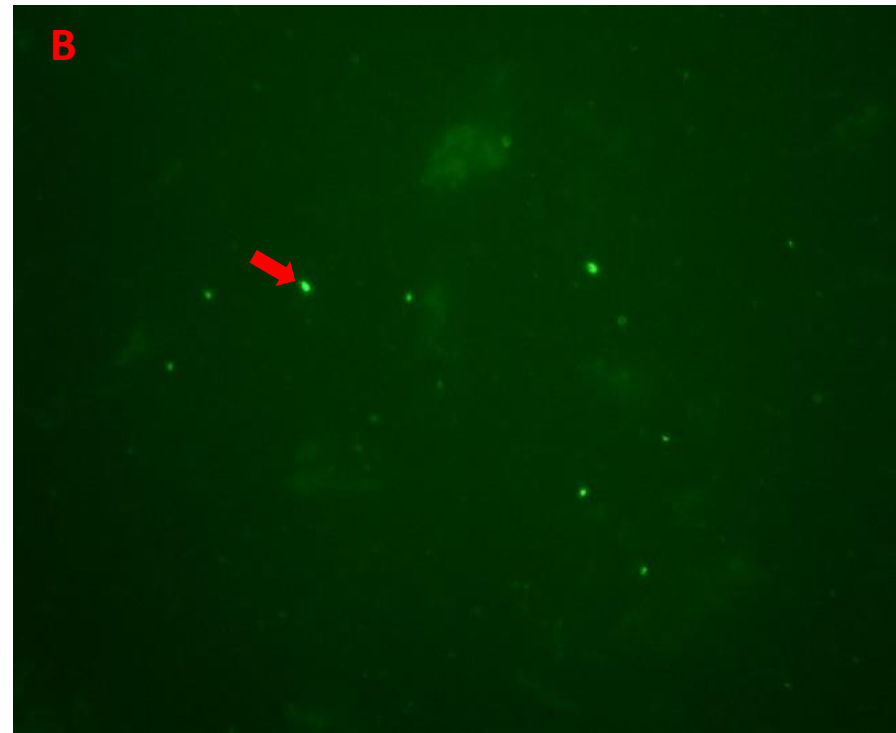
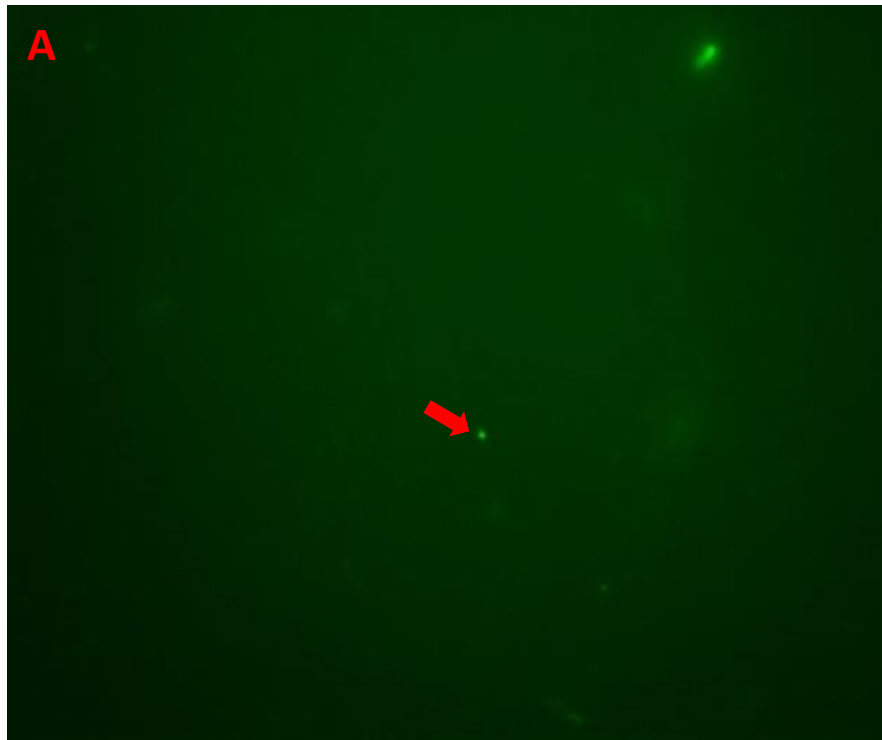


Figure 6-4 Immunofluorescence microscopy images using *M. ovipneumoniae* whole cells fixed with VECTASHIELD and probed with A) pre-immune sera or B) antisera (1:100 dilution) produced against recombinant Hypothetical protein 3 and FITC-conjugated donkey anti-sheep IgG H&L (FITC) (1:400 dilution).

The excitation wavelength was 493 nm and emission wavelength 528 nm. The microscopy photographs are representative fields of view of labelled cells at magnification 1,000x and exposure of 100 ms. Red arrows indicate location of fluorescence.

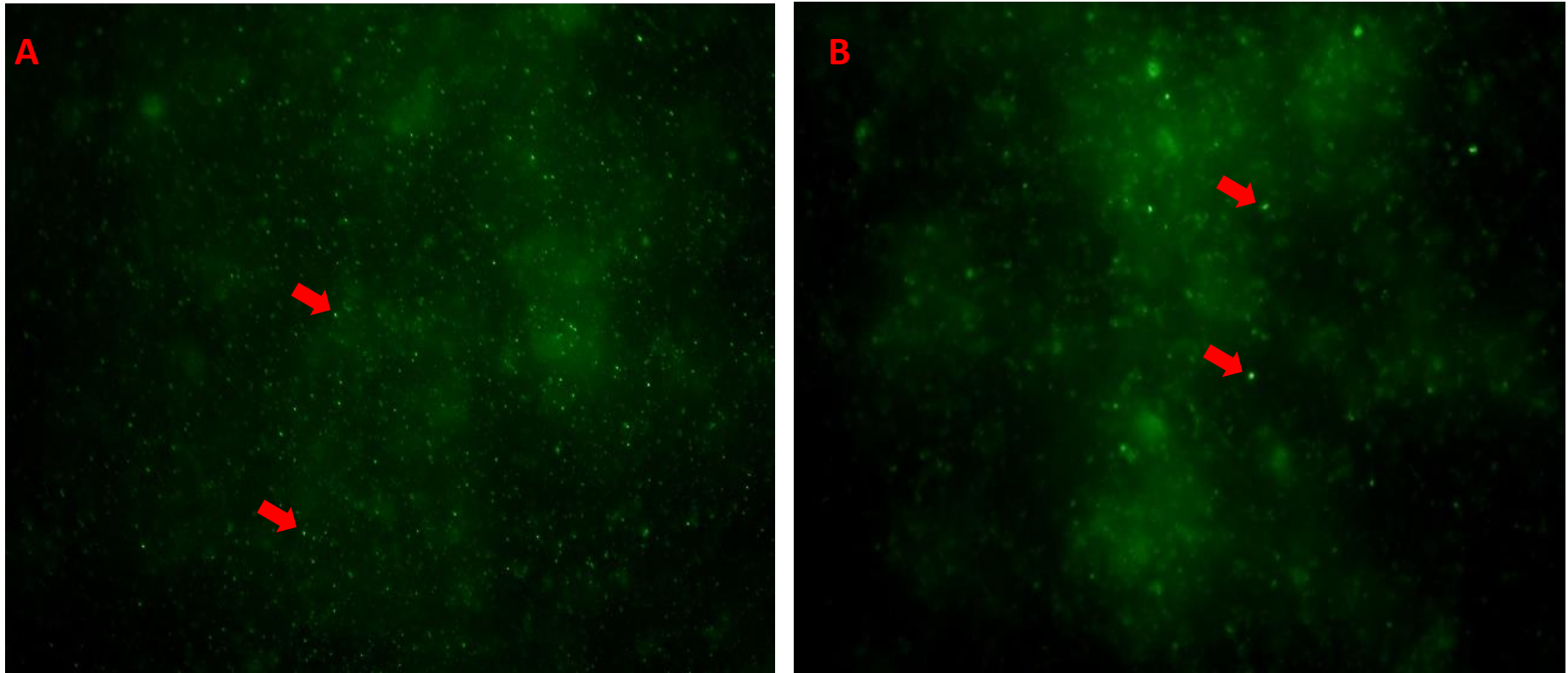


Figure 6-5 Immunofluorescence microscopy images using *M. ovipneumoniae* whole cells fixed with VECTASHIELD and probed with A) pre-immune sera or B) antisera (1:100 dilution) produced against recombinant protein P146 and FITC-conjugated Donkey Anti-Sheep IgG H&L (FITC) (1:400 dilution).

The excitation wavelength was 493 nm and emission wavelength 528 nm. The microscopy photographs are representative fields of view of labelled cells at magnification 1,000x and exposure of 100 ms. Red arrows indicate location of fluorescence.

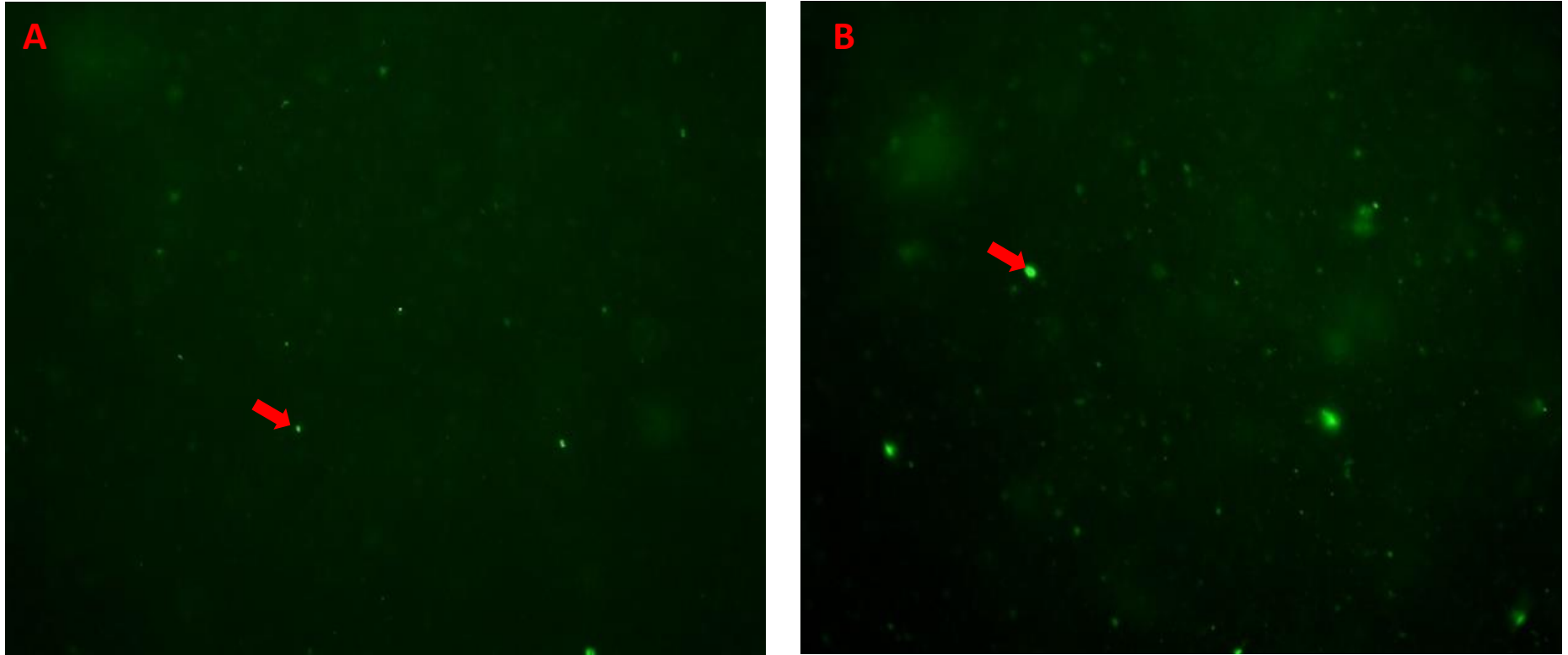


Figure 6-6 Immunofluorescence microscopy images using *M. ovipneumoniae* whole cells fixed with VECTASHIELD and probed with A) pre-immune sera or B) antisera (1: 100 dilution) produced against WCL from *M. ovipneumoniae* isolate 16 and FITC-conjugated Donkey Anti-Sheep IgG H&L (FITC) (1:400 dilution).

The excitation wavelength was 493 nm and emission wavelength 528 nm. The microscopy photographs are representative fields of view of labelled cells at magnification 1,000x and exposure of 100 ms. Red arrows indicate location of fluorescence.

6.4 Discussion

Studies in chapter 5 determined the ability of recombinant proteins from *M. ovipneumoniae* to induce an immune response in the host species, sheep. All the recombinant proteins were able to induce a strong antibody response and recombinant HP3 and P146 were shown to have potential for detecting infection of sheep with *M. ovipneumoniae* (chapter 5). Measurement of antibodies in serum or other samples by enzyme-linked immunosorbent assay ELISA can detect only if an immune response has occurred; however, it is limited in its ability to determine if the response will be protective against the disease [380]. Combination of ELISA data and observation of protective effects can help determine a 'correlate of protection' in which later ELISA data can be used to determine protective effect [380]. Two processes are commonly used to determine the ability of a vaccine to induce protective immunity in the host [381]. *In vivo* testing often consists of challenge trials, in which vaccinated animals will subsequently be inoculated with a live virulent pathogen [381]. This method provides the most direct test for the protective ability of a certain vaccine; however, due to the need to infect the animal with the pathogen and to have a control group that will not have any protection from the vaccine, it can result in a large number of animals becoming infected [381]. In the case of mycoplasma infections, euthanasia of the animals is required to determine infection status by checking the lungs [46, 60]. Ideally, this approach should only be undertaken if there is a strong support for the vaccination to have produced a protective effect. The use of recombinant proteins can provide a directed immune response to specific targets [380]; however, the identification of the correct recombinant protein or selection of recombinant proteins can be a lengthy process.

Where challenge trials are not yet appropriate, due to limited evidence for protective ability of the vaccine antigen, *in vitro* testing can be used to provide initial indications whether the vaccination has provided any protection. Both cell-mediated and humoral responses can be important for protective immunity against infectious organisms [380, 381]. As discussed in chapter 5, the recombinant proteins produced only weak cell-mediated responses and hence, in this chapter the *in vitro* experimentation was limited to testing the potential of antibodies to provide a protective effect against infection. Understanding the mechanisms by which antibodies can interact with a pathogen and reduce its ability to infect or induce disease, can help designing experiments to test the antibodies. One such method adopted in this chapter was testing the inhibition of adhesion to cells. Adherence of *M. ovipneumoniae* to the host's epithelial cells is a requirement for the successful invasion of the host and preventing the pathogen from adhering to the host airway epithelial cells will protect the animal from infection [225, 227].

6.4.1 Adhesion study

Studies were undertaken to determine if the antibodies produced against the proteins (EF-Tu, GAPDH, HP1, HP3, and P146) could interfere or reduce binding of *M. ovipneumoniae* to host epithelial cells. A sheep lung cell line was not available for the study and hence bovine endometrial epithelial cells were used. The first experiments were to determine if *M. ovipneumoniae* would adhere to these cells. The results indicated *M. ovipneumoniae* could adhere to the endometrial epithelial cells (Table 6.1). Mycoplasma did not adhere to the wells of the plates. The second part of the experiment was to determine what effect pre-incubating *M. ovipneumoniae* with antibodies produced against the recombinant proteins had on adherence of *M. ovipneumoniae* to the epithelial cells. Incubation with antibodies to the recombinant proteins did not result in a change in the ability of *M. ovipneumoniae* to adhere to the endometrial epithelial cells (Table 6.1). In contrast, antisera against WC16 prevented adherence of *M. ovipneumoniae* to the epithelial cells. Incubating *M. ovipneumoniae* with pre-immune sera did not affect adherence (Table 6.1).

The antisera produced against WC16 was used as the positive control to determine the base level of adhesion inhibition. Antibodies against whole cell should have a repertoire of anti-mycoplasma antibodies allowing for multiple surface proteins to be inhibited simultaneously. The antisera produced against WC is likely to contain antibodies against each of the recombinant proteins as well as many other antigens. The ability to attach to multiple surface proteins and interrupt the binding to epithelial cells is likely the reason that reduction in adherence was observed using antisera against WC16. Further studies using antisera against WC90 and WC103 would be useful in determining if the interference of adherence is consistent across animals vaccinated with whole cells. In the corresponding CCU experiment there was no observable change in colour in any wells tested with antisera against WC16. This shows a reduction in adhesion as all other CCU assays observed a colour change. The result suggests that incubation with antibodies against WC16 has prevented completely the adhesion of mycoplasmas to the epithelial cells as there was no colour change observed. One limitation of the protocol currently is that a sample of the final media is tested in the CCU rather than all of the media. This results in a diluted sample of mycoplasma with approximately 10^2 CCU/ml being detected. This allows for differences in the ability of the different antisera to prevent adherence to be observed. However, it cannot distinguish between reduction of adherence and prevention of adherence. A further optimisation may be to extract all the media and concentrate it into the CCU assay. This would result in all mycoplasma cells remaining after washing being available for the assay. This may provide a more accurate representation of the adherence ability of the mycoplasma.

Understanding the potential mechanisms for cell surface protein-mediated adherence of mycoplasma to host epithelial cells will assist in understanding the observed results. Attachment of

antibodies to the native EF-Tu protein on the surface of the cell has been demonstrated by the immunofluorescence microscopy. Therefore, failure to see an effect of the antibodies on adherence was not due to the antibodies not being able to recognise and bind to native EF-Tu on the surface of the mycoplasma cells. EF-Tu has been demonstrated to mediate the binding of mycoplasmas to fibronectin (Fn) [195]. Fn is a high molecular-weight glycoprotein found abundantly in fibrillary form in the extracellular matrix (ECM) [382]. The production of any ECM may be minimal due to the limited time cells were allowed to incubate in the wells (24 h) [383]. This may have reduced the effect EF-Tu had on the adhesion ability of *M. ovipneumoniae*. Analysis of endothelial cells in *in vitro* conditions have indicated that at low confluence Fn was on dorsal cell surfaces at regions of cell-cell contact only [384]. However, at complete confluence Fn was detected in an extracellular matrix underneath the cell monolayer but not on top of the monolayer [384]. This observation was echoed by Linder et al [385], with the suggestion that, at least with endothelial cells Fn is found primarily under the cells and limited Fn is found on the surface of the cells. In contrast to endothelial cells, fibroblasts have been shown to produce Fn not only underneath the cells [386, 387], but also as a fibrillar network over the cell surface [383, 386, 387]. Chen et al (1977) [383] also detected Fn on the surface of liver epithelial cells which may possess some similar properties to the endometrial epithelial cells used in this experiment; however, this was observed after a limited number of passages after collection of the cells. Once the passage of the cells exceeded 10 passages, the distribution of Fn was reduced and after 15 passages Fn was lost in the majority of cells. Cells used in this experiment have exceeded 20 passages and therefore may have lost their ability to produce Fn. In the absence of Fn, anti-EF-Tu antibodies may not have had an effect on adherence of *M. ovipneumoniae* to the epithelial monolayers. Repeating this trial with modifications to ensure production of ECM may better determine the effect anti-EF-Tu antibodies on *M. ovipneumoniae* adherence to the cells.

GAPDH has also been suggested to have a role in assisting the adherence of *M. ovipneumoniae* to host cells. Immunofluorescence microscopy has demonstrated that antibodies are able to detect and attach to the native GAPDH on the surface of the cell and antibodies not being able to recognise and bind to is not a factor to effect adherence. GAPDH is suspected to attach to mucin (Mn) the primary component in the mucosal epithelial lining [310]. In a similar limitation to testing anti-EF-Tu antibodies, anti-GAPDH antibodies would have the largest effect when the cell monolayer culture has produced a mucin layer. Due to the limited time allowed for the cells to produce a monolayer, the development of a mucin layer is unlikely. This reduces the potential effect of the antibodies on preventing adherence. This may explain the lack of response to the antibodies in the studies.

P146 has also been suggested to assist in the adhesion of mycoplasmas and has been shown to bind to cilia [322]. Compared to EF-Tu and GAPDH, this protein appears to adhere to a specific cell structure. If this is correct the endometrial epithelial cells may not have the same surface markers as developed cilia. This would again reduce the ability of antibodies to interfere with the adhesion ability of *M. ovipneumoniae*. Production of a cilia cell model would allow for a more direct testing of anti-P146 antibodies as well as other anti-*M. ovipneumoniae* antibodies because this is the natural location of *M. ovipneumoniae*. The production of a cilia cell model would be difficult, time consuming, and well beyond the scope of this project, and therefore the endometrial epithelial cells were used for the adherence studies in the thesis.

The antibodies against the epitope of HP1 and HP3 also did not appear to reduce *M. ovipneumoniae*'s adhesion ability. Because the function of these two proteins is not known their role in adhesion cannot be determined, and therefore it cannot be hypothesised as to whether antibodies against these proteins would be expected to affect the ability of *M. ovipneumoniae* to adhere to epithelial cells. Antibodies against these proteins also did not produce a strong signal in the immunofluorescence microscopy and thus the antibodies may not be binding strongly to the proteins on cell surface.

One of the major limitations to this experiment was the use of bovine endometrial epithelial cells as the target cell culture. *M. ovipneumoniae* is known to infect sheep and goats [50, 248, 249, 388], therefore the use of cells from a bovine host could introduce different receptors to the cell surface as well as loss of sheep specific receptors [389]. This could in itself reduce the ability of *M. ovipneumoniae* to adhere to the cells. The purpose of the initial experiment was to determine if *M. ovipneumoniae* cells would adhere to the epithelial cells. Despite the results showing that adherence did occur it does not confirm that adherence to the cells is by the same mechanism as would occur *in vivo* [390]. This may result in *M. ovipneumoniae* using different surface antigens to attach to the bovine cells that it would use in sheep cells [389]. This could explain why the protein specific antibodies did not have an effect on adhesion. An adaption of this procedure could be the addition of immunofluorescence microscopy analysis rather than CCU analysis as described by Svenstrup et al (2002) [225]. By analysing the adherence to individual cells, it may be possible to elucidate the effect of the antibodies on adhesion that may not be observable using the CCU analysis.

The second major limitation in this experiment is the effect of multiple adhesion sites available on the bovine endometrial epithelial cells. Incubation with antibodies against a specific protein may prevent *M. ovipneumoniae* from binding to the epithelial cells by interaction with that protein but other proteins expressed on the surface of *M. ovipneumoniae* cells may still be available to interact with targets on the epithelial cells. Combinations of antibodies against the different recombinant

proteins could be tested to determine if antibodies binding to multiple targets can overcome the adhesion ability of *M. ovipneumoniae* as observed with the anti-WC antibodies. A more direct approach would be to use *M. ovipneumoniae* cells over-expressing single membrane proteins. This would allow direct analysis of the ability of the native protein to adhere to the host cell as well as the ability of the antibodies to prevent adherence. This was the approach used by Hegde et al (2018) [227], using phase locked mutants expressing single Vpma (Variable protein of *Mycoplasma agalactiae*) lipoproteins. While this approach could be useful, it would be dependent on the ability to produce *M. ovipneumoniae* mutants able to produce selected surface antigens. Blocking *M. agalactiae* required disruption of the Xer1 gene preventing the mycoplasmas from shifting from one Vpma to another [391]. Finding a single gene able to adjust the surface proteins can allow for a simple and consistent method for testing adhesion inhibition. Adjusting the surface proteins to only express the protein of interest and allowing the mycoplasma to remain viable would require considerable work and may not be possible with current technology. Another approach could be to use the targets for each of the proteins such as Fn for EF-Tu and Mn for GAPDH. Purifying each of these proteins and binding them to plates may allow for a direct analysis of the ability of specific antibodies to block adhesion.

The undertaking of the adherence studies was hindered by two major issues. The first was the outbreak of a global pandemic caused by the SARS-COV-2 (COVID-19) virus which resulted in the country going into lockdown during the initiation of the experiment. This resulted in the ongoing experiments being stopped and prevented further experimentation for some time. This limited the time available for completion of this analysis. The second hindrance to this analysis was the growing of the endometrial epithelial cells. Growth of the cells ran as expected for the initial adhesion experiment and the first round for each of the recombinant protein tests. However, unknown contamination of cell cultures and a sudden decrease in growth rate of the endometrial epithelial cells resulted in several experiments being unable to proceed. These unforeseen limitations reduced the ability to optimise the protocols and made it difficult to confidently confirm the full effect of the antibodies on the adhesion ability of *M. ovipneumoniae*.

6.4.2 Immunofluorescence microscopy

The immunogenic proteins identified in chapter 4, were detected using Western blotting on hydrophobic membrane protein fractions. The assumption has been that these proteins are likely membrane bound or membrane associated. This view was supported by detecting potential transmembrane domains in several of the proteins indicating that at least part of the protein is located within the membrane and at least part of the protein is located outside of the cell. To further support the presence of the proteins on the cell surface, antibodies against each of the recombinant proteins were used for immunofluorescence microscopy studies. Antibodies against *M.*

ovipneumoniae surface antigens should be able to bind to the surface of *M. ovipneumoniae* cells. Labelling the cells with a FITC-conjugated anti-sheep antibody would produce a fluorescent signal observable using a microscope.

Comparison of pre-immune and antisera from an animal vaccinated with EF-Tu (Fig. 6.1) indicated no EF-Tu specific antibodies in the pre immune sera while there were several fluorescence spots observed with antisera against EF-Tu. Comparing these results with the Western blots in chapter 5 (chapter 5.3.3 Figures 5.8-5.9) suggests that the animals had no detectable anti-mycoplasma antibodies before vaccination in their sera and after vaccination had anti-EF-Tu antibodies. This suggests the spots highlighted in the antisera image are due to anti-EF-Tu antibodies providing further support for EF-Tu being membrane bound. EF-Tu has been previously shown to be membrane bound [160, 392-394], therefore this protein was used as a positive control for the experimental methods.

Pre-immune sera from animals vaccinated with GAPDH showed a limited response to the *M. ovipneumoniae* cells (Fig. 6.2) with a few bright spots observed. This was in contrast to the Western blot results in chapter five (chapter 5.3.3 Fig 5.10) which showed no response to either *M. ovipneumoniae* WCL or recombinant GAPDH. This may be due to the difference in concentration between Western blots (1/400) and immunofluorescence assay (1/100) for the primary antibody or the difference in secondary antibody 1/1000 and 1/400, respectively. Another potential reason for the differences between the immunofluorescence and Western blot images is due to cross reactivity with non-target proteins in the immunofluorescence assay. This has been observed by antibodies detecting targets in immunohistochemistry despite not detecting any targets in Western blots [395]. Comparing the pre-immune sera to the antisera, there appeared to be more pronounced fluorescence signal with the antisera. In the Western blot (chapter 5.3.3 Figure 5.10) for this animal there was an increase in the response to WCL; however, the larger response was to GAPDH. It can be suggested that the increased fluorescence observed in the sample probed with GAPDH antisera were due to the production of anti-GAPDH antibodies. GAPDH has been suggested to be membrane bound in other species [306, 307, 396]; however, this is the first evidence for GAPDH being membrane bound in *M. ovipneumoniae*.

HP1 showed limited immunofluorescence in both pre-immune and antisera images (Fig. 6.3). Western blotting did not show a strong identification of the full-size HP1 (chapter 5.3.3 Fig. 5.11, 5.12) from either pre-immune or with antisera produced against the HP1 epitope. There was; however, a low-level response to WCL in the pre immune sera and a strong response to WCL in the antisera. This response is likely due to the animal obtaining a natural infection of *M. ovipneumoniae*. This can explain the low level of response in the pre-immune image. The lack of bright fluorescence

in the sample probed with antiserum indicates antibodies against the epitope are not able to bind to the outside of the mycoplasma cell. One possibility for this lack of increase in fluorescence is that the HP1 epitopes are not on the surface of the proteins and the antibodies against the epitope are therefore not able to bind to the native protein in the context of intact cells. The second possibility is that HP1 is on the surface and the antibodies against the epitope are not able to bind to the protein in its natural state or if it is able to bind, the secondary antibodies were unable to bind to the primary antibodies. Attachment of the antibody to the protein may have resulted in a conformational change to the protein encapsulating the antibody preventing attachment of the secondary protein [397]. Analysis of the tertiary structure could allude to whether the antibodies are able to physically access the epitope. This level of analysis would require a significant amount of work and based on the experimental data from chapters five and six.

HP3 had a similar limited response as seen in case of HP1 (Fig. 6.4). Unlike HP1, HP3 was produced in as a full-length protein. This would suggest that if the protein was present on the surface of the membrane it should have been detected in the images as it would be expected that some of the antibodies against HP3 should be able to attach to the folded form of the native protein. HP3 was shown to elicit an immune response in animals vaccinated with WC of *M. ovipneumoniae* as indicated by the ELISAs in chapter 5 (chapter 5.3.4 Table 5.2). One possibility for the observed limited fluorescence could be that the conformational structure of HP3 (tertiary and quaternary) produces an immunogenic response in the host and the corresponding antibodies are able to identify the recombinant version of the protein however, antibodies against the recombinant protein may be to sections that are not conformationally present in the natural form. Due to the potential use of this protein as a diagnostic tool it may be worth conducting further analysis of this protein including identifying the function and potential interactions with other proteins.

Understanding the structure of the protein may provide insight on how the immune system interacts with the protein and may help optimise the use of this protein as a diagnostic tool.

