A study on secreted proteins of *Mycobacterium avium* subspecies *paratuberculosis* vaccine strain 316F

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Abstract

Mycobacterium avium subspecies *paratuberculosis* (MAP) strain 316F is the organism in the live attenuated vaccine Neoparasec™ which has been used to control paratuberculosis or Johne's disease in cattle and sheep.

The aim of this study was to identify novel exported proteins of MAP strain 316F, with a view to identifying immunogens that may have application in diagnostic tests. Potentially exported proteins were identified using alkaline phosphatase gene fusion technology. A partial digest of the MAP strain 316F genomic DNA was cloned into the vector pJEM11, and expressed in the surrogate hosts *E. coli* and *M. smegmatis*. The DNA inserts from selected alkaline phosphatase positive clones were partially sequenced and the sequences were analysed using public databases to identify and obtain full gene sequences and to predict the potential function of the identified proteins.

The genes from three putative exported proteins: glutamine binding protein (*glnH*, MAP3894c), sulphate binding protein (*subI*, MAP2213c) and a hypothetical protein (MAP3273c), were selected for preliminary investigation. The open reading frame of each gene was obtained by PCR amplification and was cloned into the *E. coli* expression vector pET-26b (+) for the expression of C-terminal histidine-tagged fusion proteins. The recombinant proteins were prepared and purified by immobilized-metal affinity chromatography.

Following SDS-PAGE, the three antigens were screened by Western blot analysis using sera from sheep vaccinated with Neoparasec™ and from control pre-vaccinated animals. Western blot analysis indicated that whilst antibodies could be detected in vaccinated animals to subI and the hypothetical protein, cross reactive antibodies could also be detected in some sera taken prior to vaccination. However, five out of eight animals had a strong antibody responses to glnH following vaccination with Neoparasec™ compared with one out of eight

in the pre-vaccinated control animals suggesting that this was an immunogenic protein expressed in the native host. GlnH was therefore selected for further characterisation.

Investigation into the presence of the *glnH* gene in other mycobacterial species revealed that *glnH* has a 99% identity with the extracellular solute-binding protein of *Mycobacterium avium* subspecies *avium* and similar genes exist in *M. bovis* & *M. tuberculosis* (85.1% identity), *M. ulcerans* (83.4% identity), *M. vanbaalenii* (78.9% identity), *M. smegmatis* (78.1% identity) and *M. gilvum* (78% identity).

An antibody raised in a rabbit to glnH and used in immunofluorescence and transmission electron microscopy studies for protein localisation in MAP strain 316F cells suggested that glnH is located on the surface of the native host.

In addition to antibody against glnH being detected in the sera of sheep vaccinated with Neoparasec™, Western blot analysis also showed that antibody could be detected in the sera of sheep and deer naturally infected with MAP. In order to quantify these responses, an ELISA was developed and a pilot study undertaken that confirmed that there was a significant difference ($p < 0.05$) in antibody responses to glnH between the vaccinated sheep and the unvaccinated controls. Also, serum samples collected from sheep and deer naturally infected with MAP were found to have significant ($p < 0.05$) levels of antibody to glnH compared to uninfected control animals.

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List of Abbreviations

The abbreviations commonly used are presented in the following list:

ABC	ATP binding cassette
ADC	Albumin-D-glucose/dextrose-Catalase
EDTA	Ethylenediamine tetraacetic acid
AGID	Agar gel immunodiffusion test
ATP	Adenosin triphosphate
BCIP	5-bromo-4-chloro-indolyl phosphate
BLAST	Basic local alignment search tool
bp	base pair (s)
CD	Crohn's disease
CIAP	Calf Intestinal alkaline phosphatase
CFT	Complement fixation test
CFU	Colony forming unit
CMI	Cell-mediated immune response
ConA	Concavalin-A
Cat. No.	Catalog number
cm	Centimetre(s)
^{o}C	Degrees Celcius
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTH	Delayed type hypersensitivity
EPB	Electroporation buffer
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
GTE	Glucose-Tris-EDTA
g	Acceleration due to gravity
IgG	ImmunoglobulinG
IMAC	Immobilised metal affinity chromatography
IS*900*	Insertion segment-*900*
IFN-gamma	Interferon-gamma
IM	Inner membrane
IPTG	Isopropyl-β-δ-thiogalactopyranoside
kDa	Kilo dalton
kb	Kilo base pair(s)
L	Litre(s)
LB	Luria-Bertani broth

LBA	Luria-Bertani agar
MAC	*Mycobacterium avium* complex
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MW	Molecular weight
Mb	Mega base pair(s)
M	Molar
µM	Micromolar
µg	Microgram(s)
µl	Microlitre(s)
µm	Micrometre(s)
mM	Millimolar
mg	Milligram(s)
ml	Millilitre(s)
mm	Millimetre(s)
min	Minute(s)
nm	Nanometre(s)
ng	Nanogram(s)
OD	Optical density
OM	Outer membrane
ORF	Open reading frame
PBS	Phosphate-buffered saline
PhoA	Alkaline phosphatase
PFC	Pooled faecal culture
PVDF	Polyvinylidene difluoride membrane
PPDA	Avian purified-protein derivative
PCR	Polymerase chain reaction
rpm	Revolutions per minute
SBP	Substrate binding protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOC	Super optimal broth
Tm	Melting temperature
TAE	Tris-acetate-EDTA
TE	Tris-HCl-EDTA
v/v	Volume/volume
w/v	Weight/volume
7H9-B	Middlebrook 7H9 broth
7H10-A	Middlebrook 7H10 agar

Nucleotides

A	Adenine
C	Cytidine
G	Guanosine
T	Thymidine

Amino acids

A	Alanine	I	Isoleucine	R	Arginine
C	Csyteine	K	Lysine	S	Serine
D	Aspartic acid	L	Leucine	T	Threonine
E	Glutamic acid	M	Methionine	W	Tryptophan
F	Phenyl alanine	N	Asparagine	V	Valine
G	Glycine	P	Proline	Y	Tyrosine
H	Histidine	Q	Glutamine		

CHAPTER 1

Literature review

1.1 Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is an important pathogen of ruminants causing paratuberculosis, which is also known as Johne's disease. MAP is transmitted, primarily, via the faecal-oral route, with infection occurring in animals at a young age (Mackintosh *et al*., 2004b; Stabel, 2007; Windsor & Whittington, 2009). Initial infection of young animals is followed by a lengthy subclinical stage ranging from two to five years (Cocito *et al*., 1994; Rosseels & Huygen, 2008) where no clinical signs are evident. At the advanced stage, the disease is manifested as a chronic enteritis leading to reduced production, wasting, weight loss and eventually death (Harris & Barletta, 2001).

MAP has a wide host range including domestic and many wild animals. Domestic animals include cattle, sheep, goats (Wells & Wagner, 2000; Collins, 2003; 2004; Begg *et al*., 2005; de Juan *et al*., 2005; Salem *et al*., 2005) and deer (de Lisle *et al*., 1993; Godfroid *et al*., 2000; Nebbia *et al*., 2000; Pavlik *et al*., 2000; Mackintosh *et al*., 2004). Wild species that may serve as a reservoirs of infection include bison (Buergelt *et al*., 2000), rabbits (Beard *et al*., 2001b; Daniels *et al*., 2003; Judge *et al*., 2007; Davidson *et al*., 2009), white-tailed deer (Chiodini & Vankruiningen, 1983; Woodbury *et al*., 2008), fallow deer and wild boar (Alvarez *et al*., 2005), feral cats, foxes, stoats, badgers and weasels (Beard *et al*., 2001a; Tryland *et al*., 2004; Palmer *et al*., 2005), raccoons, opossum and armadillos (Corn *et al*., 2005). Some laboratory animals, such as mice, guinea pigs, rats and hamsters, can be experimentally infected with MAP, although the lesions and clinical signs produced in these hosts are not always characteristic of paratuberculosis (Greig *et al*., 1999).

The possible involvement of MAP in human disease has raised significant public health concerns (Acheson, 2001; Chamberlin *et al*., 2001; Hermon-Taylor, 2001; DeHaven & Goldberg, 2006). This bacterium has been isolated and identified in intestinal biopsy tissue samples from patients with Crohn's disease, which is a severe inflammatory enteritis that affects the lower sections of the small intestine and the colon (Bannantine *et al*., 2004a; Ryan *et al*., 2004; Grant 2006). Similarities have also been reported between the clinical symptoms and gross pathology of Crohn's disease and Johne's disease (Rowe & Grant, 2006). This has initiated many studies to determine the role of MAP in the etiology of Crohn's disease and its zoonotic implications in human health (Grant, 2006; Feller *et al*., 2007; Uzoigwe *et al*., 2007; Behr & Kapur, 2008; Donaghy *et al*., 2009; Mendoza *et al*., 2009).

Paratuberculosis has a considerable impact on the farming industry (Hasonova & Pavlik, 2006) due to losses associated with a reduction in milk yield, decreased weight gain and general wasting in affected animals. Coussens (2001) stated an estimated loss, to the United States dairy industry, of over US$ 200 million per year. Harris and Barletta (2001) in their review estimated the loss to be from $40 to $227 per cow annually based on the percentage of culled cows with clinical signs. In Australia, a three year study of 12 infected sheep flocks, estimated the mean annual decrease in gross margin due to paratuberculosis to be between 6.4% and 8.5% equating to an average reduction of income of A$13,715 per farm per year (Bush *et al*., 2006). In New Zealand, it is estimated that annual loses in the most severely affected herd (approximately 160 cows) range up to $6,650 per herd (de Lisle, 2002). The economic cost of clinical paratuberculosis to the sheep industry in New Zealand is not accurately recorded but significant losses ($p < 0.001$) in live weight, greasy fleece weight, number of lambs born per ewe per year, and lifetime productivity of ewes, equivalent to a 46% reduction in productivity, have been reported (Morris *et al*., 2006). Additional economic costs including diagnostic testing and control measures (Coetsier *et al*., 2000) will also contribute to its economic impact. These losses, especially in the dairy industry,

make paratuberculosis rank as one of the most costly infectious diseases (Hasonova & Pavlik, 2006).

Control and eradication of paratuberculosis is difficult and is severely hampered by factors such as the limited knowledge of host factors controlling immune responses to MAP (Coussens, 2001*)* and the presence of undetected sub-clinical cases (Motiwala *et al*., 2003). During the sub-clinical stage, MAP infection is difficult to diagnose, and therefore it tends to stay undiagnosed for several years (Sohal *et al*., 2007). Furthermore, the absence of adequate MAP-specific diagnostic tools to diagnose the disease in the early subclinical stages and the lack of knowledge of strain diversity are likely impediments to successful control programs (Barrington *et al*., 2003).

Recent advances in molecular biology have provided new approaches to research into the epidemiology and diagnosis of mycobacterial diseases. Mycobacterial components interact with the host immune system (Cosma *et al*., 2003; Akira *et al*., 2006) and cell-wall associated and secreted antigens have become a focus of research for vaccine development and improved diagnosis. A range of new immunologically important MAP antigens including those associated with cell-wall or secreted antigens have been identified by the use of new technologies (Dupont *et al.,* 2005; Eckstein *et al*., 2006; Shin *et al*., 2006; Bannantine *et al*., 2007). Such findings are likely to have a major impact on the development of immunodiagnostic tools and vaccines for paratuberculosis.

1.2 *Mycobacterium avium subspecies paratuberculosis*

1.2.1 Classification

Taxonomical classifications have placed MAP under the order *Actinomycetales*, within the family *Mycobacteriaceae* and a single genus *Mycobacterium*. The genus *Mycobacterium* comprises over 130 species and subspecies (Turenne *et al*., 2007). However, the majority of species, which comprise this genus, are non-pathogenic environmental bacteria closely related to the soil bacteria, such as

Streptomyces and *Actinomyces* (Cosma *et al*., 2003; de Jonge *et al*., 2007). Only a few species are successful pathogens in both humans and animals.

Mycobacteria are aerobic, acid-fast, non-motile rods that are characterised by a complex, lipid-rich cell wall (de Jonge *et al*., 2007). Based on their growth rate, they can be divided into two major groups (Inderlied *et al*., 1993; de Jonge *et al*., 2007). The first group consists of rapidly-growing mycobacteria, which form visible colonies within seven days, under ideal culture conditions. Rapid-growing mycobacteria including *Mycobacterium smegmatis*, *Mycobacterium chelone*, *Mycobacterium abscessus*, *Mycobacterium flavescens* and *Mycobacterium vanbaalenii* are relatively easy to cultivate and are usually considered non-pathogenic although there are exceptions such as *M. chelonae* and *M. abscessus,* which are pathogenic for humans (Shinnick & Good, 1994). The second group comprises slow-growing mycobacteria, which form visible colonies after seven days or more, under suitable conditions and are more difficult to cultivate. Many species under this group such as *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium leprae* and other members of *Mycobacterium avium* complex (MAC) are pathogenic for humans and animals.

MAC is considered as a 'microcosm' of the mycobacterial genus which includes both environmental mycobacteria and host-associated pathogens with a limited capacity to survive in the environment (Turenne *et al*., 2007). The definition of MAC varies within the context in which it is discussed. Clinicians and health care workers have included *Mycobacterium avium*, *Mycobacterium intracellulare* and miscellaneous related species as a member of MAC while the taxonomists may consider the MAC to contain only *M. avium* (Turenne *et al*., 2007). Classically, there are three principal subsets of *M. avium* reported as revealed by a combination of molecular analysis, growth characteristic and biochemical tests (Thorel *et al*., 1990). These three subsets consist of *M. avium* subspecies *avium*, *M. avium* subspecies *silvaticum* and *M. avium* subspecies

paratuberculosis (MAP). In addition*, M. avium* subspecies *hominisuis* has been included as the fourth subset of *M. avium* (Mijs *et al*., 2002).

1.2.2 Relationship to other Mycobacteria

Genetically, MAP is virtually identical to *M. avium* subspecies *avium*, since they share 95% of their genes and exhibit homologies of more than 99% between these genes (Li *et al*., 2005). However, the phenotypic characteristics of MAP are different to *M. avium* subspecies *avium*. MAP grows more slowly, with a generation time of 22 to 26 hours, compared to 10-12 hours for *M. avium* subspecies *avium* (Thorel *et al*., 1990). MAP also requires mycobactin for *in vitro* growth, while *M. avium* subspecies *avium* can synthesize it (Chacon *et al*., 2004).

Rastogi and colleagues (2001) stated that while sharing many genetic similarities with *M. avium* subspecies *avium*, MAP is less closely linked to other pathogenic mycobacteria belonging to the *Mycobacterium tuberculosis* complex. Also, though not closely related to the cause of leprosy in humans, *M. leprae*, these two species have certain biological characteristics in common.

1.2.3 The MAP genome

The completion of sequencing of the MAP strain K-10 genome (Li *et al*., 2005) has led to many insights into the biology of MAP organisms and it serves as the starting point for genome-wide studies. The base composition of bacterial genomes is an essential taxonomic parameter, and is usually expressed as the percentage of guanosine plus cytidine (G + C) nucleotides in the DNA. Mycobacteria have been reported to have high (approximately 61 to 71%) genomic G + C contents. Studies carried out by Li and others (2005) and Stinear and others (2008), which compared different mycobacterial species, showed that the base composition of MAP strain K-10 DNA, isolated from a cow with paratuberculosis, is 69.3% while G + C content in the DNA of *M. avium* subspecies *avium* is 68.99%.

Current information on the complete genomes of MAP using MAP strain K-10 indicates that MAP has a size of 4.83 Mb while *M. avium* subspecies *avium* is 5.48 Mb (Li *et al.*, 2005; Stinear *et al*., 2008). The MAP genome appears to be similar in size to the *M. tuberculosis* H37Rv (Cole *et al*., 1998; Stinear *et al*., 2008) and *M. bovis* (Garnier *et al.,* 2003*)* genomes, which are 4.41 Mb and 4.35 Mb respectively. The MAP genome contains a total of 4,350 protein-coding sequences comprising of 3223 conserved proteins with assigned functions, 1088 conserved proteins with unknown function and 39 unique proteins (Stinear *et al*., 2008).

1.3 Paratuberculosis

1.3.1 History

The disorder known as Johne's disease or paratuberculosis (the term paratuberculosis will be applied throughout this review) was first described in 1895 by two German scientists, Johne and Frotingham (Rowe & Grant, 2006). At that time, they identified micro-organisms in granulomatous lesions in the intestine of a cow that had failed to gain weight. Staining of the bacteria showed an acid-fast reaction indicating some type of mycobacteria. Several years later, in 1912, Twort and Ingram successfully grew the micro-organisms in pure culture, reintroduced them into susceptible animals and classified them as a mycobacterium (Stabel, 1997; Grant, 2005).

Originally, the causative organism was named *Mycobacterium enteritidis chronicae pseudotuberculosiae bovis johne* (Twort & Ingram, 1912; cited by Chiodini, 1992). Later, in 1932, it was designated as a distinct species named *Mycobacterium paratuberculosis* (Kreeger, 1991). Under the most recent nomenclature, the organism has been reclassified as *Mycobacterium avium* subspecies *paratuberculosis* (Thorel *et al*., 1990).

In New Zealand, paratuberculosis was first diagnosed, in 1912, in an imported cow (O'Hara, 1983). However, it was not until 40 years later that the first case of

paratuberculosis was reported in sheep in Canterbury (Armstrong, 1952; cited by Begg *et al*., 2005). Since this time, it has become recognised as one of the most important production limiting infections of ruminants (Begg *et al*., 2005). In 1972, paratuberculosis was first reported in sheep flocks in the North Island (West, 2002; cited by Morris *et al*., 2006). Seven years later, in 1979, the first case of paratuberculosis was confirmed in farmed deer in New Zealand (Mackintosh *et al*., 2004a).

1.3.2 Epidemiology

According to the data presented by the Office International des Epizooties, paratuberculosis is 'categorised as a list B transmissible disease that is considered to be of socio-economic and/or public health importance within countries and it is significant in the trade of animals and animal products' (Office International des Epizooties, 2005a).

Many studies have reported the prevalence of paratuberculosis on every continent where livestock occur. The studies are mainly based on cases of paratuberculosis in dairy cattle (Grant, 2005). The most recent survey, conducted by the National Animal Health Monitoring System, reported that MAP prevalence in the United States dairy herds is 68%, which is a large increase over a 1996 estimate (USDA Animal and Plant Health Inspection Service, 2008; cited by Allen *et al*., 2009). Herd prevalence of paratuberculosis in Europe is reported to be between 7 and 55% (Bhide *et al*., 2006), whereas in Australia, the prevalence rate ranges between 9 and 22% (Manning & Collins, 2001).

In New Zealand, the prevalence of paratuberculosis in herds, especially cattle, has never been accurately recorded (de Lisle, 2002) but, according to the Council for Agricultural Science and Technology, apparent herd prevalence in New Zealand is about 60% (Council for Agricultural Science and Technology, 2001; cited by Grant, 2005). It is also interesting to note that for every clinical case of paratuberculosis existing in an infected herd, it is estimated that as many

as four to eight other animals may have sub-clinical disease and be asymptomatic carriers. Recently, a study, based on a questionnaire-based cross-sectional study on dairy farms in the North Island of New Zealand, showed that, of 427 responding farmers, 201 (47%) had suspected clinical cases of paratuberculosis in their herd in the preceding five years (Norton *et al*., 2009). In other animals, paratuberculosis has been reported to affect 70% of sheep flocks (Brett, 1998; cited by Robinson *et al*., 2008) and 6% of farmed deer (de Lisle *et al*., 2003).

1.3.2.1 Crohn's disease (CD)

CD occurs throughout the world (Uzoigwe *et al*., 2007), primarily in high-income countries (Feller *et al*., 2007). Its prevalence has increased over the past 40 years around the world including the United States and throughout many European countries (Economou & Pappas, 2008). In Australia, the incidence of CD in children is ten times greater than it was 20 years ago (Kirkwood *et al*., 2009).

CD may affect the digestive system, from the mouth to the anus, but generally it causes ulcerations of the small and large intestine (Uzoigwe *et al*., 2007). Common symptoms of CD include abdominal pain, mouth ulcers and joint pains, night sweats and loss of energy and weight (Hermon-Taylor & El-Zaatari, 2004). In many cases (more than 60%), CD patients may have diarrhoea, which contains pus and blood (Hermon-Taylor & El-Zaatari, 2004). Morphological changes found include chronic inflammation of the intestinal wall, thickening of the involved segment with narrowing of lumen, and linear ulceration of the mucosa and sub-mucosa (Uzoigwe *et al*., 2007).

The cause of CD is thought to be a combination of several factors including genetic susceptibility, undefined environmental triggers and immune dysfunction (Chamberlin *et al*., 2001; Quirke, 2001; Chacon *et al*., 2004). However, it has long been hypothesized that CD is caused by MAP. This hypothesis remains

controversial (Bannantine *et al*., 2004a; Grant, 2006) but epidemiological studies and advances in molecular techniques, such as PCR and culture methods, have enabled many researchers to demonstrate that there does appear to be an association between MAP and CD (Behr & Kapur, 2008; Mendoza *et al*., 2009). A study carried out by Autschbach and colleagues (2005) showed an MAP insertion element *IS900* DNA was detected in more than 50% of tissue from patients (52 out of 100) with CD, whereas it was less common in patients with ulcerative colitis (2 out of 100) and non-inflammatory disease control patients (5 out of 100). These results support findings from other studies (Collins *et al*., 2000; Ikonomopoulos *et al*., 2000; Ryan *et al*., 2002) which detected MAP *IS900* DNA in a proportion of cases with CD.

For the MAP organism to play a role in the pathogenesis of CD, it must first gain access to, and colonize the human intestine. Bannantine and co-workers (2004a) proposed that ingestion of either contaminated water or milk were amongst the two potential modes of MAP transmission, from the host animal to human. A study undertaken in Northern Ireland, on untreated water entering nine water treatment works, showed that of the 192 untreated water samples tested, 8% (15 out of 192) tested MAP positive (Whan *et al*., 2005). Since treated water was not tested during the study, the efficacy of water treatment to remove or inactivate the organism is unknown. Therefore, the authors stated that the efficacy of water treatment may need to be revisited in order to ensure accurate risk assessment and priority of resources to reduce public exposure to the bacteria.

Another possible route of transmission is through contaminated milk. Milk is a significant element in the diet of most people and in particular young children. Several studies have reported the presence of low levels of MAP DNA, both in raw and commercially pasteurised milk. MAP DNA has been detected in raw goat's milk (Djonne *et al*., 2003; Muehlherr *et al*., 2003), raw sheep's milk (Muehlherr *et al*., 2003) and cow's milk (Grant *et al*., 2002; O'Reilly *et al*., 2004). Using optimised decontamination protocols and immunomagnetic capture, a

study conducted by the Department of Food Science, Queen's University Belfast, found that 67 out of 567 (11.8%) samples of retail pasteurised cow's milk, in the United Kingdom, tested MAP-positive by PCR and that 1.8% of samples were MAP-positive by culture (Grant *et al*., 2002), thus suggesting that some MAP cells may survive pasteurisation.

1.3.3 Stages of MAP infection

Rosseels and Huygen (2008) summarised the stages of MAP infection in cattle (Figure 1.1), according to the severity of the clinical signs, the immune response induced and the potential to detect the infection, with available diagnostic tools. They stated that the disease progresses through four stages: preclinical, subclinical, clinical and advanced clinical infection.

Preclinical infection is characterised by 'a silent infection' as infected animals do not show clinical signs of disease. Infected susceptible animals will remain without any clinical signs, during the first two to four years after contact. The number of MAP shed in the faeces is undetectable, due to the very low levels of MAP present. In some cases, mycobacteria-specific cell-mediated immune responses (CMIs), such as interferon gamma production, lymphoproliferation and delayed type hypersensitivity (DTH) reactions might be detected.

In stage two, which is called 'the sub-clinical excretory phase', no visible clinical signs of paratuberculosis develop, but animals may shed low to moderate numbers of MAP. A strong mycobacteria-specific CMI response is developed during this stage, while antibodies to MAP are generally low. Khalifeh and Stabel (2004), in their study, reported a high expression of interferon gamma in subclinically infected animals. The high expression of interferon gamma has been reported to correlate with increased levels of interferon gamma secretion by peripheral blood mononuclear cell isolated from animals in the early stages of either natural or experimental MAP infection (Waters *et al*., 2003).

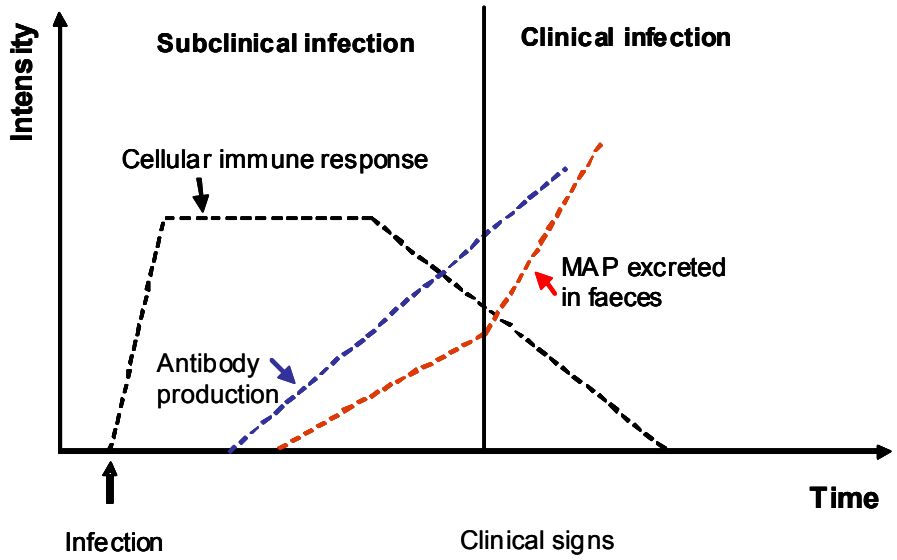


Figure 1.1. Stages of MAP infection in cattle. Adapted from Rosseels and Huygen, 2008.

In contrast to the first and the second stages, during the clinical stages, infected animals develop a progressive disease, which is characterised by the appearance of clinical signs, such as intermittent or persistent diarrhoea, gradual weight loss, reduced milk production and decreased fertility. In deer, however, the most common clinical sign of paratuberculosis is sudden death and extreme weight loss (Woodbury *et al*., 2008). Diarrhoea, which is often the first sign of the disease in cattle, may not occur in sheep or deer although several studies have reported the occurrence of diarrhoea in affected deer (Gumbrell, 1987; Mackintosh *et al*., 2004b; Glossop *et al*., 2008). During the clinical stage, most animals develop antibodies against MAP, while the mycobacteria-specific Th1-type immune responses are weak. Infected animals can profusely shed bacteria in their faeces, thus ensuring possible detection by faecal culture. Whittington and Sergeant (2001) reported that clinically infected cows may shed 10^6 to 10^8 colony forming units (CFUs) per gram of faecal material that can easily spread

the infection to new calves for which the infectious dose is 10^3 CFU/animal. When the advanced clinical stage occurs, infection can lead to the advanced form of the disease, which is characterised by persistent diarrhoea, emaciation, debilitation and eventual death.

1.3.4 Treatment

Infections, due to mycobacterial species, are amongst the most difficult to treat, using standard antimicrobial therapy (Ayele *et al*., 2001; Emery & Whittington, 2004; Hermon-Taylor & El-Zaatari, 2004). The basis for this resistance has been largely attributed to the high lipid content and complexity of the bacterial cell wall (Rastogi *et al*., 2001). Moreover, mycobacteria are capable of surviving within host macrophages, which are largely responsible for eliminating pathogenic microbes (Cocito *et al*., 1994).

Several compounds have been reported for the treatment of paratuberculosis, but the most common in use is either, clofazimine, isoniazid and rifabutin, or ethambutol, followed by a daily dose of isoniazid, for the duration of the animal's life. Many *in vivo* studies have demonstrated an improvement of clinical signs of paratuberculosis in treated animals, but the bacteria could still be detected in the faeces (Stabel, 1998).

Generally, antimicrobial therapy of livestock is impractical for several reasons. Firstly, the drugs are expensive and they need to be administered over extended periods and when the therapy is discontinued, the disease will progress (Stabel, 1998). Secondly, the use of antibiotics does not result in a complete cure, possibly due to the inaccessibility of mycobacteria to the drug *in vivo* (Cocito *et al*., 1994).

1.3.5 Transmission and pathogenesis

As the main lesions of paratuberculosis occur in the lower part of the small intestine and corresponding lymph nodes, the micro-organisms are largely

excreted in the faeces. The primary route of MAP transmission is faecal-oral, via contaminated water, milk, teats, pasture, supplements or hay and soil (Chacon *et al*., 2004; Windsor & Whittington, 2009). Another possible route is through intrauterine infection, as occurs in cows with advanced disease (Windsor & Whittington, 2009). Thompson and others (2007), in their study on intra-uterine transmission of MAP in red deer, also stated that there is a high risk of transmission of MAP organisms to the foetus during pregnancy in sub-clinically affected hinds. Sweeney (1996) reported that semen of an infected bull and embryo transfer may also be a source of infection. However, an experimental study of risk transmission of MAP, by embryo transfer of *in vivo* and *in vitro* fertilised bovine embryos, proved that MAP is unlikely to be transmitted by embryo transfer, when the embryos have been washed as recommended (Bielanski *et al*., 2006).

Young and especially newborn animals are more susceptible, than older animals and they are most at risk of infection (Stabel, 2007). A study carried out by Windsor and Whittington (2009), which reviewed works conducted by eight different groups between the years 1938 and 2006, showed that calves older than six months are less likely to develop infection, than younger calves. Their study also showed that the risk of infection progressing to paratuberculosis is high, when exposure occurs at birth, particularly in a highly contaminated environment or if the dam is infected. A number of suggestions, relating to the difference in susceptibility to MAP infection, have been proposed. Coussens (2001) stated that the immature immune system of young animals may primarily affect the ability to protect against infection. It has also been suggested that, as a new born calf has an ‘open gut’, which allows macromolecules such as colostral immunoglobulin to penetrate to the mucosa, it also allows MAP to penetrate the mucosa barrier (Sweeney, 1996). Genetic resistance to intracellular pathogens such as MAP (Koets *et al*., 2000) and the presence of a functional rumen could reduce susceptibility to paratuberculosis in older animals (Windsor & Whittington, 2009).

When animals are exposed to MAP, the bacteria cross from the lumen of the small intestine into the lymphoid system via M-cells, which are found in the dome epithelium covering Peyer's patches (Momotani *et al*., 1988; Sigurdardottir *et al*., 2001). Although limited studies are available on the role of M-cells, it is believed that M-cells provide an accessible surface for attachment of micro-organisms, since these cells lack brush-border microvilli, digestive enzymes and surface mucus (Momotani *et al*., 1988). Recent studies have shown that targeting and invasion of M-cells, by MAP organisms, is mediated by the formation of a fibronectin bridge, which is formed between the fibronectin-attachment protein of MAP and integrins on M-cells (Secott *et al*., 2004; Schleig *et al*., 2005). Once MAP organisms become localised in the lymphoid tissue of the Payer's patches of the small intestine, infection might be cleared by non-specific innate intracellular killing in some animals (Perez *et al*., 1996; Tooker *et al*., 2002). In the remaining animals, the bacteria that survive innate responses remain viable and multiply within macrophages, resulting in the initiation of specific immune responses.

The pathogenesis of paratuberculosis has been described by a number of researchers (Stabel, 2000; 2007; Koets *et al*., 2002; Koo *et al*., 2004) and they all agree that the classical pattern of the host immune response is strongly biased towards a cell-mediated response, during the early and sub-clinical stages of infection, and it later shifts to a humoral response, during the late clinical stages of the disease. There is no clear explanation as to what makes the disease progress to the clinical and terminal stages. Chacon *et al*. (2004) revealed that nutritional and hormonal factors may influence host susceptibility. In support of Chacon and co-workers (2004), Lugton (2004), in his review, summarised that excess of iron and molybdenum, deficiencies in copper and selenium and possibly other minerals and trace elements, have a role in the disease process. Physical or psychological stresses, such as calving or overcrowding, may also trigger the emergence of clinical paratuberculosis (Koets *et a*l., 2000).

1.3.6 Diagnosis

Diagnosis of paratuberculosis remains difficult (Irenge *et al*., 2009). A definitive diagnosis is often made post-mortem on relevant biological samples (Godfroid *et al*., 2005). Many specific and sensitive diagnostic methods, which allow rapid ante-mortem detection of MAP, have been reported (Collins *et al*., 2005; Salem *et al*., 2005; Tripathi *et al*., 2006; Pribylova *et al*., 2009). These methods can generally be grouped into two types, either detection of MAP (direct methods) or detection of the immune response to MAP infection (indirect methods).

1.3.6.1 Detection of MAP

1.3.6.1.1 Culture

Culture is considered to be the 'gold standard' for detection of MAP (Collins *et al*., 2005). Specimens preferentially tested by culture are faeces, which are easy to collect and can be obtained from live animals (Office International des Epizooties, 2005b). Tissue specimens, such as intestinal mucosa and the associated mesenteric lymph nodes collected at necropsy or biopsies, are cultured in the same manner as faeces.

The advantage of the faecal culture method, over serological methods, is its high specificity, since detection of the pathogen itself is the most definitive method of diagnosis (Wells *et al*., 2006). Sensitivity is reported to be approximately 38% (Whitlock *et al*., 2000). However, this is dependent on the stage of the disease, in the infected animals. Since animals at the sub-clinical stage may shed organisms intermittently in their faeces, the use of faecal culture alone, as a diagnostic method, may result in an underestimation of infection in the herd (Harris & Barletta, 2001; Sohal *et al*., 2007). In addition, culture of faeces is labour intensive (Cocito *et al*., 1994) and requires an incubation time of 12 to 16 weeks in order to produce visible colonies on solid media (Whipple *et al*., 1991). Therefore, culture is considered impractical for routine monitoring in milk, given the current short life of milk products (Tasara & Stephan, 2005). Contamination

of cultures is an added problem, when MAP is being cultured from faecal specimens (Bannantine *et al*., 2004b).

Many methods, for the culture of MAP from clinical samples, have been described but, in general, these methods include a decontamination procedure, incubation in media containing antimicrobial agents to suppress the growth of contaminants, and identification of isolates by phenotypic and genotypic means. Whittington (2009) reported several factors that may improve these culture methods. These include the use of a liquid culture system, which is reported to be more sensitive than solid media especially for culturing the 'Sheep' strain type of MAP, and the inclusion of additional antibiotic, such as ampicillin, in medium to reduce contamination. Other factors are the standardisation of the amount of samples, particularly faeces, which can have an impact on the contamination rate, and minimise the times between sampling and receipt at the laboratory in order to reduce the samples' exposure to uncontrolled environmental conditions. In addition, the application of culturing of MAP in faecal samples pooled from a number of animals, known as pooled faecal culture (PFC), has been shown to be more sensitive and economical, compared to serology, when defining the MAP infection status of whole herds or flocks (Whittington *et al*., 2000; Sergeant *et al*., 2002; Wells *et al*., 2002; Eamens *et al*., 2007). Whittington and colleagues (2000) stated that the diagnostic sensitivity of PFC, in sheep flocks with multibacillary and paucibacillary cases, is approximately between 83%-100% and 27%-73% respectively, when faeces are pooled at a rate of 1 in 50. In their study, the authors also showed that the estimated laboratory cost of PFC is approximately 30% that of serologic examination.

1.3.6.1.2 DNA probes and the Polymerase chain reaction (PCR)

Molecular biological methods, such as the combination of DNA probes and PCR, are promising diagnostic tools for paratuberculosis (Tripathi *et al*., 2006; Pinedo *et al*., 2008; Soumya *et al*., 2009), since they may be more sensitive than culture

methods. Such methods also enable detection of MAP organisms, without having to grow the bacterium.

In most studies, the main DNA target has been the *IS900* (Cousins *et al*., 1999; Stabel, 2000; Ayele *et al*., 2001; Marsh & Whittington, 2001; Ikonomopoulos *et al*., 2004; Vansnick *et al*., 2004; Tasara *et al*., 2005; Mobius *et al*., 2008). Approximately 15 to 20 copies of this sequence are integrated into the genome of MAP (Green *et al*., 1989; Murray & Moriarty, 1989). PCR methods targeting *IS900* have been widely used for detection of the presence of MAP, in faecal samples (Stabel & Bannantine, 2005; Irenge *et al*., 2009), milk samples (Djonne *et al*., 2003; Muehlherr *et al*., 2003; Ayele *et al*., 2005; Hertnek *et al*., 2008), tissue samples (Englund *et al*., 2001; Pislak *et al*., 2003) and buffy coat from the blood of cattle and sheep (Bhide *et al*., 2006).

PCR, which targets MAP-specific sequences, is useful because of its rapid turnaround time and high sensitivity (Brey *et al*., 2006). However, reduced sensitivity of PCR has also been reported and it is thought to be associated with several factors including the presence of PCR inhibitors, such as Ca^{2+} ions in the case of milk, and unsuccessful DNA isolation, due to inefficient extraction methods for mycobacterial DNA (Pislak *et al*., 2003; Herthnek *et al*., 2008). In addition, recent reports have challenged the specificity of *IS900* based assays, due to the discovery of *IS900* like sequences in mycobacteria unrelated to MAP (Englund *et al*., 2002; Motiwala *et al*., 2004; Godfroid *et al*., 2005; Tasara *et al*., 2005).

In recent years, application of real-time (RT), nested and TaqMan PCR methods, along with immunomagnetic bead separation of the micro-organism and dot blot hybridisation of PCR products to assays for the detection of MAP, have improved sensitivity and made it possible to perform semi-quantitative analyses (Fang *et al*., 2002; O'Mahony & Hill, 2002; Khare *et al*., 2004; Taddei *et al*., 2004; Schonenbrucher *et al*., 2008; Alinovi *et al*., 2009). Irenge and co-workers (2009)

carried out a triplex real-time PCR (TRT-PCR), which targeted the multi-copy *IS900*, the single-copy MAP-restricted *F57* and the unique-copy MAP-restricted *ISMAP02* genetic targets, in order to improve PCR identification of MAP. Their study showed that inclusion of the *F57* and/or the *ISMAP02* targets are an important adjunct for rapidly confirming the presence of MAP in faecal samples. Application of RT-PCR also showed a higher sensitivity, compared to culture methods and this offered faster results for better on-farm decision making (Irenge *et al*., 2009; Alinovi *et al*., 2009).

1.3.6.2 Detection of the immune response

1.3.6.2.1 Cell mediated immune response (CMI)

Cellular responses, in the host, can be used to detect the sub-clinical stage of the disease. The two most widely used methods, interferon gamma detection in peripheral blood and delayed type hypersensitivity skin testing, are briefly discussed below.

1.3.6.2.1.1 Interferon gamma (IFN-gamma) detection

IFN-gamma is considered to be one of the essential cytokines that play a number of important roles in achieving protective immunity against mycobacterial infection. The pivotal role of IFN-gamma, in immunity against mycobacteria, is well documented (Agger & Andersen, 2001; Koo *et al*., 2004; Begg & Griffin, 2005; Florido *et al*., 2005; Nagata *et al*., 2005). It has been suggested that, in experimentally infected sheep, there is a positive association between a systemic cell-mediated immune response, measured by the IFN-gamma assay and an animal's ability to control infection (Gwozdz, *et al*., 2000).

Antigen specific stimulation of IFN-gamma production in peripheral blood lymphocytes has been utilised as a diagnostic tool for the detection of both *M. bovis* and MAP infection in cattle (Wood *et al*., 1990; Wood & Rothel, 1994). Generally, the IFN-gamma produced following stimulation with antigen such as Avium purified-protein derivative (PPDA) or Johnin purified-protein derivative

(PPDJ), is captured by a monoclonal antibody on a solid support. The captured cytokine is then reacted with a second antibody conjugate that generates colour development that can be measured spectrophotometrically. The IFN-gamma-enzyme linked immunosorbent assay (ELISA) is considered to be the most suitable test for cellular immune responses, with a reported sensitivity of 50% – 75%, compared to faecal culture, when used as single test in herds with low infection status (Stabel & Whitlock, 2001). However, its specificity is reported to be quite variable (71% – 99%) amongst herds, batches of antigen and the method used to establish a cut off point (Kalis *et al*., 2003).

1.3.6.2.1.2 Delayed type hypersensitivity (DTH)

The DTH test measures a cutaneous T-cell mediated inflammatory response, due to the increase in skin thickness produced by the dermal inoculation of a specific antigen. For paratuberculosis diagnosis, the test is performed by intradermal inoculation of an extract of MAP.

According to Office International des Epizooties (2005b), increases in skin thickness (over 2 mm) should be regarded as indicating the occurrence of DTH. However, a positive reaction may not only take the form of 'a discrete circumscribed swelling' but also 'a diffuse plaque' (Office International des Epizooties, 2005b). This has been reported to occur in deer, thus making interpretation of the test more difficult. Since this test is designed to detect a cell mediated immune response, it is useful for the detection of the early stage of MAP infection. However, the sensitivity of the skin test, in animals infected with paratuberculosis, is only about 54% with a specificity of 79% (Hermel, 1998). Another study, which included 312 heifers from two different herds, also showed that the skin test lacks sensitivity, relative to the detection of MAP in faeces (Antognoli *et al*., 2007).

1.3.6.2.2 Humoral immune response

Humoral immune responses to an infection can be detected by measuring antibody production by the host, with the use of various serological methods. The serological tests most frequently employed are ELISA, agar gel immunodiffusion test (AGID) and complement fixation test (CFT). It is widely believed that serological tests can be very sensitive, when antibody production is at its highest, as in the clinical stage of paratuberculosis. However, because antibody levels are generally low during the early stages of disease, the appearance of clinical signs may be evident, before a positive test result is achieved.

AGID was amongst the first serological tests developed for the diagnosis of paratuberculosis and it was used as a supplementary method to faecal culture (Pavlik *et al*., 2000). It has a high specificity (more than 90%) in cattle with clinical signs, stage III and stage IV, of the disease while the sensitivity is estimated to be 30% in the early stage IV infections (Hermel, 1998). In comparison to ELISA and CFT, AGID is considered less sensitive (Nielsen *et al*., 2001).

CFT detects complement-fixing antibodies to MAP in the blood serum. Although it is more sensitive than AGID, it shows lower specificity, than that of both AGID and ELISA (Ridge *et al*., 1991). This test is not recommended for routine screening, since it shows many false positive and false negative results (Ayele *et al*., 2001).

ELISA is a more sensitive and specific test for serum antibodies, than any other serological tests and it has been widely used for screening herds (Rajukumar *et al*., 2001; Eda *et al*., 2006; Clark *et al*., 2008; Pinedo *et al*., 2008). Detection of infection, by the ELISA technique, appears to be dependant upon the disease stage of the animal tested (Nielsen & Toft, 2008). Dargatz and co-workers (2001), who evaluated the sensitivity of ELISA using a commercial kit, found that the results ranged from 15.4% in cattle classified as light MAP shedders, to 80%

for heavy shedders. Sergeant and others (2003) also reported a variation in the sensitivity of the ELISA in sheep with different histological lesion type, possibly indicating variation with the stage of disease. They found that sensitivity was higher in sheep with multibacillary lesions, than sheep with paucibacillary lesions.

The types of antigens used in various serological tests may also affect their accuracy (Nielsen & Toft, 2008). In conventional ELISAs, the antigens used generally consist of purified protein derivative (PPD), or lipoarabinomannan obtained by extensive physical and chemical disruption of MAP cells. The harsh conditions used for extracting the antigens may partially be responsible for the low level of diagnostic sensitivity of conventional ELISA (Eda *et al*., 2006). Current research has focused on the use of surface or secreted antigens from MAP, in an ELISA platform, for diagnosis of paratuberculosis. Eda and colleagues (2006), in their study comparing nine chemical extracting agents to extract surface antigens from MAP, found that the greatest level of IgG binding, with positive control serum, was observed when ethanol-extracted antigens were used. They also showed that ELISA using ethanol-extracted antigens identified 96.6% of the low-level faecal shedders, whereas the commercial ELISA detected only 13.7% of animals from this group. Shin and co-workers (2008) developed a novel ELISA, termed JTC-ELISA, using antigens secreted by fresh, early to mid-log phase cultures of MAP strain JTC303. Their study involved 415 faecal culture-positive cows, from seven different herds with four shedding levels, designated as slight, light, moderate and heavy. They showed that JTC-ELISA worked effectively on both serum and milk samples and it was more sensitive than commercial ELISAs, to detecting cows that were slight or light faecal shedders of MAP.

1.3.7 Disease control

Paratuberculosis control programmes are time consuming and economically costly (Pavlas, 2005). As a consequence, prevention of a herd or flock from new infection is the best option to be adopted. Prevention can be achieved by several

methods including identifying potential sources of infection, efficient management control and vaccination.

1.3.7.1 Natural reservoir

Effective disease control programmes depend on identifying potential sources of infection and routes of transmission. Cross-contamination of the feed is a major contributor to the spread of paratuberculosis and faeces are the major source of the organism. Use of improperly treated manure solids, to fertilise pastures farms, is another source of infection, since the organism can survive in the soil for up to a year. It is also important to ensure that young animals are fed uncontaminated colostrums and milk. Stabel (1997) stated that distinct watering sites must be available for infected and non-infected animals, since MAP has been found to survive in stagnant water sources for long periods.

1.3.7.2 Management

The most important management practises that are considered to be useful for controlling paratuberculosis within domestic livestock herds include overall cleanliness of the farm, manure handling and the removal of young stock from potential sources of infection, as early as possible (Kennedy *et al*., 2001). Another aspect in management practice is the removal of infected animals (culling) from a herd (Harris & Barletta, 2001), which will reduce the exposure of uninfected animals to MAP.

1.3.7.3 Vaccination

One of the characteristics of an ideal vaccine is to confer a strong and long lasting immunity. Although vaccination has been used since 1926 to control paratuberculosis (Vallee & Rinjard, 1926; cited by Rosseels & Huygen, 2008), its protective efficacy is still less than optimal (Harris & Barletta, 2001). The first-generation of vaccines, which are killed whole-cell-based and live-attenuated whole-cell-based vaccines, currently used against paratuberculosis in sheep, have been demonstrated to provide moderate levels of protection (Begg &

Griffin, 2005; Reddacliff *et al*., 2006). It is reported that these vaccines limit the severity of the disease, rather than preventing or eliminating infection (Park *et al*., 2008; Griffin *et al*., 2009). A study using a live attenuated vaccine (Neoparasec™), in lambs already infected experimentally with MAP, reported reduced numbers of lesions in the gut, compared to unvaccinated controls (Gwozdz *et al*., 2000). Similarly, Sweeney and others (2009) reported that calves vaccinated with a killed-MAP strain 316F vaccine show less extensive intestinal and lymph node tissue colonisation, compared to control calves at nine weeks, following an oral challenge with a heterologous, live field strain of MAP. The authors also stated that the early reduction in tissue colonisation by MAP, evaluated by use of culture with Herrolds egg yolk medium, may eventually result in less severe or delayed onset of clinical disease.

Another complication following vaccination is the high incidence of severe skin injection site reactions, including local oedema and swelling, abscess formation and fistulation (Windsor & Eppleston, 2006; Eppleston & Windsor, 2007). These adverse effects are not only raising animal welfare concerns but they also affect the economic return associated with condemnation of the affected carcases (Griffin *et al*., 2009). Furthermore, it has been reported that MAP vaccination may interfere with DTH skin testing for tuberculosis, due to the high degree of antigenic cross reactivity between antigens in the vaccine strain of MAP and *M. bovis* (Mackintosh *et al*., 2004; Begg & Griffin, 2005). In addition, vaccination may present a health risk to the people administering the vaccine, due to accidental self injection (Ayele *et al*., 2001; de Lisle, 2002). Windsor and others (2005) reported on six cases of accidental self-administration of Gudair™ vaccine, with adverse reactions ranging from lesions on the hand to severe leg injuries, which required extensive surgery. Furthermore the authors stated that accidental self-inoculation, while administering the vaccine in sheep, was estimated to occur once for every 500,000 doses used.

The identification of immunodominant protein antigens inducing strong Th1-type immune responses during the early stage of mycobacterial infection has led to the development of subunit-based vaccines, known as the second generation of vaccines (Sable *et al*., 2007; Rosseels & Huygen, 2008). Subunit vaccines, which include DNA and protein, can induce systemic as well as mucosal immune response (Byrd *et al*., 2005) and have certain advantages over live vaccines, including good safety profiles, the inability to multiply or revert to a virulent phenotype, fewer side effects, less cold-chain dependency and a longer shelf-life (Bramwell *et al*., 2005; Huygen, 2005; Vordermeier *et al*., 2006; Sable *et al*., 2007). The disadvantage of using the subunit vaccines is their relatively low immunogenicity, as compared to live vaccines (Vordermeier *et al*., 2006; Cendron *et al*., 2007), indicating the necessity to administer the vaccines with materials that enhance the immune response. In addition, several booster vaccinations may be required with subunit vaccines, in order to achieve effective protective immunity (Vordermeier *et al*., 2006).

1.4 Protein secretion mechanisms in bacteria

All living organisms, including bacteria, secrete a wide range of proteins with different functions. These proteins can be sorted to at least four different destinations including the cytoplasm, the cytoplasmic membrane, the cell wall and the extracellular medium (Buist *et al*., 2006). Secreted proteins which are present in the outermost lipid bilayer or in the extracellular milieu of bacteria, are the main tools that bacteria use to interact with their environment (Desvaux et al., 2009). Since protein synthesis takes place in the cytoplasm, the secreted proteins, therefore, have to be transported into or across the cytoplasmic membrane in order to be functional. Several studies have shown that the majority of bacterial virulence factors are secreted proteins (Finlay & Falkow, 1997). The importance of these proteins, in mycobacterial diseases, is illustrated by the fact that they are likely preferential targets for the immune system early in infection. Thus, a detailed understanding of the mechanism underlying bacterial protein

secretion could lead to a better understanding of the pathogenicity of micro-organisms and it could provide useful information for vaccine development.

Protein secretion has been most extensively studied in Gram-negative bacteria. However, it is generally believed that Gram-positive and Gram-negative bacteria employ similar protein secretion mechanisms, since the genome sequences show that they share the major components (van Wely *et al*., 2001). To fulfil the task of secretion, there are several pathways which can be divided into two main groups, namely general secretory (Sec) dependent and Sec independent (Kostakioti *et al*., 2005).

The Sec dependent pathway is a multi-stage reaction that can be divided into three distinct stages: protein sorting and targeting, translocation, and release and maturation (Figure 1.2) (Papanikou *et al*., 2007). Translocated proteins are synthesised as precursor (pre-) proteins at the ribosome. The pre-proteins are equipped with a signal peptide that distinguishes proteins destined for membrane translocation from cytosolic proteins and this ensures the proper targeting and translocation across the membrane (van Welly *et al*., 2001; Stephenson, 2005). A signal peptide varies in length (14-30 amino acids) and is characterised by the presence of three distinct domains termed N-, H- and C-domain (Stephenson, 2005). The signal peptide is generally cleaved from the precursor by signal peptidases, during or immediately after translocation (Tuteja, 2005; Papanikou *et al*., 2007). The N-domain is polar and it generally has an overall positive charge with basic residues (lysine or arginine residues), which promote interactions with negatively charged phospholipids and the translocation apparatus. The H-domain is important for targeting and membrane insertion and it forms a membrane-spanning α-helix that facilitates membrane insertion. The C-domain contains the cleavage site that is recognised by the signal peptidases.

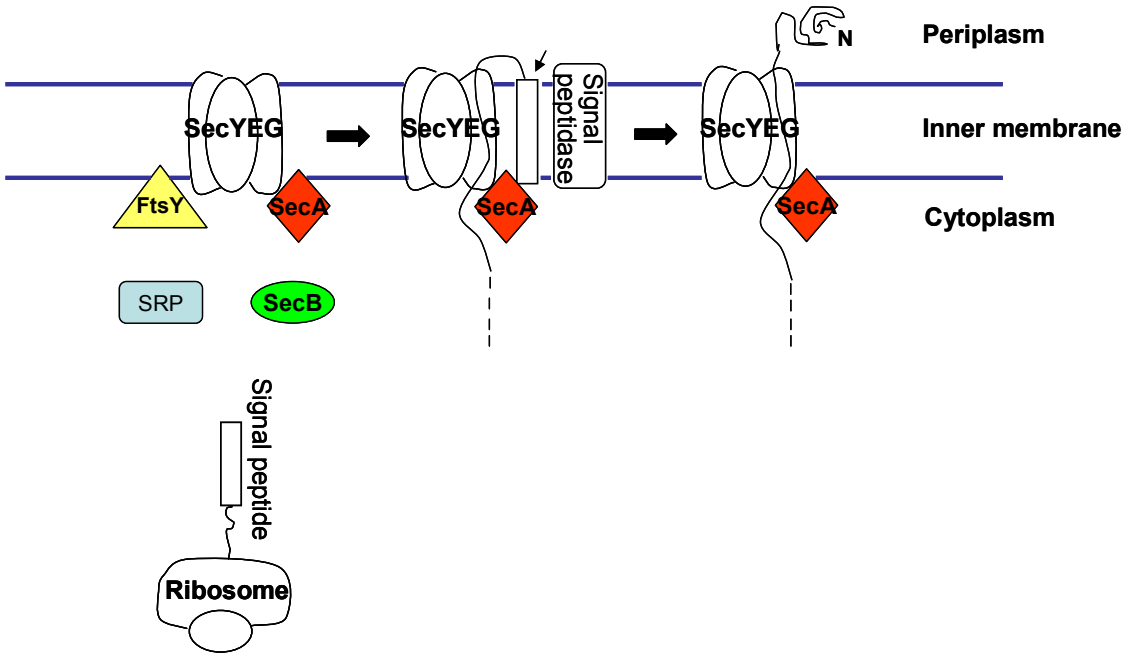


Figure 1.2 Schematic overview of protein transport across the inner membrane of *E. coli*. Adapted from Kim and Kendall, 2000. The nascent polypeptide is recognised by cytoplasmic proteins such as SRP or SecB. Using a high affinity SRP-FtsY or SecB-SecA interaction, the polypeptide is then delivered to a protein translocation channel, SecYEG, in the membrane. Subsequently, the signal peptide is then cleaved by signal peptidase, and translocation through the channel is powered by multiple rounds of SecA ATP hydrolysis.

Translocation occurs through 'a confined aqueous channel composed of a set of integral membrane proteins' called Sec-translocase. Oliver and Beckwith (1982) in their study using *E. coli*, established that translocation of pre-proteins requires at least six genes. These genes, SecA, SecB, SecD, SecE, SecF and SecY encode interacting components of a complex translocation apparatus, which consists of soluble and membrane integrated proteins. SecA performs a role in signal peptide recognition, targeting and ATP hydrolysis, in order to provide energy for polypeptide translocation (Cao & Saier, 2003; Schnell & Hebert, 2003; Hand *et al*., 2006). SecA is also reported to act as an export-specific chaperone

in *B. substilis* to help prevent folding of proteins, prior to its translocation (Scott & Barnett, 2006). Mycobacteria and several other Gram-positive bacteria, including Staphylococcus, Streptococcus and Listeria species, posses two SecA genes, namely SecA1 and SecA2 (Cole *et al*., 2001; Limia *et al*., 2001; Houben *et al*., 2006). It is not clear if both genes are essential but Braunstein and co-workers (2001) stated that SecA1 is the essential equivalent of the *E. coli* while SecA2 is not essential. In a different study, Braunstein and colleagues (2003) reported that SecA2 might contribute to a specialised secretion system involved in dealing with oxidative attack by the host.

SecB is a soluble homotetrameric protein, which functions as an export-specific chaperone and is involved in the targeting of precursor proteins to the membrane bound translocase complex (Freudl, 1992). SecD, SecF, SecE and SecY are all integral membrane proteins. While SecE and SecY are essential for the translocation of precursor proteins, the role of SecD and SecF is largely unknown, but it is speculated that they may function at a late stage of the translocation pathway (Brundage *et al*., 1990; Gardel *et al*., 1990). Nouwen and others (2005) stated that SecD and SecF form a complex with YajC and this complex could create a protected surrounding in the periplasm, so that the proteins can fold efficiently upon completion of translocation.

When pre-proteins have been synthesised at the ribosome, they are then targeted to the translocon channel in the cytoplasmic membrane. It is reported that the targeting of pre-proteins, to the translocon channel, may occur by one of two processes (van Wely *et al*., 2001; Scott & Barnett, 2006). One possibility is to bring the site of synthesis of the pre-protein in contact with the translocation channel and couple translocation to the synthesis of the secretory protein, at the ribosome. This mechanism is called co-translational translocation. The other possibility, termed post-translational translocation, is to complete the synthesis of the pre-protein, prior to its translocation. The post-translational mechanism requires the tight folding of the pre-protein in the cytosol to be prevented and

therefore this involves molecular chaperones such as SecB. As a SecB homolog is absent in Gram-positive bacteria, co-translational translocation is the only mechanism known for targeting proteins to the cytoplasmic membrane and to the translocon channel (Scott & Barnett, 2006).

Once a protein has passed the inner membrane into the periplasm, proteins are then exported into or across the outer membrane, if required. The process of exporting proteins is often achieved by the main terminal branch of the Sec pathway, which is used for the export of a wide variety of extracellular proteins, and requires at least 14 accessory proteins in order to be functional (Pugsley *et al.,* 1997).

In addition to the Sec-dependant pathways, various other secretion pathways have been discovered, which work in a Sec-independent pathway. This pathway tends to allow direct export from the cytoplasm to the extracellular-millieu in one step and it does not involve periplasmic intermediates (Kostakioti *et al*., 2005). There are three Sec-independent pathways known to exist in bacteria but the most predominant is the twin-arginine translocation (Tat) pathway (Meima & van Dijl., 2003; Bendtsen *et al*., 2005). The Tat system was first discovered in the early 1990s (Lee *et al*., 2006) and found in the cytoplasmic membranes of many bacteria, and also in the energy-transducing membranes of plant organelles (Berks *et al*., 2000; Palmer & Berks, 2003). Proteins exported by the Tat pathway are synthesised with N-terminal signal peptides, which harbour a distinctive 'twin-arginine' motif.

In *E. coli*, the minimal set of components required for Tat translocation consists of three integral membrane proteins, such as TatA, TatB and TatC (Figure 1.3) (Lee *et al*., 2006). The TatA protein is the most abundant of the Tat components consisting of 82 amino acids and is predicted to have a structure similar to TatB. The TatA may function at a late stage in translocation and likely forms the major constituent of the Tat pore itself (Gohlke *et al*., 2005). The TatB protein is longer

than TatA, by 89 amino acids. It is considered as being a mediator between TatC and TatA, since it will make contact with the substrate, after initial recognition by TatC and then it is potentially involved in a transfer to a complex consisting mainly of the TatA protein, prior to translocation (Alami *et al*., 2003). The TatC protein is the largest consisting of 258 amino acids and it is the most highly conserved Tat components. *In vitro* biochemical studies have shown that TatC serves as the initial docking site for Tat signal peptides, thus suggesting that it is strictly required for protein export by the Tat pathway (de Leeuw *et al*., 2002; Alami *et al*., 2003).

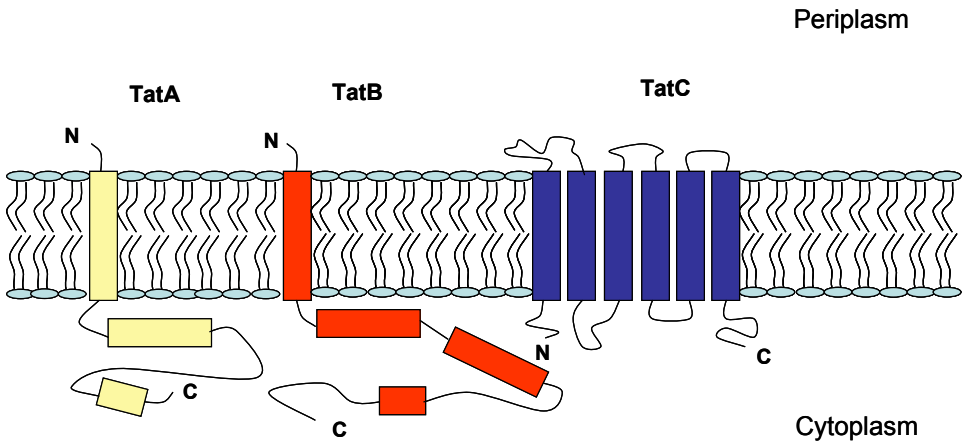


Figure 1.3 Structure and topology of the *E. coli* Tat components. Adapted from Lee *et al*., 2006. Predicted transmembrane helical regions are shown in boxes. The TatA transmembrane helices, with their N- and C-termini, are shown in yellow, the TatB in red and the TatC in blue.

Proteins secreted by bacteria must pass through a number of compartments, before they are expelled into the extracellular milieu. The cell envelope of Gram-negative bacteria consists of two membranes: an inner membrane (IM) and an outer membrane (OM), which are separated by the periplasmic space. The

passage of molecules, from the inside to the outside of the cell, can be achieved in a number of ways and to date, six separate secretion system (denominated Type I – VI) have been described (Abdallah *et al*., 2007). These types of export pathways are encoded on so-called pathogenicity islands, which are the regions of the genome that are associated with virulence. Recently, ESX sytems that exist in mycobacteria have been suggested to be named a Type-VII secretion system (Ize & Palmer, 2006; Abdallah *et al*., 2007; Simeone *et al*., 2009). These secretion systems are briefly described below.

Type I system is Sec-independent but dependent upon an ATPase-binding cassette (ABC) transporter, which is found in eukaryotes and prokaryotes and constitutes a large superfamily of multi-subunit permeases. The type I pathway is a one step process that involves protein secretion through a translocon, which spans the entire cell envelope (Holland *et al*., 2005). The translocon consists of an IM ABC transporter, with ATPase activity, a membrane-fusion protein and an OM pore.

Transport through the Type II system is a two step process (Filloux, 2004; Abdallah *et al*., 2007). The first step involves translocation across the IM, by the general Sec- or Tat-translocons (Johnson *et al*., 2006). This transport is mediated by substrates that contain an amino terminal signal sequence. Following transport into the periplasm, the signal sequence of the protein is cleaved off and folding takes place. Some proteins may undergo further modification in the periplasm, for example, disulphide bond formation, before being translocated across the OM, through a complex structure known as the secreton.

The Type III system is mediated via a structure known as injectisome, which is a needle like structure that forms a channel between the IM and the OM and extends to contact host cells (Journet *et al*., 2005; Cornelis, 2006). Virulence factors can be injected through this system into host cells.

Similarly, the Type IV process allows transport of substrate directly from the cytoplasm of the organism into surrounding host cells. This is achieved through a complex structure that spans both the inner and outer membrane and culminates in a pilus structure at the bacterial cell surface (Christie & Cascales, 2005; Christie *et al*., 2005). The process can transport both DNA and protein and can either be a one step or two step process.

Type V system involves a two-step mechanism (Henderson *et al*., 2004). The substrate protein is moved across the IM, via the Sec-translocon. Following this, a β-barrel structure, which is either part of the secreted protein or separate from it, is required for the subsequent movement across the OM. The precise nature of the OM translocator and the mechanism of its action have not been fully established.

The Type VI system is also poorly understood (Abdallah *et al*., 2007). The substrate proteins for this system do not have an amino terminal Sec-type signal sequence so the translocator system is independent of the Sec- or Tat- pathway. In addition, it has been suggested that the Type VI system might also describe substrate transport via vesicle budding of material from the OM (Abdallah *et al*., 2007). However, details of this process have yet to be established.

The type VII system (specialised secretion systems) is used by the mycobacteria for the transport of extracellular proteins across their hydrophobic and highly impermeable cell wall. There are five different specialised secretion systems (ESX-systems; named ESX1-ESX5), which appear to function independently (Abdallah *et al*., 2007). Amongst the ESX systems, ESX-1 has been the focus of research, primarily in *M. tuberculosis*, since it is involved in virulence and it is proposed to be an example of type VII secretion (Abdallah *et al*., 2007; Simeone *et al*., 2009). The ESX-1 system is encoded by the region of difference 1 (RD1), which is essential for the virulence of *M. tuberculosis* (Lewis *et al*. 2003). The RD1 comprises nine genes including the genes that encode the early secreted

antigenic target of 6 kDa (ESAT-6) and culture filtrate protein of 10 kDa (CFP-10). On the basis of protein-protein interaction studies, Abdallah and co-workers (2007) generated a working model for type VII secretion. Firstly, the ESAT-6 and CFP-10 form a tight dimer and it is targeted for secretion through the recognition of the carboxy terminal signal sequence by the cytoplasmic protein Rv3871. At the cell membrane, Rv3871 interacts with Rv3870 to form an active ATPase. The Rv3871-3870 complex forms a hexameric ring structure, with a central cavity, that propels ESX-1 substrate through the secretion channel.

1.5 Potential significance of mycobacterial secreted proteins

Secreted or surface-exposed bacterial proteins have long been known to play central roles in bacterial-host interactions. Secreted protein antigens, present in the culture filtrate of *M. tuberculosis*, have been the focus of studies, because these antigens are considered immunodominant and they are involved in inducing protective immunity (Andersen, 1997).

A number of *M. tuberculosis* complex mixture of secreted proteins, found in the culture filtrate or individual components, have been shown to impart protection against mycobacterial challenge (Sable *et al*., 2007). Amongst them are ESAT-6 and CFP-10. ESAT-6 is a small secreted protein of unknown function. It is reported that ESAT-6, delivered as a fusion protein, is able to initiate a Th1-type response in a mouse model (Menon *et al*., 2002). Moreover, vaccination of rodents and cattle, using ESAT-6 DNA vaccine, induces protection against challenge with tubercle bacilli (Kamath *et al*., 1999: Maue *et al*., 2004). Additional studies on the possible roles of ESAT-6 and CFP-10 antigens, in immunity against tuberculosis, have indicated their therapeutic and diagnostic potential (Trajkovic *et al*., 2004; Maue *et al*., 2005; Palmer & Waters, 2006).