The protein P146 had the strongest response of all the recombinant proteins in both the pre immune and the antisera images (Fig. 5). This matches the Western blots in chapter 5 (chapter 5.3.3 Fig. 5.14, 5.15), with both Western blots indicating the animals having pre-existing anti-mycoplasma antibodies in their sera before vaccination. This results in antibodies to general *M. ovipneumoniae* antigens being present in the sera, including the other recombinant proteins EF-Tu, GAPDH, HP1 HP3, in addition to antibodies to P146 from the vaccination. This makes it difficult to confirm the localisation of P146. Further analysis with antibodies against P146 would be useful to confirm the location of the protein on the membrane surface as has been observed in *M. hyopneumoniae* [322, 398]. Potentially the use of non-host species such as mice and rabbits may be useful for initial

experiments to remove the likelihood of natural infection of *M. ovipneumoniae*. P146 has shown potential as a diagnostic protein (chapter 5), and therefore further analysis on the surface location of this protein is warranted.

For the immunofluorescence assay, animals vaccinated with WC of *M. ovipneumoniae* were used as a positive control to determine the intensity of binding of antibodies against multiple *M. ovipneumoniae* antigens to the mycoplasma cells (Fig. 6.6). The pre-immune sera showed some immunofluorescence in the image with some well-defined spots of fluorescence. This could suggest a previous or current exposure to *M. ovipneumoniae* in the animals before vaccination. The lower level of response in these animals compared to the animals vaccinated with P146 may suggest that it is a previous infection with only a low level of antibodies present in the sera before vaccination. There was an increase in immunofluorescence with the antisera produced against WC indicating an increase in anti-mycoplasma antibodies, a result more similar to what was observed with the P146 antisera.

6.4.3 Additional work completed

Another approach was to test the effect of antisera obtained from sheep vaccinated with the recombinant proteins to determine whether protein-specific antibodies can inhibit the growth of *M. ovipneumoniae in vitro*. Using the method described in Jiang et al (2016) [160], antisera from animals vaccinated with EF-Tu and WC of isolate 16 were tested of their ability to inhibit mycoplasma growth. EF-Tu was selected for optimisation of the protocol because antibodies to the protein generated in mice have been shown previously to inhibit growth of *M. ovipneumoniae in vitro* [160]. WC from *M. ovipneumoniae* isolate 16 was included as a test for antibodies against the whole organism. It was expected that antisera produced against WC would be a good positive control for the assay since there should be a greater chance that the repertoire of anti-*M. ovipneumoniae* antibodies would result in inhibition of mycoplasma growth.

The growth inhibition assay was originally conducted using 10^6 CCU/ml *M. ovipneumoniae* as described by Jiang et al (2016) [160]. At this concentration, the colour of media in all wells containing *M. ovipneumoniae* changed from red to yellow during overnight incubation and did not show a measurable difference between the positive control wells and wells containing antibodies. The concentration of *M. ovipneumoniae* used in the assay was subsequently reduced 10-fold and retested. All concentrations ranging 10^5 - 10^2 produced the same result with colour in the media changing red to yellow overnight. The experiment was repeated at only 10^1 *M. ovipneumoniae*. However, at this low concentration not all positive wells changed colour during the two-week incubation. Across all concentrations of antibodies there was no measurable effect on growth of *M. ovipneumoniae*. To determine if there is any effect of antibodies on the growth of *M. ovipneumoniae*

the assay will require further optimisation, or another approach used to determine the impact of antibody on growth. Antibodies against *M. ovipneumoniae* EF-Tu produced in mice have been shown to inhibit the growth of *M. ovipneumoniae* [160]. However, this was not observed in these experiments. The cause of the discrepancy is unknown; however, either of the two major differences between these experiments and those performed by Jiang et al (2016) [160] could be the cause. First, the difference in the animal used to produce the antisera, mice versus sheep. It was observed in chapter 4 that the antisera produced in rabbits against WCL of isolates 16, 90 and 103, produced a different Western blot pattern than antisera produced in sheep against the same isolates, with the rabbit sera detecting more high molecular weight proteins and limited medium molecular weight proteins (chapter 4 Fig. 4.4,4.6). This difference in the proteins detected indicates that there are differences in the response to *M. ovipneumoniae* antigens between the different species. As rabbits and mice are not the natural hosts of *M. ovipneumoniae*, they may be able to induce immunogenic responses that are able to overcome the defence mechanisms of *M. ovipneumoniae*. It has been suggested that *M. ovipneumoniae* infections in sheep can reduce its ability to mount an immune defence on a second infection [158, 159]. This may suggest that the immune response mounted by sheep is less able to remove or prevent the growth of *M. ovipneumoniae in vitro*. To further assess the difference between sheep and rabbits, rabbit sera could be tested for growth inhibition. The second difference is the strain of *M. ovipneumoniae* used for the assay. Isolate 16 was selected for testing the impact of antibodies on growth. Isolate 16 is one of the three isolates selected from New Zealand isolates to represent the three groups of New Zealand *M. ovipneumoniae* isolates. One of the criteria for selection of these isolates was their ability to grow faster in culture relative to the growth rate of some of the other isolates. This feature facilitated producing cellular fractions for the study. However, using a faster growing organism may have made it difficult to observe differences between the positive controls and the antibody treated cells. In this case a slower growth cycle over a longer time span may have been able to tease out any small differences resulted by the presence of the antibodies. There were other isolates collected at the same time as Isolates 16, 90, and 103 which did not have the same fast growth rate. However, they would not be appropriate for this assay in the current form, as many of the isolates did not produce the acidic environment observed for other *M. ovipneumoniae* isolates. Without the change in pH from mycoplasma growth the assay would not visualise the colour change from red to yellow and the growth would not be able to be measured.

6.4.4 Future work

Further recommended work based on the experimental data collected in this chapter would be further optimisation of the adhesion experiments and confirmation of localisation of antigens on the surface of *M. ovipneumoniae* as shown by immunofluorescence studies. The later could be done

using electron microscopy. Repeating the adherence studies using the target antigen (Fn or Mn) rather than *M. ovipneumoniae* whole cells may provide a direct evaluation of the impact of antibodies on adherence. Alternatively, further optimisation of the adhesion assay may provide information on the ability of the antibodies to reduce adhesion to whole epithelial cells. Reducing the multiplicity of infection of *M. ovipneumoniae* or increasing the concentration of antibodies in the assay may demonstrate antibody-mediated reduction in adhesion.

The immunofluorescence microscopy results for EF-Tu, GAPDH, P146, and WC, showing reactivity of antibodies produced against these proteins with *M. ovipneumoniae* cells provided evidence for surface localisation of these proteins. However, due to the limited resolution of fluorescence microscopy, it was not possible to determine the exact location of the proteins on the cell membrane. This could be determined using electron microscopy.

6.5 Conclusion

This chapter aimed to determine, using *in vitro* assays, the ability of the recombinant proteins to elicit a protective effect against *M. ovipneumoniae* infection in the host. Immunofluorescence microscopy was used to determine the location of the proteins on the surface of the cell. The results support surface localisation of EF-Tu, and GAPDH; however, HP1 and HP3 and P146 could not be confirmed as located on the cell surface and further analysis would be required to determine their location. The growth inhibition experiments did not indicate a reduction in growth of *M. ovipneumoniae* in response to antibodies. This contradicts the previous work in mice, suggesting that either the New Zealand isolates grow too quickly to observe an effect of the antibodies in this current experimental setup, or sheep antibodies do not produce the same protective effect as observed in the mice model [160]. Because much of the work undertaken on *M. ovipneumoniae* has been done in non-host species, distinguishing between these possibilities will determine the potential use of non-host model species for further experiments.

Antibodies against WC of *M. ovipneumoniae* isolate 16 reduce the adhesion of *M. ovipneumoniae* to the epithelial cells used in this study. However, a reduction in adhesion was not seen due to the antibodies specifically produced against the recombinant proteins. This may be due to multiple sites of adhesion being available to *M. ovipneumoniae*, and antibodies against a single protein may not prevent adherence and hence provide a protective effect. The response to the WC antibodies supports the experimental design providing a basis for further optimisation. With increased optimisation, this assay could provide an additional method for antigens for a vaccine.

Chapter 7 General discussion

7.1 Introduction to thesis and goals of thesis

Pneumonia of sheep has been an issue in New Zealand (NZ) since the adoption of intensive farming with the first record noted in 1900 by John Gilruth [46]. The most common form of ovine pneumoniae in NZ is CNP. As the majority of lambs will be affected by some level of CNP during their first autumn [48], the cost of this infection is significant. Most affected lambs show few if any clinical signs of disease, although some animals, may have reduced weight gain, poor exercise tolerance, or an occasional cough [46]. Single strains have been observed to have limited pathogenic potential; however, the presence of multiple strains can lead to ciliostasis and allow further colonisation of additional bacteria [46, 61]. It was estimated that the average cost of pneumonia and pleurisy to the NZ sheep market was in excess of NZ\$53 million in 2008 [47]. *M. ovipneumoniae* can be carried asymptotically by domestic sheep and goats. This can make control of the disease and prevention of outbreaks difficult. There is currently no successful vaccination program for the control of *M. ovipneumoniae*.

To reduce the impact of *M. ovipneumoniae* on the commercial sheep industry, as well as to facilitate programmes such as big horn sheep wildlife conservation, further knowledge on *M. ovipneumoniae*, especially pertaining to development of vaccines and better diagnostics, is required. The overall aim of this study was to identify immunogenic proteins of *M. ovipneumoniae* and determine their potential as diagnostic agents or as antigens for a sub-unit vaccine. The work was divided into four specific objectives, corresponding to the four experimental chapters of the thesis.

1. Build and annotate the genome of NZ isolates of *M. ovipneumoniae* (chapter 3).
2. Produce membrane fractions from selected isolates of *M. ovipneumoniae* for immunogenic analysis and detect immunogenic proteins of *M. ovipneumoniae* by Western blotting using sheep antisera produced against *M. ovipneumoniae* (chapter 4).
3. Express and purify selected immunogenic proteins in *E. coli* and test their immunogenicity in lambs (chapter 5).
4. Test the ability of the antisera produced against the recombinant proteins to inhibit growth of *M. ovipneumoniae* in culture, and to interfere with adhesion of the organism to epithelial cell monolayers (chapter 6).

The following discusses the key findings from the experiments and their impact on the understanding of *M. ovipneumoniae* and the potential for improved vaccine and diagnostic targets.

7.2 Insights from genomic analysis of *M. ovipneumoniae*

7.2.1 Gene and transmembrane domain analysis

The HTS data was used to build three draft genomes of NZ isolates 16, 90, and 103. These draft genomes identified 971, 758, and 859 protein coding genes, respectively, constituting >90% of the total genes. Of them, the proteins encoded by 286, 215, and 253 genes in respective isolates were predicted to be having transmembrane domains (Table 3.1). These proteins have potential as vaccine antigens or as diagnostic targets. Further analysis to determine which proteins have single or multiple transmembrane domains at the start of the protein will provide the best options for targets and eliminate the use of proteins with multiple transmembrane domains. Proteins with just one transmembrane domain would be expected to be anchored in the cell membrane but with the majority of the protein protruding as an extracellular domain outside the cell organism [177, 178]. Conversely, proteins with multiple transmembrane domains interspersed throughout the protein sequence could impose difficulties for producing a recombinant protein. For this reason and also as their extracellular domains may be smaller, such proteins would be more lowly ranked candidates for a vaccine or diagnostics [399-401].

7.2.2 Diversity of NZ isolates

Phylogenetic analysis of the P113 gene sequence in 10 NZ isolates and the type strain (ESR) separated the isolates 16, 90, and 103 into three different groups (Fig. 3.5). This grouping of isolates 16, 90 and 103 was consistent with the original RFLP analysis undertaken on 93 isolates of *M. ovipneumoniae* from NZ sheep farms (unpublished). The ESR type strain (Australia) used in the P113 analysis; however, did not fit within any of the three NZ groups supporting the use of this gene as a typing tool for grouping isolates of *M. ovipneumoniae*. This could be useful for understanding the distribution of different isolates in different populations of domestic animals as well as distribution between domestic and wild populations [249].

7.2.3 NZ isolates of *M. ovipneumoniae* are distinct from United States of America (USA) isolates

Comparative phylogenetics using a multi-locus sequence typing (MLST) approach, that also included 15 isolates from USA [265], clustered all the NZ isolates into a single group, with the ESR strain being the closest relative (Fig. 3.6). The separation of the NZ isolates from the USA samples [265] is not unexpected due to the large geographical separation and suggests that there has not been recent movement of *M. ovipneumoniae* between these countries, at least regarding the isolates tested in this thesis. ESR was originally collected in Australia and therefore it makes sense that it is more closely related to NZ samples than to the USA samples. Within the NZ group, two sub-clades were detected. This may suggest that there has been multiple invasion events of *M. ovipneumoniae* in NZ. Further sequencing of more NZ isolates will help provide support for the sub-clades, and further

sequencing of more Australian isolates will help determine if there have been multiple movements between NZ and Australia or if there has been a single directional movement. An alternative to multiple invasions is that the NZ isolates have undergone divergent evolution.

7.3 Immunogenic surface proteins of *M. ovipneumoniae*

Membrane fractions containing putative surface proteins were prepared from three isolates 16, 90, and 103 of *M. ovipneumoniae*. By using both one-dimensional and two-dimensional Western blotting with sheep antisera produced against *M. ovipneumoniae*, and mass spectrometry together with the draft genomes of *M. ovipneumoniae* isolate 90 (chapter 3), the identity of five immunogenic proteins was revealed, either by direct comparison to *M. ovipneumoniae* proteins or through homologs in closely related species. These five proteins were elongation factor thermo unstable (EF-Tu), Heat Shock Protein (HSP70), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), multidrug and toxin extrusion protein (MATE), and adhesin like protein, P146. Additionally, three hypothetical proteins were also identified. Both EF-Tu and HSP70 have been identified as immunogenic in mice [160] and HSP70 was shown to be an immunogenic protein in sheep [194]. The studies in Chapter 4 confirmed that EF-Tu is an immunogenic protein in sheep and added six more proteins to the list of known proteins in *M. ovipneumoniae* that are immunogenic in sheep.

For each of the eight immunogenic proteins, the protein coding sequences were obtained from the draft genome of isolate 90. These sequences were used as reference to obtain the sequences from the HTS data of the other isolates. The sequences were used to predict B lymphocyte epitopes [293], transmembrane domains [294, 295], and hydrophobicity of the proteins [296]. Of the eight proteins identified, five were selected to be produced in a recombinant form for further analysis. These were EF-Tu, GAPDH, P146, an epitope of HP1, and HP3. Due to the large size of HP1, only a single epitope of the protein predicted by the B lymphocyte epitope prediction program was produced in the recombinant form. This avoided the difficulties of expressing a large protein. HSP70 has been extensively studied as a potential vaccine target for *M. ovipneumoniae* [31, 160, 194, 304]; however, to date, there have been no reports of a successful role of HSP70 as a vaccine antigen. For this reason and the lack of novelty, HSP70 was not selected for the immunogenicity trial. However, should further information promoting the use of this protein as a vaccine target emerge, it could be re-examined. The decision not to continue with the MATE protein was based on the protein having multiple transmembrane domains. This would have made the protein difficult to express, limiting the use of this target as a vaccine candidate or for diagnostic purposes. HP2 was also not included in the list of proteins studied due to the large molecular size of the protein, and lack of a known function.

7.4 Vaccination of sheep confirmed immunogenicity of the proteins

7.4.1 Ability of recombinant proteins to induce antibodies that can detect the native protein.

The five proteins were produced commercially by GenScript and one of the proteins, EF-Tu, was also produced in-house. Sheep were vaccinated with recombinant versions of the five selected *M. ovipneumoniae* proteins and serum collected for measurement of protein-specific immune responses using Western blot analysis (Fig. 5.8-5.16). The results showed that for four of the five proteins, antibodies present in the antisera reacted not only with the recombinant protein used for vaccination but also cross-reacted with the corresponding native protein presence in WCL prepared from *M. ovipneumoniae* isolates 16, 90, and 103. In the case of antisera against HP1, the antibodies reacted only with the recombinant protein but not with the native form of the protein. Antibodies against the GenScript and in-house EF-Tu were both able to detect the GenScript protein and the native protein, confirming that the in-house EF-Tu was edited correctly and able to elicit a similar response as produced by the commercial form.

7.4.2 Hypothetical protein 3 and P146 have potential for detecting antibody responses to *M. ovipneumoniae* in sheep

To study the antibody response to each of the recombinant proteins, ELISAs were also performed on the sera (Fig. 5.17). Each of the five proteins were able to elicit a strong response by Week 6 confirming the immunogenicity of the proteins in sheep, however not all proteins exhibited a further increase in response after the second (boosting) vaccination which will require further study. Of the five recombinant proteins, HP3 and P146 showed a consistent and strong antibody response in animals vaccinated with whole cells of *M. ovipneumoniae* isolates 16, 90, and 103. These proteins were studied further using sera collected from groups of sheep vaccinated with either a mixture of *M. ovipneumoniae* and *M. haemolytica* WC or *M. haemolytica* alone. HP3 and P146 showed promising results. Their response in combination as the target antigen in ELISA was superior than when they were used as individual proteins. The performance of these two antigens albeit determined in only a few animals, is promising for development of future diagnostic tests using ELISA or other diagnostic techniques such as LFD [346-350] or a Flow through assay (FTA) [351, 352] technology. These two proteins were identified through screening of sera from bacterin-vaccinated sheep, so the results need to be validated in *M. ovipneumoniae* infected animals.

7.4.3 Vaccination with individual recombinant proteins did not induce cellular responses; however, vaccination with *M. ovipneumoniae* WC produced IL-17A responses to GAPDH
The animals vaccinated with recombinant proteins produced minimal IFN- γ responses to the corresponding recombinant protein, WC or HPF in whole blood cultures (Fig 5.19). There were slightly higher IL-17A responses to the recombinant proteins and WC (Fig. 5.21) but the weak

cytokine responses in general suggest that the five recombinant proteins in the study did not induce T-cell responses in sheep.

Sheep vaccinated with a mixture of the WC of the three isolates of *M. ovipneumoniae* produced significant IL-17A responses to recombinant GAPDH when used as the recall antigen in whole blood cultures (Fig. 5.23). This ability of GAPDH to detect strong IL-17A responses in the vaccinated sheep indicates the potential of this protein to be used as a diagnostic target for determining the infection status of sheep. The over expression of IL-17A has been suspected as a cause of disease in bighorn sheep [116, 141] and GAPDH may be useful in identifying animals exhibiting elevated IL-17A responses to *M. ovipneumoniae*. In addition, animals vaccinated with WC of isolate 16 also produced a strong IL-17A response to GAPDH, although animals vaccinated with WC of isolate 90 or 103 alone did not produce a strong response to GAPDH. Only two animals were vaccinated with the single isolates so any conclusions established from the data must be made with caution; however, it may be useful to compare the different isolates for their ability to induce responses to GAPDH.

Additionally, CMI responses are likely to be different following infection versus vaccination with bacterins and this may account for the low CMI responses detected. Therefore, the antigens should be further tested in infected animals to determine their functionality as diagnostic antigens.

7.5 *In-vitro* studies using antisera from vaccinated sheep

7.5.1 Surface location of the immunogenic proteins was supported by immunofluorescence microscopy.

Immunofluorescence was used to confirm the predicted localisation of each of the recombinant proteins on the surface of the mycoplasma membrane (Fig. 5.1-5.6). While Immunofluorescence can visualise the detection of proteins by antibodies, it is unable to distinguish between different antibody-protein interactions within an individual sample. Therefore, it is important to determine what proteins the antibodies within a sample (sera) can detect. If purified polyclonal antibodies against an individual antigen are not available, then Western blot analysis can be used to determine if the sera is primarily detecting the recombinant protein used in the vaccination or reacting against additional or other mycoplasma antigens.

As EF-Tu has been previously shown to be surface localised [160, 392-394], this protein was used as a positive control for the immunofluorescence protocol. As expected, *M. ovipneumoniae* stained with pre-immune sera showed limited fluorescence whereas cells stained with antisera produced against ET-TU clearly showed fluorescently labelled cells. A similar pattern was also observed for GAPDH. In contrast, no difference in fluorescent staining was observed between pre-immune and protein-specific antisera for both HP1 and HP3. ELISA and Western blotting, using subcellular fractions showed that sheep vaccinated with the recombinant form of HP1 and HP3 produced

antibodies that recognised both recombinant and native forms of the protein. A possible explanation for the apparent lack of immunofluorescence is the antibodies were unable to bind to the native target protein in the context of the cell envelope. For HP1, antibodies produced against the relatively short epitope might not access the corresponding epitopes in the native folded protein. HP3 requires further analysis to determine its surface location. Both pre-immune and P146 antisera produced relatively strong fluorescence. The Western blots in chapter 5 also showed a comparatively high background of immunoreactivity suggesting that the animals vaccinated with P146 may have had prior exposure with mycoplasma. This made it difficult to determine the localisation of P146.

7.5.2 Bovine endometrial epithelial cells were established as a model to study adherence. Studies in Chapter 6 examined the ability of *M. ovipneumoniae* to adhere to an epithelial cell line and determined the ability of antibodies against *M. ovipneumoniae* WC or the recombinant proteins to inhibit adherence. *M. ovipneumoniae* was confirmed to adhere to the bovine -endometrial epithelial cells and the mycoplasma cells remained attached after washing the epithelial cells with phosphate-buffered saline (PBS). Pre-incubation of *M. ovipneumoniae* with antibodies against WC of isolate 16 reduced the ability of *M. ovipneumoniae* to adhere to the bovine endometrial epithelial cells. Collectively, these results confirmed the use of bovine epithelial endometrial cells as a model to study adherence of *M. ovipneumoniae*.

7.5.3 Antibodies against the immunogenic proteins did not inhibit adherence of *M. ovipneumoniae* in culture.

While antibodies against WC of isolate 16 inhibited adherence, none of the anti-recombinant protein antibodies blocked adherence of *M. ovipneumoniae* to the epithelial cells. One limitation of these experiments to test antisera against individual proteins may be the presence of multiple adherence sites on the endometrial epithelial cells. This would limit the ability of antibodies against single antigenic target to reduce the adherence of *M. ovipneumoniae* at least in an observable manner. The ability of antibodies produced against WC to reduce adherence supports the idea of multiple sites on the endometrial epithelial cells being available for mycoplasma adherence. Further optimisation of this experimental protocol and use of other cell lines may clarify the effect of the anti-recombinant protein antibodies on the adherence ability of *M. ovipneumoniae*. Combination of antibodies against multiple recombinant proteins may cause inhibition of adherence by targeting multiple sites involved in adherence simultaneously. Combination of antibodies were not tested due to time constraints as well as issues with the cultivating the bovine endometrial epithelial cells.

7.6 Significance of the study findings

Despite the common presence of *M. ovipneumoniae* there has been limited studies on the molecular analysis of New Zealand isolates. A majority of the previous studies on vaccine development work

has focused on use of the whole cells of *M. ovipneumoniae*. The work undertaken in this thesis was completed with the approach of obtaining novel immunogenic proteins for potential vaccine or diagnostic targets. The combination of genomic and immunological techniques has provided a unique methodology to identify and test potential *M. ovipneumoniae* antigens for vaccines and for diagnostics.

Using the genomes built in chapters 3, and two-dimensional Western blotting and mass-spectrometry analysis (chapter 4), eight immunogenic proteins were identified, and their full nucleotide sequence elucidated. Two of these proteins HSP70 and EF-Tu are known immunogenic proteins in *M. ovipneumoniae* which provides support for the validation of the protocols used in this thesis. The other six proteins are novel immunogenic proteins in *M. ovipneumoniae* which are considered potential antigenic targets for a vaccine or diagnostics. Combining the annotated genome and BLAST, three of the proteins were able to be matched with named mycoplasma proteins, with GAPDH and MATE previously identified in *M. ovipneumoniae* and P146 was detected as a homolog of *M. hyopneumoniae* P146. The other three proteins with no similarity to either *M. ovipneumoniae* or other mycoplasmas proteins are currently considered as hypothetical proteins. As further work is completed the functions of these proteins may become understood and their role in pathogenesis identified. This will not only be useful for increasing the general knowledge of *M. ovipneumoniae* biology but also important in understanding the biological role of HP3 as it has shown potential as a diagnostic target.

All five recombinant proteins studied in the thesis, EF-Tu, GAPDH, P146, and hypothetical proteins 1 and 3 induced strong antibody responses but produced limited cellular responses in sheep (chapter 5). As *M. ovipneumoniae* is primarily viewed as an extracellular pathogen, antibody responses may be sufficient for protection [98, 99]. However, further experimental analysis will be required to determine if antibody alone is able to provide protection against infection with *M. ovipneumoniae* or limit the severity of disease.

P146 and HP3 showed some promise as diagnostic proteins and could be investigated further. Having a test based on single or multiple targets, produced as recombinant proteins and able to detect an immune response may allow for the design of methods for quick and accurate detection of infection. Lab based analysis like ELISAs can use controls such as a blank (water/PBS) or a sera sample of known negative infection status to deduct the non-specific binding value from the unknown samples [211], reducing the chances of false positives. In the field, this approach is less convenient and the use of binary tests, like those used in pregnancy tests, can be more efficient for the rapid testing of flocks [70]. However, binary tests can be susceptible to background responses of animals and therefore the correct target antigens as well as the optimal concentrations of the target

antigen as well as the sample tested will be important in reducing the effect of background responses [70].

To determine the protective effect of vaccination against diseases an experimental challenge trial is undertaken. However, as this introduces an animal to the live virulent version of the pathogen, it is recommended to have supportive data for the protective likelihood of the vaccine, ideally limiting the number of animals contracting the disease. In this thesis, the likely protective effect of the vaccination with *M. ovipneumoniae* was investigated, looking at the effects of antibodies on adherence of *M. ovipneumoniae* to an epithelial cell model. In the adherence assay only the antibodies against the WC of isolate 16 showed inhibition of *M. ovipneumoniae* adherence to the epithelial cells. This result is encouraging because if adherence of *M. ovipneumoniae* cells, can be prevented in the lungs by vaccination then establishment of infection may be prevented. Due to the inconclusive results obtained using the methods employed in this thesis, particularly for the growth inhibition studies, further analysis is needed to investigate the protective potential of the different proteins before proceeding to test them in challenge trials. This could involve the optimisation of the experiments, using different epithelial cells to better mimic sheep airways epithelium, or specific targets such as fibronectin or extracellular matrix, to tease out any effect of the target specific antibodies has adherence ability of *M. ovipneumoniae*.

7.7 Study limitations

Due to the breadth of experimentation undertaken within this thesis, the use of novel antigens, and the time constraints of a PhD project there are a several limitations that must be accounted for. The antibody responses for each of the five proteins were only measured in 1 or 2 sheep, making it difficult to validate the results. Increasing the number of animals vaccinated with the recombinant proteins will assist in reducing the effect of individual animal immune responses. Further studies using larger groups of animals is also required to evaluate the usefulness of HP3 and P146 for detecting immune responses to *M. ovipneumoniae*.

Another limitation of using novel recombinant proteins as a vaccine target is determining the correct dosage to get an optimal immune response with minimal adverse effects. Completing additional vaccine trials with a range of antigen concentrations will determine if increasing the amount of protein in the vaccine will simulate strong antibody and also stronger cellular immune responses. The adjuvant used in this thesis was selected because of the strong responses observed in previous vaccine trials using whole cells of *M. ovipneumoniae* and *M. haemolytica*. This however does not necessarily correlate to strong responses in single antigen vaccines. Further work could be conducted to find a more suitable adjuvant for recombinant proteins.

Another limitation in the trials was that whole blood assays were done only at the final bleed in animals vaccinated with the various recombinant proteins. This meant that CMI responses for individual animals, post-vaccination could not be compared to those pre-vaccination to observe any increase in response. Collection of whole blood at multiple time points in future trials would assist in determining the kinetics of CMI responses.

Another important aspect not addressed in this thesis in the long-term immune responses to the vaccinations. For this thesis all data was collected within 7 weeks of the first vaccination, and no ongoing data was collected to determine if immune responses continued to increase or waned significantly over time. This was not undertaken as long-term analysis was outside the scope of this project and the time constraints would not allow identification of target antigens and completion of long-term studies in the same project.

Time constraints also limited the time available for certain experiments and analyses. Completion and quality assessment of sequence data was not able to be completed on all of the isolates within the time frame of the project, therefore it was decided to focus on the three isolates used as the reference strains and have them completed to a point where they could be annotated. Additionally, growth inhibition and cell adhesion assays were unable to be optimised within the time allocated to these experiments, with the cell adhesion experiments also experiencing unexpected issues with the culturing of the epithelial cells limiting the number of experiments available to be completed within the allocated time.

An overarching limitation in the study of a pathogen in an animal host, that is usually present in the majority of the animals, is the potential for natural infections occurring before or during the vaccination periods. While with the larger trials the animals would usually be tested for antibodies to *M. ovipneumoniae* before the commencement of the trials and animals with pre-existing high antibody titres, suggestive of exposure or infection being excluded, the trial for recombinant proteins did not have additional animals available. This does appear to have resulted in some animals in this trial exhibiting immune responses to *M. ovipneumoniae* in pre-immune sera suggesting a natural exposure.

7.8 Further research

The results presented in this thesis suggest several avenues for further research. One area of interest would be to further investigate the ability of specific antibodies to the recombinant proteins to inhibit growth of *M. ovipneumoniae* and adherence to host cells. One such approach would be to test different combinations of antibodies to single recombinant proteins to determine if different combinations are able to inhibit growth or adherence. Additionally, the use of a slower growing strain of *M. ovipneumoniae* may be useful in the growth inhibition assay. If a combination is found to

be effective at inhibition the next step would be to vaccinate sheep with the combined recombinant proteins and determine if the combination of antigens produces protective antibodies. If antisera from animals vaccinated with combinations of recombinant proteins are found to be effective in the *in vitro* assays, the next step would be to test the vaccine efficacy in a challenge model of ovine pneumoniae. The use of purified polyclonal antibodies (potentially in rabbits) to recombinant proteins would remove the effect of natural infections limiting responses to only the recombinant proteins. This would be useful for confirming the localisation of the recombinant proteins on the membrane using immunofluorescence microscopy or electron microscopy. Purified antibodies would also be useful in the growth inhibition and cell adhesion analysis experiments. Antigens or combinations thereof, shown to have potential to provide protection would require additional long-term immunogenicity trials. Undertaking trials with large sample sizes, different dose rates, and different adjuvants would provide the completest picture of the potential of the recombinant proteins as vaccine targets.