Several antigenic secreted proteins have been isolated from MAP organisms (Table 1.1), but only a few of these antigens have been well characterised and evaluated. Further research will no doubt identify more MAP secreted proteins

and these will be a potential resource for developing new diagnostic reagents and vaccines with therapeutic and/or prophylactic potential.

Table 1.1 Secreted antigenic components isolated from MAP.

Antigen	Comment	Reference (s)
14 kDa (MPP14)	Secreted. Induced innate interferon gamma production in young cattle. A promising candidate for inclusion in subunit vaccine preparations.	Olsen and Storset, 2001; Olsen *et al*., 2005.
16.7 kDa (CFP20)	Hypothetical thiol peroxidase gene of MAP. Cloned and expressed in *E. coli*. Considered as a potential Th1-inducing antigen, when tested in mice.	Mullerad *et al*., 2003
19 kDa lipoprotein (MAP0261c)	Conserved in mycobacteria. Secreted. Cloned, expressed and purified as a fusion protein with the maltose-binding protein in *E. coli*. Stimulates a humoral immune response, as shown in Western blot analysis, but weak interferon gamma production in infected cattle.	Huntley *et al*., 2005.
22 kDa lipoprotein (P22)	Belongs to LppX/LprAFG family of mycobacterial lipoproteins. Cloned and expressed in *M. smegmatis* and *E. coli*. The recombinant protein elicited interferon gamma secretion in blood of sheep vaccinated with Neoparasec™. Strongly recognised by MAP-infected cattle sera in Western blot analysis.	Dupont *et al*., 2005; Ridgen *et al*., 2006.
35 kDa (P35)	Surface exported protein that plays a role in invasion of epithelial cells. Well conserved amongst the two mycobacterial species, *M. leprae* and *M. avium*. Cloned and expressed in *E. coli*. Shows ability to elicit CMI responses using a mouse model. Recognised by infected cattle sera in Western blot analysis.	Banasure *et al*., 2001; Bannantine *et al*., 2003; Basagoudanavar *et al*., 2004; 2006.

Table 1.1 Continued ……

30- to 32 kDa mycolyl transferase family (Ag85 complex)	Isolated from culture filtrate. Cloned and expressed in *E. coli*. Shown to be immunodominant T-cell antigens that are recognised early in experimental and natural infection of cattle.	Rosseels *et al*., 2006; Kathaperumal *et al*., 2008;
Ferric reductase (17 kDa)	Isolated from culture filtrate. Surface-associated as well as extracellular. Enzymatic activity confirmed but its immunologic activity needs to be investigated.	Homuth *et al*., 1998.
SodC	Putative exported. Similar to SodC *M. tuberculosis* (76% identity). Cloned and expressed in *E. coli*. Enzymatic activity confirmed.	Dupont and Murray, 2001.
MAP1643 MAP3840	Predicted to be surface exposed, as determined by Western blot. Cloned and expressed in *E. coli*.	Bannantine *et al*., 2007.
MAP1087	Putative surface antigen. Cloned and expressed in *E. coli*. Strongly detected by sera (immunoblot analysis) from naturally infected cattle, in the subclinical phase of infection.	Bannantine *et al*., 2008a.
MAP0586c MAP1693c MAP2677c MAP3199 MAP4308c	Isolated from culture filtrate. Cloned and expressed in *E. coli*. MAP1693c, MAP4308c and MAP2677c are antigenic. Combination of these three antigens showed sensitivity of 94.74% and specificity of 97.92%, in an ELISA assay. Vaccination with DNA encoding MAP0586c induced strong Th1-type cytokine production and protected mice against experimental challenge with MAP.	Leroy *et al*., 2007; Roupie *et al*., 2008
MAP2609 (9 kDa) MAP2942c (15 kDa) MAP0210c (34 kDa)	Secreted. Displays strong homology to TB8.4, MPT53 and Erp of *M. tuberculosis*. Cloned and expressed in *E. coli*. Recognised by antibodies from infected cattle, both in the subclinical and clinical phase of infection.	Willemsen *et al*., 2006

Table 1.1 Continued …….

Low molecular weight proteins ranging from 20 – 50 kDa.	Found in culture filtrate. Reacts with bovine sera from animals known to be infected with MAP. DNA sequence not reported.	Pradenas *et al*., 2009
ModD Ag85C PepA MAP1693c MAP2168c	Identified in the culture filtrate of MAP by immunoblot and mass spectrometry. Cloned and expressed in *E. coli*. All five proteins were confirmed to be immunogenic by ELISA using rabbit anti-MAP culture filtrate. A higher mean ELISA A_{450} values to ModD and Ag85c was found in infected cattle, than in the control. Immunoblot analysis, using four bovine sera, showed that all proteins reacted at least with one of the selected four sera.	Cho *et al*., 2007
MAP3420c MAP1506	Member of the PPE family. Both were detected on the surface of viable MAP cells by specific polyclonal antibodies, by immunofluorescent staining. MAP3420c was cloned and expressed in *E. coli*. Strong reaction in all positive cattle sera against MAP3420c was found in Western blot. Similar result also shown in ELISA.	Newton *et al*., 2009

1.6 Studies to identify bacterial secreted proteins

The standard approach for identification of secreted proteins, in cultivable bacteria, is to analyse cell-free culture filtrates containing secreted proteins. This can be achieved by the use of electrophoretic or chromatographic separation, followed by immunological screening of the isolated components. This approach has been used to successfully identify approximately 200 secreted proteins in *M. tuberculosis*. For MAP, this approach has led to the identification and isolation of several immunologically important antigens, such as members of the 30 to 32 kDa mycolyl transferase family (Rosseels *et al*., 2006; Kathaperumal *et al*., 2008), low molecular weight proteins ranging from 20 – 50 kDa (Pradenas *et al*.,

2009) and five proteins including ModD, Ag85C, PepA, MAP1693c and MAP2168c (Cho *et al*., 2007). These methods generally target those proteins secreted to the external environment but they cannot identify proteins that are predominantly surface exposed.

Comparative genomics has been another encompassing approach used to study secreted proteins. This approach involves comparison of gene homologues in bacterial genomes known to be exported and bioinformatics analyses to predict properties of the proteins. In a study by Banasure and colleagues (2001), a 35 kDa protein from MAP was identified and characterised based on the DNA sequence information for a homologous protein of *M. leprae*. Other proteins of MAP, 16.7 kDa (Mullerad *et al*., 2003) and 19 kDa (Huntley *et al*., 2005), were also identified by DNA sequence similarities to 16.7 and 19 kDa antigens of *M. tuberculosis*.

Exported proteins can also be identified by using molecular approaches, which involve the fusion of export-competent peptides to reporter proteins such as β-lactamase and alkaline phosphatase (PhoA). These systems rely on the concomitant export and functional expression of the fused reported protein for identification of export. PhoA fusions employ recombinant *E. coli* PhoA as a marker for export. PhoA is an enzyme that normally resides in the periplasm and is synthesised as a monomer with a signal peptidase at its amino terminus. The activity of PhoA generally requires it to be exported across the plasma membrane, where it can form intramolecular disulfide bonds (Derman & Beckwith, 1995) and undergo subsequent dimerisation to form the active enzyme (Kim & Wyckoff, 1989). Due to this property of PhoA, it can be considered as a sensor for protein export signals. Manoil and others (1990), in their review, reported that two types of PhoA fusion systems have been developed. Firstly, there are plasmid vectors containing restriction sites located in the DNA of the *phoA* gene corresponding to the early part of the mature protein. With the appropriate restriction fragments, the amino termini of other proteins can be

attached to PhoA. The second type is the use of Tn*phoA*, a transposon derivative of Tn5 containing a *phoA* gene lacking its promoter, the translation initiation site, and the DNA corresponding to the signal sequence. The Tn*phoA* system has been used to directly select for transposon inserts in genes coding for secreted proteins.

PhoA fusion systems have been used for the identification of exported proteins in mycobacteria. Lim and colleagues (1995) screened *M. tuberculosis* DNA-*phoA* fusion libraries, by using a PhoA reporter shuttle plasmid pJEM11, which replicates in *E. coli* and *M. smegmatis*. They found 12 different inserts allowing *phoA* expression and they concluded that the approach was highly accurate in identifying exported proteins. Carroll and others (2000) employed a similar approach to study *M. avium* subspecies *avium* secreted proteins. Fifteen *M. avium* subspecies *avium* exported proteins were identified and found to be highly homologous to genes from *M. tuberculosis* and *M. leprae*. Furthermore, they suggested that *phoA* fusion technology is applicable to the study of atypical slow growing bacteria. In MAP cattle strain, this approach has led to the identification of several exported proteins such as SodC (Dupont & Murray, 2001) and P22 (Dupont *et al.,* 2005).

1.7 Objectives

Neoparasec™, an oil adjuvanted, freeze-dried live attenuated 316F strain of MAP, was used until 2002 in New Zealand and in France for the vaccination of ruminants primarily cattle, sheep and goats (Molina *et al*., 1996; Kohler *et al*., 2001; Begg *et al*., 2005). A protective efficacy study, carried out by Begg and Griffin (2005), indicated that there was significant protection against MAP pathology, seen in sheep, when the animals were vaccinated with Neoparasec™ vaccine at 2 weeks of age. Results in their study showed that only four out of 29 vaccinated animals, which had been challenged orally with virulent MAP developed a severe disease, while no pathology, based on gross lesions and histopathological examination were found in any of the vaccinated animals.

Moreover, the Neoparasec™ vaccinated animals had a consistently stronger response, measured by lymphocyte transformation assay, at challenge as a result of vaccination. However, to date there has been no studies on secreted proteins of the vaccine strain of MAP 316F. Therefore, this study sought to identify novel secreted proteins of MAP strain 316F, with a view to identifying those that have immunologic activity. The main objectives of this study were to:

i. Identify novel secreted protein genes in the MAP vaccine strain 316F by cloning DNA fragments into the alkaline phosphatase shuttle plasmid vector, pJEM11.
ii. Transform the heterologous strain *M. smegmatis* with the pJEM11 constructs and identify PhoA positive clones.
iii. Sequence DNA inserts and conduct database searches of identified genes and obtain full gene sequences.
iv. Express selected MAP genes in *E. coli*, and purify the recombinant protein.
v. Evaluate and compare the immunogenic properties of selected proteins, using blood from Neoparasec™ vaccinated sheep, naturally infected sheep and deer, and uninfected control animals.

CHAPTER 2

General materials and methods

2.1 Bacterial strains and plasmids

2.1.1 Bacterial strains

The bacterial strains used in this study are detailed in Tables 2.1 and 2.2.

Table 2.1 Commercial bacterial strains used in this study.

Strain	Genotype	Source/Reference
E. coli DH10B	F$^-$*mcrA Δ(mrr-hsd*RMS-*mcr*BC*) f*80 *ølacZ*ΔM15 *ΔlacX*74 *deoR rec*A1 *end*A1 *ara*D139 *Δ(ara,leu)*7697 *gal*U *gal*K *λ*$^-$ *rps*L *nup*G	Invitrogen, Life Technologies Inc., USA. Sambrook *et al*. (1989).
E. coli Top10	F$^-$*mcrA Δ(mrr-hsd*RMS-*mcr*BC*) Φ80laccZ*ΔM15 *ΔlacX*74 *rec*A1 *ara*D139 *Δ(ara-leu)*7697 *galU gal*K *rps*L *(Str^R) end*A1 *nup*G	Invitrogen, Life Technologies Inc., USA.
E. coli BL21-Codon Plus (DE3) -RP	F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte [*argU proL* Cam^r]	Stratagene, USA.

Table 2.2 Other bacterial strains used in this study.

Strain	Phenotype	Source
MAP strain 316F	Attenuated vaccine strain used in Neoparasec (Merial, France).	Merial, France.
M. smegmatis mc^2155	Electroporation-competent strain of *M. smegmatis.*	Snapper *et al*. (1990). ATTC 607

2.1.2 Plasmids

The plasmids used in this study and their relevant characteristics are shown in Table 2.3. The mycobacterial shuttle vector, pJEM11, was kindly provided by Professor Brigitte Gicquel, Unité dé Génétique Mycobactérienne, Pasteur Institute, Paris, France.

Table 2.3 Plasmids used in this study.

Plasmid	Description	Source/Reference
pCR®-Blunt II-TOPO	The vector contains the *ccd*B gene fused to the C-terminus of the LacZα fragment.	Invitrogen, Life Technologies Inc., USA.
pJEM11	*E. coli*/mycobacterial shuttle vector containing a truncated *phoA* gene, kan^r.	Lim *et al*. (1995).
pET26b	Expression vector system	Novagen, WI, USA.
pTOPO-2213c	TOPO vector harbouring MAP2213c	This study
pTOPO-3894c	TOPO vector harbouring MAP3894c	This study
pTOPO-3273c	TOPO vector harbouring MAP3273c	This study
pET26b-2213c	pET26b harbouring MAP2213c	This study
pET26b-3894c	pET26b harbouring MAP3894c	This study
pET26b-3273c	pET26b harbouring MAP3273c	This study

2.2 Growth and storage of bacterial cultures

2.2.1 Media

All media were prepared using water which had been purified by the MilliQ Reagent Water System (Millipore). The media were then sterilised by autoclaving at 121^{o}C and 15 psi for 20 min. Media prepared for bacterial culture, during this study, are listed in Table 2.4.

Table 2.4 Media.

Medium	Recipe	Reference/supplier
Luria-Bertani broth (LB)	1% (w/v) tryptone (Difco Laboratories Inc., Detroit, MI, USA) , 0.5% (w/v) yeast extract (Difco Laboratories Inc., Detroit, MI, USA), 1% (w/v) NaCl; adjust pH to 7.0 with NaOH	Sambrook *et al*., 1989.
Luria-Bertani agar (LBA)	1% (w/v) tryptone (Difco Laboratories Inc., Detroit, MI, USA), 0.5% (w/v) yeast extract (Difco Laboratories Inc., Detroit, MI, USA), 1% (w/v) NaCl; adjust pH to 7.0 with NaOH. Then add 1.5% (w/v) agar (Difco Laboratories Inc., Detroit, MI, USA).	Sambrook *et al*., 1989.
Middlebrook 7H9 broth (7H9-B)	0.47% (w/v) Middlebrook 7H9 media, 0.05% (v/v) Tween 80, 0.2% (v/v) glycerol, and 1mg/L Mycobactin J (Allied Monitor, USA).	Difco Laboratories Inc., Detroit, MI, USA.
Middlebrook 7H10 agar (7H10-A)	0.47% (w/v) Middlebrook 7H10 media, 0.05% (v/v) Tween 80, 0.2% (v/v) glycerol, and 1mg/L Mycobactin J (Allied Monitor, USA).	Difco Laboratories Inc., Detroit, MI, USA.

Sterilised media were cooled to 50^{o}C before supplementing with appropriate antibiotics/supplements. All antibiotics and supplements are listed in Table 2.5. Stock solutions of antibiotics and supplements were aseptically aliquoted into microcentrifuge tubes and kept at -20^{o}C in sterile MilliQ-treated water, unless otherwise stated. The supplemented agar media were poured immediately after mixing.

Table 2.5 Antibiotics and supplements.

Antibiotic/Supplement	Stock concentration (mg/ml)	Final concentration (µg/ml)	Supplier
Kanamycin	100	30	Sigma (Cat No. K4000)
Chloramphenicol	34*	50	Sigma (Cat No. C6455)
BCIP	40	25	Sigma (Cat No. B6149)

* dissolved in methanol

2.2.2 Growth of bacterial cultures

2.2.2.1 *E. coli* and *M. smegmatis*

E. coli isolates and *M. smegmatis* containing pJEM11 constructs were routinely grown in LB or LBA plates containing appropriate antibiotics and/or supplements. *E. coli* cultures were grown at 37^{o}C for 18-24 hours under aerobic conditions and *M. smegmatis* containing pJEM11 constructs were grown for three to four days. Liquid cultures were grown in tubes or flasks with a volume at least four times that of the culture volume, and were shaken at approximately 220 rpm on an Innova 4000 incubator shaker (New Brunswick Scientific, Edison, NJ, USA) to ensure sufficient aeration.

2.2.2.2 MAP strain 316F

The MAP microorganism was propagated in 20 ml of 7H9-B supplemented with 10% (v/v) sterile Albumin-D-glucose/dextrose-Catalase (ADC) enrichment broth (5% [w/v] bovine serum albumin {Gibco, Laboratories}, 2% [w/v] D-glucose/ dextrose and 0.003% [w/v] catalase) under standard aerobic conditions. The growth was monitored until it reached mid-log phase ($OD_{600\ nm}$ = 1.0) and then 10 ml of freshly grown mid-log phase cultures were transferred into 200 ml of fresh media. Cultures grown in this manner took three to four weeks to reach mid-log phase, at which time they were harvested.

2.2.3 Measurement of cell density of cultures

Cell density of cultures was measured using a double beam spectrophotometer (Helios α, Unicam, UK). Routinely, 1 ml of culture (or 1 in 10 dilution) was transferred to a polystyrene cuvette. The spectrophotometer was calibrated to zero at 600 nm using two blank cuvettes each containing 1 ml of appropriate media. One of the blank cuvettes was replaced with a cuvette containing culture, and the $OD_{600\ nm}$ reading was recorded. All samples were analysed in duplicate.

2.2.4 Storage of cultures

Bacterial cultures were stored at 4^{o}C for short periods (up to one week) or at -70^{o}C for longer-term storage. For longer storage, cultures were stored as glycerol stocks prepared as follows. A single colony was picked from the agar plate and inoculated into 5 ml of LB containing appropriate antibiotics. Five hundred microlitres of freshly grown culture was transferred to a 1.7 ml cryovial containing 500 µl of 50% (v/v) sterile glycerol. The mixtures were briefly vortexed and then stored.

2.3 DNA extractions and isolations

2.3.1 Isolation of plasmid DNA from *E. coli*

Plasmids were isolated from *E. coli* cultures using the QIAprep-spin plasmid Miniprep Kit (Cat. No. 27104 QIAGEN, Hildon, Germany). Briefly, a single colony was picked and grown in 5 ml LB overnight at 37^{o}C with vigorous shaking. Bacterial cells were then harvested by centrifugation and treated according to the manufacturer's instructions. Plasmid DNA was routinely eluted in 50 µl of elution buffer (10 mM Tris-Cl pH 8.0).

2.3.2 Isolation of genomic DNA from MAP strain 316F

For preparation of the MAP genomic clones, genomic DNA was extracted by chemical lysis followed by phenol:chloroform purification. The microorganisms were grown under standard conditions, as described in Section 2.2.2.2. Cells

were harvested and pelleted by centrifugation at 11,000 x *g* for 30 min, then washed twice with Glucose-Tris-EDTA (GTE) buffer pH 8.0 (0.3% [w/v] Tris, 0.4% [w/v] EDTA, 0.9% [w/v] Glucose). The pellets were frozen at -70^{o}C for a minimum of one hour and subsequently thawed at room temperature for 15 min, in order to facilitate rupture of the cells. Thawed cells were resuspended in a digestion buffer pH 8.0 (0.58% [w/v] NaCl, 0.93% [w/v] EDTA, 0.12% [w/v] Tris, 0.5% [w/v] SDS). To this suspension, 200 µg of Proteinase K (Roche Molecular Biochemicals, Germany) was added and the tube incubated at 60^{o}C for 18 hours. Genomic DNA was isolated following extraction with phenol:chloroform:isoamyl alcohol (25:24:1 [v/v], Cat No. 15593 031, Life Technologies) as follows. Six hundred microlitres of phenol:chloroform:isoamyl alcohol was added to the mixture, mixed, and centrifuged at 13,500 x *g* for 5 min. The upper aqueous phase was removed to a clean microcentrifuge tube, and phenolic extractions were repeated one more time. After the last phenol extraction, an equal volume of chloroform-isoamyl alcohol (24:1 [v/v]) was added to the samples before mixing and centrifugation. The aqueous phase was transferred to another clean tube, and an equal volume of 100% isopropanol was added to each sample. The tubes were inverted and the DNA was allowed to precipitate at -20^{o}C for 18 hours. The DNA was pelleted by centrifugation at 13,500 x *g* for 30 min at 4^{o}C. The resultant DNA pellet was washed with 1ml of 70% ice-cold ethanol and centrifuged at 13,500 x *g* for 5 min at 4^{o}C. The pellet was then air-dried at room temperature, resuspended in 20-50 ml of sterile water and stored at -20^{o}C.

2.4 DNA techniques

2.4.1 Agarose gel electrophoresis

Agarose gels of 0.8 to 2% (w/v) were prepared in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA pH 8.0). Agarose (Cat. No. 15510-027, Life Technologies) in TAE buffer was heated to boiling point for total solubilisation and cooled to 50^{o}C before pouring gels. DNA samples were mixed with 0.2 volumes of 6x DNA loading buffer (0.25% [w/v]) bromophenol blue, 30% [v/v])

glycerol, 0.25% [w/v] xylene cyanol). Samples were loaded onto solidified gels along with the appropriate ready load DNA ladder marker (100 bp Cat. No.10380-012; 1 kb plus Cat. No.12308-011, Invitrogen, Life Technologies Inc., USA). Electrophoresis was performed using Bio-Rad horizontal electrophoresis unit (Bio-Rad, Hercules, CA, USA) containing 1x TAE buffer at 90-100 Volts (Bio-Rad Power Pac). Gels were transferred to a 0.5 µg/ml solution of ethidium bromide in sterile water for 15-25 min, then destained in water for at least 2 min. DNA was visualised and photographed under UV light using the Quantity One® software (Bio-Rad) from the Bio-Rad Gel Doc 2000 imaging system. The molecular size of the DNA fragments was estimated by comparison with the DNA ladder marker.

2.4.2 Extraction of DNA from agarose gels

Restriction endonuclease-digested DNA or PCR products were loaded onto an agarose gel. After electrophoresis, gels were stained with ethidium bromide and the desired bands were excised under minimal UV light exposure using a clean and sterile scalpel blade. Purification of the excised bands was carried out using the QIAquick Gel Extraction Kit (Cat. No. 28704, Qiagen, Hildon, Germany) according to the manufacturer's instructions. The purified products were then resuspended in 10 µl of elution buffer (10 mM Tris-Cl pH 8.0) and stored at -20^{o}C.

2.4.3 DNA quantification

DNA concentrations in solution were analysed spectrophotometrically using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA). A 'blanking cycle' was first performed by loading 2 µl of appropriate buffer or solvent used with the samples onto the pedestal surface. After making an initial blank measurement, 1µl of DNA sample were loaded and measured at 260 nm. DNA concentrations were calculated automatically by multiplying the diluant factor, a constant factor 50 (double stranded DNA), and the absorbance reading at 260 nm. The nucleic acid concentration was shown as

in ng/µl. In addition, readings were also taken at 280 nm to check the purity of samples by calculating the ratio of $OD_{260/280.}$ Values ≥ 1.8 were generated by pure samples of low protein contamination.

2.4.4 Polymerase chain reaction (PCR)

2.4.4.1 Primer design

Primer length was generally between 20-30 bp. Approximate melting temperature (T_m) was calculated using the formula Tm = 4(G + C) + 2(A + T)^oC. For cloning purposes, restriction enzyme sites with a few extra nucleotides were added onto the 5' end. Custom primers were synthesised in desalted form by Invitrogen Life Technologies and were resuspended in distilled water to a final concentration of 100 µM, and stored at -20^oC. The volume of water required to resuspend the primers was calculated according to the following formula:

$$\text{Volume of water (ml)} = \frac{\text{total OD (absorbance at 260 nm)}}{\text{[(EM) x Molar concentration required]}}$$

where EM is the molar extinction coefficient

2.4.4.2 PCR conditions

PCR were carried out using Platinum® *Pfx* DNA polymerase (Cat No. 11708-013, Invitrogen, Life Technologies Inc., USA) or PrimeStar® HS DNA polymerase (Cat No. RO44A, Takara Bio Inc) in the presence of 1.5 mM Magnesium. Primers were added to a final concentration of 0.4 µM. Deoxynucleotide triphosphate (dNTP) mix was used at a final concentration of 100 µM. Reactions were carried out in 0.2 ml thin-walled PCR tubes (Invitrogen, Life Technologies Inc., USA) in a total volume of 50 µl. A negative control, with the DNA template replaced with sterile water, was also included in each PCR assay. Amplifications were achieved in 25-35 cycles using a GeneAmp Model 9600 PCR thermocycler (Perkin Elmer, Cetus, USA). The following cycling parameters were typical for a

standard PCR reaction: initial denaturation at 94^{o}C for 5 min, followed by a cycle that consisted of denaturation at 95^{o}C for 30-60 sec, annealing and elongation. The annealing temperature was selected depending on the Tm of primers and it was usually 5^{o}C below the lowest Tm of the pair of primers. Annealing was routinely performed for 15 – 60 sec. Elongation was routinely carried out at 72^{o}C and the extension time was normally 1 min per 1 kb of the expected product. Subsequently, a final elongation was performed at 72^{o}C for 7 min. The PCR amplification products were analysed by electrophoresis on agarose gel. When only one band was present, the product was directly cleaned with a PCR purification kit (Cat. No. 28104, Qiagen) or alternatively, bands were purified from the agarose gel using a gel purification kit. Purified PCR products were stored at -20^{o}C until further used.

2.5 Cloning procedures

2.5.1 DNA restriction endonuclease treatment

Restriction endonucleases used in this study were either from Roche or New England Biolabs. Restriction endonuclease digestion was routinely performed in a final volume of 20-50 µl containing 0.2-2 µg of target DNA. One to five units of enzyme, with buffer supplied by the manufacturer, were incorporated into the reaction and the digestion was performed at the recommended temperature. One unit of enzyme is defined as the amount of enzyme that will digest 1 µg of DNA to completion in one hour in the recommended buffer at the recommended temperature. Reactions were generally performed at 37^{o}C for 1-2.5 hours and stopped by the addition of 10 mM EDTA (pH 8.0). Digests were then electrophoresed in agarose gels and purified as described in Section 2.4.2.

2.5.2 Ligation of DNA into plasmid vectors

Ligation reactions between endonuclease digested-plasmid vectors and the appropriate insert were carried out in a total volume of 10 µl containing 1 unit of T4 DNA ligase (Cat. No. 481220, Roche Molecular Biochemicals, Germany) and supplied 10x ligase buffer for approximately one hour at room temperature or at

4°C overnight. Usually 20-100 ng of vector and, vector:insert ratios of 1:2 and 1:5 were used.

Where fragments of DNA carrying identical protruding termini were to be cloned, restriction enzyme-digested vector DNA was treated with calf intestinal alkaline phosphatase (CIAP) (Cat. No. 713203, Roche Molecular Biochemicals, Germany) using standard procedures (Sambrook *et al*., 1989), in order to remove the 5' phosphate group and prevent self-ligation of the vector. One unit of CIAP is the enzyme that hydrolyzes 1 µM of 4-nitrophenyl phosphatase in 1 min at 37°C. The reaction was performed in a volume of 50 µl containing CIAP and 10x dephosphorylation buffer at 37°C for one to two hours. The enzyme was then inactivated by adding 0.1 volumes of 200 mM EDTA, and heating at 65°C for 10 min. Following heating, the reaction was cleaned with the QIAquick PCR cleaning kit.

2.6 Bacterial transformations

2.6.1 Preparation of electro-competent *M. smegmatis*

M. smegmatis mc^2155 was the strain used for preparation of electroporation competent cells. The bacteria were streaked out from glycerol stock storage onto 7H10-A plates and incubated at 37°C for three to four days. Electro-competent cells were prepared according to Snapper *et al*. (1990) as follows. A single colony was picked from the agar plate and inoculated into 5 ml of 7H9-B without Mycobactin J and enriched with ADC. The culture was incubated in a rotary shaker at 37°C for three to four days until the optical density of the bacterial suspension corresponded to an absorption value of approximately 1.0 at a wavelength of 600 nm. Four millilitres of freshly growing cultures were then transferred into 300 ml of the fresh media. The cells were grown until they reached mid log phase ($OD_{600\ nm}$ = 1.0) which normally took 16-18 hours. To improve transformation efficiency, the culture was incubated on ice for 2.5 hours before they were harvested by centrifugation at 5000 rpm for 10 min at 4°C. The pellets were dissolved and washed in 300 ml of ice-cold sterile electroporation

buffer (EPB) (10% [v/v] glycerol) and re-centrifuged as above. The cell pellets were resuspended in 800 μl of ice-cold EPB and aliquoted in 100 μl volumes. Aliquots were snap-frozen for 10 sec in liquid nitrogen and were immediately stored at -70°C.

2.6.2 Transformation of *E. coli* and *M. smegmatis*

2.6.2.1 Electroporation of *E. coli*

2.6.2.1.1 Electro-transfer of purified plasmids or ligation mixture to *E.coli*

Prior to electroporation, all ligation mixtures and plasmids were dialysed for 15-20 min on 0.025μm pore 13 mm membrane filter discs (Cat. No. VSWP 01300, Millipore Corporation, MA, USA) placed in a petri-dish containing sterile distilled water. Approximately 20 ng of purified plasmid or 100 ng of ligation mixture were added to 50 μl of *E. coli* ElectroMax DH10B electro-competent cells (Cat. No. 18290-015, Invitrogen, Life Technologies Inc., USA) in a 0.2 cm gene pulser cuvette (Cat. No.1652086, Bio-Rad, USA). Electroporation was carried out using a Bio-Rad Gene Pulser apparatus (Bio-Rad, USA) set to 2.5 kV, 25 μF capacitance and 200 ohms resistance. Immediately following electroporation, 200-500 μl of SOC media (Cat. No.15544-034, Invitrogen, Life Technologies Inc., USA) was added to the cuvette. Transformed cells were incubated at 37°C for approximately one hour prior to plating on LBA plates containing the appropriate antibiotic. The plates were incubated overnight at 37°C.

2.6.2.1.2 Electro-transfer of plasmids directly from *M.smegmatis* to *E.coli*

For transference of plasmid DNA from *M. smegmatis* to *E. coli*, 5 μl of freshly grown cultures were mixed with 20 μl of *E. coli* DH10B cells in an electroporation cuvette. The mixture was then electroporated and plated out as described in section 2.6.2.1.1.

2.6.2.2 Electroporation of *M. smegmatis*

M. smegmatis bacteria were transformed with purified plasmids. Approximately 20-50 ng of dialysed plasmids were mixed with 100 µl of competent cells previously prepared (Section 2.6.1). Electroporation was carried out as for *E. coli*. After electroporation, 500 µl of LB were added to the mixture, which was then incubated for three hours at 37^{o}C. From this, 100 µl of cells were then plated on LBA containing kanamycin and BCIP for selection of PhoA positive colonies.

2.6.2.3 Heat shock

Chemically competent *E. coli* Top 10 competent cells (Cat. No. 44-0301, Invitrogen, Life Technologies Inc., USA) and *E. coli* BL21 (DE3) cells (Cat. No. 230255, Stratagene, USA) were transformed with 10-20 ng of plasmid or 10-100 ng ligation mixtures by heat shock. Briefly, 50 µl of ice-thawed competent cells were mixed with DNA by gentle tapping in a pre-cooled microcentrifuge followed by 30 min incubation on ice. Subsequently, the mixtures were heat-shocked at 42^{o}C for 30 sec and were immediately placed on ice for 2 min. After the addition of 250 µl of pre-warmed SOC media, the transformation reaction was incubated for one hour at 37^{o}C and plated on LBA containing appropriate antibiotics. The plates were incubated overnight at 37^{o}C.

2.7 DNA sequencing

Inserts cloned into plasmid vectors and PCR products were submitted to the DNA sequencing analysis unit in the Allan Wilson Centre, Massey University, Palmerston North. Plasmid DNA templates were prepared using the Qiagen plasmid purification kit and PCR products were either purified using PCR purification kit or gel purified prior to sequencing. For each sequencing reaction, 3.2-5 pmol of each primer, and 300 ng plasmid or 2 ng per 100 bp PCR products and sterile distilled water were included in a total volume of 15 µl. The reactions were submitted for automated sequencing using an ABI Prism® Big Dye™ terminator v3.1 Sequencing Ready Reaction Kit either in an ABI Prism® 377 DNA

sequencer or an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA, USA). The reaction kit uses dye-labelled dideoxynucleotides in which a specific fluorescent dye covalently attaches to each base. Products can be visualised as fluorescent colour-coded bands collected by the ABI Prism® Data collection System and then interpreted by the ABI Prism® DNA Sequencing Analysis software.

2.8 Protein preparation

2.8.1 Preparation of *M. smegmatis* cell lysates

Cell lysates were prepared from cultures grown as described in Section 2.2.2.1. Generally, 1 ml of freshly growing culture at mid-log phase was inoculated into 100 ml of fresh LB and incubated at 37^{o}C with shaking at 220 rpm. When cultures reached the mid-log phase, cells were pelleted by centrifugation at 3,000 x *g* for 30 min at 4^{o}C. The pellets were washed three times and resuspended in 10 ml of TE buffer. Cells were sonicated on ice for 5 x 30 sec using a Vibra-Cell VCX-500 sonicator (Sonics & Materials, Danbury, CT, USA) set at 40% amplitude with a 13 mm diameter probe. Insoluble debris was removed by centrifugation at 10,000 x *g* for 30 min at 4^{o}C and the supernatant was collected. Subsequently, the supernatant was aliquoted and stored at -20^{o}C.

2.8.2 Expression of recombinant proteins

In the case of each protein, the selected recombinant *E. coli* clone was streaked on LBA containing kanamycin and chloramphenicol and grown overnight at 37^{o}C. A single colony was picked and inoculated into 5 ml of LB and grown overnight at 37^{0}C while being shaken at 220 rpm on an Innova incubator shaker. Five millilitres of an overnight culture was used to inoculate 500 ml of LB (1:100 dilution of culture), which was grown until mid-log phase period ($OD_{600\ nm}$ = 0.6-0.8). To induce protein expression, isopropyl-β-δ-thiogalactopyranoside (IPTG) (Cat. No. 724815, Roche) was added to a final concentration of 1 mM and the culture was incubated at 37^{o}C at 220 rpm for five hours. After induction, the culture was divided into 250 ml centrifuge bottles and pelleted by centrifugation.

Centrifugation was carried out in a Sorvall® GSA rotor (Thermo Fisher Scientific, Waltham, MA, USA) at 7,000 x *g* for 30 min at 4°C using Sorvall® RC-5 plus centrifuge (Thermo Fisher Scientific). The pellets were stored at -70°C until further treatment.

Analysis of protein expression was carried out using 1 ml cultures removed before and after induction. Cultures were pelleted at 13,000 x *g* for 2 min, resuspended in 20 µl of 2x SDS PAGE loading buffer (0.5% [v/v] NuPAGE® 4x LDS sample buffer {Cat. No. NP007}, 0.1% [v/v] NuPAGE® 10x sample reducing agent {Cat. No. NP004}, 0.4% [v/v] distilled water) and heated at 70°C for 10 min. The samples were then analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.8.2.1 Solubility of recombinant proteins

Expressed recombinant proteins were first assessed for their solubility, prior to the purification procedure. To determine the solubility, 50 µl of an induced culture was pelleted by centrifugation at 7,000 x *g* for 30 min at 4°C. The pellet was resuspended in 5 ml of ice-cold phosphate-buffered saline pH 7.4 (PBS [140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4]) supplemented with protease inhibitor. The mixture was then sonicated and pelleted at 10,000 x *g* for 15 min at 4°C. The supernatant was collected and kept on ice. The pellet was washed twice with 2 ml of ice-cold PBS and it was resuspended in approximately 2 ml of 1% SDS by vigorous vortexing. Both the supernatant and the resuspended cells were analysed by SDS-PAGE.

2.8.3 Protein purification

2.8.3.1 Preparation of recombinant *E. coli* cell lysates

Cell lysates of recombinant *E. coli* cultures were obtained from the prepared pellets, as described in Section 2.8.2. Frozen pellets were thawed on ice and resuspended in 15 ml of chilled extraction/wash buffer containing 50 mM Na_2HPO_4, 300 mM NaCl and 6 M Urea at a pH of 7.0. The mixtures was agitated

for two hours at 4°C and then sonicated on ice with three 10 sec pulses at 25% amplitude. Following centrifugation at 8.000 x *g* for 30 min, the supernatant was separated from the cells pellet and used for protein purification.

2.8.3.2 Talon® Immobilized metal affinity chromatography (*IMAC*) Resins

The recombinant proteins, in this study, were purified under denaturing conditions using BD Talon™ metal affinity resin (Cat. No. 635502, Clontech Laboratories, Palo Alto, CA, USA). Two millilitres of resuspended resin (giving 1 ml bed volume) were equilibrated with extraction/wash buffer pH 7.0 supplemented with 6 M urea according to the manufacturer's instructions. Five millilitres of supernatant, prepared as described in Section 2.8.3.1, were added to a 15 ml Falcon tube containing the equilibrated resin. The mixture was then incubated at room temperature for 30 min on a rotary shaker to allow binding of the histidine-tagged protein to the resin. After incubation, the mixture was centrifuged at 3,000 x *g* for 10 min and the supernatant containing unbound protein was removed and labelled as the flow-through fraction. The resin with bound histidine-tagged protein was washed three times with 10 bed volumes of extraction/wash buffer with the addition of 5 mM Imidazole in the second wash in order to remove any possible contaminating histidine-rich *E. coli* proteins. Each wash consisted of a 15 min rotation at room temperature following centrifugation and removal of the supernatant labelled as wash I to III, respectively. The recombinant protein was eluted in elution buffer (pH 7.0) containing 45 mM Na_2HPO_4, 270 mM NaCl, 150 mM Imidazole (Cat. No. I2399, Sigma) and 6 M urea followed by centrifugation (as described above) and stored at -20°C.

2.8.3.3 Protein Dialysis

When proteins are purified under denaturing conditions, renaturation of proteins into their native state is required. This was achieved by dialysing the proteins in buffers containing urea. Briefly, proteins were dialysed for 24 hours at 4°C using Spectra/Por® dialysis membrane tubing in 2 L PBS containing gradually decreased urea concentration.

2.9 Protein analysis

2.9.1 Estimation of protein concentration

Concentration of the purified recombinant protein was estimated using the Bio-Rad Protein assay (Bio-Rad). The assay, based on the method of Bradford (Bradford, 1976), is a dye –binding assay that shows a variable colour change of the Coomassie® Brilliant Blue G-250 dye in response to different concentrations of the protein. A standard curve was constructed using bovine serum albumin (BSA, Pierce, Rockford, IL, USA) ranging from 1.2-10 µg/ml. Eight hundred microlitres of each standard and diluted sample solutions were mixed individually with 200 µl diluted dye reagent (1:4 dilution) in an Eppendorf tube. After mixing, each standard and sample was transferred into a cuvette. Each standard and sample was analysed in duplicate. The absorbance was read at 600 nm using the Helios α spectrophotometer. Alternatively, the purified recombinant protein concentration was estimated using NanoDrop® ND-1000 spectrofotometer (NanoDrop Technologies, Inc., Wilmington, USA) as described in Section 2.4.3.

2.9.2 Protein electrophoresis

Proteins were separated according to their molecular weight by vertical electrophoresis using denaturing SDS-PAGE (Laemmli, 1970). Electrophoresis was performed using the Xcell SureLock™ Mini-Cell (Invitrogen, Life technologies). The gels, NuPAGE® 12% Bis-Tris gel (Cat. No. NP0341BOX, Invitrogen, Life Technologies), were used as per the manufacturer's recommendation.

Prior to loading the gel, samples were mixed with NuPAGE® LDS sample buffer and NuPAGE® sample reducing agent. The mixtures were then heated at 70^{o}C for 10 min, spun at 13,000 x *g* for 30 sec and stored on ice. The gel was run for 35 min at 200 Volts in NuPAGE® MES SDS running buffer (Cat. No. NP0002-02, Invitrogen, Life Technologies). Ten microlitres of Precision Plus Protein Standards Dual Colour (Cat. No. 161-0374, Bio-Rad) were included on each gel as a size reference. After electrophoresis, the gel was stained with Simply Blue™

SafeStain (Cat. No. LC6060, Invitrogen, Life Technologies) for one hour at room temperature with gentle shaking. The gel was destained in several changes of water until the background colouration was low.

2.9.3 Western blotting analysis

The immunoblotting technique used was performed according to the method of Towbin *et al*. (1979). After electrophoresis, gels were equilibrated in NuPAGE® transfer buffer (Cat. No. NP0006-1, Invitrogen, Life Technologies) for at least 10 min. Equilibration removed SDS from the gel and counteracted the tendency of the gel to swell during electrotransfer and increased binding capacity of the membrane (Gershoni & Palade, 1982). BioTrace Polyvinylidine difluoride (PVDF) membrane (Cat. No. G66543, Gelman Sciences) was pre-incubated in 100% methanol for 1 min and then rinsed with several changes of water. After rinsing, the membrane and filter papers were also allowed to equilibrate for at least 15 min in transfer buffer. The transfer of proteins from the gel to the membrane was carried out at a constant voltage of 15 Volts for 35-40 min using a Trans-Blot® Semi-Dry (SD) electrophoretic transfer cell (Bio-Rad, USA) as per the manufacturer's instructions. Transfer was confirmed by staining the membrane with 0.2% (w/v) Ponceau S (Cat. No. P3504, Sigma) in 30% (v/v) trichloroacetic acid for 10 min with gentle shaking. Membranes were washed with distilled water until protein bands could be easily visualised.

For immunostaining, the membranes were washed multiple times in distilled water and immediately blocked with blocking buffer (Tris buffer saline pH 7.4 [20 mM Tris-HCl, 100 mM NaCl] containing 5% [w/v] skim milk and 0.1% [v/v] Tween-20) at 4^{o}C overnight or at room temperature for one hour. After washing twice in washing buffer (Tris buffer saline pH 7.4 [20 mM Tris-HCl, 100 mM NaCl] containing 0.1% [v/v] Tween-20), the membrane was cut into strips and further incubation of each strip was carried out in individual reservoirs. Each strip was incubated with 10 ml of diluted sera for one hour at room temperature or at 4^{o} C overnight. Each strip was then washed for five 5-10 min cycles with washing

buffer. After washing, a diluted secondary antibody was applied and incubated for one hour at room temperature. Strips were then washed as previously described. Immunodetection was developed by chemiluminescence with the substrate BM Chemiluminescence Blotting Substrate (POD) (Cat. No. 11500708001, Roche) as recommended by the manufacturer. Strips were exposed to Kodak BioMax MR film (Cat. No.8701302, Radiographic Supplies, Ltd) for 20 – 30 sec in the presence of a single intensifying screen (Kodak Lanex regular, Kodak). Film was developed using an automated processor (Kodak RP X-OMAT Processor Model M6B).

2.10 Interferon-gamma (IFN-gamma) assays

2.10.1 Preparation of antigens

Recombinant proteins were used at a final concentration of 15 µg/ml. PBS was used as a negative control. Avian purified-protein derivative (PPDA) (Cat. No. 63314, Prionics) was used at 12.5 µg/ml as a positive control for specific stimulation. The non-specific T-cell stimulator, concavalin A (ConA) (Cat. No. C5275, Sigma) at a final concentration of 20 µg/ml was also included for all assays in order to check cell viability.

2.10.2 IFN-gamma assays

IFN-gamma assays were performed using a commercially available kit for the detection of IFN-gamma from cattle, sheep and goats (Bovigam® Enzyme Immunoassay [EIA] test kit, Cat. No. 0300250, Prionics). This test was carried out as per the manufacturer's instructions. Generally, this test can be divided into two stages: whole blood culture and IFN-gamma EIA. Whole blood was collected from the jugular vein of each sheep into 10 ml heparinised vacutainer (Cat. No. 366480, Becton Dickinson) using 20 G x 1" vacutainer precision glide needles (Cat. No. 60214; Beckton Dickinson). The blood was mixed by gentle inversion several times and processed within four hours of collection.

In the laboratory, 1 ml of blood was dispensed into the wells of 24-well plates (NUNC) and mixed with 50 µl of each antigen. Routinely, antigens were tested in duplicate. Plates were incubated for 22 hours at 37^{o}C in a humidified incubator containing 5% CO_2. After incubation, 200 µl of plasma supernatant, from each well, were removed into 96-well plates, and stored at -20^{o}C until used. Each plasma sample was assayed in duplicate for IFN-gamma using Bovigam® EIA plates according to the manufacturer's instructions. IFN-gamma positive and negative controls included in the kit were also used in the assays. The amount of bound IFN-gamma was measured spectrophotometrically at 450 nm using an ELISA reader. Results were expressed as the mean $OD_{450\ nm}$ of antigen stimulated supernatants minus the mean $OD_{450\ nm}$ of the PBS control, unstimulated supernatants.

2.11 Rabbit details and immunisation

2.11.1 Preparation of glnH protein for immunisation

Antigen for immunisation was prepared as described in Section 2.8.3.2. Antigen was concentrated using Centriprep YM-30 centrifugal filter devices (Cat. No.4322, Amicon, Millipore Corp., MA) and stored at -80^{o}C until further used.

2.11.2 Rabbit immunisation

Animal Ethics approval (Protocol No. 08/03) had been obtained for conducting this study. A four month old male New Zealand White rabbit was individually housed in appropriate caging at the Small Animal Production Unit, Massey University, Palmerston North. Food and water were given *ad libitum* during the study period. A pre-immune blood sample was taken from the marginal ear vein and following each booster. Immunisation was carried out by subcutaneous injection at multiple sites (no more than five) behind the neck and it was dispersed under the skin by gentle rubbing. For primary immunisation, 300 µg antigen, emulsified with Freund's Complete Adjuvant (1:1 ratio with total volume not to exceed 1 ml), was injected into the rabbit. This was followed three to four weeks later by a booster injection of 150 µg antigen emulsified with Freund's

Incomplete Adjuvant. To check for antibody production, a maximum of 3 ml of blood was collected approximately 10 days following the booster injections. Serum was harvested from the clotted blood after centrifugation at 3,000 x *g* for 15 min and was stored at -80^{o}C until further assays. When antibody of sufficient titre was produced, the rabbit was euthanased and blood was collected through cardiac puncture.

2.12 Bioinformatics

DNA sequences were analysed using DNA Strider™ 1.3. Database searches, to identify DNA and translated protein sequences similarities between cloned MAP sequences and those existing on the databases, were performed on the server of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) at the National Institutes of Health (NIH, Bethesda, Maryland, USA) using the BLAST algorithm. Analyses of the MAP signal peptide cleavage sites were carried out with the use of Neural Network (NN) and Hidden Markov Model (HMM) methods of the SignalP 3.0 server (http://www.cbs.dtu,dk/ services/ SignalP/) and Phobius Server (http://phobius.cgb.ki.se/). Multiple DNA and protein alignments were created with the ClustalX 2.0 program (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) or Geneious Basic (http://geneious.com).

2.13 Statistical analysis

Basic calculations including means, standard deviation and standard error of means were performed using Microsoft Excel 2003 (Microsoft Corp, Redmond, WA, USA). The data was analysed using the generalised linear mixed model (GLMM) analysis in PROC MIXED procedure in SAS® 9.1.

CHAPTER 3

Identification of MAP strain 316F genes encoding exported proteins using PhoA fusion technology

3.1 Abstract

Mycobacterial secreted and cell-wall associated proteins are involved in essential processes including virulence and in eliciting host-immune responses. In order to identify immunogenic proteins in MAP strain 316F, alkaline phosphatase (*phoA*) gene fusions were constructed in the plasmid vector pJEM11 and transformed into *E. coli* and the closely related mycobacterial species *M. smegmatis*. Recombinant clones, which express the MAP gene sequences containing signals for transport fused to *phoA* in the correct frame for translation, were identified as blue colonies, when plated on LBA containing the colorimetric PhoA substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP). Expression of PhoA fusions was confirmed by immunoblotting of cell lysates extracted from selected recombinant *M. smegmatis*. The DNA sequences of inserts, from selected blue colonies, were analysed using DNA and protein databases to identify open reading frames encoding exported proteins.

3.2 Introduction

Bacteria produce a wide variety of proteins, which are exported out of the cytoplasm to the cell envelope, or they are secreted into the extracellular environment. These proteins have a number of functions including protection from environmental challenges, acquisition of nutrients and interaction with the host cell proteins (Carroll *et al*., 2000; Thanassi *et al*., 2005; Scott & Barnett, 2006). Thus they are potential targets for vaccine development and diagnostic tests.

In mycobacteria, exported proteins, which encompass both extracellular and cell-envelope associated proteins, have been the focus of investigation to identify antigens that may induce protective immunity or elicit immune responses of diagnostic value. The immunogenicity of exported proteins of *M. tuberculosis* and *M. bovis* has been extensively studied (Horwitz *et al*., 1995; Chiang *et al*., 1997; Wilkinson *et al*., 1997; Denis *et al*., 1998; Sharma *et al*., 1999; Brandt *et al*., 2000; Skjot *et al*., 2000; Cockle *et al*., 2002; Raja *et al*., 2002; Bahk *et al*., 2004; Olsen *et al*., 2004; Scarpellini *et al*., 2004; Ewer *et al*., 2006) resulting in the identification of key components of the pathogen, which are capable of inducing a protective immune response in the host. Research in several laboratories focusing on MAP exported proteins has also demonstrated that these proteins are promising for use in the serodiagnosis of paratuberculosis (Cho & Collins., 2006; Rosseels *et al*., 2006; Willemsen *et al*., 2006; Bannantine *et al*., 2008a). Furthermore, research carried out in our laboratory showed that a 22 kDa exported protein (P22) of MAP can elicit humoral and cell-mediated immune responses, when injected into sheep (Dupont *et al*., 2005; Ridgen *et al*., 2006).

There are several experimental approaches employed to study exported proteins. Gene fusion technology based on PhoA, coding for the *E. coli* periplasmic alkaline phosphatase (Manoil *et al*., 1990), has been successfully used to identify proteins that are exported in a number of microorganisms including *Actinobacillus actinomycetemcomitans* (Ward *et al*., 2001), *Borrelia*

burgdorferi (Kornacki & Oliver, 1998), *Treponema palidum* (Blanco *et al*., 1991), *Streptococcus pneumonia* (Pearce *et al*., 1993), Mycoplasma (Cleavinger *et al*., 1995), *Pseudomonas aeruginosa* (Lewenza *et al*., 2005), *Helicobacter pylori* (Oliaro *et al*., 2000), *M. tuberculosis* (Lim *et al*., 1995; Braunstein *et al*., 2000), *M. avium* (Carroll *et al*., 2000) and MAP (Dupont & Murray, 2001; Dupont *et al*., 2005). The important property of the *E. coli* PhoA is that it is enzymatically active, when it is exported across the cytoplasmic membrane into the periplasmic space. Successful export of the enzyme and its activity, therefore, requires the fusion of a promoterless *phoA* gene with a DNA fragment containing a functional promoter and export leader sequence. The vector pJEM11 (Figure 3.1), used in this study, allows fusions between PhoA and genes encoding exported proteins. This vector has a truncated *phoA* gene from *E. coli* without a start codon or any regulatory elements (Lim *et al.,* 1995). The multiple cloning sites allow the insertion of fragments from the gene encoding putative exported proteins, together with their regulatory elements. The enzymatic activity of the PhoA can be detected, using the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP), which is a colourless compound cleaved by PhoA to form a blue coloured compound on solid media. Using this feature as an export marker, the PhoA fusion system has become a useful tool for analysing protein secretion.

This chapter describes the application of the PhoA reporter system to the identification of exported proteins in MAP strain 316F. In this research, it was envisaged that identification of genes coding for exported proteins, from MAP strain 316F, may lead to the identification of novel antigens that may be useful for diagnosis or vaccine design.

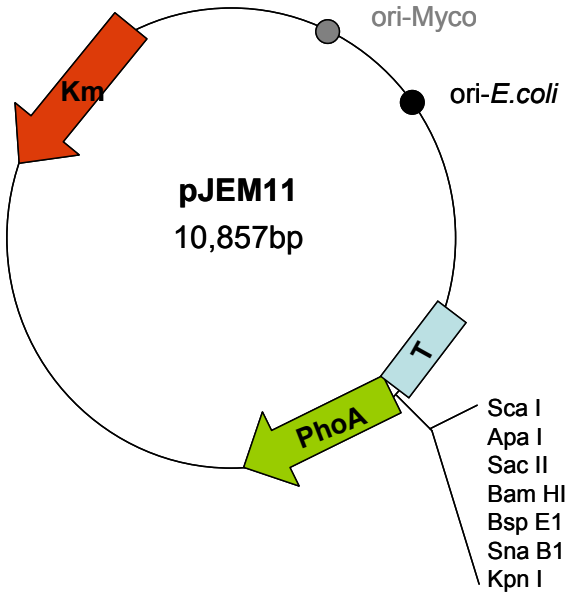


Figure 3.1 Map of the plasmid vector pJEM11 showing the major features. Adapted from Lim *et al*. (1995). Features include a kanamycin resistance marker (Km) for selection; origins of replication for both *E. coli* (ori-*E. coli*) and mycobacteria (ori-Myco); transcriptional terminator (T); multiple cloning site for insertion of DNA fragments; truncated *E. coli phoA* gene, which is devoid of the promoter, ribosomal binding site, start codon and signal sequence.

3.3 Materials and methods

3.3.1 Identification of MAP strain 316F exported proteins

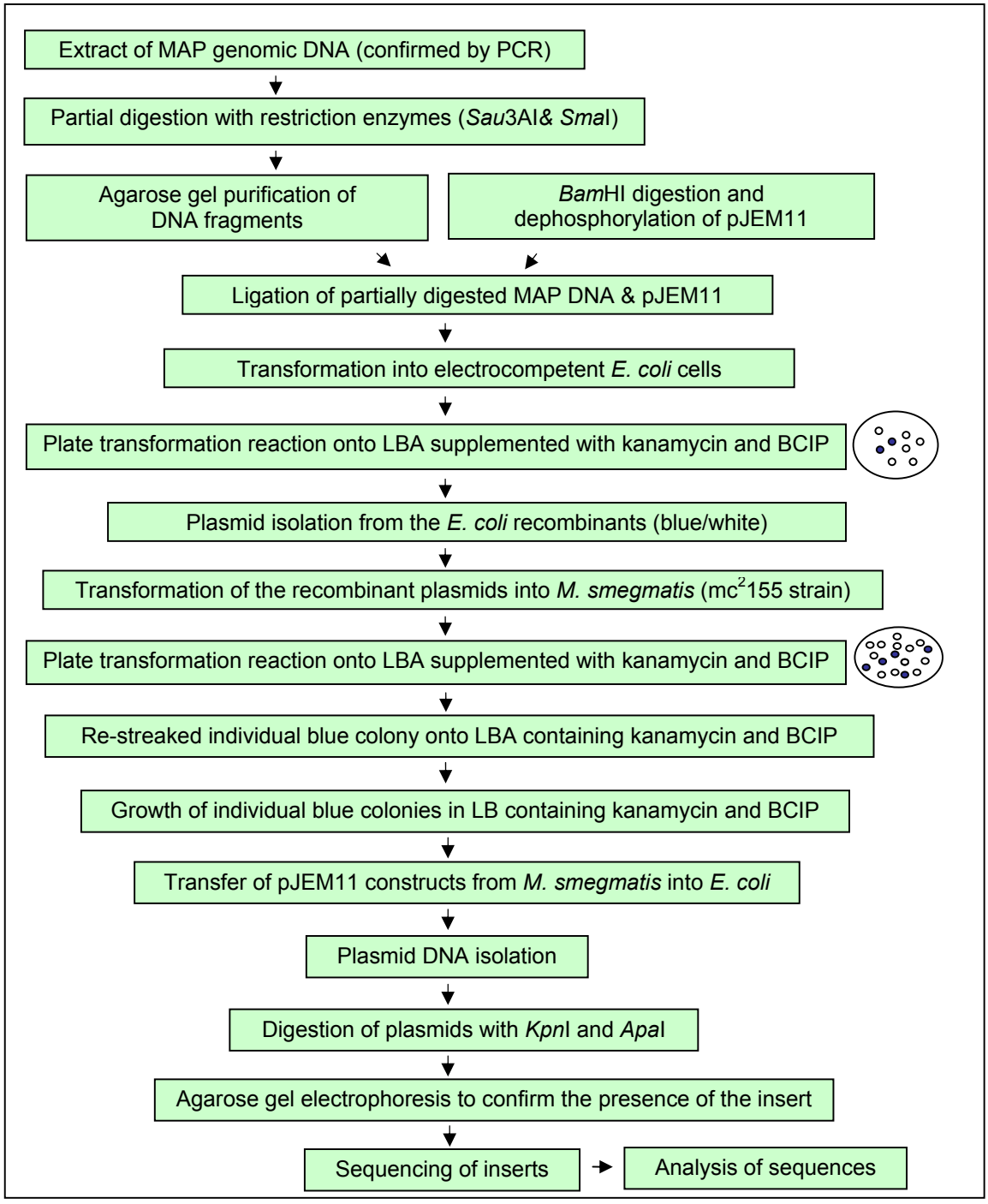


Figure 3.2 Flow chart showing steps in the identification of MAP strain 316F-pJEM11 recombinant clones and analysis of DNA inserts.

3.3.1.1 Preparation of MAP genomic DNA

MAP strain 316F was grown on 7H9-B enriched with sterile ADC broth (Section 2.2.2.2). Genomic DNA was then isolated, using the phenol:chloroform method (Section 2.3.2). Approximately 3 µg of DNA was electrophoresed on a 0.7% agarose gel (Section 2.4.1) and quantified (Section 2.4.3). To confirm the identity of isolated DNA, PCR was carried out using Platinum® *Pfx* DNA polymerase as described in Section 2.4.4.2. To confirm the genus *Mycobacterium*, two oligonucleotide primers (246 and 264) were used to amplify the 16S rRNA gene. For confirmation of MAP subspecies, the genetic elements *IS900* and *F57* were amplified using the primers *IS900*-f, *IS900*-r; *IS900*-f1, *IS900*-r1 and *F57*-f, *F57*-r. Details of the primers are shown in Table 3.1.

3.3.1.2 Preparation of pJEM11 plasmid DNA

Plasmid DNA was purified from transformed *E. coli* DH10B cells using QIAprep-spin plasmid Miniprep Kit (Section 2.3.1) and quantified (Section 2.4.3). Approximately 0.6 µg of plasmid was completely digested with 1 unit of *Bam*HI (Cat. No.10 656275 001, Roche Molecular Biochemicals, Germany) at 37^{o}C for two hours. After incubation, the digests were electrophoresed on a 1% agarose gel and extracted from the gel (Section 2.4.2). Ten microlitres of the resulting digested pJEM11plasmid DNA was then dephosphorylated using 1 unit of CIAP for one hour at 37^{o}C. This treatment prevents recircularisation of the plasmid, thus avoiding self-ligation. After the CIAP treatment, the plasmid DNA was purified by agarose gel extraction and the efficiency of phosphatase treatment was determined. Briefly, 0.25 unit of T4 DNA ligase (Cat. No. 10 481220 001, Roche Molecular Biochemical, Germany) was added to 50 ng of prepared plasmid vector, in a total volume of 10 µl, using the supplied buffer. After overnight incubation at 4^{o}C, the mixture was dialysed and introduced into 20 µl of *E. coli* DH10B competent cells by electroporation (Section 2.6.2.1.1). One hundred microlitres of transformation mixture was spread on an LBA plate containing kanamycin. As a control, the same procedure was carried out with

undigested pJEM11 vector. The plates were incubated overnight at 37^{o}C, and the number of colonies on each plate was recorded.

3.3.1.3 Partial digestion of MAP genomic DNA

To establish the conditions necessary to obtain a suitable partial digest of the genomic DNA, 6 µg of genomic DNA was digested, using either 1 unit of *Sau*3A1 or 5 units of *SmaI*, in a volume of 50 µl at room temperature. From this, 4 µl aliquots were removed at different time intervals (30 sec, 60 sec, 1.5 min, 2, 2.5, 3, 5, 10, 20, 35, 50 and 60 min) and immediately mixed with 5 µl of DNA loading dye containing 2 µl of 0.5 M EDTA (pH 8.0) to stop the reaction. All samples taken were then run on 1.5% agarose gel along with 2 µl of undigested genomic DNA, stained with ethidium bromide and photographed. A partial digest was indicated by the appearance of the predominating high molecular weight band and the appearance of a gradual smear, ranging in size from 100-10,000 bp. Digestion fragments, showing DNA ranging in size from 200-3,000 bp, were excised and purified (Section 2.4.2). The resulting DNA fragments were eluted in 10 µl of EB buffer and 4 µl of pooled DNA fragments were gel-checked. DNA concentration was measured (Section 2.4.3) and the purified digest stored at -20^{o}C until used.

3.3.1.4 Ligation of MAP DNA and pJEM11

3.3.1.4.1 *Sau*3A1 partial digests

Approximately 350 ng of pooled purified digests were ligated overnight to 0.5 µg of *Bam*HI linearized and CIAP treated pJEM11 vector, using 1 unit of T4 DNA ligase, in a 25 µl reaction at 4^{o}C. The ligation mixture was then dialysed and electroporated into 100 µl of *E. coli* DH10B cells in a 0.2 cm gene pulser cuvette as described in Section 2.6.2.1.1. To this, 400 µl of LB was added and incubated for one hour at 37^{o}C. After incubation, an aliquot of approximately 80 µl each was plated onto six LBA supplemented with kanamycin and BCIP. The plates were incubated at 37^{o}C for 18-24 hours and the number of colonies grown on each plate was counted. The total number of blue transformants was also

counted. Blue *E. coli* colonies were picked and re-streaked onto LBA plates containing kanamycin and BCIP to get a pure culture and to prepare a glycerol stock.

3.3.1.4.2 *Sma*I partial digests

Fifteen microlitres of partially digested genomic DNA were dephosphorylated using 2 units of CIAP at 37^{o}C for one hour. Subsequently, the mixture was purified with the QIAGEN PCR purification kit (Cat. No. 28104, Qiagen) and eluted in 10 µl of EB buffer. Four microlitres of CIAP-treated DNA was cloned into TOPO vector and transformed into *E. coli* Top10 chemically competent cells (Section 2.6.2.3). Transformants were plated on LBA containing kanamycin and incubated overnight at 37^{o}C. Following incubation, the number of colonies present on each plate was counted. Approximately 1.6 ml of LB was added onto each plate and colonies were scraped using a sterile spreader. Plasmid DNA was then isolated (Section 2.3.1) and quantified (Section 2.4.3). Two microlitres of pooled plasmid DNA were then digested using 5 units of *Bam* HI at 37^{o}C for 2.5 hours. The digests were run on a 1% agarose gel along with control (undigested DNA), gel purified and quantified. Purified DNA was then ligated into *Bam*HI linearized and CIAP treated pJEM11 vector as previously described in Section 3.3.1.4.1.

3.3.1.5 Plasmid isolation from the *E. coli* clones

To each of the resulting plates, 1.6 ml of LB was added and the colonies were resuspended with the aid of a sterile spreader. The mixtures from each plate were transferred into microtubes and the plasmid DNA extracted using the QIAprep-spin plasmid Miniprep Kit. DNA, in each column, was eluted with 50 µl of EB buffer. One microlitre of the total plasmid preparation was subjected to PCR amplification. PCR was carried out using the primers *IS900*-f1, *IS900*-r1 (Table 3.1) and the PCR product sequenced (Section 2.7).

3.3.1.6 Transformation of the recombinant plasmids into *M. smegmatis* mc^2155

Twenty microlitres of pooled plasmid, extracted from the *E. coli* clones, was dialysed and transformed into 100 µl of electrocompetent *M. smegmatis* cells. Cells were recovered, following electroporation, by adding 500 µl of LB to the cuvette, followed by incubation at 37°C for three hours. After incubation, approximately 85 µl samples each were spread onto 6 LBA plates containing kanamycin and BCIP. All plates were incubated at 37°C for seven days and then transferred to 4°C, to allow further development of blue colour. The number of white and blue colonies that appeared was counted and recorded. All blue colonies were picked and re-streaked onto fresh LBA plates supplemented with kanamycin and BCIP and were grown at 37°C until they turned blue. Glycerol stocks were prepared from all blue colonies (Section 2.2.4).

3.3.2 Sequencing of DNA inserts encoding putative exported proteins

3.3.2.1 Restriction endonuclease digestion of plasmids

The presence of the insert and its size in the putative exported protein was determined by rectriction endonuclease digestion of plasmid. To obtain plasmid DNA, the pJEM11 constructs were first transferred from individual *M. smegmatis* colonies into *E. coli* DH10B cells. This was done to avoid the lengthy and more difficult extraction of sufficient amounts of plasmid DNA from *M. smegmatis*. Briefly, a single colony of *M. smegmatis* was picked and grown for four days on 5 ml of LB containing kanamycin at 37°C. Five microlitres of freshly-dialysed cultures were then mixed with 20 µl of *E. coli* DH10B cells and electroporated (Section 2.6.2.1.2). Plasmid DNA was then extracted from *E. coli* using a commercial kit. Two microlitres of each plasmid was digested for 1.5 hours with 1 unit of *Kpn*I (Cat. No. 899186, Roche Molecular Biology, Germany) and 1 unit of *Apa*I (Cat. No. 899208, Roche Molecular Biology, Germany) in a reaction volume of 20 µl at 37°C. Digests were electrophoresed on 1.5% agarose gels (Section 2.4.1).