Increasing the number of genomes of New Zealand isolates as well as isolates of Australia, the location of the type strain, will increase the confidence in the phylogenetic positioning of New Zealand isolates and further evidence on likely invasion events into and out of New Zealand. Additionally, further genomic sequencing, especially by the use of long read length (Pacbio) methods, of the NZ isolates of *M. ovipneumoniae* already sequenced will aid in the completion of the genomes.

Another area of interest would be to conduct further studies to determine the suitability of P146 and HP3 as diagnostic reagents. In particular, investigate the potential use of these antigens for a rapid point-of-care test for the detection of *M. ovipneumoniae* infection under field conditions. This would require the use of animals naturally or artificially infected with *M. ovipneumoniae* to determine if these antigens were able to detect antibodies in response to infection, and not just antibodies in response to vaccination. Also, for the production of a point of care device, additional methods for obtaining antibodies, that do not require the bleeding of the animals and collection of sera, such as nasal secretions, saliva, tear fluid, would be required.

7.9 Final conclusions from the thesis

Combining an empirical approach to identify immunogenic surface proteins with genomic analysis of *M. ovipneumoniae* has resulted in the identification of six novel immunogenic proteins. Of the six novel proteins, two proteins, P146 and HP3, have shown potential as diagnostic reagents. Genomic sequencing and bioinformatic analysis have resulted in producing three draft genomes of the New Zealand isolates 16, 90, and 103. These three genomes have been annotated and will provide a large pool of information for further analysis. Bovine endometrial epithelial cells have been used to design

an adherence inhibition assay which has been validated through the use of antibodies against WC16. Further optimisation of this assay may be useful for determining the effect of antibodies to different vaccine antigens in inhibiting adherence, providing additional tools for assessing the potential of vaccine combinations in absence of challenge trials. GAPDH has been shown to have potential as a diagnostic protein for the measurement of IL-17 responses in sheep.

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Appendices

Genome paper



GENOME SEQUENCES



Draft Genome Sequence of a New Zealand Isolate of *Mycoplasma ovipneumoniae*

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ABSTRACT The genome of New Zealand *Mycoplasma ovipneumoniae* isolate 90 was sequenced and assembled using an Illumina MiSeq system and combining the built-in Geneious *de novo* and Velvet *de novo* assemblers. The 1,031,345-bp-long genome harbored 711 genes with a coding percentage of 86.6.

Chronic nonprogressive pneumonia (CNP) is one of the most prevalent and difficult-to-treat diseases to infect sheep and young lambs in New Zealand, resulting in significant economic losses to the industry (1). *Mycoplasma ovipneumoniae* is the primary cause of CNP, reducing the host's ability to prevent secondary infection by other pathogens. The draft genome presented here, designated isolate 90, is based on the sequence of a representative isolate from one of three dominant genotypes identified during a survey of farms in New Zealand which had a history of pneumonia.

Mycoplasma ovipneumoniae was isolated from pneumonic sheep according to a previously described method with slight modification (2, 3). Briefly, lungs from pneumonic sheep were washed with saline, and bronchoalveolar lavage fluid (BALF) samples were collected. BALF samples were inoculated into pleuropneumonia-like organism (PPLO) broth and incubated at 37°C until growth was evident as indicated by a phenol red pH indicator. Tenfold serial dilutions were prepared in PPLO broth from positive samples and incubated at 37°C. The highest dilution with a visible pH change was used for broth-to-plate cloning. A single drop of 25 µl of culture was layered onto PPLO agar plates. After incubation at 37°C for 72 h, colonies were observed under a stereo microscope. For plate-to-broth cloning, a single colony was picked by cutting out an agar plug using a sterile Pasteur pipette. This was inoculated into fresh PPLO broth and incubated at 37°C until a pH change was observed. The broth-to-plate and plate-to-broth cloning steps were repeated two more times to obtain pure cultures for each *M. ovipneumoniae* isolate.

Isolate 90 was cultured for sequencing in Frey's medium (4) at 37°C until a phenol red pH indicator indicated growth of the mycoplasma. Genomic DNA was extracted using a ZR fungal/bacterial DNA miniprep commercial kit (Zymo Research Corp., Irvine, CA). The resulting genomic DNA was subsequently purified using a ZR genomic DNA clean and concentrator kit. DNA was sequenced on an Illumina MiSeq system (New Zealand Genomics Limited, Dunedin, New Zealand), producing 1,401,512 paired-end (PE) reads. The raw reads were trimmed against a UniVec database (Geneious R10.0.9) (5), and short-length reads (<25 nucleotides) were removed. After trimming, 1,214,927 PE reads (Geneious R10.0.9) (5) with an average length of 171 bases per read for a 200× coverage were used for genome assembly.

Default parameters were used for all software except where otherwise noted. The

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in-house Geneious R10.0.9 (5) and Velvet 1.2.10 (6) *de novo* assemblers were used to produce the initial assemblies, which had N_{50} values of 62 kbp and 38 kbp, respectively. The initial contigs were improved by remapping the sequence reads using the Geneious R10.0.9 map-to-reference function (5) and then using the in-house Geneious *de novo* assembler to combine the contigs into larger reads. Single nucleotide polymorphisms between the combined contigs were resolved by making a final map-to-reference run using the raw reads and taking the most common nucleotide. Combining the in-house Geneious (Geneious R10.0.9) (5) and Velvet 1.2.10 (6) reads produced 14 contigs.

The draft genome sequence was 1,031,345 bp long with a GC content of 29% and an N_{50} value of 85 kbp. The genome sequence was annotated using GAMOLA2 2.0.0.14 (7), and the gene model was built using Prodigal 2.6.3 (8). Gapped Blastp was carried out with the NCBI nonredundant amino acid database. Clusters of orthologous groups (COGs) were determined using the latest COG database (2014) (9). The CDD (v3.16) (10), Pfam (release 32) (11), and TIGRFAMs (release 15.0) (12, 13) databases were used to identify the respective domains. The annotation was visualized and analyzed using a modified version of Artemis 16 (7, 14, 15). In total, 711 genes with an average length of 1,257 bp and a coding percentage of 86.6 were identified.

Data availability. This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number [VZDP00000000](https://www.ncbi.nlm.nih.gov/nuclseq/VZDP00000000). The version described in this paper is version VZDP01000000, and the BioProject number is [PRJNA573593](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA573593). The sequence reads have been deposited at the SRA under the accession number [SRR10959050](https://www.ncbi.nlm.nih.gov/sra/SRR10959050).

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Amino acid sequences and nucleotide sequences of proteins identified in Chapter 4

1 ATGGCAAAAGAAATAATTTTAGGTATTGACTTAGGAACAACAACTCAGTTGTTGCAATTATCGAA
 M A K E I I L G I D L G T T N S V V A I I E
 70 AATCAAAAACCTGTTTCTTGAAAATCCTAACGGAAAAAGAACTCCTTCTGTTGTTGCCTTC
 N Q K P V V L E N P N G K R T T P S V V A F
 140 AAAACGGTGAGGAAATTTGTTGGTGATGCTGCAAAACGTCAACTAGAACTAACCTGAAGCAATC
 K N G E E I V G D A A K R Q L E T N P E A I
 210 GCATCTGTTAAAGATTAATGGGTAGCGATAAACTGTTTCGCTAACCAAAGAGACTATAAACCT
 A S V K R L M G S D K T V R A N Q R D Y K P
 280 GAAGAATTTTCAGCAAAAATCTTGCATATTTTAAAGAATATGCTGAAAAAAGATTGGTCACAAA
 E E I S A K I L A Y L K E Y A E K K I G H K
 350 GTTTCAAAAGCTGTTATCACAGTTTCCTGCTTATTTTGATAATGCTCAACGTGAAGCGACAAAAAT
 V S K A V I T V P A Y F D N A Q R E A T K N
 420 GCCGGA AAAATCGCAGGATTAGAAGTTGAAAGAATAATTAACGAACCAACAGCAGCCGCACTTGCA
 A G K I A G L E V E R I I N E P T A A A L A
 490 TTTGGACTTGACAAAACAGAAAAAGAAATGAAAGTGCTTGTGTTTATGACTTAGGTGGTGAACCTTTT
 F G L D K T E K E M K V L V Y D L G G G T F
 560 GACGTTTCAGTTCTTGAATTATCTAACGGAACCTTTGAAGTTTATCAACAAGTGGTGATAACCAT
 D V S V L E L S N G T F E V L S T S G D N H
 630 TTAGGTGGTGATGACTGAGATAATCAAATTGTTGACTGAATGGTTAAAAGAATTAAGAAGAGTAC
 L G G D D W D N Q I V D W M V K R I K E Y
 700 GATTTTGATGCAAAAAGCGACAAAATGGCCTTACTCGTCTAAAAGAAGAAGCTGAAAAAACCAA
 D F D A K S D K M A L T R L K E E A E K T K
 770 ATTAACCTATCAAATCAAAGTGTTTCAACAATTTCTTACCATTCTTGGTCTAGGAAAAAATGGT
 I N L S N Q S V S T I S L P F L G L G K N G
 840 CCAATCAACGTTGAACTTGAACATAAAAGATCAGACTTTGAAAAAATGACAGCTCACTTAATTGAC
 P I N V E L E L K R S D F E K M T A H L I D
 910 CGAACAAGAAAACCAATCGTTGATGCCTTAAACAAGCTAAAATTGAAGCTAGTGAGCTTGACGAG
 R T R A A P I V D A L K Q A K I E A S E L D E
 980 GTTCTCTTGTGTTGGTGATCAACACGTATGCCAGCTGTTTCAGACAATGATTGAACACACTTTAAAT
 V L L V G G S T R M P A V Q T M I E H T L N
 1,050 AAAAGCCAAATCGTTCAATAAATCCTGATGAAGTTGTGGCAATTGGAGCTGCAATTCAAGGTGGA
 K K P N R S I N P D E V V A I G A A I Q G G
 1,120 GTTCTTGCTGGAGAAATTAGTGATGTTTTATTACTTGACGTTACTCCTTTAACTTTAGGAATTGAA
 V L A G E I S D V L L L D V T P L T L G I E
 1,190 ACCTTAGGCGGAGTTTTCAACCCCATTAATTCCAAGAAATACAACAATTCCGGTAACAAAATCACAA
 T L G G V S T P L I P R N T T I P V T K S Q
 1,260 ATTTTCTCAACAGCTGAGGACAAATCAAACCGAAGTTACAATCTCTGTAGTTCAAGGTGAAAGACAA
 I F S T A E D N Q T E V T I S V V Q G E R Q
 1,330 CTTGCTGCTGATAACAAAATGTTAGGTCGCTTTAATCTTTTCAGGAATTGAGCCTGCTCCTCGTGGT
 L A A D N K M L G R F N L S G I E P A P R G
 1,400 CTTCCACAAATTGAAGTTAGTTTTTCAATTGACGTTAACGGTATTACAATCTGTTTCAGCAAAAGAT
 L P Q I E V S F T I D V N G I T T V S A K D
 1,470 AAAAAAAGTGGAAAAGAACAACAAATTACAATTAATAAATACTTCAACTTTATCTGAAGAAGAAATT
 K K T G K E Q T I T I K N T S T L S E E I
 1,540 AACAGAATGATTCAAGAAGCCGAAGAAAATCGTGAAGCTGATGCAATCAAAAAAGATAAAATTGAA
 N R M I Q E A E E N R E A D A I K K D K I E
 1,610 ACAACAGTTCGTGCCGAAGGTCTTATTAATCAACTTGAAAAATCAATAACTGATCAAGGTGAAAAA
 T T V R A E G L I N Q L E K S I T D Q G E K

1,590 1,600 1,610 1,620 1,630 1,640 1,650
 ATTGATCCTAAACAAAAAGAACTTCTTGAAAAACAATTCAAGAACTCAAAGATCTTTTAAAGAA
 I D P K Q K E L L E K Q I Q E L K D L L K E
 1,660 1,670 1,680 1,690 1,700 1,710
 GAAAAAATTGACGAACTAAAAACAAATTAGACCAAATTGAGCAAGCAGCCCAAGCTTTTGCCCAA
 E K I D E L K T K L D Q I E Q A A Q A F A Q
 1,720 1,730 1,740 1,750 1,760 1,770 1,780
 GCAAGTGCTCAACAAGCTAACAGTGCAAGTGATACTGATTGAGATTCAAATACAATTGATGCTGAA
 A S A Q Q A N S A S D T D S D S N T I D A E
 1,790 1,797
 ATCAAACAAAATTAA
 I K Q N *

Appendix 2 Peptide sequence for HSP70 from isolate 90

1 10 20 30 40 50 60 70
 ATGGCAGTTGTTAAACTGGTGCAAAAAAGATTGTTGACCGTTCAAAAGAGCATATCAATTATGGGACAATT
 M A V V K T G A K K D F D R S K E H I N I G T I
 80 90 100 110 120 130 140
 GGTGATGTTGACCACGGAAAAACCACTCTAACAGCGGCAATTTCAACTGTATTATCAAAAAAGGTCTAGCT
 G H V D H G K T T L T A A I S T V L S K K G L A
 150 160 170 180 190 200 210
 GAAGCAAAAGATTATGCTTCTATTGACGCGCCCTGAAGAAAAAGCGGTGGAATTACAATCAATACAGCC
 E A K D Y A S I D A A P E E K A R G I T I N T A
 220 230 240 250 260 270 280
 CACATCGAATATAGCACAGACAAGCGTCACTATGCCCATGTTGATTGCCCTGGTCACGCCGATTATATTA
 H I E Y S T D K R H Y A H V D C P G H A D Y I K
 290 300 310 320 330 340 350 360
 AATATGATCACAGGAGCAGCAAAATGGATGGTGCCATTCTTGTGTTGCCGCAACAGATGGTCCAATGCC
 N M I T G A A Q M D G A I L V V A A T D G P M P
 370 380 390 400 410 420 430
 CAACTCGTGAGCACATTCTTTTCAAAACAAGTTGGTGTGCCAAAAATGGTTGTTTCTTAAACAAAATC
 Q T R E H I L L S K Q V G V P K M V V F L N K I
 440 450 460 470 480 490 500
 GACTTACTGAAGGTGAAGAAGAAATGGTTGACCTTGTGAGGTTGAAATTCGTGAACCTTTCTTCTATAT
 D L L E G E E E M V D L V E V E I R E L L S S Y
 510 520 530 540 550 560 570
 GATTTTGACGGAGACAACACCCCAATAATCCGTGGTTGAGCTCGTGCTCTTGAAGGAAAACCTGAATGA
 D F D G D N T P I I R G S A R G A L E G K P E W
 580 590 600 610 620 630 640
 GAAGCTAAAGTTCTTGAACATAATGGATGCAGTTGACTCATACTGACTCCCCAGTTCTGTGAAATGGACAAA
 E A K V L E L M D A V D S Y I D S P V R E M D K
 650 660 670 680 690 700 710 720
 CCATTCCTAATGGCAGTTGAAGACGTCTTTACCATACAGGTCTGTGGAACCTGTTGCTACTGGTAAAGTTGAA
 P F L M A V E D V F T I T G R G T V A T G K V E
 730 740 750 760 770 780 790
 AGAGGACAAGTTAACTAAATGAAGAGTTGAAATTGTGCGATATCGTCTGAACCTAAAAAACAGTTGTA
 R G Q V K L N E E V E I V G Y R P E P K K T V V
 800 810 820 830 840 850 860
 ACCGGAATTGAAATGTTTAAACAAAACCTTCAATCTGCAATGGCTGGAGATAATGCTGGAGTTCTTCTCGT
 T G I E M F N K N L Q S A M A G D N A G V L L R
 870 880 890 900 910 920 930
 GGTGTTGACCGTAAAGATATTGAACGTGGTCAGGTTATTGCCAAACCAAAAACCAATTATCCCCACACTAAA
 G V D R K D I E R G Q V I A K P K T I I P H T K
 940 950 960 970 980 990 1,000
 TTTAAAGCAGCAATTTACGCGCTCAAAAAAGAAAGGTGGAAGACATACTCCATTTTCAAAAATTACAAA
 F K A A I Y A L K K E E G G R H T P F F K N Y K
 1,010 1,020 1,030 1,040 1,050 1,060 1,070 1,080
 CCTCAATTTTATTTCCGTACAACCGATGTTACTGGTGAATTGAATTTGAAGCTGGCCGTGAAATGGTAATT
 P Q F Y F R T T D V T G G I E F E A G R E M V I
 1,090 1,100 1,110 1,120 1,130 1,140 1,150
 CCTGGGGATAATGTTGACCTTACTGTTGAACCTATTGCCCTATCGCTGTTGAGCAAGGTACAAAATTCCTCA
 P G D N V D L T V E L I A P I A V E Q G T K F S
 1,160 1,170 1,180 1,190 1,200 1,209
 ATTCGGAAGGTGGAAGAACTGTTGGAGCCGGAACAGTAACTGAAATTTATTAATAA
 I R E G G R T V G A G T V T E I I K *

Appendix 3 Peptide sequence for EF-Tu from isolate 90

1 ATGAAAAAATTGCAATTAATGGCTTTGGAAAGAATCGGTAGATTAGCACTTCGCCAACTTCTAGAT
 M K K I A I N G F G R I G R L A L R Q L L D
 70 CTTAAAGATGAAAATTTAGAGGTTGTTGCAATTAATGATCTGACTGATGCATCTGTCTTAGCTCAC
 L K D E N L E V V A I N D L T D A S V L A H
 140 CTTTTTAATACGACTCTGCCAAGGAAGATTTTCTGGAAGTGTAGCGTTTTTAAAGAAGATGAC
 L F K Y D S A Q G R F S G T V S V L K E D D
 200 AAGAATTATCTTGTAATCAATGACAAAAAATTCGCGTTTTTCAGAAAAAGACCCTGAAATGTTG
 K N Y L V I N D K K I R V F S E K D P E M L
 270 CTTGAGGAGAACTAGAAATTTAGTTCTTGAATGTACAGGGCGTTATGCATCAAAAGCTGGG
 P W G E L E I D L V L E C T G R Y A S K A G
 340 GCTACTTTACACCTTAATGCTGGTGCAAAAAAAGTTCTAGTTTCTGCTCCTGCTGGAAGTGATGTA
 A T L H L N A G A K K V L V S A P A G S D V
 400 AAAACTATCGTTCAATGTAACTGCCACACTGTTGATGAAAATGATAAAATTTTATCTTCTGCA
 K T I V H N V N C H T V D E N D K I L S S A
 470 TCATGTACAATAATGCGCTTGCTCCACTTGTAATGCGCTTGAAAAAGAATTTGGAATAAGCCAT
 S C T T N A L A P L V N A L E K E F G I S H
 530 GGATTTATGACAACAGTTTCATGCTTATACTGCGACCAAGAAATTCAAGATGCACCACATAAAGAT
 G F M T T V H A Y T A D Q R I Q D A P H K D
 600 CTAAGAAGAGCAGTGCTGCTGCTGTTAACTTAGTACCTTCTTCAACTGGTGCTGCAAAAGCAATT
 L R R A R A A V N L V P S S T G A A K A I
 670 GGTCTTGTTGTTCTTCTTAACTGGAAAATTAGACGGAATTGCTATAAGAGTGCCGGTAATTACA
 G L V V P S L T G K L D G I A I R V P V I T
 730 GGTCTTTTTGTTGACTTAAGTGTGGAATTAATCATCACCTTCTATTGAAGAACTTAATAAAGCT
 G S F V D L S V E L K S S P S I E E L N K A
 800 ATTAATAAATACGAAAGCGAATCCTTTTATTATTGCGACGAACCTATTGTTTCTAGCGACATTATC
 I K K Y E S E S F Y Y C D E P I V S S D I I
 860 GGGGACACATGGTTCAATTTTGTGATGCAACCTTAACAAAATTTGTTGAAGTTGATGGTAAGA
 G D T H G S I F D A T L T K F V E V D G K R
 930 TTATACAACTATACACTTGATACGACAATGAATCTTCATTTGTTGCACAATTCGTTAGAGTTATC
 L Y K L Y T W Y D N E S S F V A Q F V R V I
 1,000 AGATACTTTATTCAAAAATAG
 R Y F I Q K *

Appendix 4 Peptide sequence for GAPDH from isolate 90

1 ATGCATTTTCAATAAAAAATTTTTCCTGATTCGCCAGAAATGTGGAAAAATTTGCCAAAATT
 M H F S I K K F F P D S P E M W K K F A K I
 70 ACTATCCAGTAATTTTGGCAACGCTTTTATTTGATAAAATTTTATTGATAATTTTCATGGTT
 T I P V I L A T L F I S I N N F I D N F M V
 140 TCCCAATCAGTGGTGGTATTACGGCTGTTGGGATGGCCAATTTTGAACAGGAATTGTTTTTCT
 S Q I S G G I T A V G M A N F W T G I V F S
 210 TTTATAATTTGATTAACACAATCGGGTCAATTTTGTCCCAATACTGAGGAAAAAAGAATTT
 F I I S I N T I G S I I F A Q Y W G K K E F
 280 AAACGGCCCGCAAGTAAATAATATTAGATATATTTCTTTCTTTTATTGTTATTTTATTTTTTGCT
 K L A R Q V N N I R Y I L S F I V I L F F A
 350 GTTCTCTCGTGAGCTTTTCTGAAAGTCTGCTCAGAGTCGTTTCAGTGAAGACGGGGTTCGTCAAAT
 V L S W A F P E S L L R V V Q W R R G S S N
 420 TTAGACCAGAGTATTTTGGACGAGGCAAAAAGTTACTTATTTATAAAGTTTCTTGAATCTTG
 L D Q S I F D Q A K S Y L F I I S F S W I L
 490 TTTGCTTACATTGTCACTTCTCAGGAATTCTGCGTGAAATGGGGTAAAGTAAAGTTTCTGTTCTT
 F A Y I V T S S G I L R E I G V M K V S V L
 560 TTTAACACTTTGACCTTAGTAACGAATATTGGCCTTAATTTTACTAATTCCTTAATGGGAGTC
 F N T L T L V T N I G L N F L L I P L M G V
 630 TCTGGTTCAGCGCTTGCAACTGTAATTTCAAGACTAATAACCTCTTTTTTAATGTATCTTTATCAA
 S G S A L A T V I S R L I T S F L M Y L Y Q
 700 CTTTTTATAGAAAAGAAATTAGTCTCTCAATTTTAAAAATGTTTCAAATTGACAGACAAATTTGA
 L F Y R K E I S L S I L K M F Q I D R Q I W
 770 AAGCAGTTTTTGGCCGTTTTGTGGAATGTGCTGTGTAACCTTTGCTTCTTTAATTTTTCAGTC
 K Q F F A R F V G M S L V T I A S L I I S V
 840 CGTTTCAGTTCTTTGATCCCAAAGTTTGGCCGCGTTCAATTTGGAATTTGACGATGGCAGTGGTTTT
 R S V L W S Q S L P P G S I G I D D G S G F
 910 TATAAATTTTGAGGTATTGGATTTTAACTATTTTCAAGGAATAATTCTCACAATTTGCTAACATTTT
 Y K F W G I G F L T I S G I I L T I A N I F
 980 TTTACAACTTTTGCCTCAATTCAGACAAGCGTTTTCGATTGTTGTCGGTCAAAATTTAGGTCAAAAT
 F T T F A S I Q T S V S I V V G Q N L G Q N
 1,050 AAAATTGAACTAGCCAAAAAAACGCTGCAATGCTCAAAGGATTTTTATTTGTTGTTTCAGTTGTT
 K I E L A K K N A A M L K G F L F V V S V V
 1,120 ATGTCAGTTGTGGCTTTTATTATCGTTTTTGTCTATCACAACAACTGACTTGATCACAACGGGAATT
 M S V V A F I I V F A I T K T D L I T T G I
 1,190 AAAGAACAAGTTCAAAAAGGATTGTCTGATTATTTTGGCGAAAATAATTTGCCAGTTGACCAAAGT
 K E Q V Q K G L S D Y F A E N N L P V D Q S
 1,260 ATTATTGACGCCAAAAAGTTCTAGCCGTTGATTTTATCTAAATCAGATTTTATAATCTGTATA
 I I D A Q K V L A V D F Y L N Q I F I I C I
 1,330 GTTATAATTTTAATTAATCCAAATTTGGGTTCAAATTAATGCCTCTTTAAACAATAATTAAAGCCGGC
 V I I L I N P I W Q I N A S L T I I K A G
 1,400 GGACGGGCTAATTTTGTAGTTGGTGAGATTTTTTACCAGGTATAGGTCAACTTGTGTTGACAAATT
 G R A N F A S W D F F T G I G Q L V W Q I
 1,470 CTTATAGTTTTAGTTTTTATCCCGCTGATTTCTGAGGTTTCAATTTAAACTTTTTATTTCCTGACA
 L I V L V F I P L I S E V H L K L F I S L T
 1,540 GTTTTTTATCTTTCAGATATTCTAAAGTGAATTATTTTGAACATAATTTATCTAAAAAGTAATTGG
 V F Y L S D I L K W I I F E L I Y L K S N W
 1,610 GCGATTAATTTGACGCTTGAAAATTCAGCCTTAAAGAATCTATCTAA
 A I N L T L E N S A L K E S I *

1 ATGCCTAAAAATACTAATAGATTAGCAATAGCAATAACCGCTGTTGGCGGTGTTGCTATTTTTCGA
 M P K N T N R L A I A I T A V G G V A I F A
 70 ACGACTATTGGGCTTGTAATTCGAATTCGCTATACAGGAGAAAAATCCCCGAGCTGAATTAGAAAGT
 T T I G L V T R I R Y T G E N P R A E L E S
 140 TTAGTTTCGCGAGTCCAAAATGTTGCTTTAAATCTGATGTCTTTGATGATTCTACCACATACAGC
 L V S R V Q N V A F K S D V F D D S T T Y S
 210 CAAATAAAATCACAACTTTTGACCAAAGTGGAAAATTATTAGCTGGCACAGATTTAAATAAATTT
 Q I K S Q L F D Q S G K L L A G T D L N K F
 280 ATATCTTTTACACACAGGTTAACTCAAACCTACGTAAATTTGAGCCAACTTTTGACCAAATAAG
 I S F Y T Q V N S K L R K F E P T F A P N K
 350 CCATTTTGTAGATTTATTGACTTAATTCCAAATGATAATGATCAAAGTTTTGAGCTTCGTTTTTCGT
 P F L E F I D L I P N D N D Q S F E L R F R
 420 GCAAAGCACCAAGTTGATAACAATCACACAGCATTTTCAACCATTATTCAAAAAAGGTGTCAATT
 A K H Q V D N N H T A F S T I I S K K V S F
 490 GCACAACGTTTACAATTTGCTCTTGCCGAATTTAATTCAAACTTAGAAAAAATCACCAAAAGTTTC
 A Q R S Q F A L A E F N S N L E K I T K S F
 560 AAAGAAAAATATCAAAATCTAAGAAGAACAGATTTTGTAGCTCAAGTGTATATAATCCAAGTTTAGTT
 K E N I Q N L R R T D F S S S V I N P S L V
 630 GATCAAAAAATTGCATCGCTAACTCGTCGAGAAGATTTTGGCGCTGATATTAATAGAGCCCGAAT
 D Q K I A S L T R A E D F A A D I N R A R T
 700 CAAAGCGAGGCTGTTGAAAAATAAGTCAATTTCCCTGATTTTCAAAAAATAATTAACGATTTA
 Q S E A V E K I S Q Y F P D F Q K I I N D L
 770 AACTTTGACAAAAATAATTTTCACTTTTAAACAAGGAACAATTTACAATTTTAGTTTAGAAAAA
 N F D K N N F F T F K Q G T I Y N F S L E K
 840 CATCCTGGAATAATAATTTTATTTTCAAGTTGGTCCAAATTCAGTTCCAAAGTTTTTTAGTTAAGGCT
 H P G T N N F I S V G P N S V P S F L V K A
 910 GAATTAAGTGTATGATGCAAAATTTGTGCTTAATAAATTTTAATATTGAAGATGCCCAATTGCTTGAA
 E L S D D A K F V L K N F N I E D A Q L L E
 980 AAAATTGATTTAGTTTCCGCGAGGCTAGTTTCAGGTTCTGAGTCAAGCCAAAGGTGAAGAAAAACAAGCT
 K I D L V P Q A S S G S E S S Q G E E K Q A
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 K Q A T Y F A D V D D I L S K I S L R K L Q
 1,120 TTTACTGATTTTAAAGTTGGAAGTCAGCCAGTTACTAATACTCAAGTTCAAGCTCAGGCTAGCTTA
 F T D F K V G S Q P V T N T Q V Q A Q A S L
 1,190 TTAAGTTCTGGAGATATTAAGCAACTAGTTCAAGTTTCTCTGAAGTTACCCAAAGTCAACCTCAA
 L S S G D I K Q L V Q V S S E V T Q S Q P Q
 1,260 GCTCAAGCTGAAGGAGGAGCGGGAGGAGGAACGGAACAAGCAACTTCAGGAACAAGTGAACA
 A Q A E G G A G G T G Q Q A T S G T T G T
 1,330 GACGCGGGATCAACTGGGACAGGTGGAGTGGCAGGCGCAGGAGCACCCGACTCAGGAACAGGATCA
 D A G S T G T G G V A G A G A P D S G T G S
 1,400 ACTGGATCAACACAGATCAGCAAACTAAACGGAGAATCTGGCACAGAACACCTTCTGTAACTACT
 T G S T T D Q Q T N G E S G T E Q P S V T T
 1,470 CCAGCTGAACCTGAAGATAAAACAAAGGAATTAGTCAATATCTTGAAATTTCAAATCGCACTACA
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 1,540 AATGAATTTTAGCTAGTTTAAATAAAAAATATATTCTTCTATTAGAGAAAAATCTAAAGAAGTA
 N E F L A S F N K K I Y S S I R E K S K E V
 1,610 GTAGATAAAATAAATTTGAAATTATTAATTAAGCCAATTTCCCTTGATTTTGGTGATTTTAATCAG
 V D K I N S E L L I K P I S L D F G D F N Q
 1,680 TATTTTGACAAAAACAACCTCAAGGTGTTGATTTTATTTAGATCTTTCTAAAGCACAGAAAAA
 Y F D K K Q P Q G V D F Y L D L S K A Q E K
 1,750 GATGGCGTTAATACAGACAATTCAAACCTTGAATCCCGGTTGTTATTAATCTTTATTCAAGTTTT
 D G V N T D N S N L E I P V V I N L Y S S F
 1,820 TTTGGTGAAGTCAAGAAAAACAACCTTTAGGATCTAAACCTTCAGTTTTTGAATTTCTAATTTCAAG
 F G D S E N K L L G S K T S V F E I P N F K

AAGGTTGGAACACAAACTGGTTTCAGCGGGAACCTACAGATCTCTCTTCTAATTTAGATACAGAGCGG
 K V G T Q T G S A G T T D L S S N L D T E R
 1,850 1,860 1,870 1,880 1,890 1,900 1,910
 AAAACATTCTAATCTTGACGGTTTACCTACAACCTTCGGCTTCTCAACCACTTCAGTTTCTGTT
 K T F Y N L D G L P T T S A S Q P T S V S V
 1,920 1,930 1,940 1,950 1,960 1,970 1,980
 TCTGCTTCAACAACAGGTGCGCAAACTACCCAAATAAACAATTAGAATCGGGTCAACAACAGCT
 S A S T T G A Q T T Q N K I R I G S T T A
 1,990 2,000 2,010 2,020 2,030 2,040
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 Y I S K T E L E N L I G T S T T G S T S E G
 2,050 2,060 2,070 2,080 2,090 2,100 2,110
 ACCGGACAACTACTGAATCAAAAGCTCCTAAATTTGAAGAAATTAAGAAAATTTATCGGAAATCCT
 T G Q T T E S K A P K F E E I K K I I G N P
 2,120 2,130 2,140 2,150 2,160 2,170
 TTCCAATATGCATATGATTTTGTATGCAAAATGAGTCAATGCTAAAAGCTTGAGTTGGACAGCAAAAA
 F Q Y A Y D F D A N E S M L K A W V G Q Q K
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 F P K L E D F A S F T K N N F I A S D Y K I
 2,250 2,260 2,270 2,280 2,290 2,300 2,310
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 K S L K S D K F F K N E Y D V A S F Y A Y L
 2,320 2,330 2,340 2,350 2,360 2,370
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 I Q M E P A Q V L N Y L F E I A K A N G L V
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 V K F N T T E T N P V Y S L D F N N Q F L G
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 T F D S R G W I S N L F L P K T V W Q K T N T
 2,580 2,590 2,600 2,610 2,620 2,630 2,640
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 2,650 2,660 2,670 2,680 2,690 2,700
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 S G T S D D L Y K T L Q D A A K I K E A L
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 2,840 2,850 2,860 2,870 2,880 2,890 2,900
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 T Q P L S T L K D L L L A F Y F K A K E L T
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 2,980 2,990 3,000 3,010 3,020 3,030
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 K L T Y Y Y K V F D K K T G V A K Y Q T P K
 3,110 3,120 3,130 3,140 3,150 3,160
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 I D L N L W V N K Q D T Q R T E R D E L N K
 3,170 3,180 3,190 3,200 3,210 3,220 3,230
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 A V L S I P P S F S L F Y L K K E D F D K L
 3,240 3,250 3,260 3,270 3,280 3,290 3,300
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 K K G D S T E N T E G K K F E E T A V F K E
 3,310 3,320 3,330 3,340 3,350 3,360
 ATTCAAGAAAAATCCAAGAAAAACAATAAAGATCTCAAACCTCAGTTGTTTCAATAGATGAAGAT
 I Q E K I Q E N N K D L K L S V V S I D E D
 3,370 3,380 3,390 3,400 3,410 3,420 3,430
 AAAATCAGTCCGCAAGATTTAAAGTTGTGAATTTGCAAAATCGAAAAAACTACACCAACTACTGAA
 K I S P Q R F K V V N L Q I E K T T P T T E
 3,440 3,450 3,460 3,470 3,480 3,490
 GGTCTTCAACTGGTCAGTCAGTTAAGTCAAGTCTTAGTTTCCAAGTTAGAATTGCACTAGACGAT
 G S S T G Q S V K S S L S F Q V R I A L D D
 3,500 3,507
 ACACAATAA
 T Q *

1 ATGCCTAAAAATACTAATAGATTAGCAATAGCAATAACCGCTGTTGGCGGTGTTGCTATTTTGTCA
 M P K N T N R L A I A I T A V G G V A I F A
 70 ACGACTATTGGGCTTGTAACTCGAATTCGCTATACAGGAGAAAAATCCCCGAGCTGAATTAGAAAGT
 T T I G L V T R I R Y T G E N P R A E L E S
 140 TTAGTTTCGCGAGTCCAAAATGTTGCTTTAAATCTGATGTCTTTGATGATTCTACCACATACAGC
 L V S R V Q N V A F K S D V F D D S T T Y S
 210 CAAATAAAATCACAACTTTTGACCAAAGTGGAAAATTATTAGCTGGCACAGATTTAAATAAATTT
 Q I K S Q L F D Q S G K L L A G T D L N K F
 280 ATATCTTTTACACACAGGTTAACTCAAACCTACGTAAATTTGAGCCAACTTTTGACCAAATAAG
 I S F Y T Q V N S K L R K F E P T F A P N K
 350 CCATTTTGTAGATTTTATTGACTTAATTCCAAATGATAATGATCAAAGTTTTGAGCTTCGTTTTCGT
 P F L E F I D L I P N D N D Q S F E L R F R
 420 GCAAAGCACCAAGTTGATAACAATCACACAGCATTTTCAACCATTATTCAAAAAAGGTGTCAATT
 A K H Q V D N N H T A F S T I I S K K V S F
 490 GCACAACGTTTACAATTTGCTCTTGCCGAATTTAATTCAAACTTAGAAAAAATCACCAAAAGTTTC
 A Q R S Q F A L A E F N S N L E K I T K S F
 560 AAAGAAAAATATCAAAATCTAAGAAGAACAGATTTTGTCTCAAGTGTATATAATCCAAGTTTAGTT
 K E N I Q N L R R T D F S S S V I N P S L V
 630 GATCAAAAAATTGCATCGCTAACTCGTCGAGAAGATTTTGGCGCTGATATTAATAGAGCCCGAAT
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 700 CAAAGCGAGGCTGTTGAAAAATAAGTCAATTTCCCTGATTTTCAAAAAATAATTAACGATTTA
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 770 AACTTTGACAAAAATAATTTTCACTTTTAAACAAGGAACAATTTACAATTTTAGTTTAGAAAAA
 N F D K N N F T F K Q G T I Y N F S L E K
 840 CATCCTGGAATAATAATTTTATTTCAAGTTGGTCCAAATTCAGTTCCAAAGTTTTTTAGTTAAGGCT
 H P G T N N F I S V G P N S V P S F L V K A
 910 GAATTAAGTGATGATGCAAAATTTGTGCTTAATAAATTTTAATATTGAAGATGCCCAATTGCTTGAA
 E L S D D A K F V L K N F N I E D A Q L L E
 980 AAAATTGATTTAGTTCCGCGAGGCTAGTTCAAGTTCTGAGTCAAGCCAAAGGTGAAGAAAAACAAGCT
 K I D L V P Q A S S G S E S S Q G E E K Q A
 1,050 AAACAGGCAACTTATTTTGCTGATGTAGACGATATTTTGTCAAAAAATTTCACTTAGAAAAATTACAA
 K Q A T Y F A D V D D I L S K I S L R K L Q
 1,120 TTTACTGATTTTAAAGTTGGAAGTCAGCCAGTTACTAATACTCAAGTTCAAGCTCAGGCTAGCTTA
 F T D F K V G S Q P V T N T Q V Q A S L
 1,190 TTAAGTTCTGGAGATATTAAGCAACTAGTTCAAGTTTCTCTGAAGTTACCCAAAGTCAACCTCAA
 L S S G D I K Q L V Q V S S E V T Q S Q P Q
 1,260 GCTCAAGCTGAAGGAGGAGCGGGAGGAGGAACGGAACAAGCAACTTCAGGAACAAGTGAACA
 A Q A E G G A G G T G Q Q A T S G T T G T
 1,330 GACGCGGGATCAACTGGGACAGGTGGAGTGGCAGGCGCAGGAGCACCCGACTCAGGAACAGGATCA
 D A G S T G T G G V A G A G A P D S G T G S
 1,400 ACTGGATCAACACAGATCAGCAAACTAAACGGAGAATCTGGCACAGAACACCTTCTGTAACTACT
 T G S T T D Q Q T N G E S G T E Q P S V T T
 1,470 CCAGCTGAACCTGAAGATAAAACAAAGGAATTAGTCAATATCTTGAAATTTCAAATCGCACTACA
 P A E P E D K T K E L V K Y L E I S N R T T
 1,540 AATGAATTTTAGCTAGTTTAAATAAAAAATATATTCTTCTATTAGAGAAAAATCTAAAGAAGTA
 N E F L A S F N K K I Y S S I R E K S K E V
 1,610 GTAGATAAAATAAATTTCTGAATTTAATTAAGCCAATTTCCCTTGATTTTGGTGATTTTAATCAG
 V D K I N S E L L I K P I S L D F G D F N Q
 1,680 TATTTTGACAAAAACAACCTCAAGGTGTTGATTTTATTTAGATCTTTCTAAAGCACAGAAAAA
 Y F D K K Q P Q G V D F Y L D L S K A Q E K
 1,750 GATGGCGTTAATACAGACAATTCAAACCTTGAATCCCGGTTGTTATTAATCTTTATTCAAGTTTT
 D G V N T D N S N L E I P V V I N L Y S S F
 1,820 TTTGGTGAAGTCAAGAAAAACAACCTTTAGGATCTAAACCTTCAGTTTGTGAATTTCTAATTTCAAG
 F G D S E N K L L G S K T S V F E I P N F K

1.790 1.800 1.810 1.820 1.830 1.840
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 D A K K Q K T F T E V A K L F I L D L G M
 1.850 1.860 1.870 1.880 1.890 1.900 1.910
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 E P E S A S A L Q K Y I D L N K P R F E P T
 1.920 1.930 1.940 1.950 1.960 1.970 1.980
 TCTAAAGACAGTGGTAGCGAAAAACCAGCTGAAGGTGAGAAAAAACAGAAGAAGGTGAGAAAAAA
 S K D S G S E K P A E G E K K T E E G E K K
 1.990 2.000 2.010 2.020 2.030 2.040
 CCTGAAGAAGGTGCAACAACTGGAAGTCAATCAGCAGATTCTCAACCATCAACCGGTGTTCCAGCT
 P E E G A T T G S Q S A D S Q P S T G V P A
 2.050 2.060 2.070 2.080 2.090 2.100 2.110
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 2.120 2.130 2.140 2.150 2.160 2.170
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 A G G A G A T T S T G A Q A T S T T G
 2.180 2.190 2.200 2.210 2.220 2.230 2.240
 TCCCAACAACCTCATCAACAACAGCATTGGAAGATGATTCAACTGCTAAAACTAATGATGATTCA
 S Q T T S S T T A F E D S T A K T N D D S
 2.250 2.260 2.270 2.280 2.290 2.300 2.310
 ACTACTGAAACTAAAACTGAAACAAATGAAGTTTCAACTTTTAAAGGTTTAGGTGTTGAACTTTGA
 T T E T K T E T N E V S T F K G L G V E L W
 2.320 2.330 2.340 2.350 2.360 2.370
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 2.450 2.460 2.470 2.480 2.490 2.500
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 S N P K A I F S I T Q L E N D A D Y D V L R
 2.510 2.520 2.530 2.540 2.550 2.560 2.570
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 S Y N P T V F F D F R Q N Q K T D G S D K I
 2.580 2.590 2.600 2.610 2.620 2.630 2.640
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 T K I Q P L N R G D V K I D L E N D K D N V
 2.650 2.660 2.670 2.680 2.690 2.700
 GCAACTAAAGATGGGTTATTAGTAAAAAAAGCTGTTGAAATTAAGGAGGTTCCGGCAAGTCCACAG
 A T K D G L L V K K A V E I K E V P A S P Q
 2.710 2.720 2.730 2.740 2.750 2.760 2.770
 GAAGCGGCGGCTGTAAACGGCAACTGAAGCAAGTACAACAGTAGCAACTACTGAAACCACAGCACCA
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 2.780 2.790 2.800 2.810 2.820 2.830
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 T T T T T G T P T P T P T T T T E T P T
 2.840 2.850 2.860 2.870 2.880 2.890 2.900
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 P T P I T E T T P T T T A T T A I D S K
 2.910 2.920 2.930 2.940 2.950 2.960 2.970
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 K Q I L E S G V I F L A F Q P R N I K D N K
 2.980 2.990 3.000 3.010 3.020 3.030
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 K H Y L I A S G D G K G I F I K K T K L G D
 3.040 3.050 3.060 3.070 3.080 3.090 3.100
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 G K T E K F A L G L D E N P G S N G G S K V
 3.110 3.120 3.130 3.140 3.150 3.160
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 D S I V A L I S G V D G E A D T R L Q F E E
 3.170 3.180 3.190 3.200 3.210 3.220 3.230
 CCAACTTCTGGTTCTAATGGCGCGGTGGCGGTGGTTCAAATAATGGGTCTACTGCTGAGGCAAAA
 P T S G S N G G G G G S N N G S T A E A K
 3.240 3.250 3.260 3.270 3.280 3.290 3.300
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 N I I Q L V F G V P A P D P F R Q T N V N K
 3.310 3.320 3.330 3.340 3.350 3.360
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 E D A D D F D F I K D G D L I F L T L I K N
 3.370 3.380 3.390 3.400 3.410 3.420 3.430
 CAAAATAAATGGACAATTTGACTTACTTCATCAAAAAGCAAAAAACCTTACAGTCAAGAATTTCT
 Q N K W T I W L T S S K A K N P Y S Q R I S
 3.440 3.450 3.460 3.470 3.480 3.490
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 S T L D L T L G G H F D H S K N L T W T H L

3,500 3,510 3,520 3,530 3,540 3,550 3,560
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 G P K A E A T T S S G G T E A Q T Q T N
 3,570 3,580 3,590 3,600 3,610 3,620 3,630
 ACAGCCCCAACCTCATCTCCAACCTCAAATCTTGTTCAAAGGTTTTGCAGTTTATGACTCACCTACT
 T A P T S S P T Q I L F K G F A V Y D S P T
 3,640 3,650 3,660 3,670 3,680 3,690 3,696
 TTAGCAACAAATGTTGAGACAGTCTCAACCTTAAATAATCTTTTTATTAAAAAATTCATTAAATAA
 L A T N V E T V S T L N N L F I K K F I K *

Appendix 7 Peptide sequence for Hypothetical protein 1 from isolate 90

1 10 20 30 40 50 60
 GTGACGAAAAGTACAATCACTAAAAAACTACTAACTAAAAAAATATTTTTCTTTTAGGGCTAAGT
 V/M T K S T I T K K L L T K K N I F L L G L S
 70 80 90 100 110 120 130
 ACTATTGCTGGTGGAGTAATTATGGCACTCCGCTACTCGTTTTTGCAAATTTAGAACTAAAAAAC
 T I A G G V I I A T P L L V F A N L E L K N
 140 150 160 170 180 190
 CCCCGAATTGATGTGCAAAACCAAGCAAAATCAATTTCTTTTCATTAGCATAAAGGATAAATATTTA
 P R I D V Q N Q A K S I S F I S I K D K Y L
 200 210 220 230 240 250 260
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 N A N S D Y L D L K K K L L N D D N T K K T
 270 280 290 300 310 320 330
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 D I N L T D F F D F Y Q T N N S S I P V N F
 340 350 360 370 380 390
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 S T D H N W K P F K L E I F D I K P D D N E
 400 410 420 430 440 450 460
 CAAACATTTGAGGTTTTTTTGACGAGTTTTACAAAAATTAGACGATGGTAAACAGCAACTTCTGAC
 Q A T F E V F W R V L Q K L D D G K T A T S D
 470 480 490 500 510 520
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 L F K Q K V A Y N Y V P D Y S L S N F S T Y
 530 540 550 560 570 580 590
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 S E D Q L K K L R P Y T N S E V N F S F K K
 600 610 620 630 640 650 660
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 670 680 690 700 710 720
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 730 740 750 760 770 780 790
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 K S F Y F E S D S G I K K P R Y D I N L V K
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 GATCAAATTTATACTGATCGATATTTAGTAAAAACAGCAACGCCTGGGATTATATAAATTTGACCTTT
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 860 870 880 890 900 910 920
 GTTGCCAGTTTTCTTCTGATTTTTCAAAGGAAATTTGCTGCTGATTTTAAACAGGATTCCAATTT
 V A Q F S S D F S K E I A A D F N K D S K F
 930 940 950 960 970 980 990
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 F V T T Q L N L S N S F L D K S I S D D I V
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 D F L N S L I E K I V K T P L I S K I R F Q
 1,190 1,200 1,210 1,220 1,230 1,240 1,250
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 N N L A N L N F N Q I S K F L D V Q I K L D
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 T E Q V N L D F K D N N V V A R I N G D I V
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 1,520 1,530 1,540 1,550 1,560 1,570 1,580
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 I R V I I L E H T S S L L L K K *