3.3.2.2 Sequencing and analysis of DNA inserts

MAP inserts present in pJEM11 constructs were sequenced (Section 2.7), either with primers JEM1 and JEM2 (Table 3.1), or JEM 2 alone. Primer JEM1 binds to the complementary sequence of the transcriptional terminator, approximately 100 bp from the *Bam*HI restriction site of the vector, whereas JEM2 binds to the coding sequence of the *phoA* gene 141 bp from the *Bam*HI site of pJEM11 (Figure 3.3). Primer JEM2 was used to sequence across the fusion site and the 3' end of the MAP insert DNA.

Nucleotide sequences were edited using DNA strider™ 1.3 software. Bioinformatics analysis for nucleotide and deduced amino acid sequences was performed using the database described in Section 2.12. The N-terminal amino acid of fusion proteins were predicted using BlastP data analysis.

Table 3.1 Design of oligonucleotide primers used to analyse MAP DNA.

Primer	Sequence (5' to 3')	Product size (base pairs)	Reference
246 264	AGAGTTTGATCCTGGCTCAG TGCACACAGGCCACAAGGGA	1,030	Boddinghaus *et al*., 1990.
IS900-f *IS900*-r	TCCTTACCTTTCTTGAAGGG CTGAATGACGTTGTCAAGCC	1,451	Green *et al*., 1989.
IS900-f1* *IS900*-r1*	CCGCTAATTGAGAGATGCGATTGG AATCAACTCCAGCAGCGCGGCCTCG	229	Stabel & Bannantine, 2005.
F57-f *F57*-r	GGATCTCGGCCCCGATAG ATC TCAGACAG GGCAGG	620	Poupart *et al*., 1993.
JEM1 JEM2	CGAGCTGCAGTGGGATGACC TCGCCCTGAGCAGCCCGGTT	Variable	Pasteur Institute, Paris.

* Primer *IS900*-f1 binds at position 140-163 and primer *IS900*-r1 binds at position 344-368 within *IS900* element.

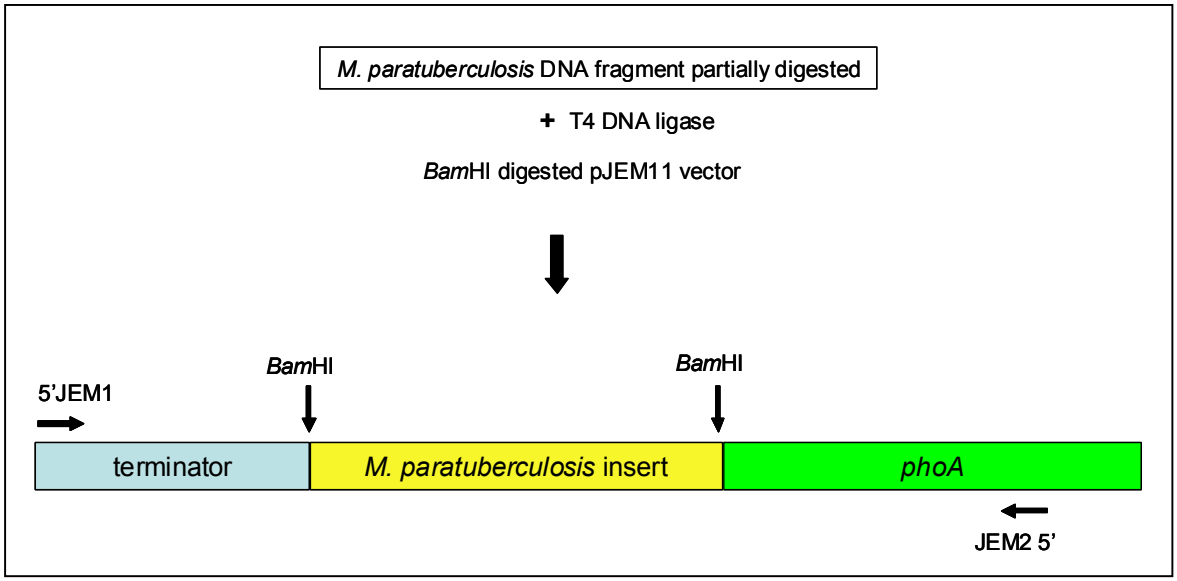


Figure 3.3 Schematic diagram showing the location of the oligonucleotide primers used for sequencing the MAP insert in the pJEM11 construct. JEM1 binds to the complementary strand of the transcriptional terminator and JEM2 binds to the coding strand of the *phoA* gene.

3.3.3 Western blot analysis of exported proteins

To detect the alkaline phosphatase moeity of exported fusion proteins, Western blot analysis (Section 2.9.3) was carried out using cell lysates of selected PhoA positive colonies. Briefly, approximately 5 µg of cell lysate protein, prepared from each recombinant *M. smegmatis* colony (Section 2.8.1), was electrophoresed on NuPAGE® pre-cast 12% Bis-Tris gel (Section 2.9.2) and transferred onto PVDF membrane. Commercial *E. coli* alkaline phosphatase (Cat. No. E2110Y, Amersham Pharmacia Biotech) was used as a positive control. Non-specific sites of the membrane were blocked overnight at 4 ^{o}C, and the membrane was incubated at room temperature with 1:20,000 diluted rabbit anti *E. coli* alkaline phosphatase (Cat. No.100-4134, Rockland, Gilbertsville, PA, USA), for one hour. After washing, the membrane was incubated at room temperature with 1:20,000 diluted peroxidase-labelled goat anti-rabbit IgG (Cat. No. A0545, Sigma), for one

hour. Following washing, the bands were observed by immunodetection as described in Section 2.9.3.

3.4 Results

3.4.1 MAP strain 316F exported proteins

MAP DNA sequences encoding exported proteins were identified, by the preparation of MAP-*phoA* fusions in the pJEM11 vector and subsequent expression in *E. coli and M. smegmatis*. A schematic diagram, showing the basis of the PhoA fusion construction, is illustrated in Figure 3.4.

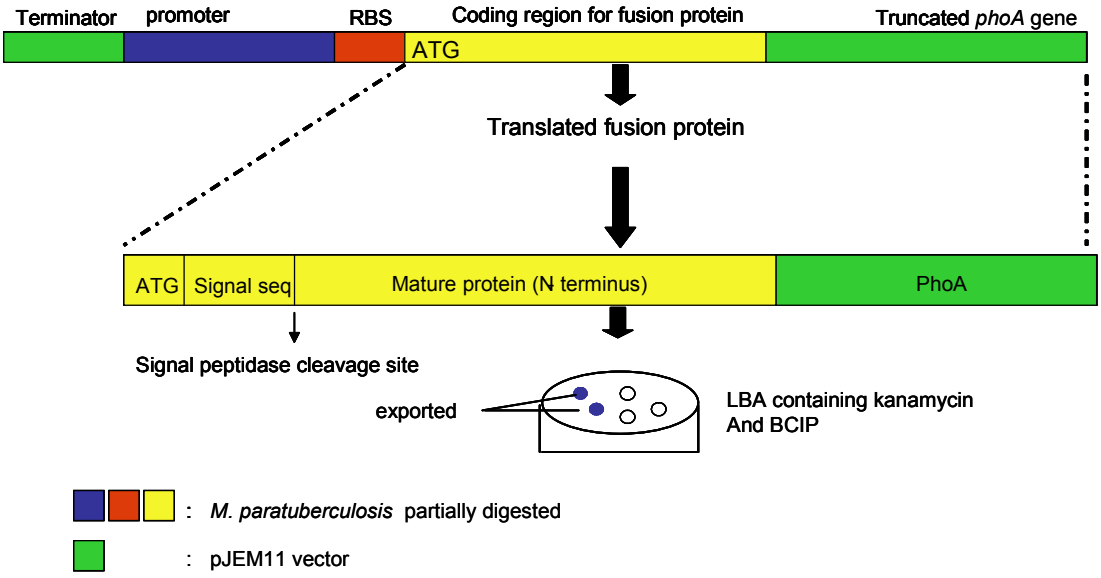


Figure 3.4 Schematic representation illustrating the basis of the PhoA fusion construction. MAP partially digested fragments were cloned into the *Bam*HI site of the pJEM11 vector. MAP cloned sequences, containing a promoter, ribosome binding site (RBS), start codon (ATG) and signal sequence in-frame with the *phoA* gene, are expressed as PhoA fusion protein in *E. coli* and *M. smegmatis*. The PhoA fusion protein exported across the cytoplasmic membrane would turn the recombinant colony blue, when grown on the media containing the substrate BCIP.

3.4.1.1 Growth measurement of MAP culture

The growth measurement of MAP culture (Section 2.2.2.2), taken at weekly intervals, is presented in Figure 3.5. The figure shows that the optical density reading ($OD_{600\ nm}$) of mid-log phase ($OD_{600\ nm}$ = 1.0) was reached five weeks after the initial culture was set up. The cells harvested at this time point were used for further analysis.

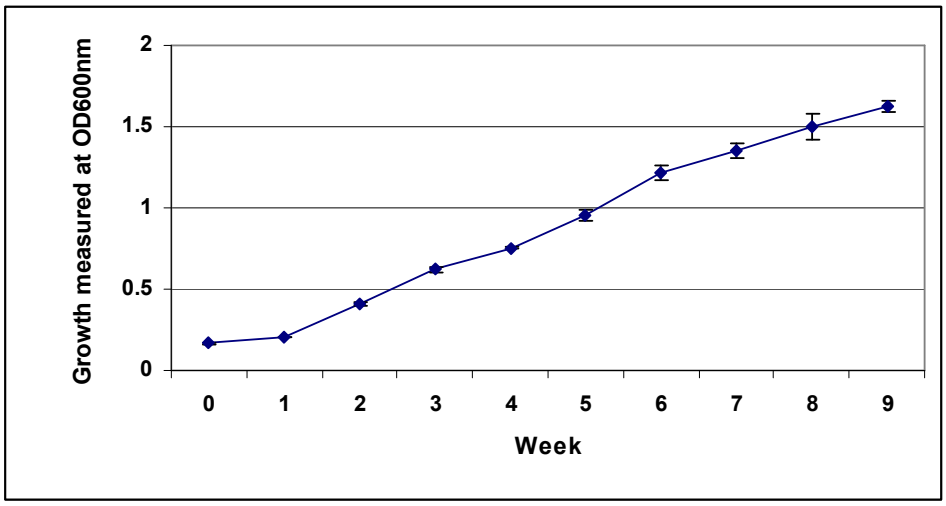


Figure 3.5 Growth curve generated for MAP strain 316F strain.

3.4.1.2 Confirmation of MAP DNA for cloning

In order to confirm the identity of the genomic DNA extracted, the DNA was used as a template to amplify, by PCR, the mycobacterial genus specific gene and the species-specific genetic elements of MAP. The results are presented in Figure 3.6. Amplification of 16S rRNA genus specific gene showed a PCR product bigger than 1,000 bp on agarose gel. The PCR product at 620 bp is consistent with the size of *F57* genetic element. *IS900* fragments of the appropriate size were detected in lanes 3 (1451 bp) and 4 (229 bp). Sequencing of both species-specific genetic elements confirmed that the amplified products were *IS900* and *F57*.

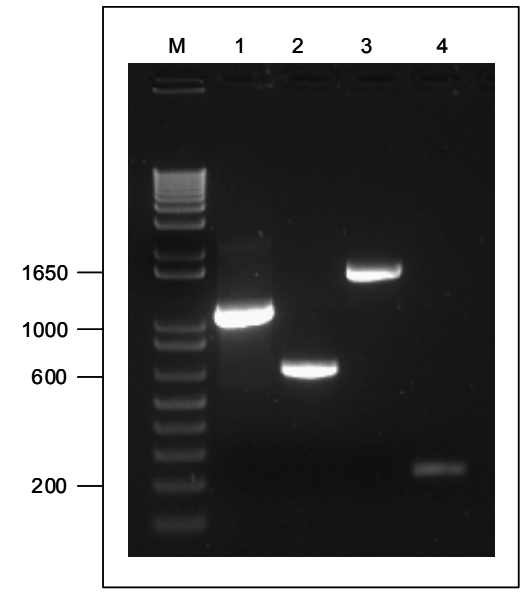


Figure 3.6 PCR amplification of MAP genetic elements. Lane M, 1 kb plus DNA ladder marker (bp); lane 1, the mycobacterial genus-specific 16S rRNA product; lane 2, *F57* species-specific elements; lane 3 and 4, *IS900* species-specific element. PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide and photographed under UV light.

3.4.1.3 Subcloning of MAP DNA into the vector pJEM11 and its expression in *E. coli*

Figure 3.7 shows that digestion of genomic DNA with the two restriction enzymes resulted in a different profile of digestion. Fragment sizes ranged from 200-3,000 bp for *Sau*3AI (1.5 to 10 min digestion) (lanes 4-9). *Sma*I digestion was less well defined, but showed variable fragment sizes seen following 10 to 60 min incubation (lanes 9-13). Partially digested genomic DNA fragments were then subcloned into pJEM11 vector as described in Section 3.3.1.4. It has been reported that *M. smegmatis* mc^2155 has a 10^4 lower rate of transformation efficiency, compared to *E. coli* (Snapper *et al*., 1990). Thus, the plasmids were first transformed into *E. coli* competent cells to ensure the highest possible proportion of constructs could be recovered.

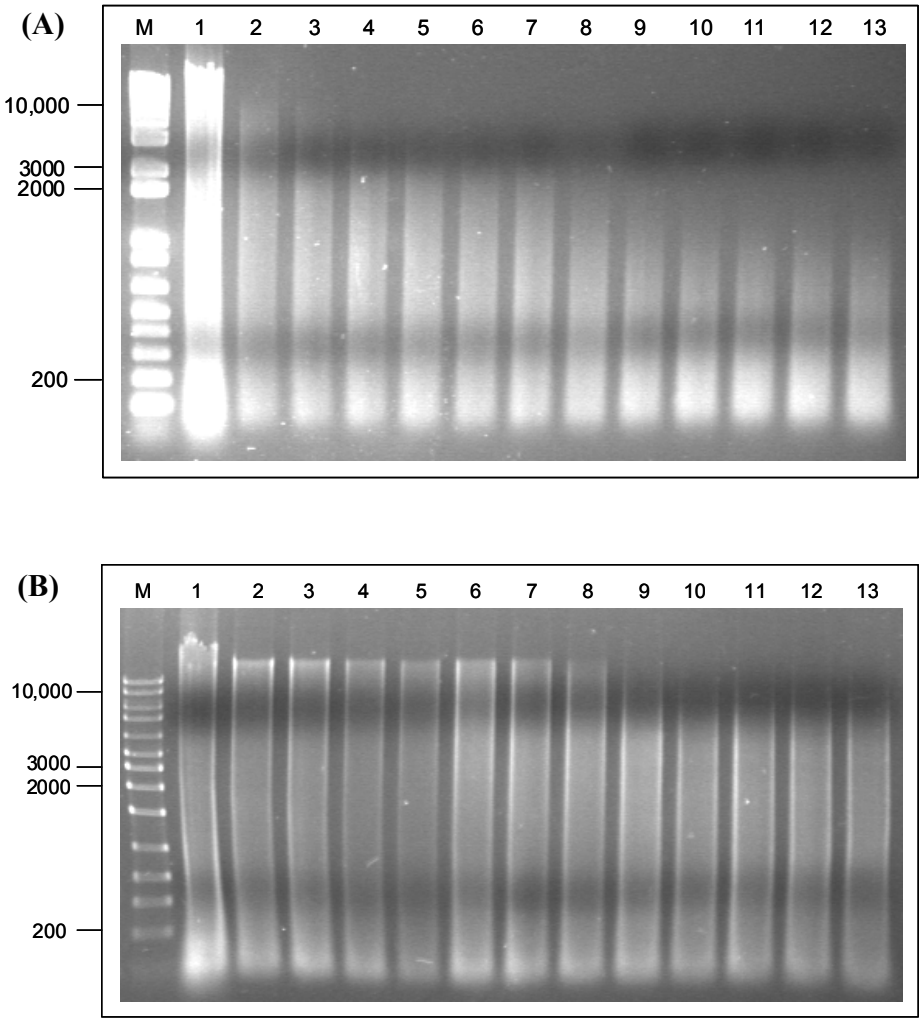


Figure 3.7 Partial restriction digests analysis of MAP genomic DNA at different time intervals. (A) Digestion of genomic DNA with *Sau*3AI. (B) Digestion of genomic DNA with *Sma*I. Lane M, 1 kb DNA ladder marker; lane 1, undigested genomic DNA; lane 2, 30 sec; lane 3, 60 sec; lane 4, 1.5 min; lane 5, 2 min; lane 6, 2.5 min; lane 7, 3 min; lane 8, 5 min; lane 9, 10 min; lane 10, 20 min; lane 11, 35 min; lane 12, 50 min; lane 13, 60min. Digested genomic DNA was electrophoresed on 1.5% agarose gel along with DNA size standard. The gels were stained with ethidium bromide and photographed under UV light.

After an overnight incubation at 37^{o}C, a colony count of 9,000 was obtained from six plates of *Sau*3AI digests. Of these, only one colony was blue and it was designated Ecosau-1. Approximately 12,000 colonies were obtained from all plates of *Sma*I digests in which two colonies were blue. They were designated Ecosma-1 and Ecosma-2.

Plasmid DNA, from a representative 10 *E. coli* transformant, was subjected to restriction analysis. For *Sau*3AI digests, nine out of ten transformants contained inserts in the size range of 200-600 bp (data not shown). A similar number of transformants, which had inserts, were also found in *Sma*I digests. However, the size of these inserts ranged from 200 to 1800 bp (data not shown). A background of five colonies was found on the control plates (vector only).

3.4.1.4 Expression of the genes in *M. smegmatis*

The transformants showing blue phenotype in *E. coli* (Ecosau-1, Ecosma-1 and Ecosma-2) were each electroporated into *M. smegmatis* mc^{2}155 competent cells and incubated at 37^{o}C on LBA plates containing kanamycin and BCIP. Following 30 days observation, no blue colour developed.

To the remaining white colonies, total plasmid from each digest was isolated and electroporated individually into *M. smegmatis*. After seven days of incubation at 37^{o}C, 18 blue colonies (from a total of approximately 9,000 colonies) appeared on the plates, in which the genomic DNA was digested with *Sau*3AI. One-hundred and seventy two blue colonies (from a total of approximately 25,000 colonies) appeared on the plates, in which the genomic DNA was digested with *Sma*I. On day 8, all plates were transferred to 4^{o}C. Over 30 days of observation, a total of 138 blue colonies were collected from *Sau*3AI digest plates, while SmaI plates gave a total number of 283 blue colonies. In summary, there were 421 blue colonies, from both *Sau*3AI and *Sma*I digests that were designated Mptb1 to Mptb421.

To obtain a single colony, each blue colony (Figure 3.8a) was picked and restreaked onto fresh LBA supplemented with kanamycin and BCIP. From the total of 421 blue colonies, 123 colonies grew consistently blue, on BCIP containing medium. The colony colour phenotype of one representative clone is shown in Figure 3.8b.

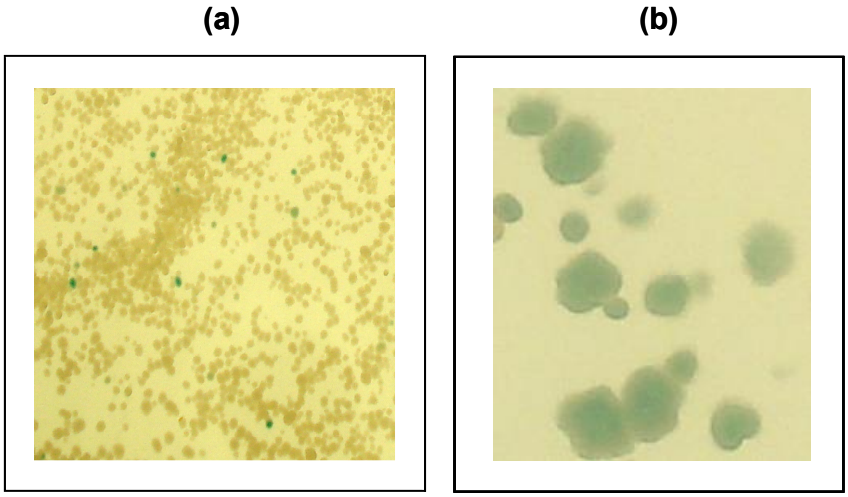


Figure 3.8. Recombinant *M. smegmatis* colonies from the MAP pJEM11 clones. PhoA positive colonies turn blue in the presence of the substrate BCIP. (a) PhoA positive recombinant *M. smegmatis* colonies on a plate containing BCIP from original clones. (b) Selected *M. smegmatis* colony (Mptb241) upon restreaking on BCIP containing media.

3.4.2 Sequence of DNA inserts encoding putative exported proteins

3.4.2.1 Restriction endonuclease digestion of plasmids

Following electroporation of PhoA positive *M. smegmatis* into *E. coli*, plasmid DNA was isolated from *E. coli* and then subjected to restriction endonuclease digestion. Figure 3.9 shows the results of restriction endonuclease digestion with *KpnI* and *ApaI*, of selected PhoA positive clones. The average insert size was calculated from PhoA positive constructs analysed by agarose gel electrophoresis. From 123 plasmids analysed, the average insert size was approximately 950 bp, with a range of 200 to 2500 bp.

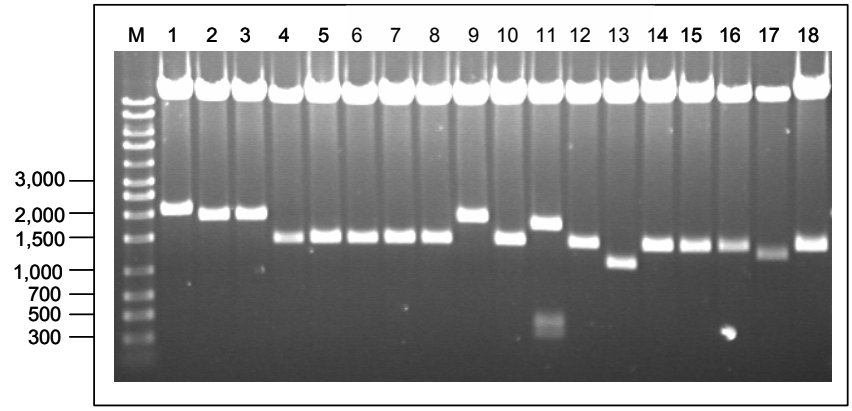


Figure 3.9 Restriction endonuclease digests of selected recombinant plasmids isolated from *E. coli*. Digested plasmids were electrophoresed on 1.5% agarose gel along with DNA size standard, stained with ethidium bromide and photographed under UV light. Insert sizes were deduced, by comparison with the DNA standard size marker. Lane M, DNA ladder marker (bp); lane 1 to 18, restriction endonuclease digest of plasmid from PhoA positive clones Mptb9, Mptb10, Mptb11, Mptb13, Mptb14, Mptb15, Mptb278, Mptb50, Mptb51, Mptb57, Mptb60, Mptb65, Mptb131, Mptb169, Mptb170, Mptb205, Mptb81 and Mptb219 respectively.

3.4.2.2 Analysis of DNA inserts

DNA isolated from 123 plasmids that contained inserts were sequenced to identify the genes encoding exported proteins. The resulting nucleotide sequences and the corresponding predicted amino-acid sequences were analysed using DNA Strider™ 1.3 software. Identification of the open reading frames (ORF), responsible for expression of the PhoA fusion products, was carried out by submitting DNA sequences and/or deduced amino-acid sequences of the inserts to the databases. The result of these findings revealed that unique PhoA fusions accounted for only 18% (22 out of 123) while the remaining 82% were redundant. All unique PhoA fusions, which were in frame with PhoA, could be categorised into three groups of *phoA* gene fusions. The results are presented in Table 3.2, 3.3 and 3.4 respectively. The first group, as listed in Table 3.2, consisted of 94 clones encoding 13 unique proteins carrying signal

sequence predicted for their N-terminus. This group included sulphate binding protein (NP_961147), RNA polymerase sigma factor sigB (NP_961760), glutamine binding protein (NP_962828), mycobacterial integration host factor (NP_960056), acetyl-CoA acetyltranferase (NP_961370), enoyl-CoA hydratase (NP_959817), NADH dehydrogenase subunit L (NP_962146) and seven hypothetical proteins (NP_962038, NP_959867, NP_963244, NP_960415, NP_960818 and NP_962207). Of 94 clones belonging to the first group, the majority (36 clones) showed homology to glutamine binding protein.

The second group of 19 clones, representing four unique proteins (Table 3.3), had no predictable signal sequence. BlastP of three of these proteins were highly homologous to hypothetical proteins of MAP strain K-10 (NP_963210, NP_962733 and NP_959134) with no assigned function. Unexpectedly, one fusion protein exhibited a similarity to *M. avium* subspecies *avium* protein (MAV1037). Ten sequences (listed in Table 3.4) had very short sequences fused to the *phoA* gene and therefore it was not possible to determine the N-termini and the export signal. BlastP analysis, using translated amino-acids, showed no significant identity to any of the annotated database. Searches were then carried out using a different frame translation. BlastP analysis revealed a high degree of homology with a number of MAP strain K-10 sequences, which included exodeoxyribonuclease III XthA, urydylate kinase PyrH and three hypothetical proteins.

Table 3.2 MAP strain 316F PhoA fusion proteins carrying signal peptide predicted for N-terminus.

Clone	Insert size (bp)	Protein identity[a]	Species	Gene (synonyms)	% ID	% SIM	Comments
Mptb81 Mptb271	1300 1500	Sulphate binding protein NP_961147	MAP2213c (MAP K-10) Rv2400c (*M. tuberculosis*) MAV1782 (*M. avium*) MI0615 (*M. leprae*) Mb2422c (*M. bovis*)	*subI*	100 77 99 78 77	100 86 99 84 86	Involved in the active transport across the membrane of multiple sulphur-containing compound including sulfate and thiosulfate.
Mptb371	300	Hypothetical protein NP_962038	MAP3104c (MAP K-10) MAV3922 (*M. avium*) Rv3054c (*M. tuberculosis*) Mb3080c (*M. bovis*)	Not assigned	100 97 77 77	100 99 89 89	Hypothetical protein of unknown function
Mptb46 Mptb60 Mptb102 Mptb169 Mptb219 Mptb229 Mptb51 Mptb11 Mptb13 Mptb57 Mptb66	1500 1800 1500 1500 1500 1500 2000 2000 1500 1500 2500	Glutamine binding protein NP_962828	MAP3894c (MAP K-10) Rv0411c (*M. tuberculosis*)	*glnH*	100 85	100 93	Involved in active transport of glutamine across the membrane (import). Interacts with the glutamine-transport system (Braibant *et al*., 2000). 99% ID and 100% SIM to bacterial extracellular solute-binding protein (MAV4750) in *M. avium*.

Table 3.2 Continued ……

Mptb205	1500	Glutamine binding					
Mptb15	1500	protein NP_962828					
Mptb287	1500	(continued……)					
Mptb14	1500						
Mptb324	1500						
Mptb335	1300						
Mptb328	1300						
Mptb334	1300						
Mptb322	2500						
Mptb330	1300						
Mptb332	1300						
Mptb333	1300						
Mptb337	1300						
Mptb325	1500						
Mptb336	1300						
Mptb5	1500						
Mptb50	1500						
Mptb90	1500						
Mptb10	2000						
Mptb12	1500						
Mptb52	1500						
Mptb65	1500						
Mptb170	1500						
Mptb331	1300						
Mptb9	2100						
Mptb131	1200	RNA polymerase	MAP2826 (MAP K-10)	*sigB*	100	100	Promotes attachment of the RNA
Mptb282	2000	sigma factor SigB	MAV3603 (*M. avium*)		99	99	polymerase to specific initiation sites.
		NP_961760	Rv2710 (*M. tuberculosis*)		94	95	
			MI1014 (*M. leprae*)		90	92	May control the regulation of stationary phase and general stress resistance.

Table 3.2 Continued ……

Mptb393 MPtb351 Mptb385 Mptb16 Mptb243	650 500 650 500 500	Mycobacterial integration host factor NP_960056	MAP1122 (MAP K-10) MAV3386 (*M. avium*) Rv1388 (*M. tuberculosis*) Mb1423 (*M. bovis*)	*mlHF*	100 98 96 96	100 99 99 99	Protein of unknown function
Mptb413 Mptb396 Mptb400 Mptb403 Mptb395 Mptb368 Mptb391 Mptb412	500 650 650 650 650 500 650 650	Acetyl-CoA acetyltransferase NP_961370	MAP2436c (MAP K-10) MAV1544 (*M. avium*) Rv1323 (*M. tuberculosis*) Mb1358 (*M. bovis*) Ml1158 (*M. leprae*)	*fadA4*	98 98 89 89 80	100 99 95 95 88	Catalyses the synthesis of acetoacetyl coenzymeA from two molecules of acetyl coenzymeA.
Mptb416	200	Enoyl-CoA hydratase NP_959817	MAP0883 (MAP K-10) MAV1060 (*M. avium*)	*echA12_1*	100 98	100 99	35% ID and 50% SIM to ech_12 in *M. tuberculosis* (Rv1472) involved in lipid metabolism and could possibly oxidize fatty acids using specific components (catalytic activity).
Mptb420 Mptb392 Mptb389 Mptb390 Mptb343 Mptb366 Mptb419 Mptb344 Mptb372	400 500 500 500 400 300 400 400 300	Hypothetical protein NP_959867	MAP0933 (MAP K-10)	Not assigned	100	100	97% ID and 99% SIM to Rieske (2Fe-2S) domain containing protein in *M. avium* (MAV1116). 39% ID and 57% SIM to lppY probable conserved lipoprotein in *M. tuberculosi* (Rv2999) with unknown function.

Table 3.2 Continued ……

Mptb409	200	Hypothetical protein NP_963244	MAP4310c (MAP K-10) MAV5773 (*M. avium*)	Not assigned	100 98	100 99	30% ID and 47% SIM to fadE36 possible Acyl-CoA in *M. tuberculosis* (Rv3761c) possibly involved in lipid degradation.
Mptb341 Mptb388 Mptb370 Mptb376 Mptb364 Mptb363 Mptb360 Mptb365 Mptb358 Mptb378 Mptb377 Mptb387 Mptb382 Mptb386 Mptb407 Mptb381 Mptb373 Mptb374 Mptb384 Mptb379	850 1000 700 850 850 850 850 750 850 700 850 1000 1000 1000 1000 1000 700 850 1000 700	Hypothetical protein NP_960415	MAP1481c (MAP K-10)	Not assigned	100	100	98% ID and 99% SIM to ATP-dependent RNA helicase in *M. avium* (MAV2956). 43% ID and 57% SIM to probable ATP-dependent RNA helicase in *M. tuberculosis* (Rv3211). 32% ID and 42% SIM to heat shock protein in *M. leprae* (Ml2490).
Mptb305 Mptb348 Mptb347 Mptb398 Mptb399	500 300 300 300 300	Hypothetical protein NP_960818	MAP1884c (MAP K-10) MAV2352 (*M. avium*) Rv1128c (*M. tuberculosis*) Mb1159c (*M. bovis*)	Not assigned	100 97 70 70	100 98 79 79	Hypothetical protein of unknown function

Table 3.2 Continued ……

Mptb257	1100	Hypothetical protein	MAP3273c (MAP K-10)	Not assigned	100	100	Hypothetical protein of unknown function
Mptb92	1100	NP_962207	MAV4111 (*M. avium*)		98	99	
			Rv3131 (*M. tuberculosis*)		66	76	
			Mb3155 (*M. bovis*)		66	76	
Mptb259	300	NADH	MAP3212 (MAP K-10)	*nuoL*	98	100	Involved in aerobic/an aerobic respiration (catalytic activity: NADH + Ubiquinone = NAD $^{(+)}$ + ubiquinol).
Mptb207	300	dehydrogenase	MAV4044 (*M. avium*)		99	99	
		subunit L	Rv3156 (*M. tuberculosis*)		86	91	
		NP_962146	Mb3180 (*M. bovis*)		86	91	

[a]Accesion number based on MAP strain K-10 database.
% ID, percent identity; % SIM, percent similarity

Table 3.3 MAP strain 316F PhoA fusion proteins without predicted signal peptide.