Appendix 8 Peptide sequence for Hypothetical protein 2 from isolate 90

```

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M  A  I  K  L  K  K  L  R  N  L  F  I  F  S  T  P  I  V  F  L  V

70     80     90    100    110    120    130
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A  T  S  C  G  V  S  H  K  I  A  D  K  L  G  P  S  V  S  F  D  V

140    150    160    170    180    190
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N  G  N  Y  V  F  T  I  K  N  D  K  F  K  D  A  T  E  V  V  A  H

200    210    220    230    240    250    260
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F  E  G  E  N  D  G  T  N  T  A  N  N  A  R  K  I  T  A  E  V  S

270    280    290    300    310    320    330
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G  T  I  N  N  L  T  A  S  F  N  T  V  G  K  L  Q  Y  D  Y  R  W

340    350    360    370    380    390
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T  L  K  K  I  K  V  G  S  E  I  S  C  Q  D  Q  F  P  D  D  L  K

400    410    420    430    440    450    460
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D  D  I  W  F  T  E  K  K  I  A  I  V  K  Q  D  T  S  L  W  I  D  P

470    480    490    500    510    520
CAAACCCAACTTTTAAAAAATTGAATAAATACTGAAGCAAATGTTGAGTTAAAAGCTGAATTTATT
Q  T  Q  L  L  K  N  W  I  N  T  E  A  N  V  E  L  K  A  E  F  I

530    540    550    560    570    580    590
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R  I  D  D  P  N  K  P  T  T  E  T  T  E  I  I  D  G  V  E  V  K

600    610    620    630    640    650    660
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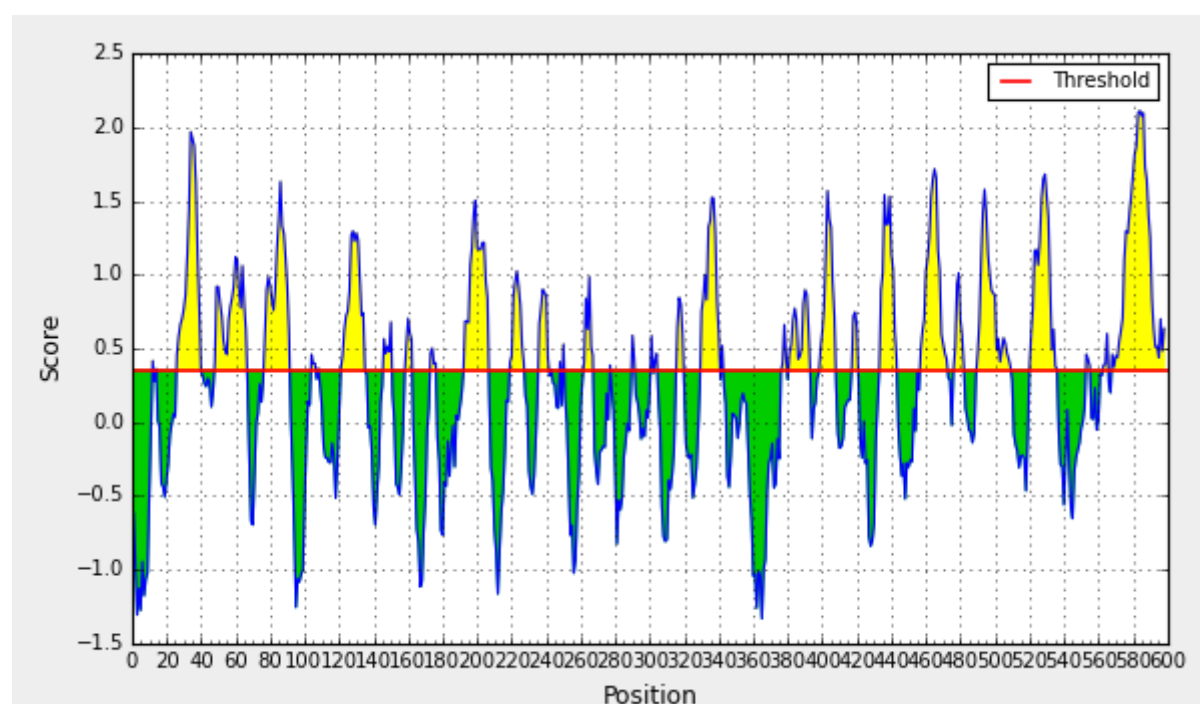
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730    740    750    756
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T  I  S  L  P  Y  K  P  F  *

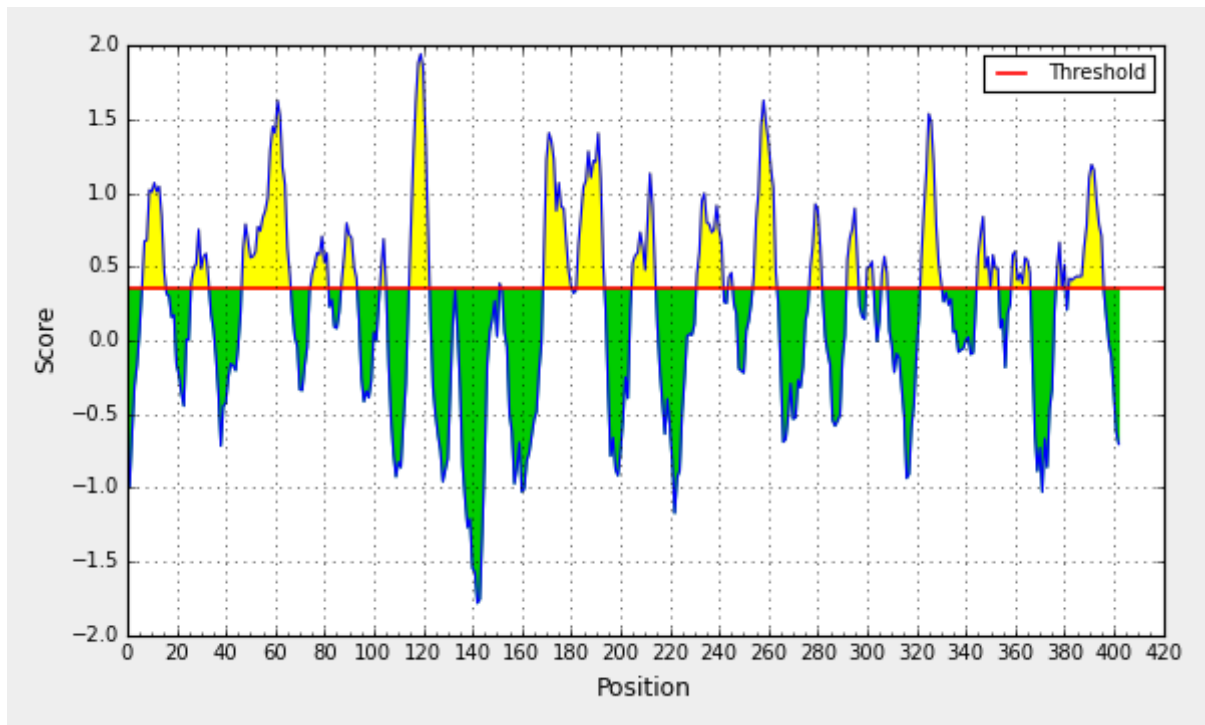
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Appendix 9 Peptide sequence for Hypothetical protein 3 from isolate 90

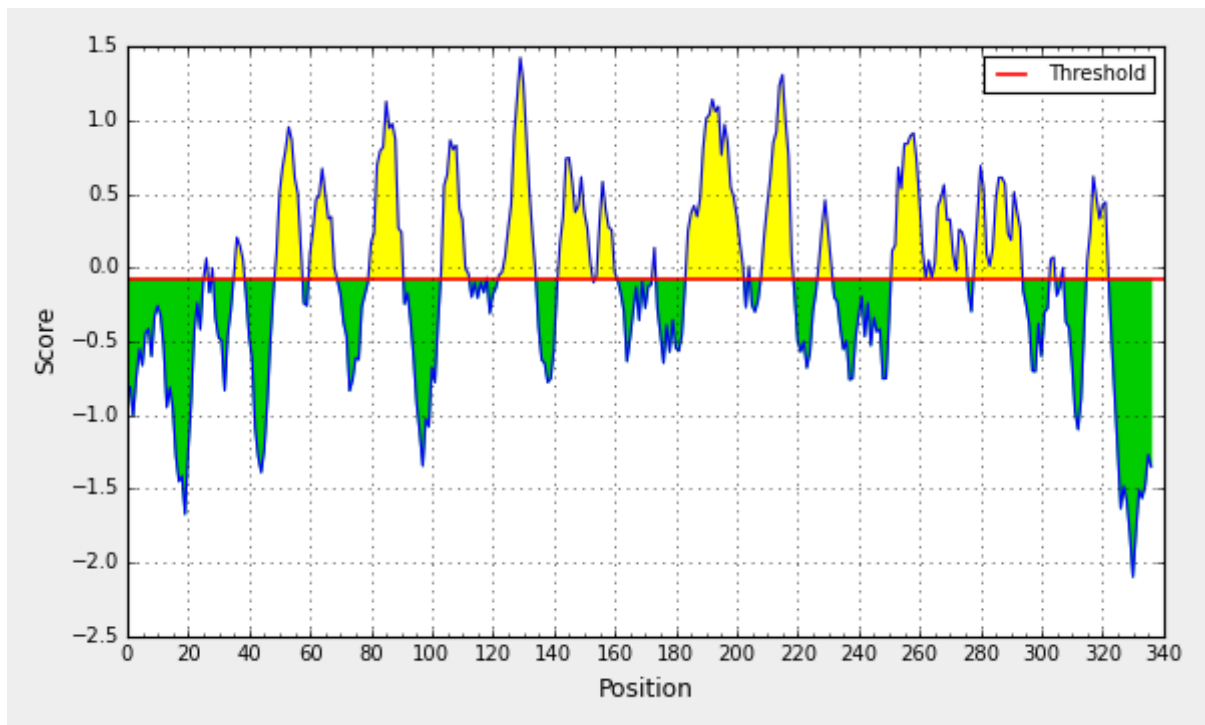
Predicted B-cell epitopes on proteins identified in Chapter 4



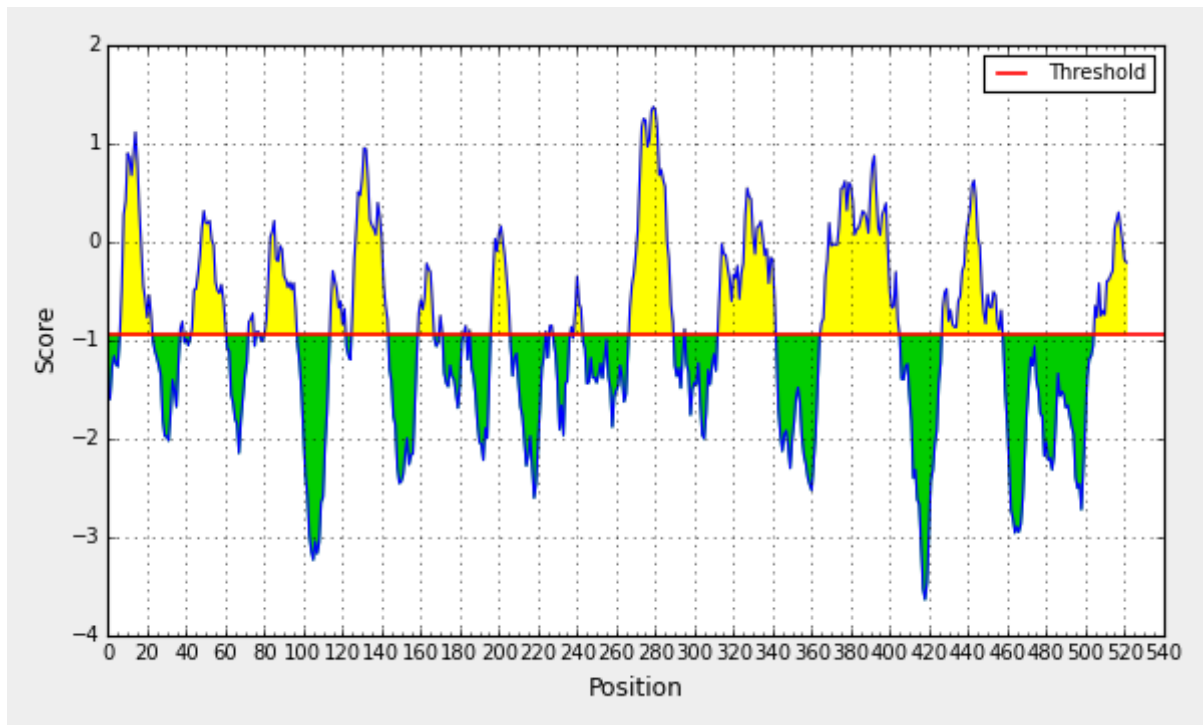
Appendix 10 B cell epitopes of HSP70 Bepipred-1.0 Linear Epitope Prediction [293]



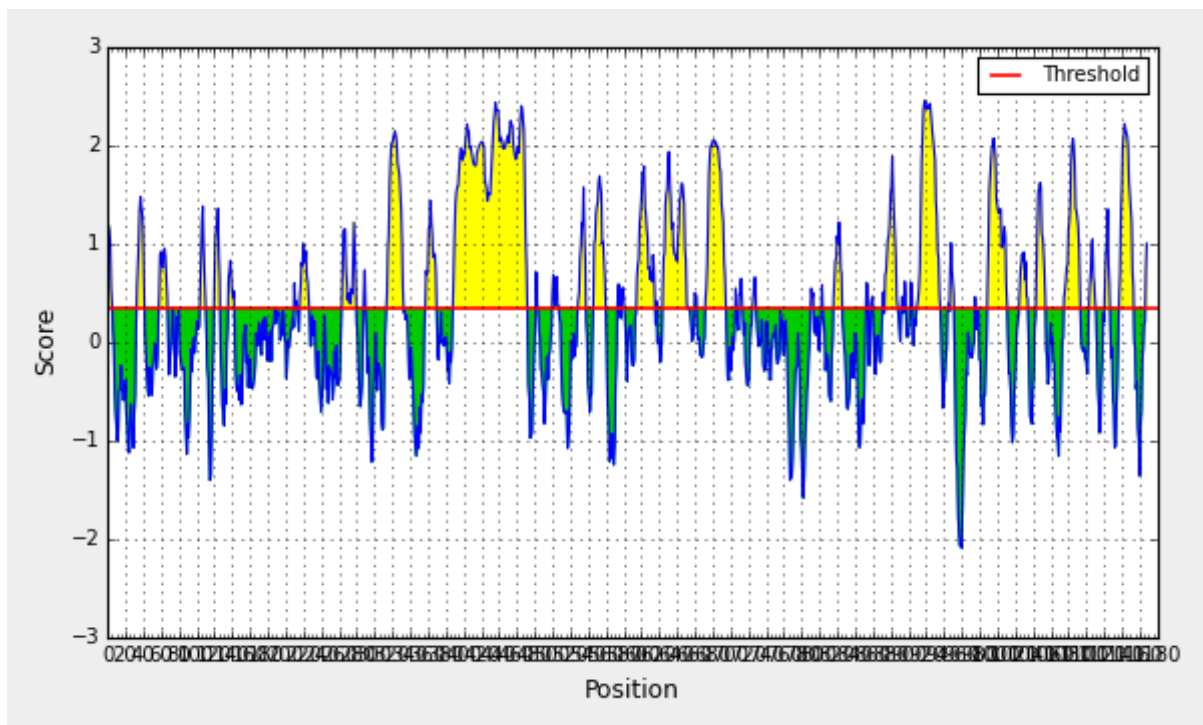
Appendix 11 B cell epitopes of EF-Tu Bepipred-1.0 Linear Epitope Prediction [293]



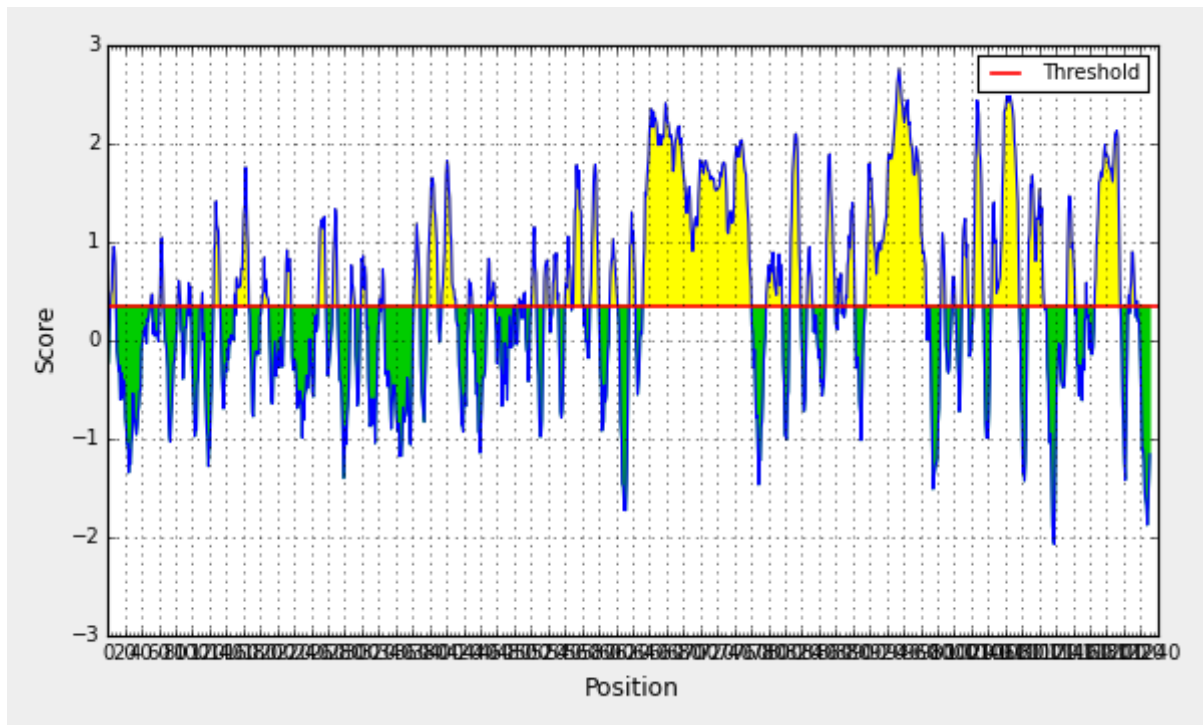
Appendix 12 B cell epitopes of GAPDH Bepipred-1.0 Linear Epitope Prediction [293]



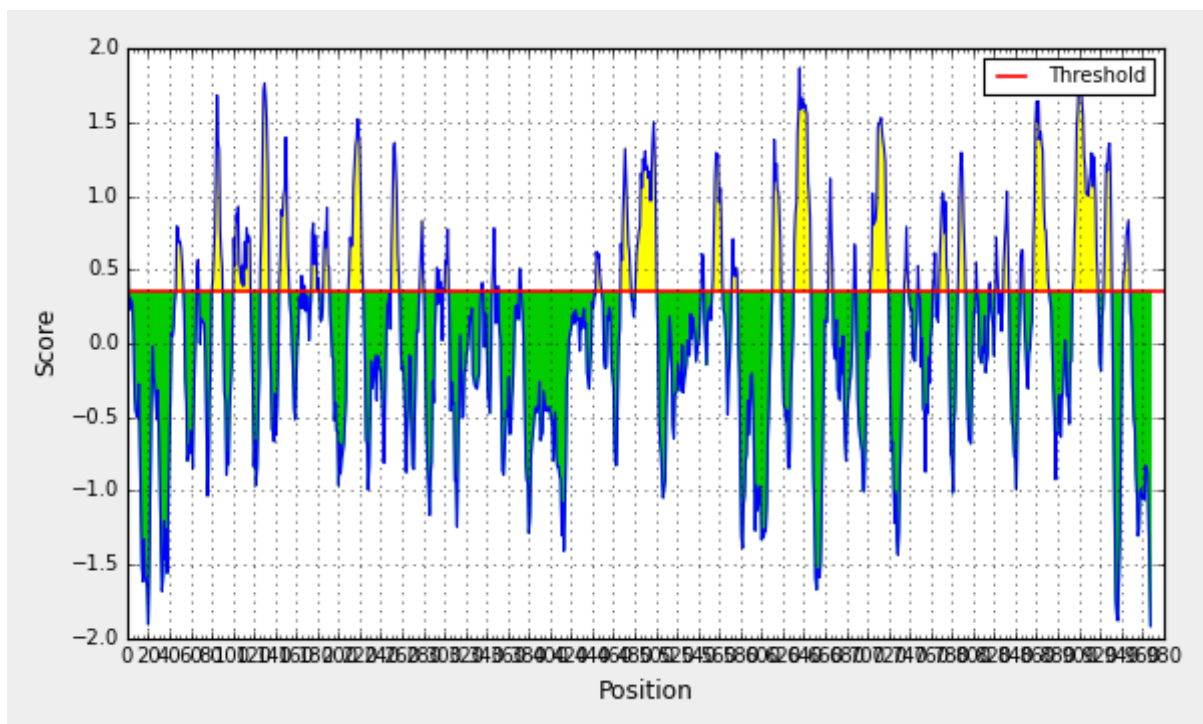
Appendix 13 B cell epitopes of MATE Bepipred-1.0 Linear Epitope Prediction [293]



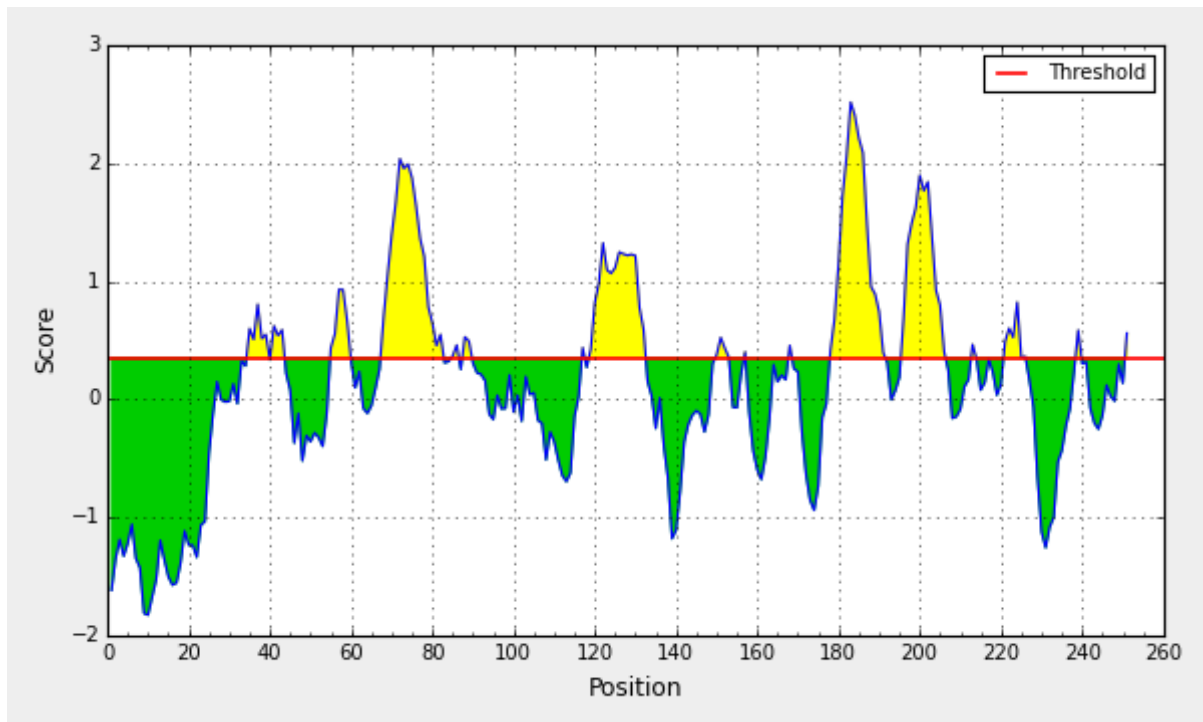
Appendix 14 B cell epitopes of P146 Bepipred-1.0 Linear Epitope Prediction [293]



Appendix 15 B cell epitopes of HP1 Bepipred-1.0 Linear Epitope Prediction [293]

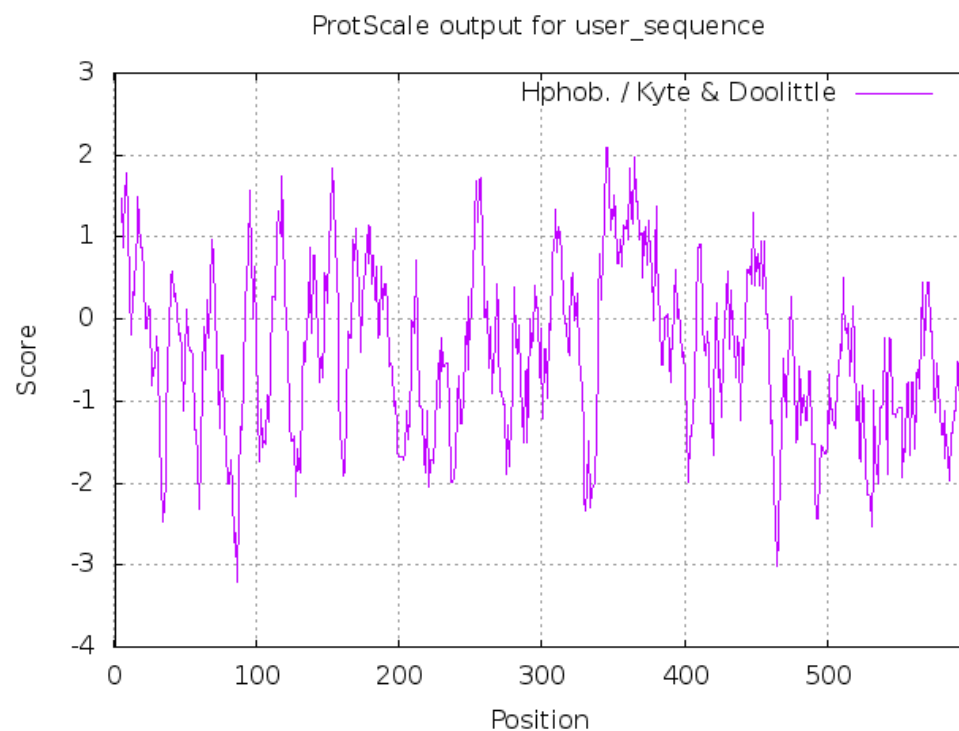


Appendix 16 B cell epitopes of HP2 Bepipred-1.0 Linear Epitope Prediction [293]

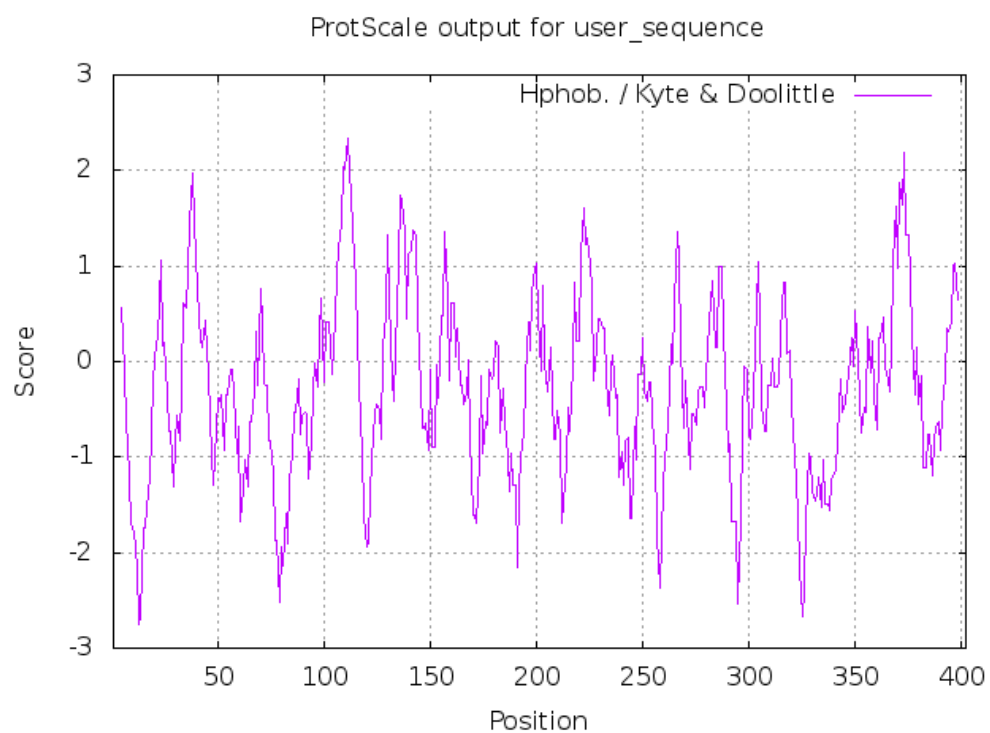


Appendix 17 B cell epitopes of HP3 BepiPred-1.0 Linear Epitope Prediction [293]

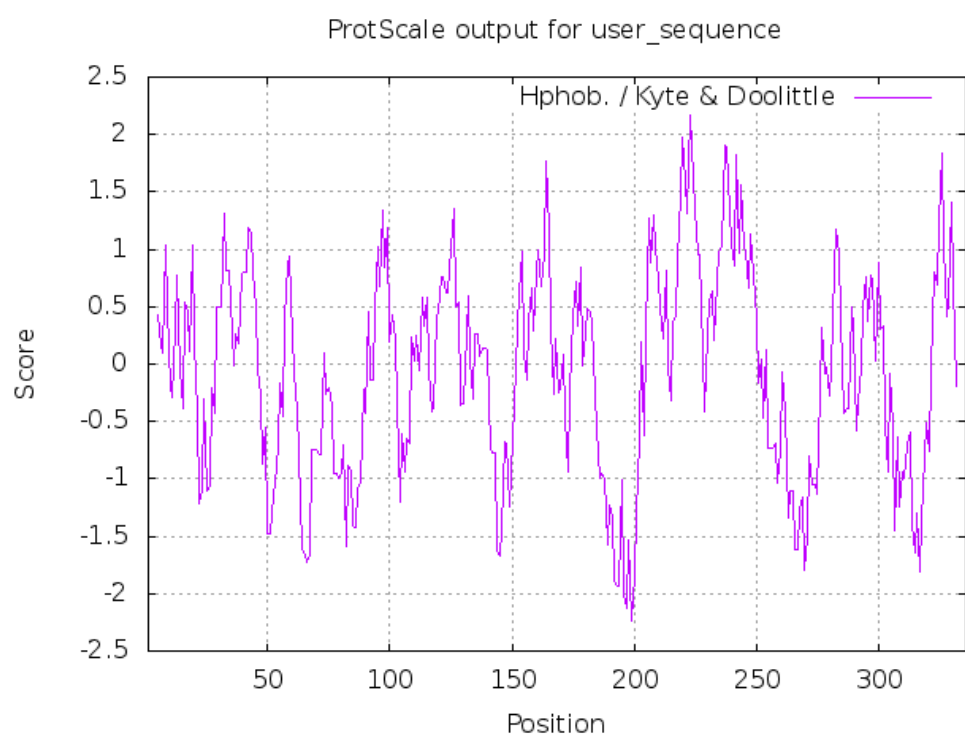
Kyte and Doolittle plots of proteins identified in Chapter 4



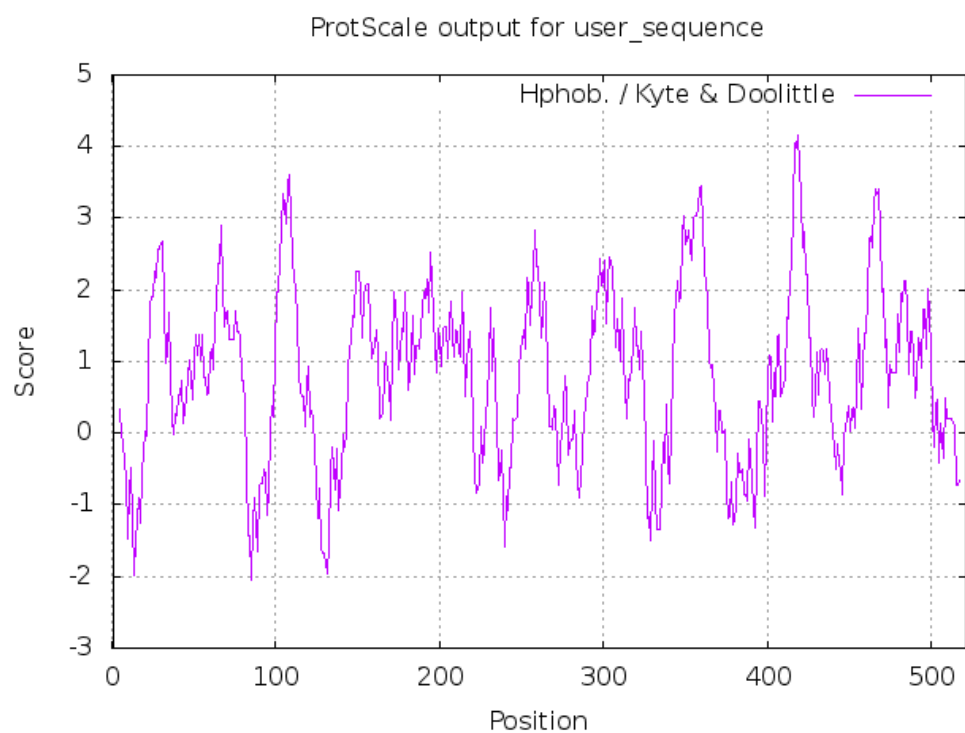
Appendix 18 Kyte/Doolittle analysis of HSP70



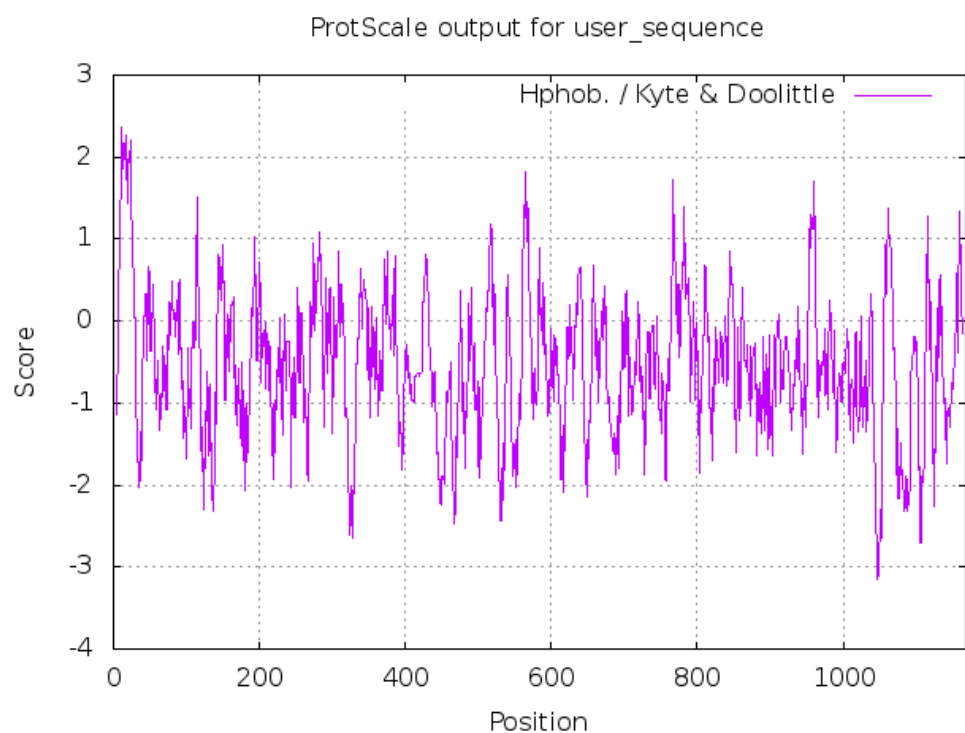
Appendix 19 Kyte/Doolittle analysis of EF-Tu



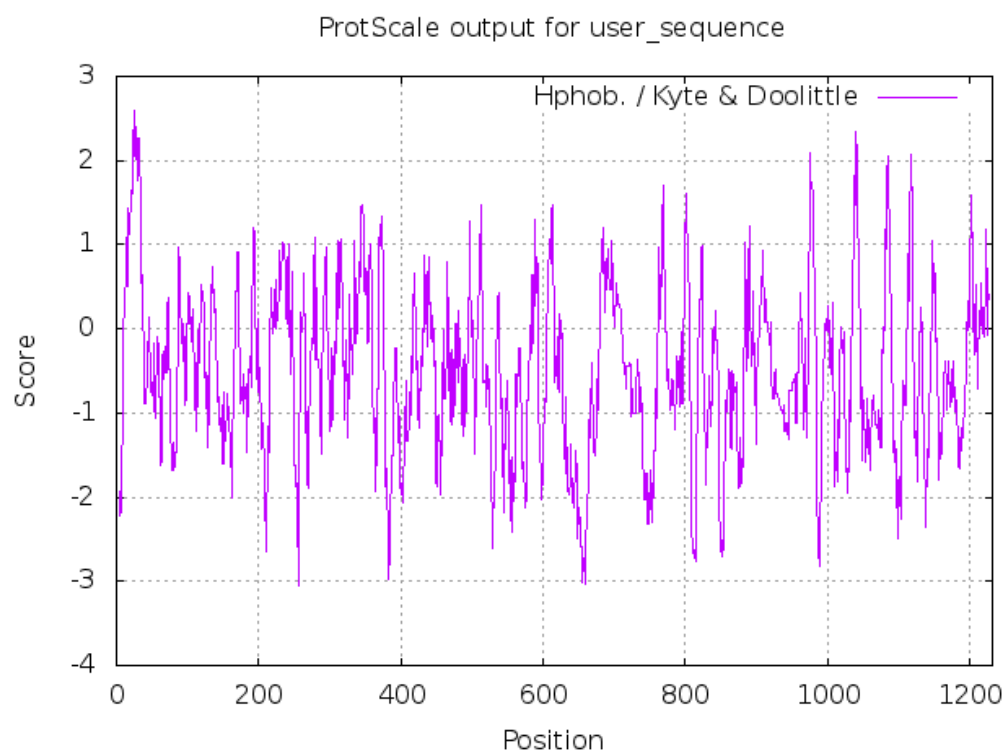
Appendix 20 Kyte/Doolittle analysis of GAPDH



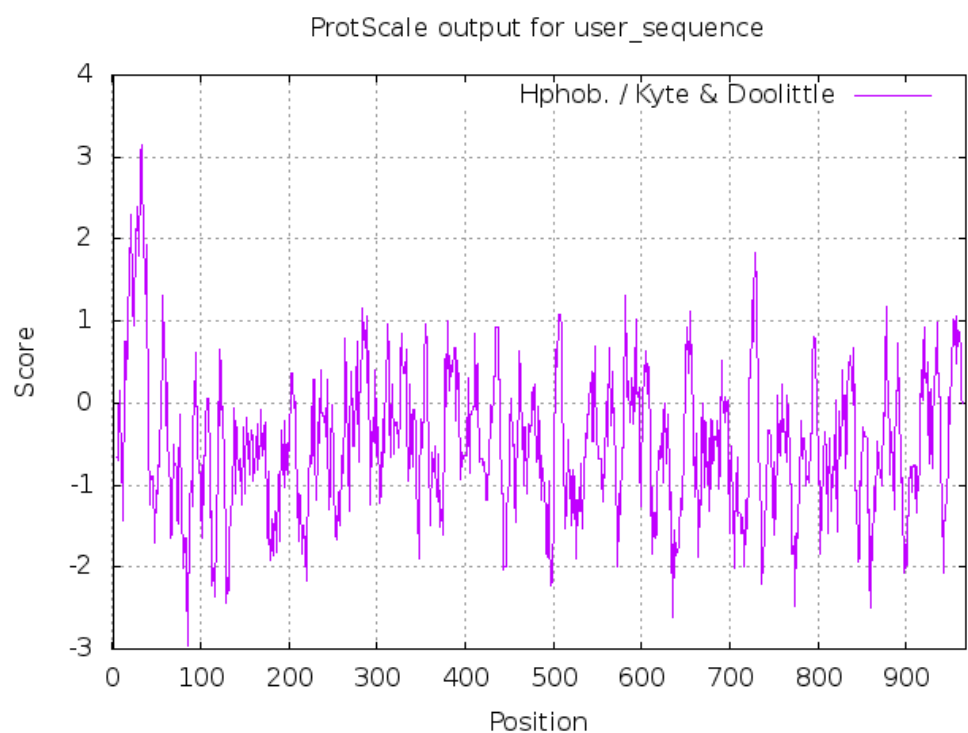
Appendix 21 Kyte/Doolittle analysis of MATE



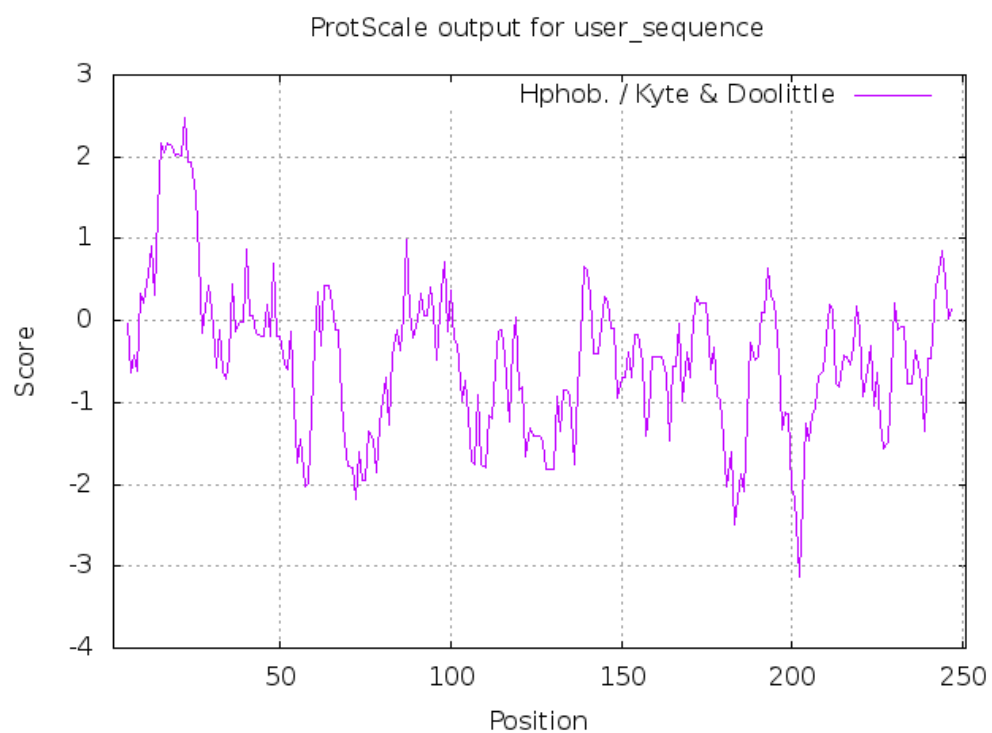
Appendix 22 Kyte/Doolittle analysis of P146



Appendix 23 Kyte/Doolittle analysis of HP1

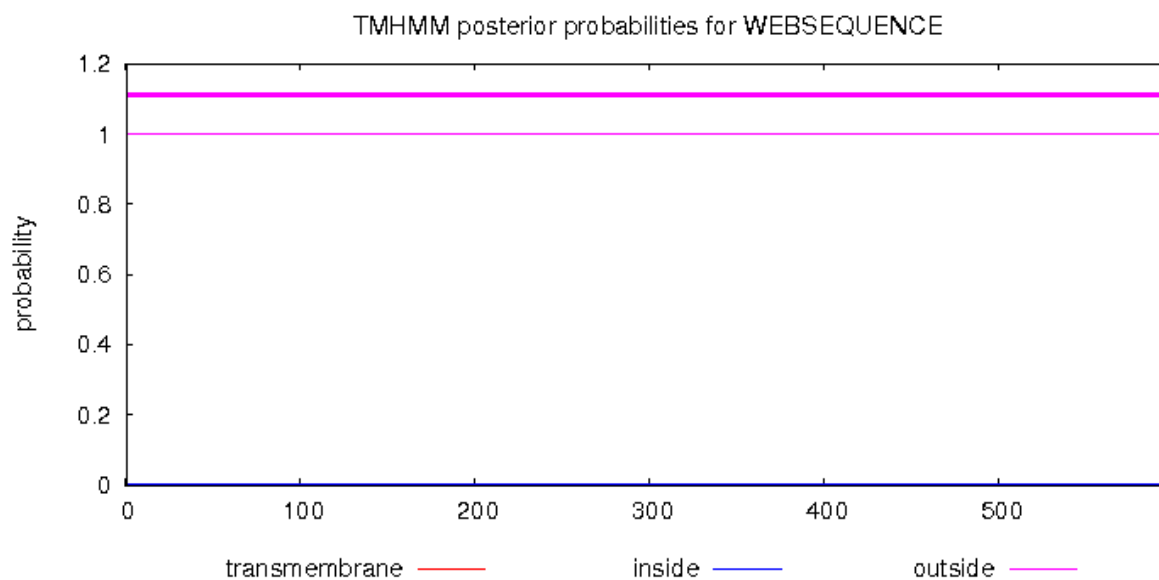


Appendix 24 Kyte/Doolittle analysis of HP2

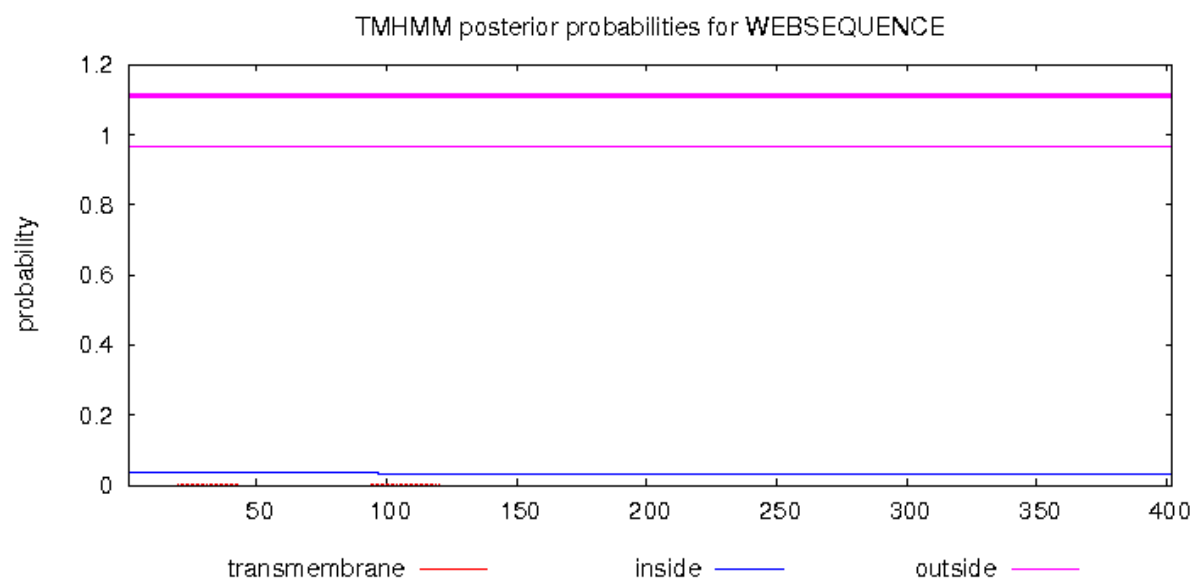


Appendix 25 Kyte/Doolittle analysis of HP3

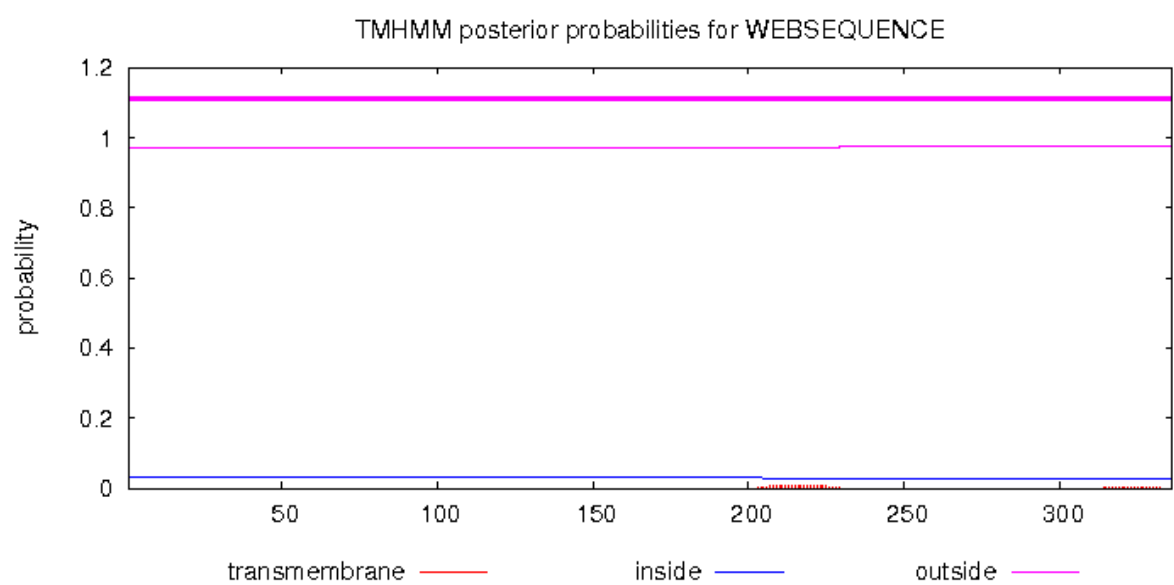
Transmembrane analysis of proteins identified in Chapter 4



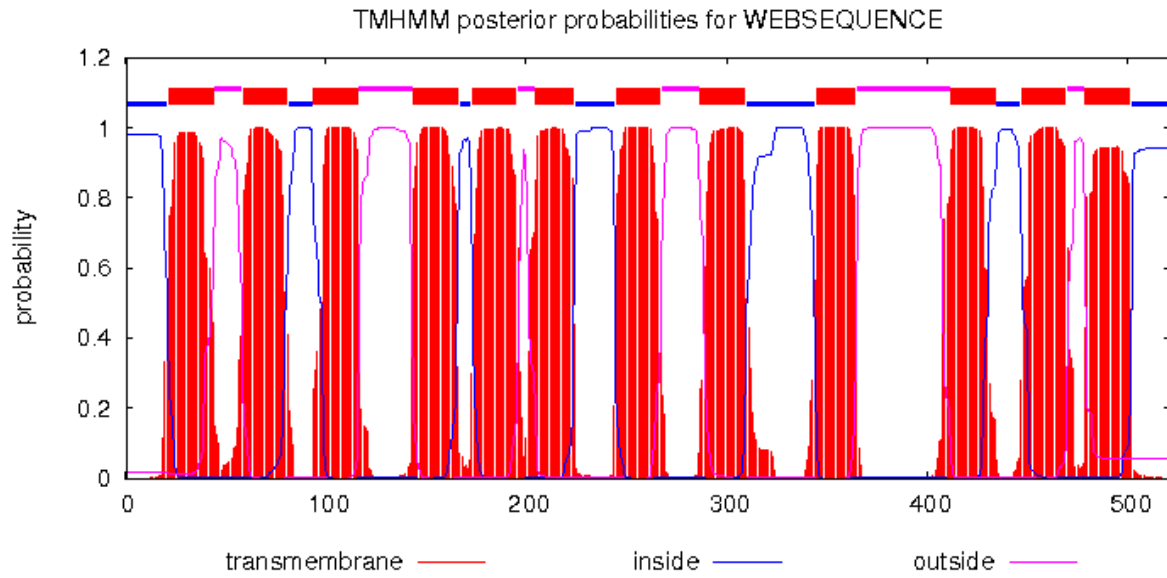
Appendix 26 Transmembrane analysis of HSP70



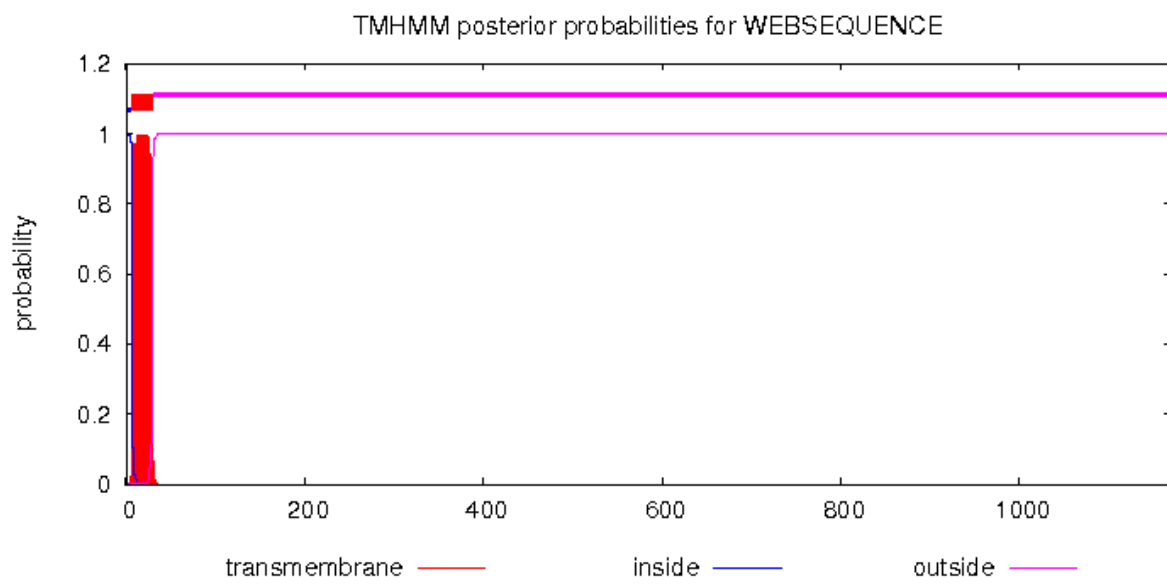
Appendix 27 Transmembrane analysis of EF-Tu



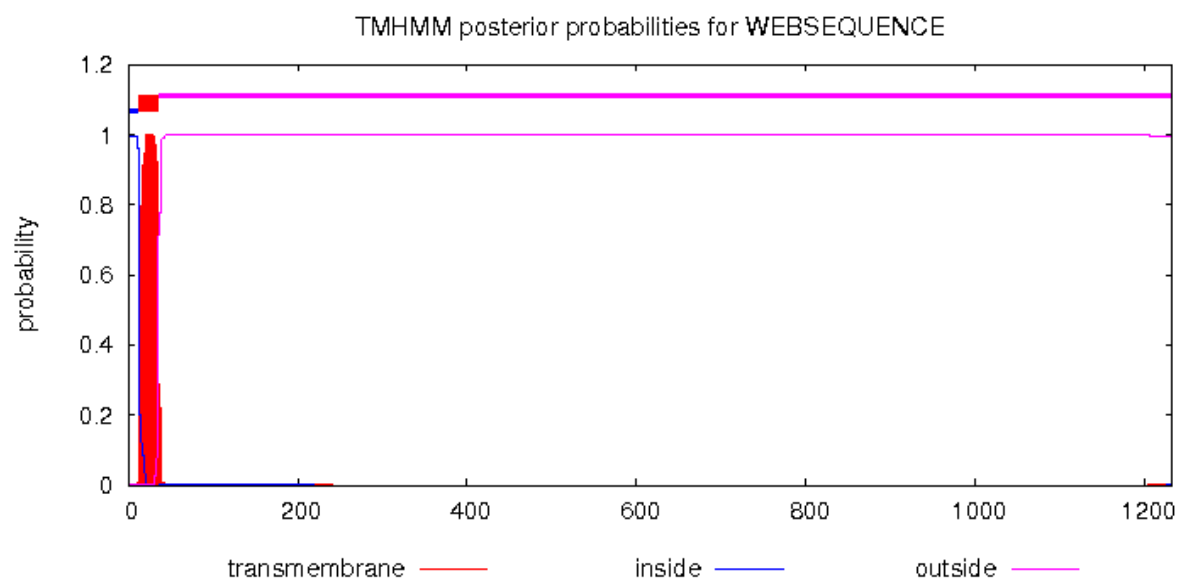
Appendix 28 Transmembrane analysis of GAPDH



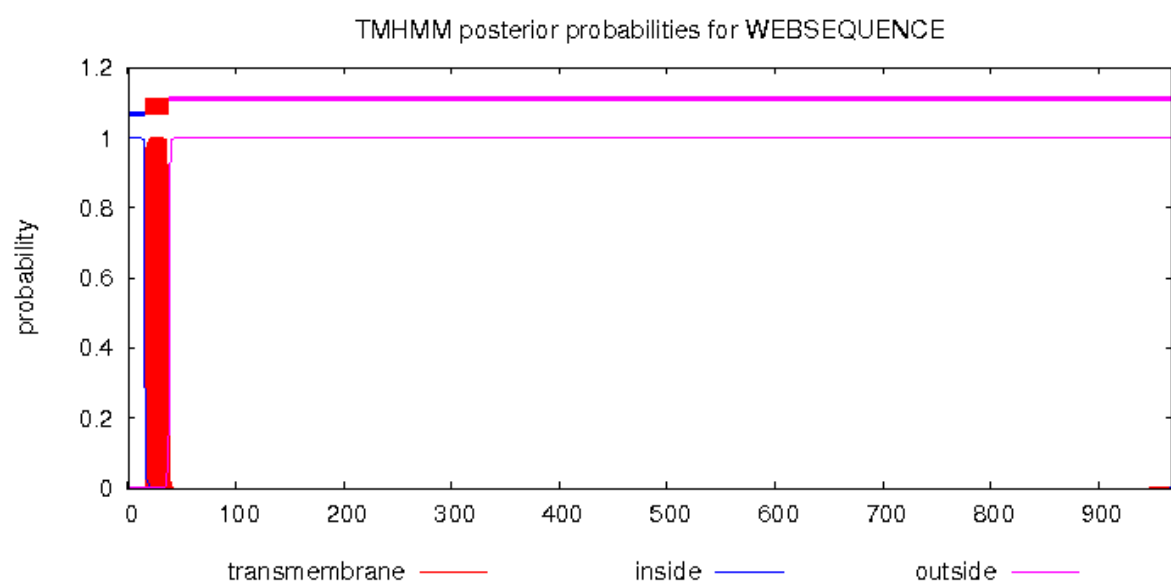
Appendix 29 Transmembrane analysis of MATE



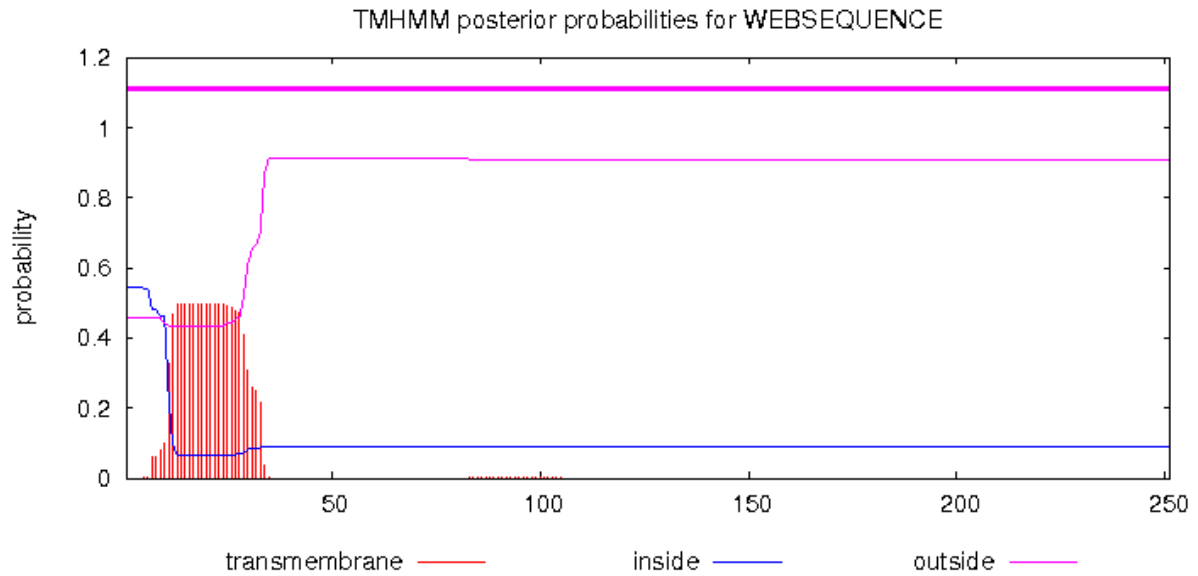
Appendix 30 Transmembrane analysis of P146



Appendix 31 Transmembrane analysis of HP1



Appendix 32 Transmembrane analysis of HP2



Appendix 33 Transmembrane analysis of HP3

B-cell epitopes of proteins identified in Chapter 4

No.	Start	End	Peptide	Length	Hydrophobicity
1	26	39	PVVLENPN GKRTTP	14	Hydrophilic
2	48	66	EEIVGDAAKRQLETNPEAI	19	Hydrophilic
3	77	91	KTVRANQRDYKPEEI	15	Hydrophilic
4	121	134	YFDNAQREATKNAG	14	Hydrophilic
5	146	150	NEPTA	5	Hydrophobic
6	159	162	KTEK	4	Hydrophilic
7	173	176	GGTF	4	Hydrophobic
8	193	206	TSGDNHLGGDDWDN	14	Hydrophilic
9	219	227	EYDFDAKSD	9	Hydrophilic
10	236	241	EEAEKT	6	Hydrophilic
11	262	267	KNGPIN	6	Hydrophilic
12	330	340	NKKPNRSINPD	11	Hydrophilic
13	381	392	TPLIPRNTTIPV	12	-
14	399	407	STAEDNQTE	9	Hydrophilic
15	417	420	RQLA	4	Hydrophilic

No.	Start	End	Peptide	Length	Hydrophobicity
16	433	443	GIEPAPRGLPQ	11	Hydrophilic
17	457	472	TVSAKDKKTGKEQTIT	16	Hydrophilic
18	476	482	TSTLSEE	7	Hydrophilic
19	490	509	EAEENREADAIKKDKIETTV	20	Hydrophilic
20	520	536	KSITDQGEKIDPKQKEL	17	Hydrophilic

Appendix 34 B cell epitope analysis of HSP70

No.	Start	End	Peptide	Length	Hydrophobicity
1	7	15	GAKKDFDRS	9	Hydrophilic
2	26	33	HVDHGKTT	8	Hydrophilic
3	47	66	LAEAKDYASIDAAPEEKARG	20	Hydrophilic
4	75	81	EYSTDKR	7	Hydrophilic
5	87	93	DCPGHAD	7	Hydrophilic
6	115	122	TDGPMPQT	8	-
7	169	180	DFDGDNTPIIRG	12	Hydrophilic
8	183	193	RGALEGKPEWE	11	Hydrophilic
9	205	214	SYIDSPVREM	10	Hydrophilic
10	231	241	RGTVATGKVER	11	-
11	254	263	GYRPEPKKTV	10	Hydrophilic
12	277	281	AMAGD	5	Hydrophilic
13	292	296	RKDIE	5	Hydrophilic
14	322	330	KEEGGRHTP	9	Hydrophilic
15	345	353	DVTGGIEFE	9	-
16	359	366	VIPGDNVD	8	-
17	382	395	KFSIREGGRTVGAG	14	Hydrophilic

Appendix 35 B cell epitope analysis of EF-Tu

No.	Start	End	Peptide	Length	Hydrophobicity
1	35	38	DLTD	4	Hydrophobic

No.	Start	End	Peptide	Length	Hydrophobicity
2	49	57	DSAQGRFSG	9	Hydrophilic
3	60	68	SVLKEDDKN	9	Hydrophilic
4	80	90	FSEKDPEMLPW	11	Hydrophilic
5	103	112	GRYASKAGAT	10	Hydrophilic
6	122	134	VLVSAPAGSDVKT	13	Hydrophobic
7	142	152	HTVDENDKILS	11	Hydrophilic
8	154	160	ASCTTNA	7	Hydrophobic
9	184	202	AYTADQRIQDAPHKDLRRA	19	Hydrophilic
10	208	218	NLVPSSTGAAK	11	Hydrophobic
11	227	231	LTGKL	5	Hydrophobic
12	251	275	ELKSSPSIEELNKAIKKYESESFY	25	Hydrophilic
13	278	293	EPIVSSDIIGDTHGSI	16	-
14	315	322	WYDNESSF	8	Hydrophilic

Appendix 36 B cell epitope analysis of GAPDH

No.	Start	End	Peptide	Length	Hydrophobicity
1	7	22	KFFPDSPMWKKFAKI	16	Hydrophilic
2	43	60	MVSQISGGITAVGMANFW	18	Hydrophobic
3	81	96	QYWGKKEFKLARQVNN	16	Hydrophilic
4	114	121	WAFPEPLL	8	Hydrophobic
5	125	143	QWRRGSSNLDQSIFDQAKS	19	Hydrophilic