Clone	Insert size (bp)	Protein identity[a]	Species	Gene (synonyms)	% ID	% SIM	Comments
Mptb241	700	Hypothetical protein NP_963210	MAP4276 (MAP K-10)	Not assigned	100	100	Hypothetical protein of unknown function
Mptb238	700		MAV4358 (*M. avium*)		99	99	
Mptb408	650		Rv3413c (*M.tuberculosis*)		65	82	
Mptb307	650						
Mptb240	650						
Mptb43	650						
Mptb308	700						
Mptb319	600						
Mptb320	600						
Mptb357	200	Hypothetical protein NP_962733	MAP3799 (MAP K-10)	Not assigned	95	100	Hypothetical protein of unknown function
			MAV4850 (*M. avium*)		95	99	
			Rv0311 (*M. tuberculosis*)		79	91	
			Mb0319 (*M. bovis*)		79	91	
Mptb356	400	Hypothetical protein NP_959134	MAP0200c (MAP K-10)	Not assigned	100	100	Hypothetical protein of unknown function
Mptb402	400		MAV0195 (*M. avium*)		99	99	
Mptb361	300		Rv3831 (*M. tuberculosis*)		77	77	
			Mb3861 (*M. bovis*)		77	77	
Mptb206	1500	Hypothetical protein	MAV1037 (*M. avium*)	Not assigned	98	100	Hypothetical protein of unknown function
Mptb222	1500						
Mptb166	1500						
Mptb176	1500						
Mptb278	1500						
Mptb277	1500						

[a]Accesion number based on MAP strain K-10 database.
% ID, percent identity; % SIM, percent similarity

Table 3.4 MAP strain 316F PhoA fusion proteins for which N-terminus and signal sequences could not been determined.

Clone	Insert size (bp)	Protein identity[a]	Species	Gene (synonyms)	% ID	% SIM	Comments
Mptb327	850	Hypothetical protein NP_960077	MAP1143c (MAP K-10)	Not assigned	100	100	not in frame with PhoA (frames = +2)
Mptb345 Mptb367 Mptb354 Mptb342 Mptb355	200 200 200 200 200	Hypothetical protein NP_961738	MAP2804 (MAP K-10)	Not assigned	100	100	not in frame with PhoA (frames = +2)
Mptb284 Mptb268	300 200	Exodeoxyribonuclease III Protein NP_962850	MAP3916c (MAP K-10)	*xthA*	100	100	not in frame with PhoA (frames = +3)
Mptb315	650	Hypothetical protein NP_962655	MAP3721 (MAP K-10)	Not assigned	100	100	not in frame with PhoA (frames = +2)
Mptb380	700	Uridylate kinase NP_961880	MAP2946c (MAP K-10)	*pyrH*	100	100	not in frame with PhoA (frames = +2)

[a]Accesion number based on MAP strain K-10 database.
% ID, percent identity; % SIM, percent similarity

3.4.2 Detection of PhoA fusion proteins from recombinant *M. smegmatis*

Seven PhoA positive *M. smegmatis* transformants and an *M. smegmatis* containing non-recombinant pJEM11 were subjected to immunoblot analysis (Figure 3.10). This figure shows that all cell lysates extracted from PhoA positive transformants showed reactivity to a band having molecular weights that are equal, or slightly higher than that of commercial alkaline phosphatase. Faint banding was observed in the cell lysates prepared from two *M. smegmatis* recombinants, Mptb356 and Mptb357. No similar band was detected in the non-recombinant transformant (lane 9).

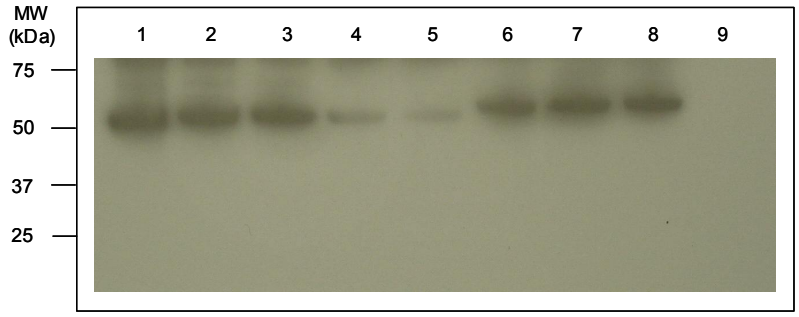


Figure 3.10 Western Blot analysis of PhoA fusion proteins. Approximately 5 µg of lysate protein of each transformant was separated on NuPAGE® pre-cast 12% Bis-Tris gel and transferred to PVDF membrane for Western blotting with rabbit anti-PhoA IgG. Lane 1, commercial alkaline phosphatase; lane 2 to 8, recombinant *M. smegmatis* transformants (Mptb409, Mptb416, Mptb356, Mptb357, Mptb81, Mptb13, and Mptb52 respectively); lane 9, non-recombinant *M. smegmatis*.

3.5 Discussion

Proteins released by some mycobacterial species, to the extracelluler environment, may be a target for immune responses in the infected hosts. In mycobacterial research, there are two major experimental approaches commonly employed to identify these proteins. One is to analyse the protein composition of mycobacterial culture filtrates targeting antigenic components. The other is a genetic approach, which involves the screening of libraries of mycobacterial fusion proteins that become enzymatically active, upon translocation across the cell membrane. Since many exported proteins remain attached to the cell membrane and are therefore not found in culture supernatants, the use of a genetic method, such as the PhoA reporter system, is very useful (Lim *et al*., 1995).

In this study, a MAP strain 316F PhoA fusion clones were constructed and partially characterised, in order to identify novel exported proteins from this micro-organism. The genes were expressed in both *E. coli* and in the non-pathogenic and fast-growing mycobacterial species *M. smegmatis*. More than a two-fold increase in the number of total colonies was observed, when original clones expressed in *E. coli* transformed into *M. smegmatis*. This was a result of amplification of the plasmids in *E. coli*, before being transferred into *M. smegmatis*. Thus, multiple copies of the plasmids, which may lead to clone redundancy, were expected to be present in the *M. smegmatis* clones. Redundant clones were shown, through analysis of the pJEM11 plasmids constructs described in Table 3.2, 3.3 and 3.4. Similar studies, carried out by other researchers, have also showed the presence of redundant representations of many different inserted fragments of *M. tuberculosis*, because the cloned plasmids were propagated in *E. coli*, before they were transferred to *M. smegmatis* (Wiker *et al*., 2000). Western blotting of selected PhoA positive *M. smegmatis* transformants (Figure 3.10) confirmed the presence of the PhoA moeity in the randomly selected fusion proteins. A number of bands with molecular weights, similar or slightly higher than 50 kDa (positive control), were

seen in all PhoA positive fusion proteins. The weak response observed in two out of seven PhoA positive clones may suggest that the fusion proteins are rapidly degraded. A number of studies that support this finding have demonstrated that many secreted fusion proteins are highly sensitive to proteolytic degradation in the periplasmic space (Manoil & Beckwith, 1985; Gomez *et al*., 2000).

Ten of the 123 sequences analysed (Table 3.4) by the BLAST algorithm were not similar to any annotated nucleic acid or protein in the databases. The mechanism by which they may have been exported, leading to blue colour development, is unknown. These fusion-proteins are probably false positives associated with the PhoA fusion technique. Spontaneous lysis of bacteria, folding of cytoplasmic PhoA to active conformation in cells that are no longer growing and leakage of cytoplasmic PhoA fusions from healthy cells, are all possible ways that can generate false positives colonies in the PhoA system (Derman & Beckwith, 1995; *Lim et al*., 1995; Tullius *et al*., 2001; Lewenza *et al*., 2005). Furthermore, it has been reported that fusions near the N-terminal part of cytoplasmic loops may give false positive signals, because they do not have enough positively charged amino acids acting as 'topogenic signals' (Prinz & Beckwith, 1994).

Proteins that are destined to be secreted are normally synthesised bearing an N-terminal hydrophobic signal peptide (Pool, 2005). The presence of a signal peptide was predicted in 13 of the 17 fusion proteins, for which the N-terminal could be identified (Table 3.2 and Table 3.3). Signal peptides can be predicted using two methods: Neural Network (NN) and Hidden Markov (HMM). Both methods are not only designed to identify signal peptides, but they can also predict the cleavage site. The majority of fusion proteins, in this study (Appendix 1), had a predicted cleavage site based on the HMM method. Wiker and colleagues (2000), based on their study using both NN and HMM to predict the cleavage site on secreted proteins of *M. tuberculosis*, found that the HMM, when applied to mycobacterial sequences, gives a better prediction than the NN method, because relatively few mycobacterial proteins were included for training

in the NN method. When the N-terminal was evident, 89 out of 113 (79%) proteins listed in Table 3.2 and 3.3 possessed ATG coding for methionine (M) as the start codon. Ten (9%) had GTG, which encodes valine (V), while 14 (12%) started with TTG coding for leucine (L). O'Donnell and Janssen (2001) stated that the most frequently used start codon in prokaryotes is ATG, while approximately 8% start with GTG and 1% starts with TTG.

Four fusion proteins identified in this study (Table 3.3) had no predicted signal peptide, but one of those had a predicted transmembrane segment. Transmembrane segments may allow the insertation of proteins in the cell membrane and function like signal peptides. Unlike a signal peptide that is proteolytically removed by signal peptidase shortly after translocation, transmembrane segments are not removed and they serve to anchor proteins to the membrane. For fusion proteins, this can lead to sequestration of PhoA outside the cell membrane, resulting in phosphatase activity. This is the principle upon which PhoA fusions have been used to study membrane protein topology (Manoil & Beckwith, 1986). The mechanism, by which the three fusion proteins with no predicted signal sequences in this study may have been exported, is not known. Lim and others (1995) reported the presence of superoxide dismutase of 23 to 28 kDa, with no characteristic signal peptide sequence and is found in short term culture filtrates of *M. tuberculosis*. These authors suggested that there may be a specific pathway for secretion of this enzyme by mycobacteria. In addition, some mycobacterial proteins including dnaK, heat shock protein GroES and ESAT-6 have been reported to be secreted without signal peptide (Sonneberg & Belisle, 1997; Weldingh *et al*., 1998; Abdallah *et al*., 2007).

BlastP analysis of the deduced amino acid sequences showed that the majority of the fusion proteins were higly homologous with proteins of MAP strain K-10 and they were similar, in a lesser extent, to proteins of other mycobacterial species including *M. avium*, *M. tuberculosis*, *M. leprae* and *M. bovis*. A study showed that approximately 71% of the 4,350 annotated proteins from the

sequencing of MAP strain K-10 are currently listed as hypothetical proteins (Li *et al*., 2005). Amongst those hypothetical proteins, one protein (MAP3273c) from the MAP strain 316F clones is of particular interest. Cho and colleagues (2006) in their study to identify proteins of potential diagnostic value from culture filtrate of MAP showed that this protein was amongst 14 different proteins that reacted with antibodies in the sera of MAP-infected cattle, as shown by immunoblot of 2-DE gels and mass spectrometry. The authors further stated that the identified proteins may represent potential antigens for improved antigen-antibody based diagnostic tests for bovine paratuberculosis. Investigation of the immunological activity of this protein, in other paratuberculosis infected animals, would be of interest.

Proteins, other than hypothetical proteins found in this study, include proteins known to be responsible for a number of cellular processes. These included enoyl-CoA hydratase and acetyl-CoA acetyltransferase, which are important in fatty acid metabolism (Bahnson *et al*., 2002) and NADH dehydrogenase, which play roles in energy metabolism, in addition to substrate modification (Yagi & Matsuno-Yagi, 2003). The importance of the remaining proteins, which include miHF, RNA polymerase and two lipoproteins, will be briefly summarised below.

In bacteria, bacterial integration host factors play central roles in the cellular processes of recombination, DNA replication, transcription and bacterial pathogenesis. In mycobacteria, such as *M. smegmatis* and *M. tuberculosis* they are known as mycobacterial integration host factors (miHF), which stimulates integration of mycobacteriophage L5, a temperate phage of the mycobacteria (Pedulla *et al*., 1996). It has been reported that *miHF* gene, in *M. smegmatis* and *M. tuberculosis*, is highly similar, suggesting that the proteins perform important functions in the mycobacteria (Pedulla *et al*., 1996). Since this protein is most abundant, just prior to the entry of the stationary phase, one possible function may be to regulate the expression of stationary phase specific genes (Pedulla &

Hatfull, 1998). It would be of interest to investigate the role of this mycobacterial protein in MAP.

Bacterial sigma factors are classified as principal and alternative sigma factors (Lee *et al*., 2008). Principal sigma factors are essential for bacterial survival, whilst alternative sigma factors mediate the adaptive response of bacteria to the extracelluler environment. Members of alternative sigma factors are of interest, as potential regulators of virulence factors in bacterial pathogens (Lee *et al*., 2008). In this study, protein encoding for sigma factor SigB, which belongs to the alternative sigma factor group, was identified. This gene has been reported to play a role in general stress conditions including heat and cold shock of *M. tuberculosis* (Lee *et al*., 2008). A different study, which analysed the role of this gene in *Brevibacterium flavum*, also showed that disruption of the gene leads to a substantially diminished growth of mutant *in vitro* (Halgasova *et al*., 2001).

Lipoproteins have long been considered immunomodulators (Huntley *et al*., 2005) and some lipoproteins of MAP have been shown to induce both cellular and humoral immune responses (Dupont *et al*., 2005; Huntley *et al*., 2005; Gioffre *et al*., 2006). Two lipoproteins, which are essential for growth *in vitro*, were found in this study. One is sulphate binding lipoproteins subI, previously referred to as sbpA (Cole *et al*., 1998), involved in sulphate uptake. Sulphate, once transported into the mycobacterial cell, is reduced into sulphide, followed by incorporation into cysteine and methionine (Wooff *et al*., 2002). Disruption of subI has been reported to be a cause of methionine auxotrophy, which in many pathogens, including mycobacteria, leads to attenuation of virulence (Bermudez *et al*., 1999; Wooff *et al*., 2002). Another lipoprotein identified in this study is glutamine binding lipoprotein glnH. GlnH gene is predicted to encode a glutamine-binding lipoprotein that is involved in the glutamine import system (Cole, 1999; Av-Gay & Everett, 2000). The availability of glutamine, in sufficient level in mycobacteria, has been suggested to be crucial for growth and survival both *in vitro* and within macrophages (Nguyen *et al*., 2005). The glnH gene was

also included in a vaccine preparation for the intracellular enteropathogen, *Lawsonia intracellularis* (Rosey *et al*., 2005). Its role in the pathogenesis of MAP has yet to be investigated.

In summary, the application of the PhoA reporter system, for the identification of MAP strain 316F exported proteins in this study, has led to the identification of 17 individual proteins. Three of the identified proteins, which include a hypothetical protein (MAP3273c) and two lipoproteins (glnH and subI), were selected for further studies. Details of these proteins and the work undertaken with them are described in Chapter 4.

CHAPTER 4

Cloning of MAP strain 316F genes encoding exported proteins into pET-26b (+) expression vector and immunogenicity testing

4.1 Abstract

The genes from three exported proteins, *glnH* (MAP3894c), *subI* (MAP2213c) and a hypothetical protein (MAP3273c), were cloned into the *E. coli* expression vector pET-26b (+) and the proteins were purified and assessed for their immunoreactivity. The open reading frame of each gene was cloned and expressed as a carboxy-terminal histidine-tagged recombinant protein. All three recombinant proteins were successfully purified by immobilized-metal affinity chromatography. The purified recombinant proteins were evaluated in Western blot analyses for reactivity against sera from sheep vaccinated with Neoparasec™ and from control unvaccinated animals. Sera from five out of eight vaccinated animals reacted with glnH, seven out of eight reacted with subI and five out of eight reacted with the hypothetical protein (MAP3273c). However, reactivity was not solely due to vaccination because (either faint or more intense) bands were found in two out of eight pre-vaccination sera reacting with glnH, seven out of eight pre-vaccination sera reacting with subI and four out of eight pre-vaccination sera reacting with the hypothetical protein (MAP3273c).

4.2 Introduction

Although a considerable amount of research has been devoted to the control of paratuberculosis, it is generally accepted that there are still no satisfactory therapeutic agents or an efficacious vaccine to combat the disease (Leroy *et al*., 2007; Bannantine *et al*., 2008b; Park *et al*., 2008; Rosseels & Huygen, 2008). Diagnostic tests, which detect the cell-mediated and humoral immune responses to infection by MAP, are available but they lack specificity and exhibit poor sensitivity (Shin *et al*., 2004; Willemsen *et al*., 2006; Leroy *et al*., 2007; Alvarez, 2009). To enable development of improved tests, Leroy and co-workers (2009) affirmed that many laboratories have chosen to substitute the current common "crude extract antigen" with purified MAP-specific subunit antigen. Thus, identification and purification of novel antigenic proteins specific to MAP is an important research focus for paratuberculosis.

Proteins are frequently produced by recombinant means, due to difficulties encountered in purifying them from their native host cells (Sorensen & Mortensen, 2005). Recombinant proteins are produced by cloning the foreign DNA encoding the protein of interest into an appropriate plasmid expression vector that will allow its expression in a desired heterologous host. The successful expression of recombinant protein, in sufficient quantities, is an essential prerequisite to any detailed downstream application. For many years, recombinant protein production technique has greatly facilitated research on the identification of antigen proteins, which may be recognised by antibodies and T cells, since it has enabled a single protein to be easily produced in various hosts (Sorensen & Mortensen, 2005). In mycobacterial research, this field was first reported by Bloom and others in 1985, who demonstrated a high-level expression of mycobacterial DNA fused to the structural protein β-galactosidase in *E. coli* (Bloom *et al*., 1985; cited by Mustafa, 2005). Since then, a number of studies have shown that mycobacterial proteins can be expressed in heterologous hosts, such as *E. coli* and purified to homogeneity for functional

studies (Ahmad *et al*., 2003; Al-Attiyah *et al*., 2004; Bannantine *et al*., 2004b; Cho *et al*., 2007).

MAP produces components that interact with the host immune system (Mullerad *et al*., 2003). Secreted or surface-exposed mycobacterial proteins are more available for interaction with antigen-presenting cells, and T and B lymphocytes than other components, indicating that they might have potential for inclusion in diagnostic tests and vaccines (Cho *et al*., 2006).

In the previous chapter, 17 exported proteins of MAP strain 316F were identified. The following three proteins were selected for further study: the glutamine binding lipoprotein (glnH) (MAP3894c), the sulphate binding lipoprotein (subI) (MAP2213c) and a hypothetical protein (MAP3273c). The proteins were selected because glnH and subI are both lipoproteins and have been shown to be crucial for growth and survival of *M. tuberculosis*, either *in vitro* or within macrophages (Av-Gay & Everett, 2000; Wooff *et al*., 2002; Nguyen *et al*., 2005). Other workers also reported the inclusion of glnH in the vaccine preparation for *L. intracellularis* (Rosey *et al*., 2005). In addition, lipoproteins of MAP have been shown to induce cellular and humoral immune responses (Dupont *et al*., 2005; Gioffre *et al*., 2006). The third protein selected was the hypothetical protein (MAP3273c), which was amongst 14 immunogenic proteins suggested to be a strong candidate for use as an antigen for diagnosis of bovine paratuberculosis (Cho *et al*., 2006). Further studies carried out by Cho and colleagues (2007) demonstrated that naturally infected cattle had significantly ($p = 0.0377$) higher mean ELISA A_{450} value to purified recombinant hypothetical protein (MAP3273c), than infection free cattle. However, the immune responses to this hypothetical protein have not been investigated in sheep.

The objective of the current study was to express and purify the three selected proteins. The open reading frame encoding the entire protein was PCR amplified, cloned into the pET-26b (+) expression vector and expressed in *E. coli* BL21-

CodonPlus® (DE3)-RP cells as a C-terminus histidine-tagged recombinant protein. The immune response to the purified recombinant proteins was evaluated using sera from sheep vaccinated with Neoparasec™ and unvaccinated control animals.

4.3 Materials and methods

4.3.1 PCR amplification of the three selected genes from MAP strain 316F

To obtain the complete nucleotide sequences of *glnH*, *subI* and MAP3273c genes from MAP strain 316F, oligonucleotide primers were first designed to the flanking regions of all genes. The forward primers were designed to be approximately 100 bp upstream of the 5' end of the predicted ORF, using the sequence obtained from the pJEM11 constructs. The reverse primers were designed to be approximately 100 bp downstream of the 3' end of the ORF, using DNA sequence available for the MAP strain K-10 genome (genome accession number NC_002944). Details of the primers are shown in Table 4.1. PCR reactions were carried out using genomic DNA template extracted from MAP strain 316F (Section 3.3.1.1), in a reaction volume of 50 µl. The conditions used were an initial denaturation step for 5 min at 95^{o}C, followed by 30 cycles of 30 sec denaturation at 95^{o}C, 30 sec primer annealing at 56^{o}C – 63^{o}C depending on melting temperature of each primer (see Table 4.1), 1 min extension at 72^{o}C, and a final extension for 7 min at 72^{o}C using Platinum® *Pfx* DNA Polymerase. A negative control, without genomic DNA added into the reaction, was also included in each PCR assay. The presence of amplification products was assessed by running 5 µl of each reaction on 1.5% agarose gels. The gel was stained with ethidium bromide and the image was captured under UV light using the Bio-Rad Gel Doc 2000 imaging system (Section 2.4.1). For the sequencing purposes, the PCR products were purified using the QIAquick Gel Extraction Kit (Section 2.4.2), quantified (Section 2.4.3) and submitted along with the primers to

the Allan Wilson Sequencing Facility of Massey University (Section 2.7). The remaining purified DNA was stored at -20°C until used.

4.3.2 Cloning of the ORFs from the three selected genes

The sequence of the ORF for each gene was amplified with a primer pair that was designed to cover the entire mature exported portion. Each primer pair, as listed in Table 4.1, included restriction enzyme sites for directional ligation into the pET-26b (+) expression vector (Cat No. 69862-3, Novagen, Madison WI). A *Nco*I site was incorporated to the 5' end of the forward primers for cloning of all genes. For the reverse primers, a *Xho*I site was added for *glnH* and *subI*, while a *Not*I site was added for MAP3273c. The stop codon (TGA) was omitted, to allow read-through to produce the histidine x 6 tag coded by the vector. PCR reactions were performed using MAP strain 316F genomic DNA as a template (Section 4.3.1). Following DNA purification, the PCR product of each gene was cloned into pCR®-Blunt II-TOPO vector, as recommended by the manufacturer. Recombinant clones were then transformed into *E. coli* Top10 chemically competent cells (Section 2.6.2.3). Following transformation of the *E. coli* Top 10 cells, the sequences were confirmed using M13 forward and reverse sequencing primers (Table 4.1). The recombinant plasmids, having the correct sequence confirmed by sequencing, were purified and subsequently digested with restriction endonuclease enzymes at room temperature for 2.5 hours (Section 2.5.1). Digests were run on the gel, gel-purified and eluted in 10 µl of distilled water. A 5 µl sample was electrophoresed in a 1% agarose gel to check its recovery and the remainder was kept at -20°C.

4.3.3 Cloning of the three selected genes into the pET-26b (+) expression vector

4.3.3.1 Preparation of the pET-26b (+) vector

The expression vector pET-26b (+) was used to express recombinant proteins in *E. coli.* This vector, depicted in Figures 4.1 and 4.2, carries an N-terminal *pelB* signal sequence for potential periplasmic localisation. It also contains a T7

promoter and multiple cloning sites for the over-expression of carboxy-terminal histidine-tagged proteins. The primers were designed so that the ORF of each protein would be in frame with the pelB leader at the N-terminus and the histidine tag at the C-terminus.

E. coli DH5α clones, harbouring pET-26b (+) plasmids, were streaked out from glycerol stocks onto LBA plates supplemented with kanamycin and incubated overnight at 37^{o}C. A single colony was used to inoculate 5 ml of LB containing kanamycin. After an overnight incubation at 37^{o}C with constant shaking, the pET26-b (+) plasmid was extracted using the QIAprep-spin plasmid Miniprep Kit (Section 2.3.1). For cloning of *glnH* and *subI*, five micrograms of pET-26b (+) vector was digested with 10 units of *Nco*I (Cat No. R0193S, New Englands Biolabs, Inc) and 10 units of *Xho*I (Cat No. R0146S, New Englands Biolabs, Inc). Similar amount of vector was also digested with 10 units of *Nco*I and 10 units of *Not*I (Cat No. R0189L, New Englands Biolabs, Inc) for cloning of MAP3273c. Digests were in a reaction volume of 20 µl at 37^{o}C for 2.5 hours. The digest was run on a 1% agarose gel, extracted using the QIAquick Gel Extraction Kit and kept at -20^{o}C for further assay.

Table 4.1 Details of primers used to amplify and to sequence the three selected genes from MAP strain 316F.

Primers[a]	Sequence (5' to 3') [b]	Reference	Annealing temperature (°C)	Product size (bp)
glnHflank-F glnHflank-R	CCGCCATCGCGGTGGCGCTG GTCGTCGCCCACCTCGGCCG	This study	59	1119
sublflank-F sublflank-R	GCCCGTGTGCGGTCTGGTTC GTGGTGCCGCGGCCGCGTC	This study	58	1234
MAP3273cflank-F MAP3273cflank-R	CCGAAAGTCCCTCCGGCTAGAG GCAGGTGCTGCCGTGGACCAG	This study	56	1179
glnHpET-F glnHpET-R	CCATGGATATGCTGGTCGCGGCGGGGTG CTCGAGGTCCAGATACCTCGGCGTGGGCG	This study	63	941
sublpET-F sublpET-R	CCATGGATGCCGTCGGCGGCACCGGG CTCGAGTCCGGTGGCCTGCGTGTAGAT	This study	61	950
MAP3273cpET-F MAP3273cpET-R	CCATGGATATGAACACCGGCTTC GCGGCCGCGCCGACCCACTC	This study	57	988
M13-F M13-R	GTAAAACGACGGCCAG CAGGAAACAGCTATGAC	Zero Blunt® PCR cloning kit user manual, 2004 (Invitrogen)	N/A	N/A
T7 promoter primer T7 terminator primer	TAATACGACTCACTATAGG GCTAGTTATTGCTCAGCGG	Novagen, Madison WI.	N/A	N/A

[a]F, forward; R, reverse
[b]The sequences underlined are restriction sites for the enzymes *Nco*I (CCATGG), *Xho*I (CTCGAG) and *Not*I (GCGGCCGC). Extra bases (blue) were added to the 5' ends of glnHpET-F, sublpET-F and MAP3273cpET-F to be in frame with the *pel*B leader.

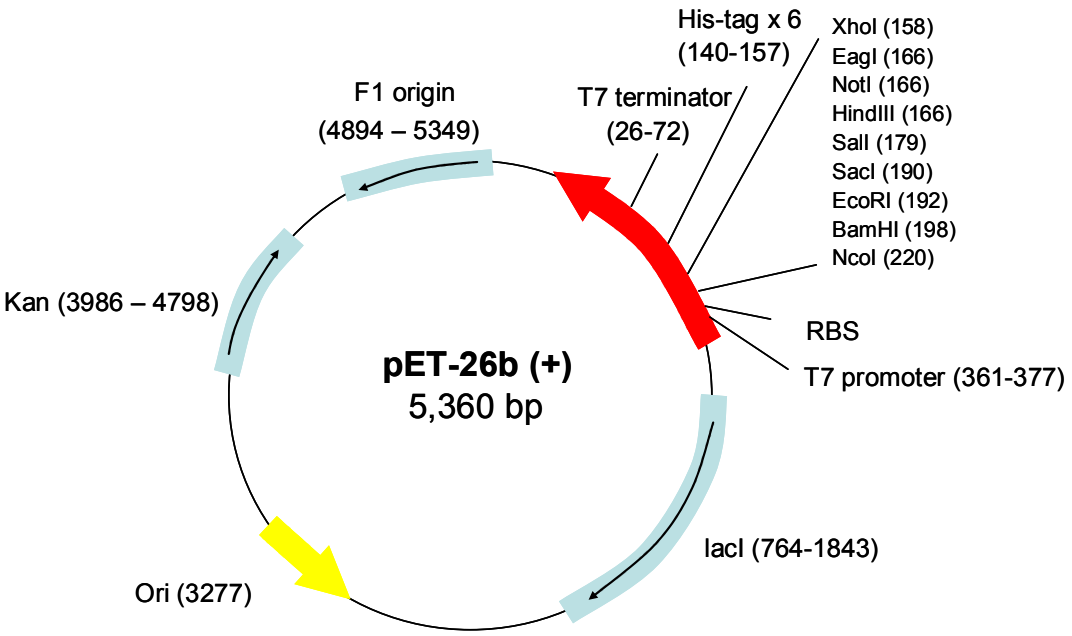


Figure 4.1 Map of the pET-26b (+) expression vector. Adapted from the pET vector manual (www.emdbiosciences.com). Important features include the presence of an *E. coli* origin (Ori, 3277), lacI coding sequence (764-1843), bacteriophage T7 promoter (361-377), multiple cloning sites (*Nco*I –*Xho*I, 158-225), C-terminal histidine x 6 tag (140-157), T7 terminator (26-72), F1 phage origin (4894-5349) and kanamycin resistance (3986-4798).

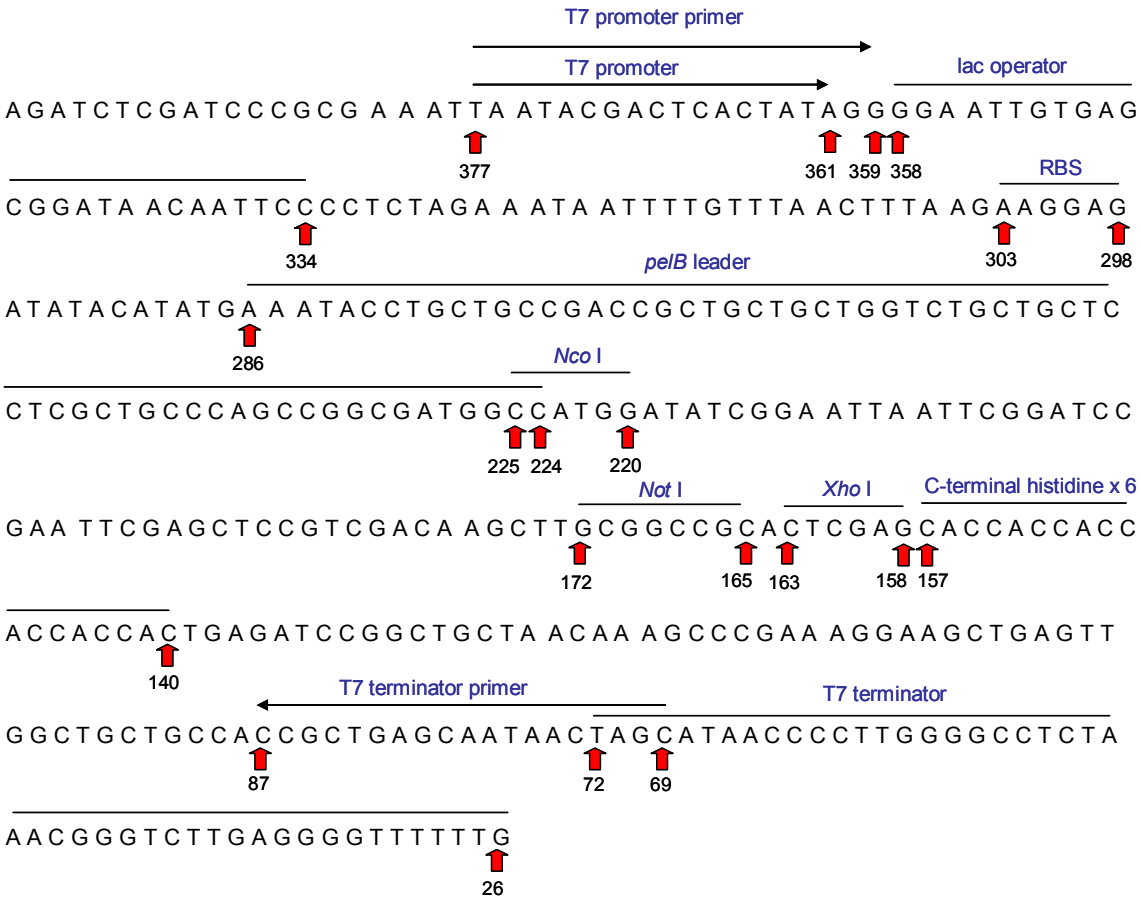


Figure 4.2 The cloning/expression region of the coding strand of pET-26b (+) vector. Adapted from the pET vector manual (www.emdbiosciences.com). T7 promoter (361-377) and its primer (359-377), T7 transcription start (360), lac operator (334-358), ribosomal binding site (RBS, 298-303), *pelB* leader (224-286), multiple cloning sites; *Nco*I site (220-225), *Not*I site (165-172), *Xho*I site (158-163), C-terminal histidine x 6 tag (140-157), T7 terminator (26-72) and its primer (69-87) are shown.

4.3.3.2 Cloning of the three selected genes into the expression vector

Fifty nanograms of digested pET-26b (+) vector and 100 ng of gel-purified products of each gene, prepared previously (Section 4.3.2), were ligated using 1:2 vector:insert ratio using Rapid DNA ligation kit (Cat No. 11635379001, Roche Applied Science) as per the manufacturer's recommendations. Ligation was performed at room temperature for 10 min in a total reaction of 21 µl. The recombinant plasmids were transformed by heat

shock transformation (Section 2.6.2.3) into *E. coli* BL21-CodonPlus® (DE3)-RP competent cells (Cat No. 230255, Stratagene, USA), which carry λDE3 lysogen containing T7 RNA polymerase under control of lacUV5 promoter for expression. Following transformation, the mixture was incubated with 900 ml of SOC media at 37°C for one hour with shaking at 225 rpm and then plated on LBA supplemented with kanamycin and chloramphenicol. The plates were incubated overnight at 37°C, and ten transformant colonies, from each pET26b-glnH, pET26b-subI and pET26b-MAP3273c, were picked and inoculated into 5 ml of LB containing kanamycin and chloramphenicol. Plasmid DNA was extracted (Section 2.3.1) from 4 ml of broth culture and resuspended in 50 µl of elution buffer. Three microlitres of plasmid was subjected to restriction enzymes endonuclease digestion (Section 2.5.1) and analysed on a 1% agarose gel, to select the plasmids potentially carrying correctly orientated inserts. The reading frame and the sequence of the inserts were confirmed, by sequencing a selected plasmid of each transformation. The plasmid-specific primers, T7 promoter and T7 terminator primers (Table 4.1), were used for sequencing. Colonies containing the correct insert were grown in LB containing appropriate antibiotics and stored as glycerol stocks at -80°C (Section 2.2.4).

4.3.4 Expression and purification of the selected recombinant proteins from *E. coli*

Glycerol stocks of *E. coli* BL21-CodonPlus® (DE3)-RP cells, which carry pET26b-glnH, pET26b-subI and pET26b-MAP3273c plasmids, were used for the expression of histidine-tagged fusion proteins as described in Section 2.8.2. The expressed proteins were analysed by SDS-PAGE (Section 2.8.2), and the solubility of the recombinant proteins was tested as described in Section 2.8.2.1. The expressed proteins were insoluble; hence they were purified under denaturing conditions with buffer containing 6M urea using BD Talon™ Metal Affinity Resin (Section 2.8.3.2). The flow through, wash and eluted fractions were electrophoresed on NuPAGE® 12% Bis-Tris gel and stained with Coomassie blue (Section 2.9.2). Fractions containing each of the purified protein were pooled and dialysed against six changes of PBS containing decreasing amount of urea (6 M to 0 M). Dialysis was carried out

as described in Section 2.8.3.3 using Spectra/Por® Regenerated Cellulose Membranes (Spectrum Lab. Inc., Rancho Dominguez, CA, USA) with a molecular-weight-cut-off (MWCO) of 12-14 kDa (Cat No. 132678). The dialysed samples were filter-sterilised through a 0.22 µm syringe filter, gel-checked and quantified. The proteins were then aliquoted and kept at -20°C for future use.

4.3.5 Western blot analyses of the selected recombinant proteins

Approximately 250 ng of purified protein was loaded on to each lane of NuPAGE® 12% Bis-Tris gel (Section 2.9.2) and electrophoresed. The resulting protein bands were transferred onto PVDF membrane by the semi-dry transfer method (Section 2.9.3). The efficiency of transfer was visualised by staining with Ponceau red. For detection of histidine-tagged proteins, mouse anti-histidine x 6 conjugated monoclonal antibody (Cat. No.OB05, Calbiochem, Germany) was added to blots at 1:1000 (v/v) dilutions and incubated at room temperature for one hour with gentle shaking. Blots were subsequently washed for five 5-10 min cycles, with washing buffer, and developed.

For detection of antibody to the three selected recombinant proteins, approximately 1 µg of each recombinant protein was electrophoresed alongside a molecular weight marker on NuPAGE® 12% Bis-Tris gels. Following transfer to PVDF membrane, the membrane was stained with Ponceau S to visualise the bands. The lanes were then cut into strips, numbered and subjected to Western blotting. Briefly, each single strip was incubated overnight at 4°C with 10 ml of serum (see Section 4.3.6) diluted 1:200 in blocking buffer on an individual small tray. Following washing, the strips were then incubated with 10 ml of peroxidase-labelled goat anti-sheep whole IgG antibody (diluted 1:20,000) for one hour at room temperature. After another cycle of washing, the immuno-reactive protein bands were developed with chemiluminescence reagent as described in Section 2.9.3. Western blots were done twice with each sample.

4.3.6 Serum samples

Sera for Western blot analyses were collected from a sheep vaccination trial carried out in 2007 approved by the Animal Ethics Committee of Massey University in Palmerston North (MUAEC # 07/13 dated on 16/02/07). Fourteen Romney wethers, purchased through Agricultural Research Services of Massey University, were ear-tagged for identification, housed at the Massey University Animal Research Facilities and fed on pasture with water *ad libitum* throughout the study. At eight months of age, eight sheep were chosen randomly (with negative responses to PPDA using the IFN-gamma Bovigam® assay) and vaccinated subcutaneously in the right side of the upper neck with Neoparasec™ (Merial, France), as recommended by the vaccine manufacturer. The remaining six sheep were kept as unvaccinated controls. The delegated sheep numbers used in the vaccination trial are shown in Table 4.2.

Table 4.2 Sheep used in the 2007 vaccination trial.

Sheep number	
Neoparasec vaccinated	**Unvaccinated (control)**
102	101
114	117
119	124
129	125
135	139
154	142
155	
163	

A blood sample was taken from the jugular vein of each sheep using non-heparinised 10 ml vacutainers (Cat No. 366430; Betckton Dickinson) and was allowed to clot at room temperature for two hours. Following centrifugation at 3,000 x *g* for 30 min, the serum was collected and aliquoted into 200µl volumes before storage at -80^{o}C.

4.4 Results

4.4.1 Sequence analysis of the three selected genes

The PCR products of the selected genes and their flanking region are depicted in Figure 4.3. Three major bands, of approximate sizes 1119 bp, 1234 and 1179 bp, were observed for *glnH*, *subI* and MAP3273c genes respectively. All PCR products were sequenced in the TOPO plasmid and the alignment of the nucleic acid sequences confirmed that *glnH*, *subI* and MAP3273c genes amplified from MAP strain 316F showed 100% identity to MAP strain K-10. The DNA and translated sequence of the defined ORF of all three genes is presented in Figure 4.4 to Figure 4.6.

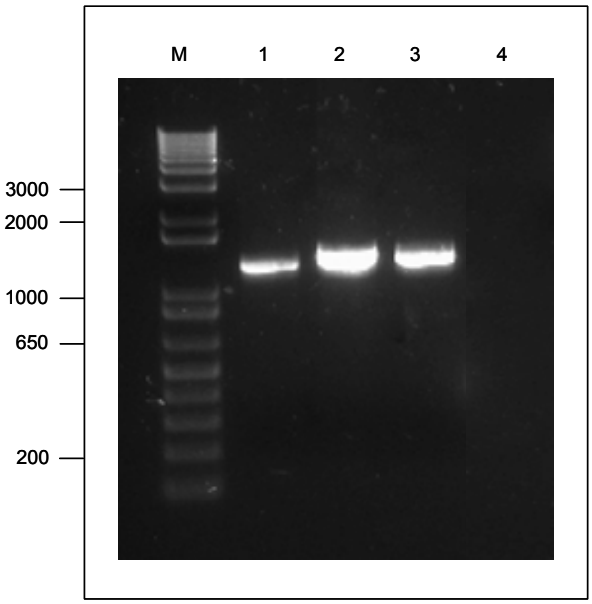


Figure 4.3 Agarose gel showing PCR amplification of *glnH*, *subI* and MAP3273c genes and their flanking regions from MAP strain 316F genomic DNA. Five microlitres of each PCR product was electrophoresed on 1.5% agarose gels, stained with ethidium bromide and examined under UV light. Lane M, 1 kb plus DNA ladder marker (bp); lane 1, *glnH* gene and its flanking region; lane 2, *subI* gene and its flanking region; lane 3, MAP3273c gene and its flanking region; lane 4, negative control (water).

atg	ctg	gtc	gcg	gcg	ggg	tgc	ggc	cac	acc	gag	tcg	ctg	cgg	gtg
M	L	V	A	A	G	C	G	H	T	E	S	L	R	V
gcc	agc	gtg	ccg	acg	ttg	ccg	ccg	ccc	acc	ccg	gtc	ggc	atg	gag
A	S	V	P	T	L	P	P	P	T	P	V	G	M	E
cag	ctg	ccg	ccg	cag	ccg	ccg	ctg	ccg	ccc	gac	ggc	ccg	gac	cag
Q	L	P	P	Q	P	P	L	P	P	D	G	P	D	Q
aac	tgc	gac	ctg	acg	gcc	agc	ctg	cga	ccc	ttc	ccc	acc	aag	gcg
N	C	D	L	T	A	S	L	R	P	F	P	T	K	A
gag	gcc	gac	gcc	gcg	gtg	gcc	gac	atc	cgc	gcc	cgc	ggc	cgg	ctg
E	A	D	A	A	V	A	D	I	R	A	R	G	R	L
atc	gtc	ggg	ctc	gac	atc	ggc	agc	aac	ctg	ttc	agc	ttc	cgc	gac
I	V	G	L	D	I	G	S	N	L	F	S	F	R	D
ccg	atc	acc	ggc	gag	atc	acc	ggc	ttc	gac	gtc	gac	atc	gcc	ggc
P	I	T	G	E	I	T	G	F	D	V	D	I	A	G
gag	atc	gcc	cgc	gac	atc	ttc	ggc	gcg	ccc	tcg	cac	gtc	gag	tac
E	I	A	R	D	I	F	G	A	P	S	H	V	E	Y
cgg	atc	ctg	tcg	tcg	gac	gag	cgg	gtc	acc	gcg	ctg	cag	cgc	ggc
R	I	L	S	S	D	E	R	V	T	A	L	Q	R	G
gag	gtc	gac	gtc	gtg	gtc	aag	acc	atg	acc	atc	acc	tgc	gac	cgg
E	V	D	V	V	V	K	T	M	T	I	T	C	D	R
cgc	aag	cag	gtg	aac	ttc	tcc	acc	gtg	tac	ctc	gat	gcc	aac	cag
R	K	Q	V	N	F	S	T	V	Y	L	D	A	N	Q
cgc	atc	ctg	gcc	cca	cgc	gac	tcg	ccg	atc	acc	aag	gtc	tcc	gac
R	I	L	A	P	R	D	S	P	I	T	K	V	S	D
ctg	tcc	ggc	aaa	cgc	gtc	tgc	gtg	gcc	aag	ggc	acc	acg	tcg	ctg
L	S	G	K	R	V	C	V	A	K	G	T	T	S	L
cac	cgg	atc	cgg	cag	atc	gat	ccc	ccg	ccg	atc	gtg	gtg	tcg	gtg
H	R	I	R	Q	I	D	P	P	P	I	V	V	S	V
gtc	aac	tgg	gcc	gat	tgc	ctg	gtc	gcc	atg	cag	cag	cgc	gag	atc
V	N	W	A	D	C	L	V	A	M	Q	Q	R	E	I
gac	gcc	gtc	agc	acc	gac	gac	tcg	atc	ctg	gcc	ggg	ttg	gtc	gaa
D	A	V	S	T	D	D	S	I	L	A	G	L	V	E
gag	gac	ccg	tac	ctg	cac	atc	gtc	ggg	ccg	aac	atg	gcc	acc	cag
E	D	P	Y	L	H	I	V	G	P	N	M	A	T	Q
ccc	tac	ggc	atc	ggg	atc	aac	ctg	aac	aac	acc	ggg	ttg	gtg	cgc
P	Y	G	I	G	I	N	L	N	N	T	G	L	V	R
ttc	gtc	aac	gga	acg	ctg	gag	cgg	atc	cgc	cgg	gac	ggc	acc	tgg
F	V	N	G	T	L	E	R	I	R	R	D	G	T	W
aac	acg	ttg	tac	cgc	aag	tgg	ttg	acc	gtg	ctg	ggc	ccc	gcg	ccg
N	T	L	Y	R	K	W	L	T	V	L	G	P	A	P
gcc	ccg	ccc	acg	ccg	agg	tat	ctg	gac	tga					
A	P	P	T	P	R	Y	L	D	*					

Figure 4.4 Sequence analysis of the *glnH* ORF. The predicted start codon is in red. The cloned portion of the ORF in the original pJEM11 phoA^{+} construct (Mptb57) is indicated in green. The entire ORF is 930 bp in length. The corresponding translated 310 amino acid sequence is shown as in one-letter and derived from the DNA sequence.

atg	gac	atc	agg	acc	gca	gcg	cgc	tgg	cgg	cct	gtt	ctc	gcc	ctt
M	D	I	R	T	A	A	R	W	R	P	V	L	A	L
gtt	ctc	acc	gcc	ggt	gtc	gtg	gcc	gga	tgc	cac	ggc	ggc	gcc	agc
V	L	T	A	G	V	V	A	G	C	H	G	G	A	S
gat	gcc	gtc	ggc	ggc	acc	ggg	ccg	gcc	gac	gcg	cgc	acc	agc	atc
D	A	V	G	G	T	G	P	A	D	A	R	T	S	I
acg	ctg	gtc	gcc	tac	tcg	gtc	cca	gaa	ccc	gga	tgg	agc	aag	ata
T	L	V	A	Y	S	V	P	E	P	G	W	S	K	I
att	ccg	gcg	ttc	aac	gcc	tcc	gac	gag	ggc	aag	ggc	atc	cag	gtg
I	P	A	F	N	A	S	D	E	G	K	G	I	Q	V
gtc	acc	tcc	tac	ggg	gcc	tcc	ggt	gac	caa	tcc	cgc	ggc	gtg	gtg
V	T	S	Y	G	A	S	G	D	Q	S	R	G	V	V
gac	ggc	aaa	ccc	gcc	gac	gtg	gtg	aac	ttc	tcc	gtc	gaa	ccc	gac
D	G	K	P	A	D	V	V	N	F	S	V	E	P	D
atc	gct	cgc	ctg	gtc	aag	gcc	ggc	aag	gtc	gcc	aag	gat	tgg	aac
I	A	R	L	V	K	A	G	K	V	A	K	D	W	N
acc	gac	gcc	acc	aag	ggg	atc	ccg	ttc	ggc	tcg	gtg	gtc	acg	ctg
T	D	A	T	K	G	I	P	F	G	S	V	V	T	L
gtg	gtg	cgc	aag	ggc	aat	ccg	aag	cac	atc	aag	gat	tgg	gac	gac
V	V	R	K	G	N	P	K	H	I	K	D	W	D	D
ctg	ttg	cgg	ccc	ggc	gtc	gag	gtg	atc	acg	ccc	agc	ccg	ctc	agt
L	L	R	P	G	V	E	V	I	T	P	S	P	L	S
tcc	ggg	tcg	gcc	aag	tgg	aat	ctg	ctg	gcg	ccg	tac	gcc	gtc	aag
S	G	S	A	K	W	N	L	L	A	P	Y	A	V	K
agc	gag	ggc	ggc	gcg	cac	ggc	gac	gcc	ggc	gtg	gac	ttc	atc	cgg
S	E	G	G	A	H	G	D	A	G	V	D	F	I	R
aag	ttg	gtg	acc	gaa	cac	gtc	aag	ttg	cgc	ccg	gga	tcg	ggg	cgc
K	L	V	T	E	H	V	K	L	R	P	G	S	G	R
gag	gcc	acc	gac	gtc	ttc	gtg	cag	ggc	agc	ggt	gac	gtg	ttg	atc
E	A	T	D	V	F	V	Q	G	S	G	D	V	L	I
agc	tac	gag	aac	gag	gcc	atc	gcc	acc	gag	cgg	gcg	gga	aag	ccg
S	Y	E	N	E	A	I	A	T	E	R	A	G	K	P
gtc	gag	cac	ctc	aat	ctg	gcg	cag	acc	ttc	aag	atc	gac	aac	ccg
V	E	H	L	N	L	A	Q	T	F	K	I	D	N	P
gtc	gcg	gtg	gtc	aac	acc	agc	ccg	cac	ctg	cag	gcc	gcc	gtc	gcg
V	A	V	V	N	T	S	P	H	L	Q	A	A	V	A
ttc	aag	aac	ttc	cag	tac	acc	gcg	gcg	gcc	caa	aag	gtt	tgg	gcg
F	K	N	F	Q	Y	T	A	A	A	Q	K	V	W	A
caa	gcc	ggt	ttc	cgg	ccg	gtc	gac	ccg	gcc	gtc	gcc	gcc	gac	ttc
Q	A	G	F	R	P	V	D	P	A	V	A	A	D	F
cgc	gac	cag	tat	ccg	gtg	ccg	gcg	aaa	ctg	tgg	acc	atc	gcc	gat
R	D	Q	Y	P	V	P	A	K	L	W	T	I	A	D
ctg	ggc	ggc	tgg	agc	gcc	gcg	gac	ccg	cag	ctg	ttc	gac	aag	aac
L	G	G	W	S	A	A	D	P	Q	L	F	D	K	N
acc	ggc	agc	atc	acc	aag	atc	tac	acg	cag	gcc	acc	gga	tga	
T	G	S	I	T	K	I	Y	T	Q	A	T	G	*	

Figure 4.5 Sequence analysis of the *subI* ORF. The predicted start codon is in red. The cloned portion of the ORF in the original pJEM11 phoA^{+} construct (Mptb57) is indicated in green. The entire ORF is 930 bp in length. The corresponding translated 310 amino acid sequence is shown as in one-letter and derived from the DNA sequence.

atg	aac	acc	ggc	ttc	ccg	gat	ccg	gaa	acg	gtc	cgc	acg	gca	ctg
M	N	T	G	F	P	D	P	E	T	V	R	T	A	L
gcg	ctg	gcg	tct	cga	gcc	ccc	tcg	gtg	cac	aac	acc	caa	ccg	tgg
A	L	A	S	R	A	P	S	V	H	N	T	Q	P	W
cgg	tgg	cgc	atc	gat	ccg	gcc	ggc	ctg	cac	ctg	tac	gcc	gac	ccc
R	W	R	I	D	P	A	G	L	H	L	Y	A	D	P
gcc	cgg	caa	ctg	ccc	cac	acc	gac	ccc	gac	ggc	cgc	gac	ttg	atc
A	R	Q	L	P	H	T	D	P	D	G	R	D	L	I
ctg	agt	tgc	ggc	atc	gcc	ctg	cag	cac	tgc	gtc	acc	gcg	ttc	gcc
L	S	C	G	I	A	L	Q	H	C	V	T	A	F	A
gcc	gtc	ggc	tgg	cgg	tcc	agg	gtg	cgc	agg	ctg	ccg	gat	ccc	gac
A	V	G	W	R	S	R	V	R	R	L	P	D	P	D
gac	ccg	gat	cac	ctt	gcg	acg	ctt	gag	ttt	tcg	ccc	cag	acc	gcc
D	P	D	H	L	A	T	L	E	F	S	P	Q	T	A
gac	tac	gtc	gac	gtc	gcg	ctc	gcc	gcc	gcg	atc	ccg	cgc	cgg	cgc
D	Y	V	D	V	A	L	A	A	A	I	P	R	R	R
acc	gac	cgg	cgc	cac	tac	agc	tgt	tgg	ccg	gtg	ccg	gtc	ggc	gac
T	D	R	R	H	Y	S	C	W	P	V	P	V	G	D
atc	gcg	ctg	atg	gcg	gcc	cgg	gcg	gcc	cgc	gcc	ggg	gtg	acg	ctg
I	A	L	M	A	A	R	A	A	R	A	G	V	T	L
tac	cag	gtg	cag	gac	gtc	gac	ggc	ctg	cac	gac	atc	gtc	gcg	caa
Y	Q	V	Q	D	V	D	G	L	H	D	I	V	A	Q
tcg	atc	cgc	gac	cac	ctg	acc	cag	gat	tat	ctg	gcc	gag	ctg	acc
S	I	R	D	H	L	T	Q	D	Y	L	A	E	L	T
acc	tgg	agt	ggg	cgc	tac	ttc	tcg	gtg	tcc	ggg	gtg	ccc	gcc	cgc
T	W	S	G	R	Y	F	S	V	S	G	V	P	A	R
aac	acc	ccg	ttc	ccg	gat	ccg	gcc	gcc	aag	atc	ccg	acc	cgg	ttg
N	T	P	F	P	D	P	A	A	K	I	P	T	R	L
ttc	gcc	ggc	gcg	gcg	ctg	ccg	atg	gcg	cag	ggg	tcg	tcg	gcc	gcc
F	A	G	A	A	L	P	M	A	Q	G	S	S	A	A
gac	gac	aac	gcc	gtg	gtg	ctc	gcg	ctg	ggc	acc	cgc	cac	gac	gac
D	D	N	A	V	V	L	A	L	G	T	R	H	D	D
cgg	ctg	gcg	cgg	ctg	cgc	gcc	ggc	gag	gcc	acc	ggg	gtg	gtg	ctg
R	L	A	R	L	R	A	G	E	A	T	G	V	V	L
ctc	acc	gcc	acc	tcg	gcg	ggg	ctg	gcc	agc	tgc	ccg	gtc	acc	gaa
L	T	A	T	S	A	G	L	A	S	C	P	V	T	E
ccg	ctg	gag	acg	ccg	cag	acg	cgc	gcg	gcg	gtg	cgc	gcc	gac	atc
P	L	E	T	P	Q	T	R	A	A	V	R	A	D	I
ttc	ggt	gac	ggc	cac	cat	ccg	cag	atg	ctg	ctg	cgg	gtg	ggt	tgg
F	G	D	G	H	H	P	Q	M	L	L	R	V	G	W
gcg	ccg	atc	aac	gcg	gac	ccg	ttg	ccg	gcg	acc	ccg	cgc	cgc	gac
A	P	I	N	A	D	P	L	P	A	T	P	R	R	D
ctc	gcc	gac	ttc	gtc	gag	tgg	gtc	ggc	tga					
L	A	D	F	V	E	W	V	G	*					

Figure 4.6 Sequence analysis of the MAP3273c ORF. The predicted start codon is in red. The cloned portion of the ORF in the original pJEM11 phoA^{+} construct (Mptb257) is indicated in green. The entire ORF is 975 bp in length. The corresponding translated 325 amino acid sequence is shown as in one-letter and derived from the DNA sequence.

4.4.2 Cloning of the selected genes

To express recombinant proteins, the three selected genes were PCR amplified as described in Section 4.3.2. PCR products were cloned separately into pCR®-Blunt II-TOPO vector and subsequently transformed into *E. coli* Top 10 competent cells. Plasmid DNA of each gene was sequenced to ensure that no base pair changes in the sequences had occurred. DNA sequencing confirmed the identity of the cloned genes. Each insert having correct sequence was ligated to the linearised vector (Figure 4.7) and the construct was used to transform BL21-CodonPlus® (DE3)-RP *E. coli* cells. DNA sequencing of the inserts confirmed the identity of the cloned genes and that the correct reading frame was in place for expression.

4.4.3 Expression and purification of selected recombinant proteins

To aid purification and detection of the recombinant proteins, glnH, subI and the hypothetical protein (MAP3273c) were expressed with a C-terminal histidine x 6 tag, by induction of the expression vector. As shown in Figure 4.8, all three recombinant proteins were successfully expressed, as histidine-tagged fusion proteins, after IPTG induction of *E. coli* BL21-CodonPlus® (DE3)-RP cells carrying plasmids pET26b-glnH, pET26b-subI and pET26b-MAP3273c. The molecular masses prediction of histidine-tagged glnH, subI and the hypothetical protein (MAP3273c), calculated using ExPASY protein tool website, were 34.74 kDa, 34.11 kDa and 36.67 kDa respectively. SDS-PAGE analysis showed that the purified recombinant hypothetical protein (MAP3273c) was of the expected size but the glnH and subI migrated slower than predicted.

The yield of purified proteins, from 500 ml culture media, were variable ranging from 2.82 mg to 8.7 mg. Purification of the recombinant hypothetical protein (MAP3273c) with TALON affinity resin gave the highest total protein (8.7 mg). The glnH and subI recombinant proteins gave total protein levels of 3.51 mg and 2.82 mg respectively.

SDS-PAGE analysis, presented in Figure 4.9, revealed that the proteins were purified in sufficient quantity and purity for further analysis. The recombinant proteins were clearly recognised by the monoclonal antibody against tetra-histidine in immunoblot analysis (Figure 4.10).

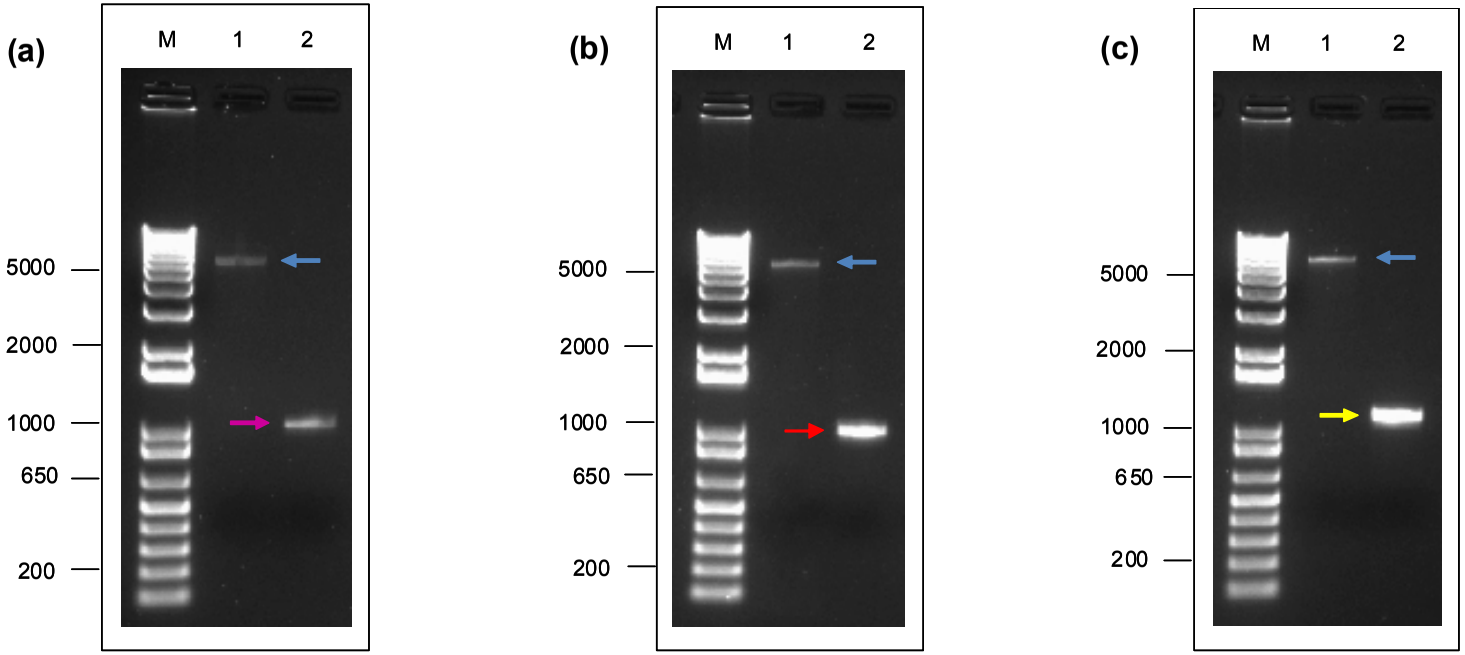


Figure 4.7 Agarose gel showing the pET-26b (+) vector and PCR products, *glnH*, *subI* and MAP3273c, used for cloning. The products were electrophoresed on a 1% agarose gel, stained with ethidium bromide and photographed under UV light. **(a)** Lane M, 1 kb plus DNA ladder marker (bp); lane 1, 3 µl of *Nco*1 and *Xho*1 digested vector; lane 2, 5 µl of *Nco*1 and *Xho*1 digested and gel purified *glnH* product. **(b)** Lane M, 1 kb plus DNA ladder marker (bp); lane 1, 3 µl of *Nco*1 and *Xho*1 digested vector; lane 2, 5 µl of *Nco*1 and *Xho*1 digested and gel purified *subI* product. **(c)** Lane M, 1 kb plus DNA ladder marker (bp); lane 1, 3 µl of *Nco*1 and *Not*1 digested vector; lane 2, 5 µl of *Nco*1 and *Not*1 digested and gel purified MAP3272c product. Blue, pink, red and yellow arrows indicate pET-26b (+) vector, PCR product *glnH*, PCR product *subI* and PCR product MAP3273c respectively.

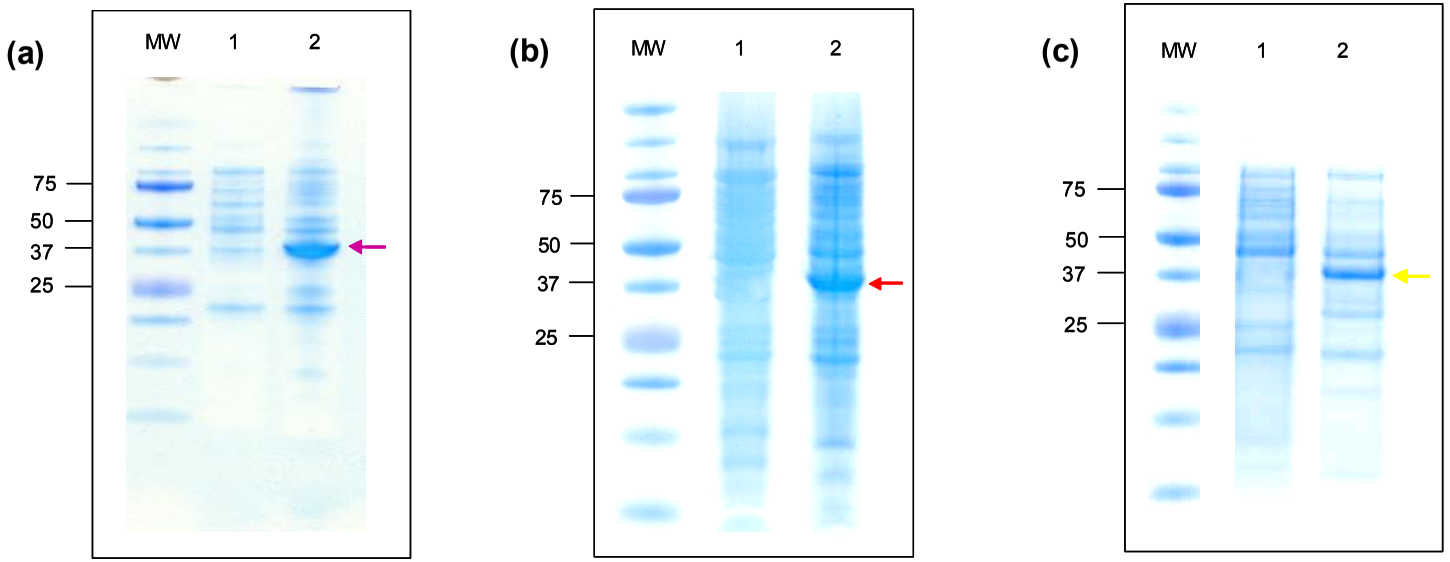


Figure 4.8 Acrylamide gels showing over-expression of the three selected proteins as histidine-tagged recombinant proteins in pET-26b (+) from *E. coli*. Protein expression was induced with 1mM IPTG and the cells were harvested before and 5 hours after induction. Twelve microlitres of resuspended cells from before and after induction were run on NuPAGE® 12% Bis-Tris gel and stained with Simply Blue™ Safe stain. **(a)** glnH recombinant protein. **(b)** subI recombinant protein. **(c)** recombinant hypothetical protein (MAP3273c). Lane MW, Precision Plus Protein Standards Dual Color (kDa); lane 1, uninduced sample of the glnH, sub I and the hypothetical proteins; lane 2, 5 hours induced sample of the glnH, sub I and the hypothetical proteins. Pink, red and yellow arrows indicate the positions of recombinant proteins glnH, subI and the hypothetical protein (MAP3273c) respectively.

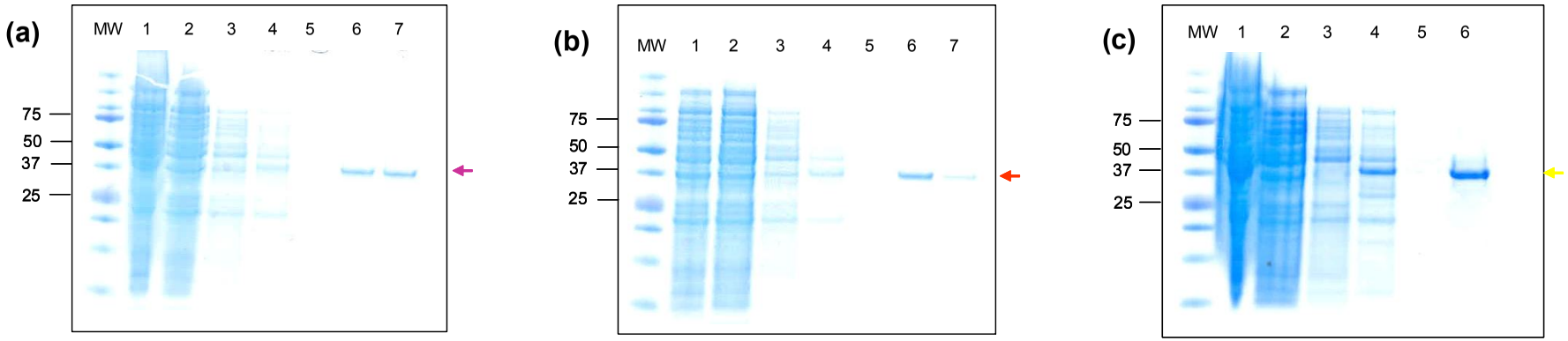


Figure 4.9 Acrylamide gels showing purification of the three selected proteins as histidine-tagged recombinant proteins from *E. coli*. Sonicated cell lysates of *E. coli* harbouring the plasmids pET26b-glnH **(a)**, pET26b-subI **(b)**, and pET26b-MAP3273c **(c)** were purified by immobilized-metal affinity chromatography under denaturing conditions. Eight microlitres of each fraction collected was analysed by SDS-PAGE and stained with Coomassie blue. Lane MW, Precision Plus Protein Standards Dual Color (kDa); lane 1, cell lysates; lane 2, flow-through fraction; lane 3, wash I fraction; lane 4, wash II fraction; lane 5, wash III fraction; lane 6, elution I fraction; lane 7, elution II fraction. Pink, red and yellow arrows indicate the positions of recombinant proteins glnH, subI and the hypothetical protein (MAP3273c) respectively.