6	159	166	VTSSGILR	8	Hydrophobic
7	196	205	MGVSGSALAT	10	Hydrophobic
8	239	242	RQIW	4	Hydrophilic
9	267	289	VLWSQSLPPGSIGIDGSGFYKF	23	-
10	312	341	FASIQTSVSIIVGQNLGQNKIELAKKNAAM	30	-
11	365	404	TKTDLITGTIKEQVQKGLSDYFAENNL PVDQSIIDAQKVL	40	-
12	427	457	WVQINASLTIIKAGGRANFASWWDFFTIGIGQ	31	Hydrophobic

Appendix 37 B cell epitope analysis of MATE

No.	Start	End	Peptide	Length	Hydrophobicity
1	1	5	MPKNT	5	-
2	33	41	YTGENPRAE	9	Hydrophilic
3	58	67	VFDDSTTYSQ	10	Hydrophilic
4	104	110	PTFAPNK	7	Hydrophilic
5	120	126	PNDNDQS	7	Hydrophilic
6	136	142	QVDNNHT	7	Hydrophilic
7	210	213	DFAA	4	Hydrophilic
8	216	226	NRARTQSEAVE	11	Hydrophilic
9	263	279	EKHPGTNNFISVGPNSV	17	-
10	314	332	PQASSGSESSQGEEKQAKQ	19	Hydrophilic
11	358	369	VGSQPVTNTQVQ	12	Hydrophilic
12	388	471	SEVTQSQPQAQAEQGAGGGTGQQATSGTTGTDAGSTGTGGVA GAGA PDSGTGSTGSTTDQQTNGESGTEQPSVTTTPAEPEDKTK	84	Hydrophilic
13	500	507	REKSKEVV	8	Hydrophilic
14	529	537	YFDKKQPQG	9	Hydrophilic
15	546	560	KAQEKDGVNTDNSNL	15	Hydrophilic
16	578	581	NKLL	4	Hydrophilic
17	595	615	KVGTQTGSAGTTDLSSNLDTE	21	Hydrophilic
18	624	651	GLPTTSASQPTSVSVSASTGAQTTQNK	28	-
19	672	695	GTSTTGSTSEGTGQTTESKAPKFE	24	Hydrophilic
20	710	713	DFDA	4	Hydrophilic
21	725	728	QKFP	4	Hydrophilic
22	816	825	KFNTTETNPV	10	Hydrophilic
23	874	887	PVKVETSSGTSDDL	14	Hydrophilic
24	910	935	LKAGNGSGSGGGGAGGSDSTTQTSI	26	Hydrophilic
25	947	951	TQPLS	5	Hydrophilic
26	989	1011	HSGSTGSSSGTSETLTTPSGLED	23	Hydrophilic

No.	Start	End	Peptide	Length	Hydrophobicity
27	1025	1034	TGVAKYQTPK	10	Hydrophilic
28	1044	1054	QDTQRTERDEL	11	Hydrophilic
29	1075	1093	FDKLKKGDSTENTEGKKFE	19	Hydrophilic
30	1103	1110	EKIQENNK	8	Hydrophilic
31	1121	1126	EDKISP	6	Hydrophilic
32	1137	1153	EKTPPTTEGSSTGQSVK	17	Hydrophilic

Appendix 38 B cell epitope analysis of P146

No.	Start	End	Peptide	Length	Hydrophobicity
1	4	9	KENKSK	6	Hydrophilic
2	62	65	STFS	4	Hydrophilic
3	83	86	KDLS	4	Hydrophilic
4	125	133	KANASGTEV	9	Hydrophilic
5	152	166	QTDLKGFTQSDKADG	15	Hydrophilic
6	182	190	LTGTKNSNL	9	Hydrophilic
7	210	216	TRSKSQA	7	Hydrophilic
8	249	258	KTVENQKAKQ	10	Hydrophilic
9	266	271	DSDTKR	6	Hydrophilic
10	288	292	DQAGT	5	Hydrophilic
11	300	305	KDPSGK	6	Hydrophilic
12	362	368	KTDNNPV	7	Hydrophilic
13	378	390	TQNSQQNGRQSQV	13	Hydrophilic
14	395	411	FQDSGQNSQANNAKVDI	17	Hydrophilic
15	427	430	FQVP	4	Hydrophilic
16	449	457	TQAQKDKLV	9	Hydrophilic
17	501	506	LGKGND	6	Hydrophilic
18	516	520	SSKSN	5	Hydrophilic
19	526	532	DYDENQR	7	Hydrophilic

No.	Start	End	Peptide	Length	Hydrophobicity
20	540	547	EQNSEDFT	8	Hydrophilic
21	551	561	KIDPQDPKFEN	11	Hydrophilic
22	570	580	EFKENPNGPKL	11	Hydrophilic
23	593	602	VEDAKKQKTF	10	Hydrophilic
24	616	623	MEPESASA	8	Hydrophilic
25	632	760	KPRFEPTSKDSGSEKPAEGEKKTEEGERKPEEGATTGSQSADSQPS TGVPAAGAGAATGEAVAAGGGTAATGGAGGAGATTSTSTTGAQAT STTGSQTTSTTAFEDDSTAKTNDSTTETKTETNEVS	129	Hydrophilic
26	778	796	YSALGQTDYQAEVVTKSDS	19	Hydrophilic
27	806	818	TKTETEQKDSNPK	13	Hydrophilic
28	826	832	LENDADY	7	Hydrophilic
29	849	859	NQKTDGSDKIT	11	Hydrophilic
30	863	866	PLNR	4	Hydrophilic
31	872	882	DLENDKDNVAT	11	Hydrophilic
32	895	967	KEVPASPQEAATAVTEASTTVATTETTAPTTTTT GTPTPTPTTTTTETPTPTPTITETTTPTTTTATTATDS	73	Hydrophilic
33	984	989	RNIKDN	6	Hydrophilic
34	1009	1016	KLGDGKTE	8	Hydrophilic
35	1023	1034	DENPGSNGGSKV	12	Hydrophilic
36	1044	1077	VDGEADTRLQFEEPTSGSNGGGGGGSNNGSTAEA	34	Hydrophilic
37	1087	1106	VPAPDPFRQTNVNKEDADDF	20	Hydrophilic
38	1133	1142	SKAKNPYSQR	10	Hydrophilic
39	1166	1196	LGPKEATSTSSGGTEAQTQNTAPTSSPT	31	Hydrophilic
40	1208	1214	SPTLATN	7	-

Appendix 39 B cell epitope analysis of HP1

No.	Start	End	Peptide	Length	Hydrophobicity
1	46	53	RIDVQNQA	8	Hydrophilic
2	80	90	LNDDNTKKTDI	11	Hydrophilic

No.	Start	End	Peptide	Length	Hydrophobicity
3	101	116	TNNSSIPVNFSDHNW	16	Hydrophilic
4	126	134	IKPDDNEQT	9	Hydrophilic
5	144	153	KLADGKTATS	10	Hydrophilic
6	175	181	TYSEDQL	7	Hydrophilic
7	186	191	PYTNSE	6	Hydrophilic
8	210	223	QKEVNSAKNDTEAR	14	Hydrophilic
9	250	257	DSGIQKPR	8	Hydrophilic
10	277	280	ATPG	4	Hydrophobic
11	301	304	DINK	4	Hydrophilic
12	444	448	KRNDK	5	Hydrophilic
13	467	476	QNDPDFAASV	10	Hydrophilic
14	481	501	LTIQPNQETVAKDKQGIPK	21	Hydrophilic
15	554	563	SNVDDAASKG	10	Hydrophilic
16	572	577	FNSDEK	6	Hydrophilic
17	611	618	TKWPEDLN	8	Hydrophilic
18	631	646	IKPTKQPRGQIGDENS	16	Hydrophilic
19	663	667	NKPLE	5	Hydrophilic
20	703	720	NKFTQPNFSSTDGKNNIE	18	Hydrophilic
21	735	739	SKQKS	5	Hydrophilic
22	768	776	ALEKSNDQN	9	Hydrophilic
23	786	793	GPVDKNGD	8	Hydrophilic
24	829	834	STFSPN	6	Hydrophilic
25	857	872	AKSKENEPVSVDKEVE	16	Hydrophilic
26	895	918	AKQSQTDTDSNATTMQTTDTDIS	24	Hydrophilic
27	924	931	ITSPASPV	8	-
28	942	948	KNNPKIL	7	Hydrophilic

Appendix 40 B cell epitope analysis of HP2

No.	Start	End	Peptide	Length	Hydrophobicity
1	35	39	KLGPS	5	Hydrophilic
2	55	59	DKFKD	5	Hydrophilic
3	68	82	EGENDGTNTANNARK	15	Hydrophilic
4	119	132	SEEISQDQFPDDLK	14	Hydrophilic
5	150	153	LWID	4	Hydrophilic
6	178	191	IDDPNKPTTETTEI	14	Hydrophilic
7	196	206	EVKKPDPTKRR	11	Hydrophilic
8	221	226	APLQEG	6	Hydrophilic

Appendix 41 B cell epitope analysis of HP3

[illegible]

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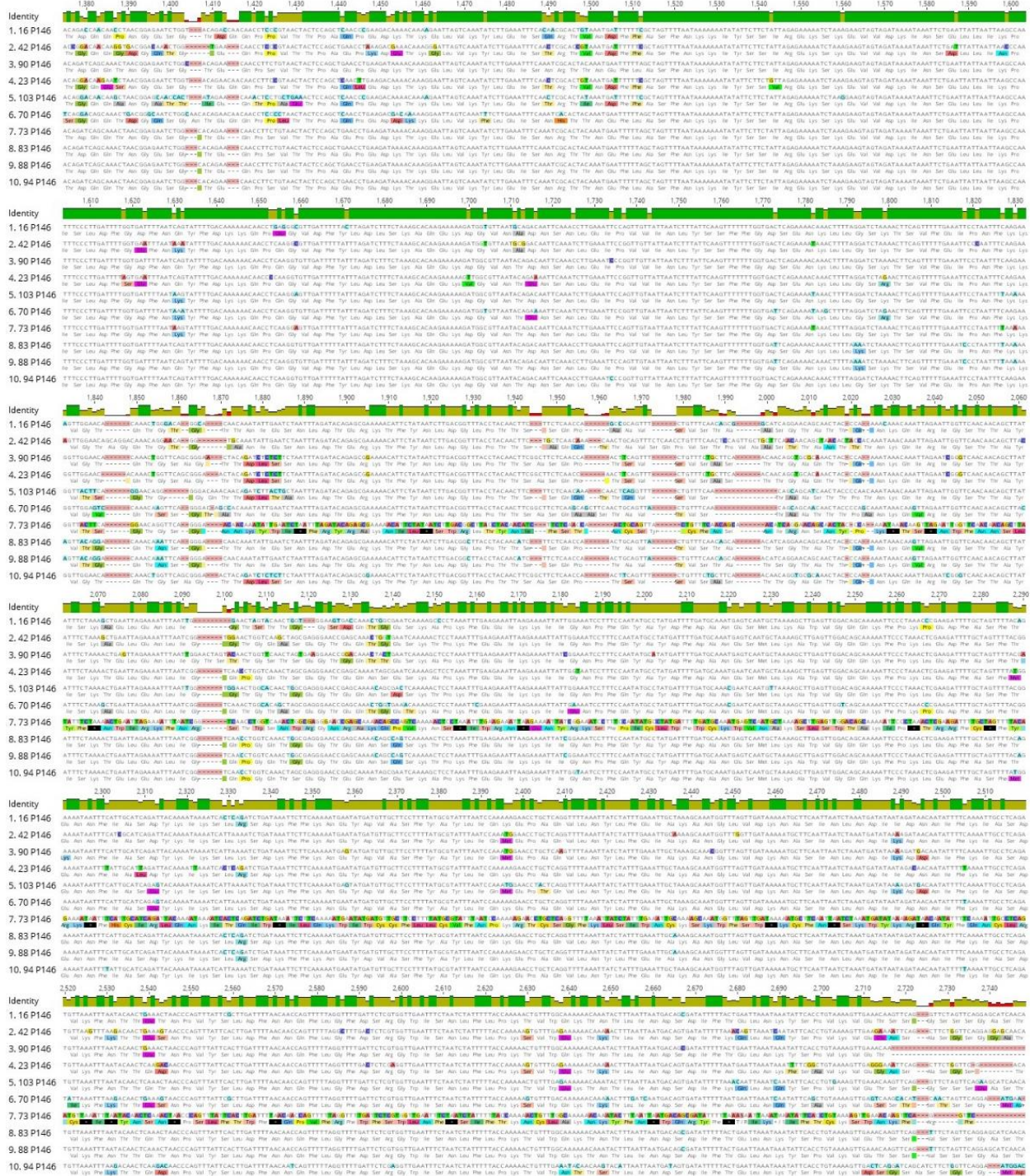
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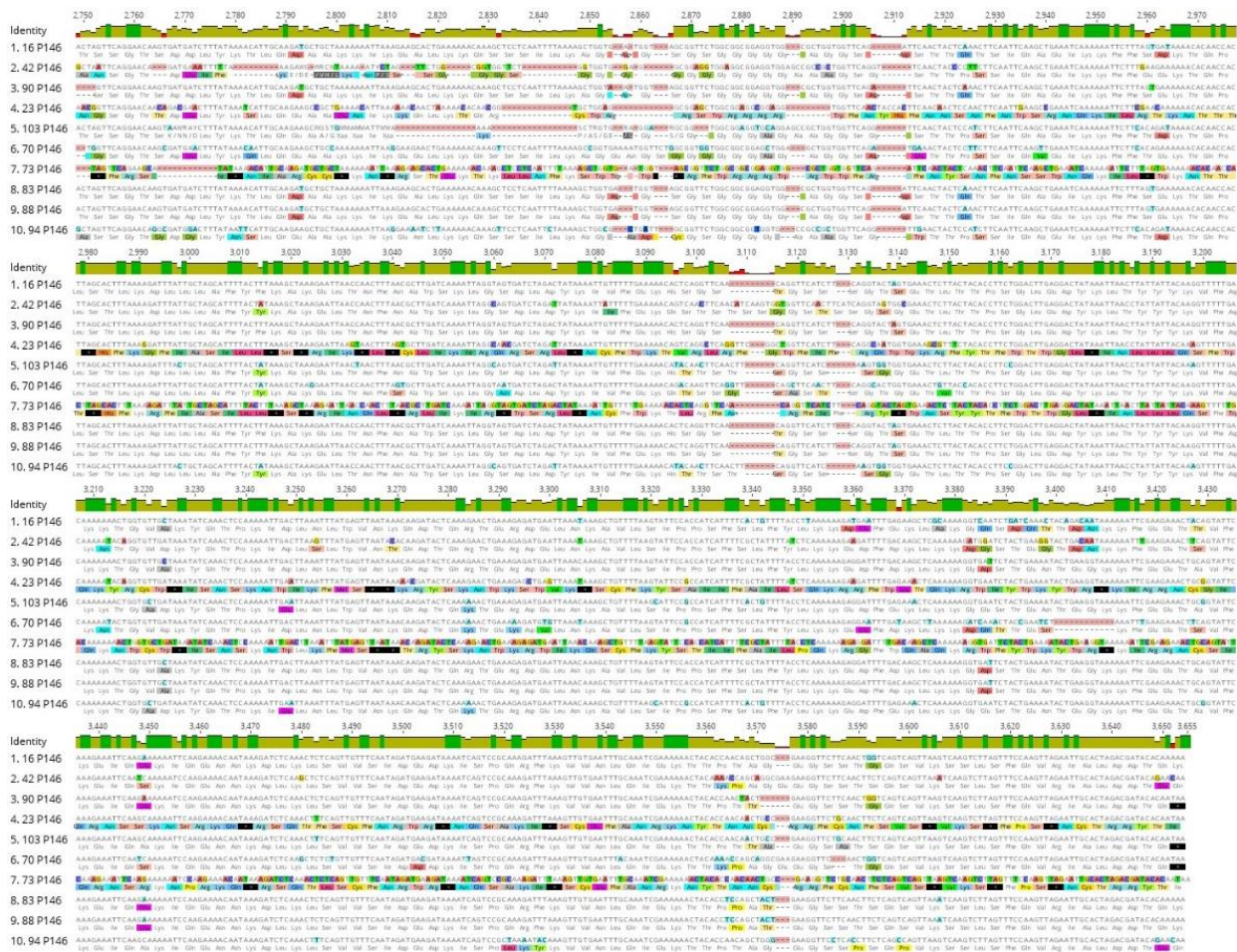
Identity		620	630	640	650	660	670	680	690	700	710
1.16 EF-Tu Frame 1	TC	TACATTGACTCGCCAGTTCGTGA	AAATGGATAAACCAATCTTAAT	GCGCATTTGAAGACGCTTTT	ACCATTACAGGTCGTGGAAC	TGTTGCTACTGGTAA					
2.42 EF-Tu Frame 1	TC	TACATTGACTCGCCAGTTCGTGA	AAATGGACAAACATCTTAA	TGCGCATTTGAAGACGCTTTT	ACCATTACAGGTCGTGGAAC	TGTTGCTACTGGTAA					
3.90 EF-Tu Frame 1	TC	ATCATTTGACTCCAGTTCGTGA	AAATGGACAAACATCTTAA	TGCGCATTTGAAGACGCTTTT	ACCATTACAGGTCGTGGAAC	TGTTGCTACTGGTAA					
4.23 EF-Tu Frame 1	TC	ATCATTTGACTCGCCAGTTCGTGA	AAATGGACAAACATCTTAA	TGCGCATTTGAAGACGCTTTT	ACCATTACAGGTCGTGGAAC	TGTTGCTACTGGTAA					
5.103 EF-Tu Frame 1	TC	ATCATTTGACTCGCCAGTTCGTGA	AAATGGACAAACATCTTAA	TGCGCATTTGAAGACGCTTTT	ACCATTACAGGTCGTGGAAC	TGTTGCTACTGGTAA					
6.70 EF-Tu Frame 1	TC	ATCATTTGACTCGCCAGTTCGTGA	AAATGGACAAACATCTTAA	TGCGCATTTGAAGACGCTTTT	ACCATTACAGGTCGTGGAAC	TGTTGCTACTGGTAA					
7.73 EF-Tu Frame 1	TC	ATCATTTGACTCGCCAGTTCGTGA	AAATGGACAAACATCTTAA	TGCGCATTTGAAGACGCTTTT	ACCATTACAGGTCGTGGAAC	TGTTGCTACTGGTAA					
8.83 EF-Tu Frame 1	TC	ATCATTTGACTCGCCAGTTCGTGA	AAATGGACAAACATCTTAA	TGCGCATTTGAAGACGCTTTT	ACCATTACAGGTCGTGGAAC	TGTTGCTACTGGTAA					
9.88 EF-Tu Frame 1	TC	ATCATTTGACTCCAGTTCGTGA	AAATGGACAAACATCTTAA	TGCGCATTTGAAGACGCTTTT	ACCATTACAGGTCGTGGAAC	TGTTGCTACTGGTAA					
10.94 EF-Tu Frame 1	TC	ATCATTTGACTCCAGTTCGTGA	AAATGGACAAACATCTTAA	TGCGCATTTGAAGACGCTTTT	ACCATTACAGGTCGTGGAAC	TGTTGCTACTGGTAA					
Identity		720	730	740	750	760	770	780	790	800	810
1.16 EF-Tu Frame 1	GT	TGAAGAGGACAAGTTAACTAA	ATGAAGAGGTTGAATGTG	CGTTACCGTTCAGAACCTA	AAAAAACAGTTGTAACCG	GAATGAAATGTTTAA	CAAA				
2.42 EF-Tu Frame 1	GT	TGAAGAGGACAAGTTAACTAA	ATGAAGAGGTTGAATGTG	CGTTACCGTTCAGAACCTA	AAAAAACAGTTGTAACCG	GAATGAAATGTTTAA	CAAA				
3.90 EF-Tu Frame 1	GT	TGAAGAGGACAAGTTAACTAA	ATGAAGAGGTTGAATGTG	CGTTACCGTTCAGAACCTA	AAAAAACAGTTGTAACCG	GAATGAAATGTTTAA	CAAA				
4.23 EF-Tu Frame 1	GT	TGAAGAGGACAAGTTAACTAA	ATGAAGAGGTTGAATGTG	CGTTACCGTTCAGAACCTA	AAAAAACAGTTGTAACCG	GAATGAAATGTTTAA	CAAA				
5.103 EF-Tu Frame 1	GT	TGAAGAGGACAAGTTAACTAA	ATGAAGAGGTTGAATGTG	CGTTACCGTTCAGAACCTA	AAAAAACAGTTGTAACCG	GAATGAAATGTTTAA	CAAA				
6.70 EF-Tu Frame 1	GT	TGAAGAGGACAAGTTAACTAA	ATGAAGAGGTTGAATGTG	CGTTACCGTTCAGAACCTA	AAAAAACAGTTGTAACCG	GAATGAAATGTTTAA	CAAA				
7.73 EF-Tu Frame 1	GT	TGAAGAGGACAAGTTAACTAA	ATGAAGAGGTTGAATGTG	CGTTACCGTTCAGAACCTA	AAAAAACAGTTGTAACCG	GAATGAAATGTTTAA	CAAA				
8.83 EF-Tu Frame 1	GT	TGAAGAGGACAAGTTAACTAA	ATGAAGAGGTTGAATGTG	CGTTACCGTTCAGAACCTA	AAAAAACAGTTGTAACCG	GAATGAAATGTTTAA	CAAA				
9.88 EF-Tu Frame 1	GT	TGAAGAGGACAAGTTAACTAA	ATGAAGAGGTTGAATGTG	CGTTACCGTTCAGAACCTA	AAAAAACAGTTGTAACCG	GAATGAAATGTTTAA	CAAA				
10.94 EF-Tu Frame 1	GT	TGAAGAGGACAAGTTAACTAA	ATGAAGAGGTTGAATGTG	CGTTACCGTTCAGAACCTA	AAAAAACAGTTGTAACCG	GAATGAAATGTTTAA	CAAA				
Identity		820	830	840	850	860	870	880	890	900	910
1.16 EF-Tu Frame 1	AA	CTTCAATTCGAATGGCTGGAG	AATAATGCTGGAGTCTCT	CTCTGCTGGTGTGACCG	TAAAGATATTGAAC	GTGTCAGGTTATTG	CCAAACCAAA	AAACA			
2.42 EF-Tu Frame 1	AA	CTTCAATTCGAATGGCTGGAG	AATAATGCTGGAGTCTCT	CTCTGCTGGTGTGACCG	TAAAGATATTGAAC	GTGTCAGGTTATTG	CCAAACCAAA	AAACA			
3.90 EF-Tu Frame 1	AA	CTTCAATTCGAATGGCTGGAG	AATAATGCTGGAGTCTCT	CTCTGCTGGTGTGACCG	TAAAGATATTGAAC	GTGTCAGGTTATTG	CCAAACCAAA	AAACA			
4.23 EF-Tu Frame 1	AA	CTTCAATTCGAATGGCTGGAG	AATAATGCTGGAGTCTCT	CTCTGCTGGTGTGACCG	TAAAGATATTGAAC	GTGTCAGGTTATTG	CCAAACCAAA	AAACA			
5.103 EF-Tu Frame 1	AA	CTTCAATTCGAATGGCTGGAG	AATAATGCTGGAGTCTCT	CTCTGCTGGTGTGACCG	TAAAGATATTGAAC	GTGTCAGGTTATTG	CCAAACCAAA	AAACA			
6.70 EF-Tu Frame 1	AA	CTTCAATTCGAATGGCTGGAG	AATAATGCTGGAGTCTCT	CTCTGCTGGTGTGACCG	TAAAGATATTGAAC	GTGTCAGGTTATTG	CCAAACCAAA	AAACA			
7.73 EF-Tu Frame 1	AA	CTTCAATTCGAATGGCTGGAG	AATAATGCTGGAGTCTCT	CTCTGCTGGTGTGACCG	TAAAGATATTGAAC	GTGTCAGGTTATTG	CCAAACCAAA	AAACA			
8.83 EF-Tu Frame 1	AA	CTTCAATTCGAATGGCTGGAG	AATAATGCTGGAGTCTCT	CTCTGCTGGTGTGACCG	TAAAGATATTGAAC	GTGTCAGGTTATTG	CCAAACCAAA	AAACA			
9.88 EF-Tu Frame 1	AA	CTTCAATTCGAATGGCTGGAG	AATAATGCTGGAGTCTCT	CTCTGCTGGTGTGACCG	TAAAGATATTGAAC	GTGTCAGGTTATTG	CCAAACCAAA	AAACA			
10.94 EF-Tu Frame 1	AA	CTTCAATTCGAATGGCTGGAG	AATAATGCTGGAGTCTCT	CTCTGCTGGTGTGACCG	TAAAGATATTGAAC	GTGTCAGGTTATTG	CCAAACCAAA	AAACA			
Identity		920	930	940	950	960	970	980	990	1,000	1,010
1.16 EF-Tu Frame 1	AT	TATTCGCCACATAAATTAAG	CAGCAATTTACGCGCTCA	AAAAAGAAAGGTGGA	AGACATACTCCATTTT	CAAAAATACAAAC	CTCAATTTAT				
2.42 EF-Tu Frame 1	AT	TATTCGCCACATAAATTAAG	CAGCAATTTACGCGCTCA	AAAAAGAAAGGTGGA	AGACATACTCCATTTT	CAAAAATACAAAC	CTCAATTTAT				
3.90 EF-Tu Frame 1	AT	TATTCGCCACATAAATTAAG	CAGCAATTTACGCGCTCA	AAAAAGAAAGGTGGA	AGACATACTCCATTTT	CAAAAATACAAAC	CTCAATTTAT				
4.23 EF-Tu Frame 1	AT	TATTCGCCACATAAATTAAG	CAGCAATTTACGCGCTCA	AAAAAGAAAGGTGGA	AGACATACTCCATTTT	CAAAAATACAAAC	CTCAATTTAT				
5.103 EF-Tu Frame 1	AT	TATTCGCCACATAAATTAAG	CAGCAATTTACGCGCTCA	AAAAAGAAAGGTGGA	AGACATACTCCATTTT	CAAAAATACAAAC	CTCAATTTAT				
6.70 EF-Tu Frame 1	AT	TATTCGCCACATAAATTAAG	CAGCAATTTACGCGCTCA	AAAAAGAAAGGTGGA	AGACATACTCCATTTT	CAAAAATACAAAC	CTCAATTTAT				
7.73 EF-Tu Frame 1	AT	TATTCGCCACATAAATTAAG	CAGCAATTTACGCGCTCA	AAAAAGAAAGGTGGA	AGACATACTCCATTTT	CAAAAATACAAAC	CTCAATTTAT				
8.83 EF-Tu Frame 1	AT	TATTCGCCACATAAATTAAG	CAGCAATTTACGCGCTCA	AAAAAGAAAGGTGGA	AGACATACTCCATTTT	CAAAAATACAAAC	CTCAATTTAT				
9.88 EF-Tu Frame 1	AT	TATTCGCCACATAAATTAAG	CAGCAATTTACGCGCTCA	AAAAAGAAAGGTGGA	AGACATACTCCATTTT	CAAAAATACAAAC	CTCAATTTAT				
10.94 EF-Tu Frame 1	AT	TATTCGCCACATAAATTAAG	CAGCAATTTACGCGCTCA	AAAAAGAAAGGTGGA	AGACATACTCCATTTT	CAAAAATACAAAC	CTCAATTTAT				
Identity		1,020	1,040	1,060	1,080	1,100	1,120	1,140	1,160	1,180	1,200
1.16 EF-Tu Frame 1	TT	CCGTACAACCTGATGTTAC	CGGTGGAATGAAATG	AAAGCTGGCCGTGAA	TGGTAATTCCTGGG	GATAATGTTGAC	CTTACTGTTG	AACCTATTG	CCCCCT		
2.42 EF-Tu Frame 1	TT	CCGTACAACCTGATGTTAC	CGGTGGAATGAAATG	AAAGCTGGCCGTGAA	TGGTAATTCCTGGG	GATAATGTTGAC	CTTACTGTTG	AACCTATTG	CCCCCT		
3.90 EF-Tu Frame 1	TT	CCGTACAACCTGATGTTAC	CGGTGGAATGAAATG	AAAGCTGGCCGTGAA	TGGTAATTCCTGGG	GATAATGTTGAC	CTTACTGTTG	AACCTATTG	CCCCCT		
4.23 EF-Tu Frame 1	TT	CCGTACAACCTGATGTTAC	CGGTGGAATGAAATG	AAAGCTGGCCGTGAA	TGGTAATTCCTGGG	GATAATGTTGAC	CTTACTGTTG	AACCTATTG	CCCCCT		
5.103 EF-Tu Frame 1	TT	CCGTACAACCTGATGTTAC	CGGTGGAATGAAATG	AAAGCTGGCCGTGAA	TGGTAATTCCTGGG	GATAATGTTGAC	CTTACTGTTG	AACCTATTG	CCCCCT		
6.70 EF-Tu Frame 1	TT	CCGTACAACCTGATGTTAC	CGGTGGAATGAAATG	AAAGCTGGCCGTGAA	TGGTAATTCCTGGG	GATAATGTTGAC	CTTACTGTTG	AACCTATTG	CCCCCT		
7.73 EF-Tu Frame 1	TT	CCGTACAACCTGATGTTAC	CGGTGGAATGAAATG	AAAGCTGGCCGTGAA	TGGTAATTCCTGGG	GATAATGTTGAC	CTTACTGTTG	AACCTATTG	CCCCCT		
8.83 EF-Tu Frame 1	TT	CCGTACAACCTGATGTTAC	CGGTGGAATGAAATG	AAAGCTGGCCGTGAA	TGGTAATTCCTGGG	GATAATGTTGAC	CTTACTGTTG	AACCTATTG	CCCCCT		
9.88 EF-Tu Frame 1	TT	CCGTACAACCTGATGTTAC	CGGTGGAATGAAATG	AAAGCTGGCCGTGAA	TGGTAATTCCTGGG	GATAATGTTGAC	CTTACTGTTG	AACCTATTG	CCCCCT		
10.94 EF-Tu Frame 1	TT	CCGTACAACCTGATGTTAC	CGGTGGAATGAAATG	AAAGCTGGCCGTGAA	TGGTAATTCCTGGG	GATAATGTTGAC	CTTACTGTTG	AACCTATTG	CCCCCT		
Identity		1,130	1,140	1,150	1,160	1,170	1,180	1,190	1,200	1,209	
1.16 EF-Tu Frame 1	AT	GCGGTTGAGCAAGGAACAA	AATTTCTCAATTCGCG	GAAGGTGGAAGAACT	GTTGGTGCGCGGAAC	GTAAGTAATTTA	TAATTA				
2.42 EF-Tu Frame 1	AT	GCGGTTGAGCAAGGAACAA	AATTTCTCAATTCGCG	GAAGGTGGAAGAACT	GTTGGTGCGCGGAAC	GTAAGTAATTTA	TAATTA				
3.90 EF-Tu Frame 1	AT	GCGGTTGAGCAAGGAACAA	AATTTCTCAATTCGCG	GAAGGTGGAAGAACT	GTTGGTGCGCGGAAC	GTAAGTAATTTA	TAATTA				
4.23 EF-Tu Frame 1	AT	GCGGTTGAGCAAGGAACAA	AATTTCTCAATTCGCG	GAAGGTGGAAGAACT	GTTGGTGCGCGGAAC	GTAAGTAATTTA	TAATTA				
5.103 EF-Tu Frame 1	AT	GCGGTTGAGCAAGGAACAA	AATTTCTCAATTCGCG	GAAGGTGGAAGAACT	GTTGGTGCGCGGAAC	GTAAGTAATTTA	TAATTA				
6.70 EF-Tu Frame 1	AT	GCGGTTGAGCAAGGAACAA	AATTTCTCAATTCGCG	GAAGGTGGAAGAACT	GTTGGTGCGCGGAAC	GTAAGTAATTTA	TAATTA				
7.73 EF-Tu Frame 1	AT	GCGGTTGAGCAAGGAACAA	AATTTCTCAATTCGCG	GAAGGTGGAAGAACT	GTTGGTGCGCGGAAC	GTAAGTAATTTA	TAATTA				
8.83 EF-Tu Frame 1	AT	GCGGTTGAGCAAGGAACAA	AATTTCTCAATTCGCG	GAAGGTGGAAGAACT	GTTGGTGCGCGGAAC	GTAAGTAATTTA	TAATTA				
9.88 EF-Tu Frame 1	AT	GCGGTTGAGCAAGGAACAA	AATTTCTCAATTCGCG	GAAGGTGGAAGAACT	GTTGGTGCGCGGAAC	GTAAGTAATTTA	TAATTA				
10.94 EF-Tu Frame 1	AT	GCGGTTGAGCAAGGAACAA	AATTTCTCAATTCGCG	GAAGGTGGAAGAACT	GTTGGTGCGCGGAAC	GTAAGTAATTTA	TAATTA				

Appendix 43 Alignment of EF-Tu for *M. ovipneumoniae* isolates

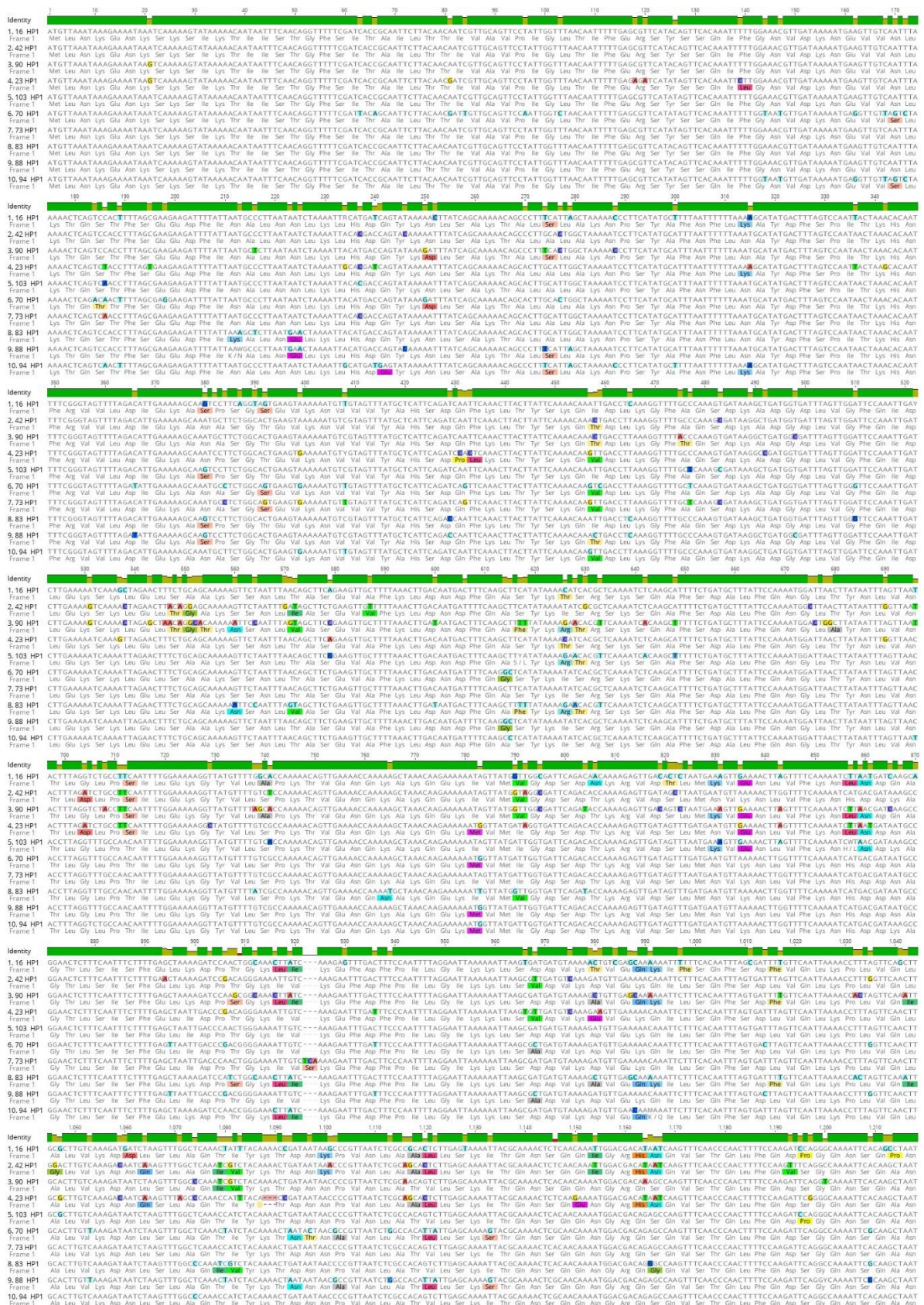
1. 16 GAPDH Frame 1	ATG	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
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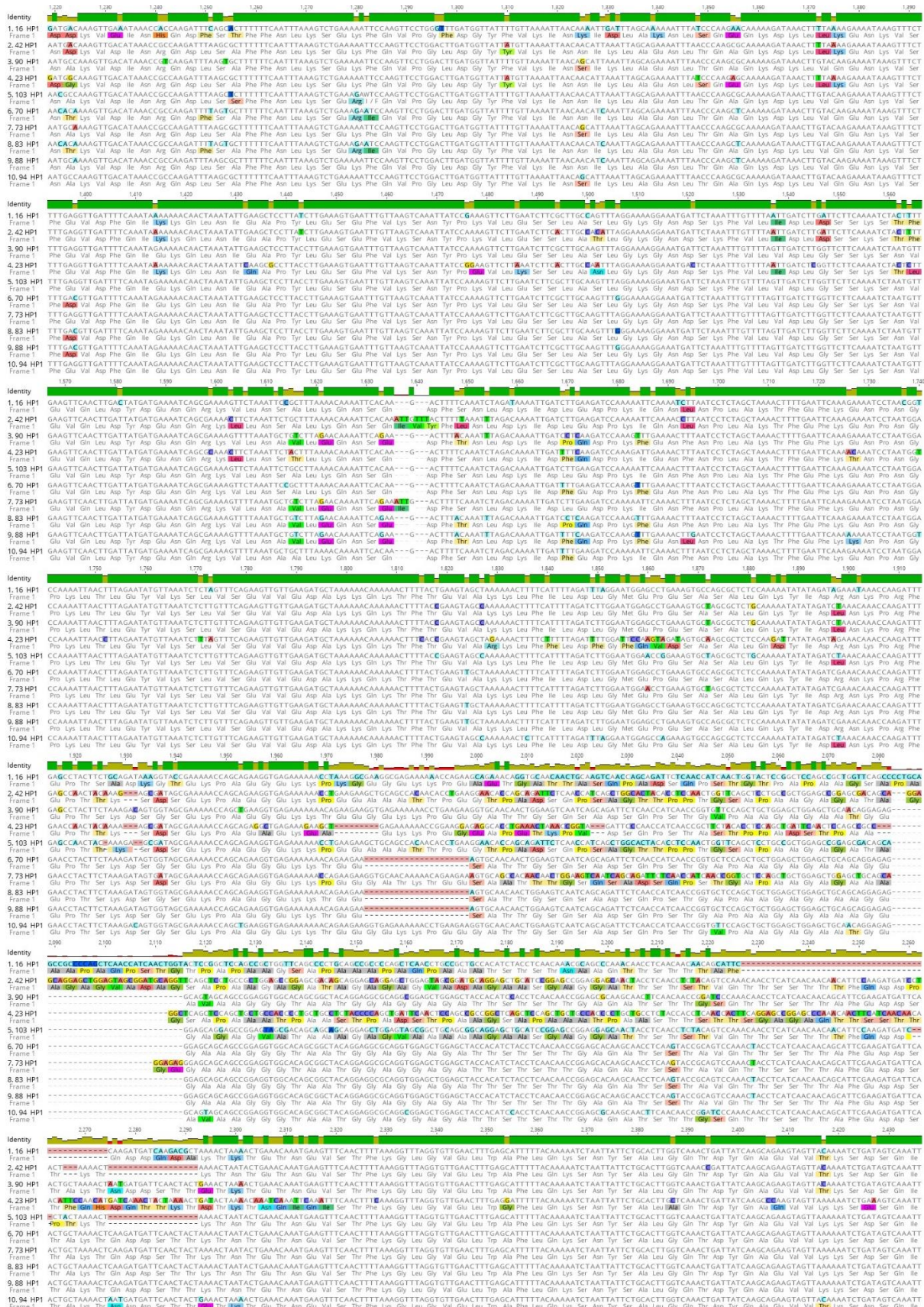
248





Appendix 46 Alignment of P146 for *M. ovipneumoniae* isolates



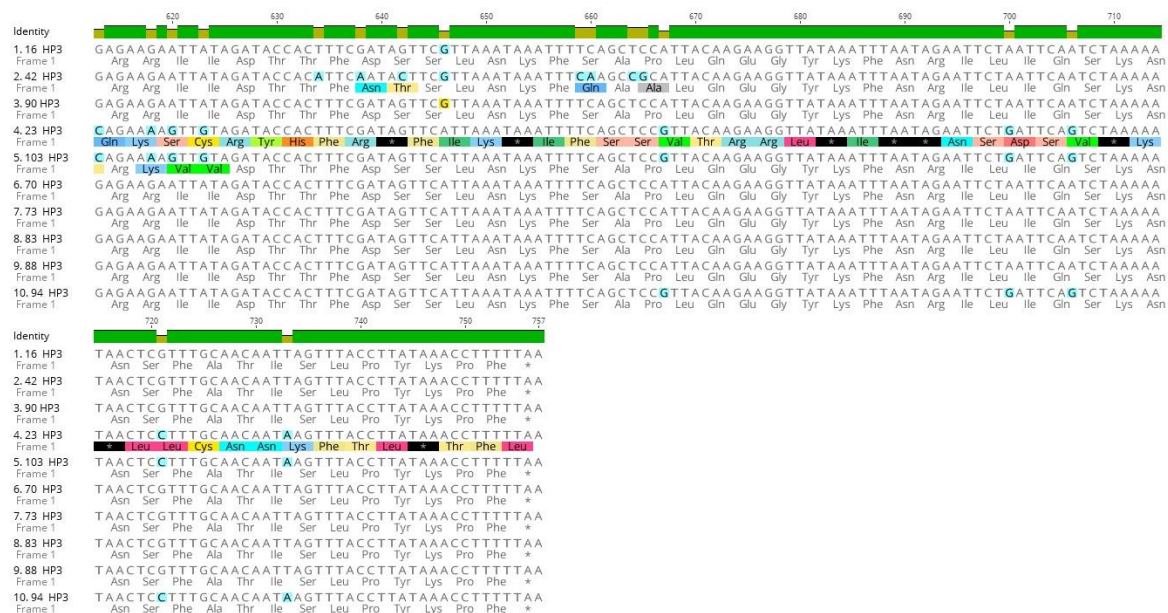






Appendix 48 Alignment of HP2 for *M. ovipneumoniae* isolates

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Appendix 49 Alignment of HP3 for *M. ovipneumoniae* isolates

Details of proteins produced by GenScript:

```

ATGCACCACCACCATCACCACATGGCTGTAGTTAAACAGGAGCTAAGAAGGACTTTGACCGTAGC
M H H H H H H M A V V K T G A K K D F D R S
AAGGAGCACATCAATATCGGCACCATCGGCCACGTTGATCACGGCAAGACCACCTGACCGCGGCG
K E H I N I G T I G H V D H G K T T L T A A
ATTAGCACCGTGTCTGAGCAAGAAAGGTCTGGCGGAAGCGAAGGATTACGCGAGCATTGATGCGGCG
I S T V L S K K G L A E A K D Y A S I D A A
CCGGAGGAAAAAGCGCGTGGCATCACCATTAAACACCGCGCACATTGAGTACAGCACCGACAAGCGT
P E E K A R G I T I N T A H I E Y S T D K R
CACTATGCGCACGTTGATTGCCCGGGTCACGCGGACTATATCAAAAACATGATTACCGGTGCGGCG
H Y A H V D C P G H A D Y I K N M I T G A A
CAGATGGATGGTGCATCCTGGTGGTTGCGGCGACCGATGGTCCGATGCCGCGAGACCCGCTGAGCAC
Q M D G A I L V V A A T D G P M P Q T R E H
ATTCTGCTGAGCAAGCAAGTGGGTGTTCCGAAATGGTGGTTCCTGAACAAGATCGATCTGCTG
I L L S K Q V G V P K M V V F L N K I D L L
GAAGGCGAGGAAGAGATGGTTGACCTGGTGGAAAGTTGAGATTCGTGAGCTGCTGAGCAGCTACGAC
E G E E E M V D L V E V E I R E L L S S Y D
TTTGATGGTGACAACACCCCGATCATTCTGTGGTAGCGCGCGTGGCGCGCTGGAAGGCAAGCCGGAA
F D G D N T P I I R G S A R G A L E G K P E
TGGGAGGCGAAAGTTCTGGAGCTGATGGATGCGGTGGACAGCTATATCGATAGCCCGGTGCGTGAA
W E A K V L E L M D A V D S Y I D S P V R E
ATGGACAACCGTTCTGATGGCGGTGGAGGATGTTTTTACCATCACCGGTCGTGGCACCGTGGCG
M D A K P F L M A V E D V F T I T G R G T V A
ACCGGCAAGGTTGAACGTGGTCAAGTGAAGTGAACGAAGAGGTGGAATCGTTGGCTACCGTCCG
T G K V E R G Q V K L N E E V E I V G Y R P
GAGCCGAAGAAAACCGTGGTTACCGGTATTGAGATGTTCAACAAGAACCTGCAGAGCGCGATGGCG
E P K K T V V T G I E M F N K N L Q S A M A
GGTGACAACGCGGTGTTCTGCTGCGTGGCGTGGATCGTAAAGACATCGAACGTGGTCAAGTTATT
G D N A G V L L R G V D R K D I E R G Q V I
GCGAAGCCGAAAACCATCATTCGCGACACCAAGTTCAAAGCGGCGATCTATGCGCTGAAGAAAGAA
A K P K T I I P H T K F K A A I Y A L K K E
GAGGGTGGCCGTACACCCCGTTCTTTAAGAACTACAAACCGCAGTTCTATTTTCGTACCACCGAT
E G G R H T P F F K N Y K P Q F Y F R T T D
GTTACCGGTGGCATCGAATTTGAGGCGGGCCGTGAAATGGTGGTTCGGGTGATAACGTGGACCTG
V T G G I E F E A G R E M V I P G D N V D L
ACCGTTGAACTGATCGCGCCGATTGCGGTGGAGCAAGGCACCAAATTCAGCATCCGTGAGGGTGGT
T V E L I A P I A V E Q G T K F S I R E G G
CGTACCGTTGGCGCGGGCACCGTTACCGAAATCATCAAAATAATGA
R T V G A G T V T E I I K * *

```

Appendix 50 Sequence analysis of EF-Tu.

The start codon is in orange. The His tag is indicated in purple. The terminator is in red. Sequence is 1233 base pairs in length. The corresponding translated 4410 amino acid sequence is shown as derived from the DNA sequence using Geneious.

```

ATGCCACCACCATCACCACATGAAGAAGATAGCAATAAATGGATTTGGTCGTATTGGCCGCTG
M H H H H H H M K K I A I N G F G R I G R L
GCGCTGCGTCAACTGCTGGACCTGAAGGATGAGAACCTGGAAGTGGTTGCGATCAACGACCTGACC
A L R Q L L D L K D E N L E V V A I N D L T
GATGCGAGCGTGCTGGCGCACCTGTTCAAATATGACAGCGCGCAGGGTCGTTTTAGCGGCACCGTG
D A S V L A H L F K Y D S A Q G R F S G T V
AGCGTTCTGAAAGAGGACGATAAAAACTACCTGGTGATCAACGATAAGAAAATTCGTGTTTTTCAGC
S V L K E D D K N Y L V I N D K K I R V F S
GAGAAGGACCCGGAATGCTGCCGTGGGGTGAGCTGGAATCGATCTGGTTCTGGAATGCACCGGT
E K D P E M L P W G E L E I D L V L E C T G
CGTTATGCGAGCAAGGCGGGTGCGACCTGCACTGAACGCGGGTGCGAAGAAAGTGCTGGTTAGC
R Y A S K A G A T L H L N A G A K K V L V S
GCGCCGGCGGGTAGCGATGTGAAGACCATCGTGACACGTTAACTGCCACACCGTTGACGAGAAC
A P A G S D V K T I V H N V N C H T V D E N
GATAAAATTCGTGAGCAGCGCGAGCTGCACCAACGCGCTGGCGCCGCTGGTGAACGCGCTGGAG
D K I L S S A S C T T N A L A P L V N A L E
AAGGAATTCGGTATCAGCCACGGCTTTATGACACCGTTACGCGTACACCGCGGACCAGCGTATT
K E F G I S H G F M T T V H A Y T A D Q R I
CAAGATGCGCCGCACAAAGACCTGCGTCGTGCGCGTGCGGCGGGTGAACCTGGTTCCGAGCAGC
Q D A P H K D L R R A R A A A V N L V P S S
ACCGGTGCGGCGAAGGCGATTGGCCTGGTGGTTCCGAGCCTGACCGGTAAACTGGATGGCATCGCG
T G A A K A I G L V V P S L T G K L D G I A
ATTCGTGTGCCGGTTATTACCGGTAGCTTCGTGGACCTGAGCGTTGAACCTGAAGAGCAGCCCGAGC
I R V P V I T G S F V D L S V E L K S S P S
ATCGAGGAAGTGAACAAAGCGATTAAGAAATATGAGAGCGAGAGCTTCTACTATTGCGATGAGCCG
I E E L N K A I K K Y E S E S F Y Y C D E P
ATCGTGAGCAGCGACATCATTGGTGATACCCACGGCAGCATTTCGATGCGACCTGACCAAGTTT
I V S S D I I G D T H G S I F D A T L T K F
GTGGAAGTTGACGGCAAGCGTCTGTACAACTGTATACCTGGTACGACAATGAGAGCAGCTTCGTG
V E V D G K R L Y K L Y T W Y D N E S S F V
GCGCAGTTTCGTTCTGTGTTATCCGTTACTTTATTTCAGAAAATAATGA
A Q F V R V I R Y F I Q K * *

```

Appendix 51 Sequence analysis of GAPDH.

The start codon is in orange. The His tag is indicated in purple. The terminator is in red. Sequence is 1035 base pairs in length. The corresponding translated 343 amino acid sequence is shown as derived from the DNA sequence using Geneious.

```

GGATCCAAACCGCGCTTTGAACCGACCGAGCAAGGACAGCGGCGAGCGAAAAACCGGCGGAAGGCGAG
G S K P R F E P T S K D S G S E K P A E G E
AAGAAGACCGAAGAAGGCGAGAAGAAGCCGGAGGAAGGCGGACCGGTAGCCAGAGCGCGGAC
K K T E E G E K K P E E G A T T G S Q S A D
AGCCAACCGAGCACCGGCGTGCCGGCGGGCGGGTGCGGGTGCGGCGACCGGCGAGGCGGTTGCGGCG
S Q P S T G V P A A G A G A A T G E A V A A
GGTGGCGGTACCGCGGCGACCGGCGGTGCGGGCGGTGCGGGCGCGACCAACAGCACCAGCACCACC
G G G T A A T G G A G G A G A T T S T S T T
GGCGCGCAGGCGACCAACCGGTAGCCAAACCAACAGCAGCACCACCGCGTTTGAAGACGAT
G A Q A A T S T T G S Q T T S S T T A F E D D
AGCACCGCGAAAAACCAACGACGACAGCACCACCGAAACCAAAACCGAGACCAATGAAGTTAGCTAA
S T A K T N D D S T T E T K T E T N E V S *
TGA
*
```

Appendix 52 Sequence analysis of HP1.

The start codon is in orange. The His tag is indicated in purple. The terminator is in red. Sequence is 399 base pairs in length. The corresponding translated 131 amino acid sequence is shown as derived from the DNA sequence using Geneious. HP1 also had a large GST added to the start of the fragment (Not Shown)

ATG CACCACCACCATCACCAC ACTTCATGTGGAGTAAGTCACAAAATAGCGGATAAACTGGGTCCG
 M H H H H H H T S C G V S H K I A D K L G P
 AGCGTTAGCTTTGACGTGAACGGTAACCTACGTGTTCCACCATCAAGAACGACAAGTTTAAAGATGCG
 S V S F D V N G N Y V F T I K N D K F K D A
 ACCGAAGTGTTGCGCACTTCGAGGTGAAAACGACGGCACCAACACCGCGAACAACGCGGTAAA
 T E V V A H F E G E N D G T N T A N N A R K
 ATCACCGCGGAAGTGAGCGGCACCATTAACAACCTGACCGCGAGCTTTAACACCGTTGGCAAGCTG
 I T A E V S G T I N N L T A S F N T V G K L
 CAGTACGACTATCGTTGGACCCTGAAGAAAATCAAAGTTGGCAGCGAGGAAATTAGCCAGGATCAA
 Q Y D Y R W T L K K I K V G S E E I S Q D Q
 TTCCCGGACGATCTGAAGGACGATATTTGGTTTGAAAAGAAAATCGCGATTGTGAAACAAGACACC
 F P D D L K D D I W F E K K I A I V K Q D T
 AGCCTGTGGATCGATCCGCAGACCCAACTGCTGAAGAAGTGGATTAAACACCGAGGCGAACGTTGAA
 S L W I D P Q T Q L L K N W I N T E A N V E
 CTGAAGGCGGAGTTTCATCCGTATTGACGATCCGAACAACGACACCGAACACCGAAATCATT
 L K A E F I R I D D P N K P T T E T T E I I
 GACGGTGTGGAAGTTAAGAAACCGGATCCGACCAACGTCGTATCATTGACACCACCTTCGATAGC
 D G V E V K K P D P T K R R I I D T T F D S
 AGCCTGAACAAGTTTAGCGCGCCGCTGCAGGAAGGTTATAAATTTAACCGTATCCTGATCCAGAGC
 S L N K F S A P L Q E G Y K F N R I L I Q S
 AAAACAATAGCTTCGCGACCATTAGCCTGCCGTACAAACCGTTCT**TAATGA**
 K N N S F A T I S L P Y K P F * *

Appendix 53 Sequence analysis of HP3.

The first 23 amino acids were removed to remove predicted signal peptides. The start codon is in orange. The His tag is indicated in purple. The terminator is in red. Sequence is 711 base pairs in length. The corresponding translated 235 amino acid sequence is shown as derived from the DNA sequence using Geneious.

ATG CACCACCACCATCACCAC AGGATAAGATATACAGGAGAAAATCCCCGTGCGGAGCTGGAAAGC
 M H H H H H H R I R Y T G E N P R A E L E S
 CTGGTTAGCCGTGTTCAAAACGTGGCGTTCAAGAGCGACGTTTTTGACGATAGCACCACTATAGC
 L V S R V Q N V A F K S D V F D D S T T Y S
 CAGATCAAAAGCCAACCTGTTTCGACCAGAGCGGCAAGCTGCTGGCGGGTACCGATCTGAACAAATTC
 Q I K S Q L F D Q S G K L L A G T D L N K F
 ATTAGCTTTTACACCCAGGTGAACAGCAAGCTGCGTAAATTCGAGCCGACCTTTGCGCGAACAAG
 I S F Y T Q V N S K L R K F E P T F A P N K
 CCGTTCCTGGAATTTATCGACCTGATTCCGAACGACAACGATCAGAGCTTCGAGCTGCGTTTTTCGT
 P F L E F I D L I P N D N D Q S F E L R F R
 GCGAAACACCAAGTGGATAACAACACCGCGTTTAGCACCATCATTAGCAAGAAAGTTAGCTTC
 A N K H Q V D N N H T A F S T I I S K K V S F
 GCGCAACGTAGCCAGTTTCGCGCTGGCGGAGTTTAAACAGCAACCTGGAAGATCACCAAGAGCTTC
 A Q R S Q F A L A E F N S N L E K I T K S F
 AAGGAGAACATTCAAAACCTGCGTCGTACCGACTTCAGCAGCAGCGTGATCAACCCGAGCCTGGTT
 K E N I Q N L R R T D F S S S V I N P S L V
 GATCAGAAGATTGCGAGCCTGACCCGTGCGGAAGACTTTGCGGCGGATATCAACCGTGCGCTACC
 D Q K I A S L T R A E D F A A D I N R A R T
 CAAAGCGAGGCGGTGGAAAAGATTAGCCAATATTTCCCGGACTTTTCAGAAAATCATTACGACCTG
 Q S E A V E K I S Q Y F P D F Q K I I N D L
 AACTTCGATAAGAACAACCTTCTTTACCTTCAAGCAGGGTACCATCTACAACCTTCAGCCTGGAGAAG
 N F D K N N F F T F K Q G T I Y N F S L E K
 CACCCGGGCACCAACAACCTTTATTAGCTGGGTCCGAACAGCGTTCCGAGCTTCCTGGTGAAAGCG
 H P G T N N F I S V G P N S V P S F L V K A
 GAACTGAGCGACGATGCGAAGTTCGTGCTGAAAACTTTAACATCGAGGACGCGCAGCTGCTGGAA
 E L S D D A K F V L K N F N I E D A Q L L E
 AAAATTGATCTGGTTCCGCAAGCGAGCAGCGGCGAGCGAAGCAGCCAGGGCGAGGAAAAACAAGCG
 K I D L V P Q A S S G S E S S Q G E E K Q A
 AAGCAGGCGACCTACTTCGCGGACGTTGACGATATCCTGAGCAAGATTAGCCTGCGTAAACTGCAA
 K Q A T Y F A D V D D I L S K I S L R K L Q
 TTCACCGATTTTAAAGTTGGCAGCCAGCCGGTGACCAACACCCAGGTTCAAGCGCAGGCGAGCCTG
 F T D F K V G S Q P V T N T Q V Q A Q A S L
 CTGAGCAGCGGTGACATCAACAACCTGGTGAGGTTAGCAGCGAGGTGACCCAAAGCCAAACCGCAA
 L S S G D I K Q L V Q V S S E V T Q S Q P Q
 GCGCAGGCGGAAGGTGGCGGGGTGGCGGTACCGGTCAGCAAGCGACCGGTACCAACCGGTACC
 A Q A E G G A G G G T G Q Q A T S G T T G T
 GATGCGGGTAGCACCGGTACCGGCGGTGTTGCGGGTGCGGGTGCGCCGGACAGCGGTACCGGTAGC
 D A G S T G T G V A G A G A P D S G T G S

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ACCGGTAGCACCACCGATCAGCAAAACCAACGGCGAGAGCGGTACCGAACAAACCGAGCGTGACCACC
T G S T T D Q Q T N G E S G T E Q P S V T T
CCGGCGGACCGGAAGACAAGACCAAGAGCTGGTTAAGTATCTGGAAATCAGCAACCGTACCACC
P A E P E D K T K E L V K Y L E I S N R T C C C
AACGAGTTCCTGGCGAGCTTTAAACAAGAAAATCTACAGCAGCATTTCGTGAGAAGAGCAAAGAAGTG
N E F L A S F N K K I Y S S I R E K S K E V
GTTGACAAATTAACAGCGAAGCTGCTGATCAAGCGATTAGCCTGGACTTCGGCGATTTCACCAQA
V D K I N S E L L I K P I S L D F G D F N Q
TACTTCGATAAGAAGCAACCGCAGGGTGTGGACTTCTACCTGGATCTGAGCAAGGCGCAGGAGAAA
Y F D K K Q P Q G V D F Y L D L S K A Q E K
GACGGTGTAAACACCGATAACAGCAACCTGGAAATCCCGGTGGTTATTAACCTGTACAGCAGCTTC
D G V N T D N S N L E I P V V I N L Y S S F
TTTGGCGACAGCGAGAACAAGCTGCTGGGTAGCAAAACAGCGTGTTCGAAATCCCGAACTTTAAG
F G D S E N K L L G S K T S V F E I P N F K
AAAGTTGGTACCCAAACCGGTAGCGCGGTACACCGACCTGAGCAGCAACCTGGATACCGAGCGT
K V G T C Q T G S A G T T D L S N L D T E R
AAAACCTTCTATAACCTGGATGGTCTGCCGACCACAGCGCGAGCCAGCCGACCAGCGTGAGCGTT
K T F Y N L D G L P T T S A S Q P T S V S V
AGCGGAGCACCACCGGTGCGCAAAACACCCAGAACAAGCAAAATCCGTATTGGTAGCACCACCGG
S A S T T G A Q T T Q N K Q I R I G S T T A
TACATCAGCAAAACCGAGCTGGAAAACCTGATTGGCACCAGCACCACCGGTAGCACCAGCGAGGGT
Y I S K T E L E N L I G T S T T G S T S E G
ACCGGTCAAAACACCGAAGCAAGCGCCGAAGTTTCGAGGAATCAAGAAAATCATTGGCAACCCG
T G Q T T E S K A P K F E E I K K I I G N P
TTCCAGTATGCGTACGACTTTGATGCGAACGAGAGCATGCTGAAGGCGTGGGTGGGTCAACAAAAA
F Q Y A Y D F D A N E S M L K A W V G Q K
TTTCCGAAGCTGGAAGACTTTCGCGAGCTTTACCAAAACAACTTCATCGCGAGCGATTATAAGATT
F P K L E D F A S F T K N N F I A S D Y K I
AAAAGCCTGAAAAGCGACAAGTTCTTTAAAAACGAGTACGATGTTGCGAGCTTTTATGCGTACCTG
K S L K S D K F F K N E Y D V A S F Y A Y L
ATCCAAATGGAGCCGGCGCAGGTGCTGAACCTACCTGTTTCGAAATTGCGAAAGCAACGGTCTGGTT
I Q M E P A Q V L N Y L F E I A K A N G L V
GACAAGAACGCGAGCATCAACCTGAACGATATCAAGGACGATAACATCTTCAAGATCGCGAGCGAC
D K N A S I N L N D I K D D N I F K I A S D
GTGAAGTTCAACACCAACCGAAGCAACCCGGTTTACAGCCTGGACTTTAACAACCAATTCTTGGGC
V K F N T T E T N P V Y S L D F N N Q F L G
TTTGATAGCCGTGGTTGGATCAGCAACCTGTTCTGCGAAGACCGTTTGGCAGAAAAACCAACACC
F D S R G W I S N L F L P K T V W Q K T N T
CTGATCAACGACAGCGATATTTTACCGAGCTGAACAAGTATAGCCCGGTGAAAGTTGAAACAGC
L I N D S D I F T E L N K Y S P V K V E T S
AGCGGTACCAGCGACGATCTGTACAAGACCCTGCAAGATGCGGCGAAGAAAATCAAAGAGGGCGCTG
S G T S D D L Y K T L Q D A A K K I K E A L
AAGAAACAGAGCAGCAGCATTCTGAAAGCGGTAAACGGTAGCGGTAGCGGGTGGCGGGTGGCGG
K K Q S S I L K A G G N G S G S G G G G G A
GGTGGCAGCGACAGCACCCCAAAACAGCATCCAGGCGGAGATTAAGAAATTCTTTAGCGAAAAA
G G S D S T T Q T S I Q A E I K K F F S E K
ACCGACCGCTGAGCACCTGAAGGATCTGCTGCTGGCGTTCTATTTTAAGGCGAAAGAGCTGACC
T Q P L S T L K D L L A F Y F K A K E L T
AACTTTAACGCGTGGAGCAAACCTGGGCAGCGACCTGGATTACAAGATCGTGTTTCGAAAAACATAGC
N F N A W S K L G S D L D Y K I V F E K H S
GGTAGCACCGGTAGCAGCAGCGGCACCAAGCAACCTGACCAACCCGAGCGGTCTGGAAGACTAT
G S T G S S G T S E T L T T P S G L E D Y
AACTGACCTACTATTACAAGGTGTTTGATAAGAAAACCGGTGTTGCGAAGTACCAGACCCCGAAA
K L T Y Y Y K V F D K K T G V A K Y Q T P K
ATCGACCTGAACCTGTGGGTGAACAAGCAGGACACCCAACTACCGAGCGTGATGAAGTGAACAAA
I D L N L W V N K Q D T Q R T E R D E L N K
GCGGTTCTGAGCATTCCGCCGAGCTTCAGCCTGTTTTACCTGAAGAAAGAAGACTTCGATAAGCTG
A V L S I P P S F S L F Y L K K E D F D K L
AAGAAAGGCGACGACACCGGAGAACACCGAAGGTAAGAAATTCGAGGAAACCGGGTTTTAAGGAG
K K G D S T E N T E G K K A F E E T A V F K E
ATCCAAGAAAAAATTCAGGAGAACAAAGGATCTGAAACTGAGCGTGGTTAGCATCGACGAAGAT
I Q E K I Q E N N K D L K L S V V S I D E D
AAAATTAGCCCGCAACGTTTCAAGGTGGTTAACCTGCAGATCGAGAAAAACCCCGACCCGAA
K I S P Q R F K V V N L Q I E K T T P T T E
GGTAGCAGCACCGGTACAGAGCGTTAAGAGCAGCCTGAGCTTTCAAGTGCCTATTGCGCTGGACGAC
G S S T G Q S V K S S L S F Q V R I A L D D
ACCGAATTAATGA
T Q * *

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Appendix 54 Sequence analysis of P146.

The first 30 amino acids were removed to remove predicted transmembrane domain. The start codon is in orange. The His tag is indicated in purple. The terminator is in red. Sequence is 3444 base pairs in length. The corresponding translated 1146 amino acid sequence is shown as derived from the DNA sequence using Geneious.



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We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Benjamin Bridgeman
Name/title of Primary Supervisor:	Dr Rao Dukkupati
In which chapter is the manuscript /published work:	Chapter 3
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Date:	06-Apr-2021
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