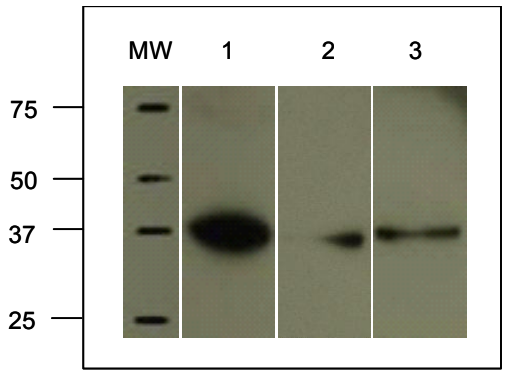


Figure 4.10 Immunodetection of purified recombinant proteins with anti-histidine monoclonal antibody. Mouse anti-histidine x 6 conjugated monoclonal antibody was used at 1:1000. Lane MW, Precision Plus Protein Standards Dual Color (kDa); lane 1, recombinant hypothetical protein (MAP3273c); lane 2, recombinant glnH protein; lane 3, recombinant subI protein.

4.4.4 Humoral immune responses to the purified recombinant proteins

Immune responses to the purified recombinant proteins were investigated using Neoparasec™ vaccinated animals (Section 4.3.6). All the sera samples collected pre-vaccination and 8 weeks post-vaccination were used in the Western blot analysis (Figure 4.11 to Figure 4.13). A summary of Western blot results is given in Table 4.3.

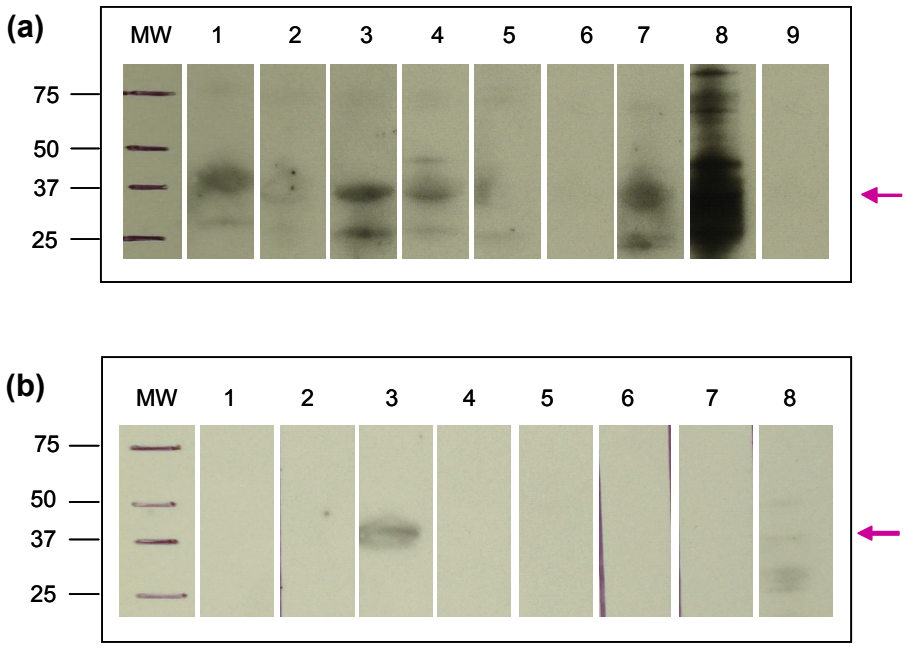


Figure 4.11 Immunodetection of serum IgG antibodies against recombinant glnH in sheep vaccinated with Neoparasec™. Blots of recombinant glnH were individually incubated with 1:200 dilution of sera. Anti-sheep IgG peroxidase conjugated antibody was used at 1:20,000. **(a)** Sera from sheep 8 weeks after vaccination with Neoparasec. **(b**) Sera from pre-vaccinated sheep. Numbers at the left indicate molecular masses in kDa. Lane MW, Precision Plus Protein Standards Dual Color marker; lane 1 to 8, sheep number 102,114, 119, 129, 135, 154, 155 and 163 respectively; lane 9, control conjugate (secondary antibody was omitted). Pink arrows point to the position of recombinant glnH bands.

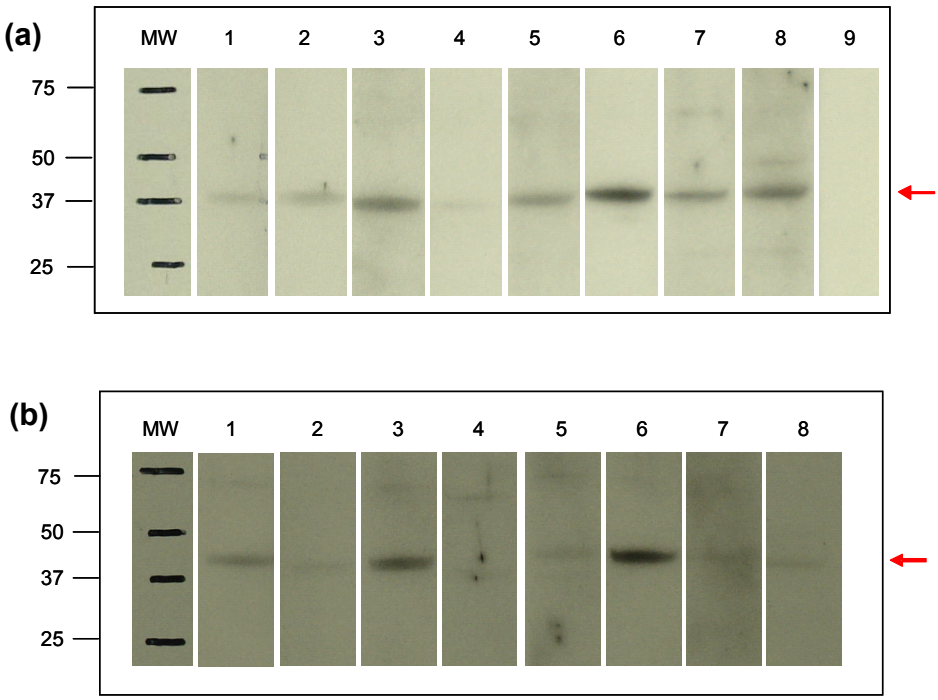


Figure 4.12 Immunodetection of serum IgG antibodies against recombinant subI in sheep vaccinated with Neoparasec™. Blots of recombinant subI were individually incubated with 1:200 dilution of sera. Anti-sheep IgG peroxidase conjugated antibody was used at 1:20,000. **(a)** Sera from sheep 8 weeks after vaccination with Neoparasec. **(b**) Sera from pre-vaccinated sheep. Numbers at the left indicate molecular masses in kDa. Lane MW, Precision Plus Protein Standards Dual Color marker; lane 1 to 8, sheep number 102,114, 119, 129, 135, 154, 155 and 163 respectively; lane 9, control conjugate (secondary antibody was omitted). Red arrows point to the position of recombinant subI bands.

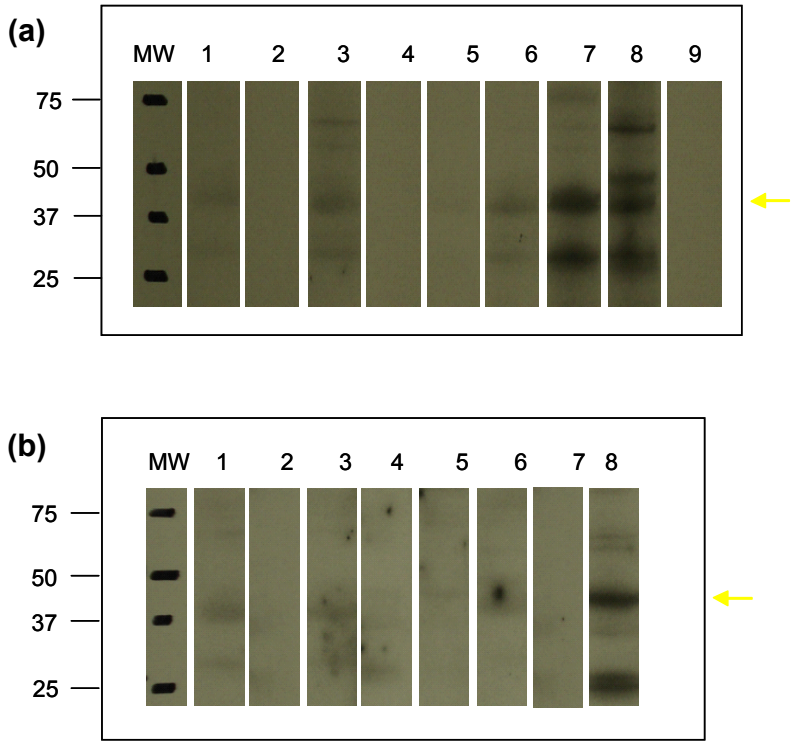


Figure 4.13 Immunodetection of serum IgG antibodies against recombinant hypothetical protein (MAP3273c) in sheep vaccinated with Neoparasec™. Blots of recombinant hypothetical protein were individually incubated with 1:200 dilution of sera. Anti-sheep IgG peroxidase conjugated antibody was used at 1:20,000. **(a)** Sera from sheep 8 weeks after vaccination with Neoparasec. **(b)** Sera from pre-vaccinated sheep. Numbers at the left indicate molecular masses in kDa. Lane MW, Precision Plus Protein Standards Dual Color marker; lane 1 to 8, sheep number 102,114, 119, 129, 135, 154, 155 and 163 respectively; lane 9, control conjugate . Yellow arrows point to the position of recombinant hypothetical protein (MAP3273c) bands.

Table 4.3 Western blot analysis of purified recombinant proteins using sera obtained 8 weeks after Neoparasec™ vaccination and pre-vaccinated control sheep.

Sheep number	Reactivity of serum to the following recombinant protein:		
	glnH	**subI**	**Hypothetical protein (MAP3273c)**
Vaccinated			
102	+	+	+ (faint)
114	-	+	-
119	+	+	+
129	+	-	-
135	-	+	-
154	-	+	+
155	+	+	+
163	+	+	+
Pre-vaccinated			
102	-	+	+
114	-	+ (faint)	-
119	+	+	+ (faint)
129	-	-	-
135	-	+ (faint)	-
154	-	+	+ (faint)
155	-	+ (faint)	-
163	+ (faint)	+ (faint)	+

Table 4.3 shows that sera from five vaccinated animals reacted with the glnH recombinant protein. However, two of the pre-vaccination animals (number 119 and 163) also reacted to the glnH. A higher number of vaccinated animals (seven out of eight) reacted to subI protein but reactivity also occurred in the corresponding sera taken from the animals before vaccination. Antibodies to the hypothetical protein (MAP3273c) were detected in five of eight vaccinated sheep but four pre-vaccination animals showed similar response.

4.5 Discussion

Identification and characterisation of immunodominant MAP-specific antigens would assist in the development of specific diagnostic tests and, possibly, Th1-inducing subunit vaccines (Rosseels & Huygen, 2008). In the present study, three genes encoding *glnH*, *subI* and MAP3273c identified as exported proteins were cloned and produced as recombinant histidine-tagged proteins in *E. coli* and the immunoreactivity of each recombinant protein was evaluated.

For each protein, the ORF of the full-length gene was individually amplified from MAP strain 316F genomic DNA. Sequencing results showed that *glnH* (Figure 4.4), *subI* (Figure 4.5) and MAP3273c (Figure 4.6) genes amplified from MAP strain 316F matched the nucleotide sequence of the complete genome of MAP strain K-10 (genome accession No. NC_002944). To be able to express and purify all proteins on a large scale, the ORF of each gene was cloned into the pET-26b (+) expression vector and subsequently transformed into *E. coli*. Results presented in this study showed that all three recombinant proteins, as depicted in Figure 4.8, were successfully expressed as polyhistidine tag fusion proteins in *E. coli*. Gaberc-Porekar and Menart (2001) in their review stated that histidine tags seem to be compatible with all expression systems used and therefore, histidine-tagged proteins can usually be successfully produced in prokaryotic and eukaryotic organisms. However, Bannantine and co-workers (2004b), who evaluated two different affinity tags, polyhistidine and Maltose Binding Protein (MBP) for expression of MAP proteins in *E. coli*, found that all of the 21 MBP fusion proteins were successfully expressed, while only 5 of 21 MAP proteins were successfully expressed by using the polyhistidine tags. The authors believed that the cloning and expression of each of the coding regions may yield varying results with each tag as co-expression with the protein can profoundly influence stability, solubility and expression levels.

Each affinity tag has distinct advantages and disadvantages, and therefore it may be difficult to decide on the best fusion system for a specific protein of

interest. Generally, expression of recombinant proteins, as a fusion with large peptides or proteins such as MBP, can overcome problems such as toxicity in *E. coli*, low expression levels and insolubility (Sachdev & Chirgwin, 2000; Terpe, 2003). The disadvantage is that the size and immunogenicity of affinity tags may complicate any downstream immunoassays (Bannantine *et al*., 2004b). Therefore, where the protein of interest cannot be separated from the affinity tag, the inclusion of the tag itself as a control in experiments is necessary (Bannantine *et al*., 2004b). Conversely, the use of a very small peptide tags, such as a polyhistidine tag, may not be as immunogenic as large tags, and can often be used directly as an antigen in antibody production (Terpe, 2003).

A wide range of affinity tags that can facilitate purification and detection of the recombinant proteins have been developed (Stevens, 2000; Jana *et al*., 2005). The polyhistidine, which consists of six consecutive histidine residues attached to the amino or carboxy terminals of the expressed protein, is one of the most commonly used affinity tags (Gaberc-Porekar & Menart, 2001; Sorensen & Mortensen, 2005) and provides the capability of high affinity purifications using metal-chelate resins (Terpe, 2003; Tsuji *et al*., 2009). In this study, purification of recombinant histidine-tagged protein, from cellular extract using Talon® IMAC Resins, was efficient with a yield ranging from 2.82 to 8.7 mg per 500 ml culture media. The recombinant proteins after affinity purification were relatively well enriched and any co-purification contaminants were of insufficient quantity to be detected by Coomassie Brilliant blue staining (Figure 4.9). This study confirmed the usefulness of polyhistidine tag as a tool for molecular analysis and purification of recombinant protein. It has been reported that histidine tagging in combination with IMAC has become routinely used in the isolation of newly expressed proteins and in most cases, the use of histidine tags may not affect protein folding and not interfere significantly with the biological functionality (Gaberc-Porekar & Menart, 2001; Bucher *et al*., 2002). In addition, several MAP-specific antigens, such as 16.7 kDa (Mullerad *et al*., 2003), 9, 15 and 34 kDa (Willemsen *et al*., 2006), P22 (Ridgen *et al*., 2006), MAP-specific antigens

encoding MAP 1693c, MAP4308c, MAP0586c, MAP3199, MAP2677c (Leroy *et al*., 2007), MAP3968, MAP3184 and MAP1518 (Bannantine *et al*., 2008c) and MAP-specific antigens referred to antigen 5, 6 and 7 (Leroy *et al*., 2009), have been purified from *E. coli* as a polyhistidine-tagged fusion protein with a great success.

SDS-PAGE analysis revealed that two of the three purified recombinant proteins migrated slower than predicted. However, Western blot analysis with anti histidine-tag monoclonal antibody (Figure 4.12) confirmed that the over-expressed proteins were correctly identified. Size discrepancy, between the predicted size of the mature protein based on its amino acid sequence and the observed size on SDS-PAGE, has been reported previously for other proteins, for example, the mature native P22 from MAP (Dupont *et al*., 2005), ModD of MAP (Cho *et al*., 2007) and the purified recombinant U2 protein (Mukherjee *et al*., 2005). SDS-PAGE is rapid, low cost and reproducible method for quantifying, comparing and characterising protein (Gumber *et al*., 2007) but anomalous mobilities on SDS-PAGE are more common than usually acknowledged (Marshak, 1996).

Size discrepancy may be attributed to several factors. This could be an artefact of the SDS-PAGE gel, an indication of post-translational modification (Bigi *et al*., 1997; Mukherjee *et al*., 2005) or may be due to high proline composition on protein (Cho *et al*., 2007). Liljeqvist and Stahl (1999), in their review article on the production of recombinant subunit vaccine, stated that one potential drawback of using bacteria including *E. coli*, *Salmonella typhimurium*, *Vibrio cholera* and *Bacillus brevis* as production hosts is that they are unable to carry out post-translational modification. However, other beneficial properties, particularly the cost efficient production systems, make bacteria the dominating hosts for production of subunit vaccine candidates. For accurate assessment of the molecular weight of recombinant protein, it is necessary to use techniques such

as mass spectroscopy that can determine the nature of post-translational modification (if any).

A preliminary study to evaluate the capacity of the selected recombinant proteins to induce a humoral response was obtained by screening sera, obtained from sheep eight weeks after Neoparasec™ vaccination. This group was chosen, because previous work in our laboratory showed that sheep vaccinated with Neoparasec™ exhibit significant humoral and cell-mediated immune responses as early as 4 weeks after vaccination (Dupont *et al*., 2005; Ridgen *et al*., 2006). In addition, a study carried out by Waters and colleagues (2003) reported that an unknown MAP protein can be detected by antibodies from cattle just three weeks after experimental infection. Western blot analysis using purified recombinant proteins as summarised in Table 4.3 indicated that the glnH was recognised by some vaccinated animals. Five of the eight sera taken from vaccinated sheep showed a prominent band with different intensities on the blots. The differences in band intensity observed could be a reflection of the presence of varying amounts of IgG antibodies in the different sheep in response to the antigen in vaccination. A faint band was produced with one of the pre-vaccination sheep sera to the glnH recombinant protein and a more intense band was seen with another. This may be cross reactive antibody and due to exposure to other mycobacterial species. There is a possibility that epitopes may be conserved with protein from other mycobacteria and may therefore react with sera from animals exposed to other mycobacteria (Bannantine *et al*., 2004b).

Naturally MAP-infected cattle have been reported to show higher mean antibody responses (ELISA A_{450} value) to the purified hypothetical protein (MAP3273c) than uninfected animals (Cho *et al*., 2007). Western blot analysis (Figure 4.13), carried out in the study described in this chapter, showed that five out eight vaccinated sheep gave immunoreactive bands to the hypothetical protein (MAP3273c). However, bands were also seen in four (50%) of the control pre-vaccinated sheep. This result is not entirely inconsistent with the results of Cho

and colleagues because 5 out of 15 infection-free cattle in their study gave A_{450} OD values of approximately 0.5 or above (Cho *et al*., 2007). Due to the finding of antibodies in pre-vaccinated sheep to both MAP3273c protein and subI, and limited resources, these two proteins were not investigated further.

The glnH was chosen for further study. Chapter 5 describes characterisation of the MAP glutamine binding lipoprotein.

CHAPTER 5

Characterisation of the MAP glutamine-binding lipoprotein

5.1 Abstract

Preliminary results obtained from the study described in Chapter 4 indicated that the recombinant glnH protein is immunogenic as shown by Western blotting. Therefore, this recombinant protein was selected further for characterisation. Immunohistochemistry studies demonstrated that antibodies raised to the recombinant glnH protein recognised the glnH epitopes at the surface of the MAP strain 316F cells. Neoparasec™ vaccinated sheep and naturally MAP infected sheep and deer showed immunoreactive bands corresponding to the glnH protein in Western blots. However, peripheral blood mononuclear cells (PBMC) from Neoparasec™ vaccinated sheep failed to produce detectable levels of IFN-gamma in the presence of recombinant glnH. The results of ELISAs using sera obtained from vaccinated sheep, naturally MAP infected sheep and deer demonstrated that there was a significantly different ($p < 0.05$) antibody response in vaccinated sheep and naturally infected sheep and deer when compared to unvaccinated and uninfected controls.

5.2 Introduction

GlnH is thought to encode the substrate binding protein involved in glutamine importing systems and belongs to the sub-family 4, amino acid transporters, of mycobacterial ATP binding cassette (ABC) transporters (Braibant *et al*., 2000). ABC transporters are found in all species from microbes to humans. The basic structure of ABC transporters was first proposed in 1986 by Higgins and co-workers (Higgins *et al*., 1986) and it is believed that all species share a structural organisation of the transporters, which consist of a hydrophobic membrane-spanning domain (MSD) and a hydrophilic nucleotide-binding domain (NBD) (Content & Peirs, 2008). ABC transporters are generally classified into two main functional categories; exporters and importers (Braibant *et al*., 2000; Dassa & Bouige, 2001; de Jonge *et al*., 2007). The exporters are found in both prokaryotes and eukaryotes and are potentially involved in secretion of various molecules from the cytoplasm. The importers are found in prokaryotes and facilitate the uptake of extracellular molecules. In many ABC transporters, the presence of auxiliary domains, substrate binding protein (SBPs), is an essential component for transport and is required for optimal function (Davidson *et al*., 2008). SBPs are soluble proteins located in the periplamic space between the inner and outer membranes of Gram-negative bacteria. In Gram-positive bacteria, which lack an outer membrane, they are anchored by a lipid tail (Figure 5.1) in the cytoplasmic membrane (Braibant *et al*., 2000; Sutcliffe & Harrington, 2004).

ABC transporters play a wide variety of physiological roles and are of considerable medical and economic importance (Higgins, 2001). Traditionally, they have been considered to play roles in nutrient uptake and drug resistance. However, there is increasing evidence that these transport systems may have immunogenic properties or may play either direct or indirect roles in the virulence of bacteria (Garmory & Titball, 2004). There are several examples of immunogenic ABC transporter proteins from both Gram-negative and Gram-positive micro-organisms. In terms of SBPs, they include *Campylobacter jejuni* CjaA and CjaC (Pawelec *et al*., 1997; 1998), *Actinobacillus pleuropneumonia* ApaA (Martin & Hulks, 1999), *Lactobaccillus*

fermentum BspA (Turner *et al*., 1997), *Bacillus anthracis* protein (Ariel *et al*., 2002), *Streptococcus pneumonia* PsaA, PiuA and PiaA (Ogunniyi *et al*., 2000; Rapola *et al*., 2000; Brown *et al*., 2001) and *M. tuberculosis* PstS (Lefèvre *et al*., 1997).

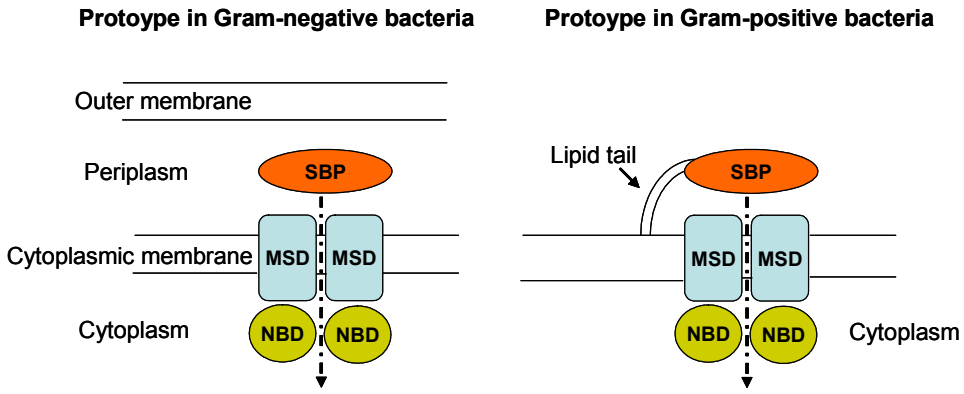


Figure 5.1 Topological organisation of prototypical importers of ABC transporters. Adapted from Braibant *et al*., 2000. MSD encodes transmembrane spanning domains. NBD encodes nucleotide binding domains and SBP indicates the substrate binding protein. The direction of translocation of substrate is indicated by an interrupted arrow.

To date, there are no published reports on the immunogenicity of the MAP glnH protein. A preliminary study described in the previous chapter (Chapter 4) to assess the immunogenicity of the three selected recombinant proteins identified as exported proteins showed that Neoparasec™ vaccinated sheep produced antibody that recognised the glnH protein in Western blot analysis. The purpose of the present study was to further characterise the glnH protein. Immunohistochemistry studies were performed using polyclonal antibodies raised against the glnH protein in rabbit. In addition, IFN-gamma, Western immunoblot and ELISA assays were carried out to evaluate cell-mediated and humoral immune responses against the glnH protein.

5.3 Materials and Methods

5.3.1 Preparation of recombinant glnH protein

The purified recombinant glnH protein described in Section 4.3.4 was used as the antigen in this study unless otherwise stated.

5.3.2 Protein sequence confirmation of the recombinant protein by mass spectrometry

Analysis of the recombinant glnH protein was carried out by the Centre for Protein Research of Otago University, New Zealand, using the MALDI tandem Time-of-Flight (MALDI-TOF) mass spectrometry procedure. Approximately 500 ng of the purified recombinant protein was run on NuPAGE® 12% Bis-Tris gel and stained as described in Section 2.9.2. The Coomassie-stained glnH protein was excised from the gel (Section 2.9.4) and mixed with 100 µl of ultra pure distilled water DNAse and RNAse free (Cat. No.10977-015, Gibco, Invitrogen Corporation) in a sterile eppendorf tube to avoid dryness during shipping. Upon receipt, the excised bands were subjected to in-gel digestion with trypsin. Procedures for in-gel trypsin digestion can be accessed through www. biochem. otago. ac. nz/cpr/ protocols.html. The resulting peptides were resuspended in 30% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid in water. One microlitre of peptide solution was premixed with an equal amount of matrix (10 mg/ml alpha cyano-4-hydroxycinnamic acid dissolved in 65% [v/v] aqueous acetonitrile containing 0.1% [v/v] trifluoroacetic acid and 10 mM ammonium hydrogen phosphate). Sample/matrix mixtures (0.8 µl) were spotted onto a MALDI sample plate and air-dried. MALDI-TOF was performed using a 4800 MALDI tandem Time-of-Flight Analyzer. Data were analysed by the Centre by submitting mass spectrometry data to the Swiss-Prot amino acid sequence database using the Mascot search engine.

5.3.3 Generation of polyclonal antibody raised to glnH

Polyclonal antibody to glnH was generated by immunising a New Zealand White rabbit with the glnH recombinant protein as described in Chapter 2 (Section 2.11.2). Pre-immune sera were collected prior to the first

immunisation. Similarly, serum was collected at 10 days after the first boost (7 weeks after initial immunisation), 12 days after the second boost (11 weeks after initial immunisation) and a final bleed 13 weeks after initial immunisation. Both pre-immune and immune sera were tested for the presence of antibodies against purified recombinant glnH protein using Western blot and ELISA.

5.3.3.1 Purification of IgG from rabbit serum

Antibody was purified using an ammonium sulphate precipitation method followed by an ion-exchange chromatograpy procedure. Three millilitres of sera obtained from the final bleed were mixed with an equal volume of PBS (0.1 M, pH 7.4). Saturated ammonium sulphate, equal to the volume of serum plus PBS (0.1 M, pH 7.4), was added slowly to the mixture while stirring. After ammonium sulphate addition, the mixture was continuously stirred for another 30 min at room temperature and centrifuged at 10,000 x g for 15 min at 4^{o}C. The supernatant was removed carefully and the pellet was resuspended in 3 ml of sodium phosphate buffer (70 mM, pH 6.3). The suspension was then dialysed against 500 ml of sodium phosphate buffer (at least two changes) at 4^{o}C over a 24 hour period.

A DEAE Sepharose™ Fast Flow (Cat. No. 17-0709-10, GE Healthcare, Sweden), a weak anion exchanger, was used to further purify the dialysate after ammonium sulphate precipitation. Two millilitres of DEAE matrix supplied in suspension in 20% ethanol were packed in a 3 ml syringe barrel with two layers of GF/A glass micro- fibre filters discs (Cat. No. 1820-055, Whatman, GE Healthcare, Sweden) at the bottom of the barrel. The column was then equilibrated with three column volumes of sodium phosphate buffer (70 mM, pH 6.3) at room temperature. One millilitre of the dialysate was applied on to the column, followed by washing with sodium phosphate buffer. Wash fractions, which would contain IgG, were collected and the absorbance of each fraction was monitored at 280 nm (A_{280}) to estimate its concentration using NanoDrop® ND-1000 spectrophotometer (Section 2.4.3). Fractions containing high IgG concentration were pooled and aliquoted into 50μl volumes before storage at -20^{o}C.

5.3.4 Multiple sequence alignment of nucleotides and amino acids of glnH

In the previous chapter (Chapter 4), primers were synthesized to amplify by PCR the *glnH* gene from MAP strain 316F genomic DNA (Section 4.3.2). The gene was then cloned and expressed in *E. coli* (Section 4.3.3 and Section 4.3.4). BlastN and BlastP searches were performed against mycobacterial genomes and all sequences were aligned with the use of the Geneious Basic version 4.7.5 software (http://geneious.com).

5.3.5 Immunohistochemistry for surface localization

5.3.5.1 Immunofluorescence assay

An indirect immunofluorescence assay was performed using exponentially growing MAP strain 316F culture (Section 2.2.2.2). A 1 ml aliquot of the culture was harvested by centrifugation at 10,000 x g for 5 min, washed three times in PBS (0.1 M, pH 7.4) and resuspended in 200 µl of PBS (0.1 M, pH 7.4). The bacterial cell suspension was homogenised with sterile glass balls (2.5 – 3.5 nm) (Cat. No.332124G, BDH Laboratory Supplies) with vigorous vortexing to break up large clumps and the resulting cell suspension was recovered by pipetting. Approximately 4 µl of the cell suspension was placed on to poly-L-lysine-coated glass slides, air-dried, and fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. Following fixation, slides were washed with PBS (0.1 M, pH 7.4) to remove all traces of PFA. Prior to immunofluororesence staining, a circle was drawn around the cells using a Super PAP Pen (Cat. No. 72112, Ladd Research, Williston, USA) to create a water repellent barrier. The defined section was then incubated with 100 µl of blocking buffer (PBS [0.1 M, pH 7.4] containing 3% [w/v] BSA and 0.1% [v/v] Tween-20) for 30 min at room temperature and washed 3 x 2 min in washing buffer (PBS [0.1 M; pH 7.4] containing 0.1% [v/v] Tween-20). One hundred microlitres of purified rabbit IgG antibody raised to the glnH protein or pre-immune sera were added at a dilution of 1:40 in blocking buffer. After 3 x 2 min washes with washing buffer, 100 µl of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cat. No. 111-095-003, Jackson ImmunoResearch Lab. Inc. USA) diluted 1:100 in blocking buffer was added. All antibody incubations were performed in the humid chamber, prepared by

placing three to four circles of moist filter paper in a petri dish, for one hour at room temperature. As the fluorescein label is light sensitive, the chamber was placed in the dark during the secondary antibody incubation. Slides were washed for 3 x 2 min in washing buffer and 1 x 2 min in PBS (0.1 M; pH 7.4). Subsequently, the signal was viewed under a Zeiss Axiophot photomicroscope (Zeiss, Germany) equipped with a camera and Spot RT Software 3.0 (Diagnostic Instrument, Inc) for image acquisition and processing.

5.3.5.2 Immunoelectron microscopy

Immunogold electron microscopy was carried out according to a method described by Rioux and colleagues (2001) with a few modifications. Approximately 200 µl of cell suspension (Section 5.3.5.1) was pelleted by centrifugation at 10,000 x g for 5 min and resuspended in 200 µl of blocking buffer (PBS [0.1 M; pH 7.4] containing 3% [w/v] BSA and 0.1% [v/v] Tween-20). After 30 min incubation at room temperature with gentle rotation, the blocking buffer was recovered by centrifugation at 10,000 x g for 5 min and the cells were washed two times in washing buffer (PBS [0.1 M; pH 7.4] containing 0.1% [v/v] Tween-20). Two hundred microlitres of purified rabbit IgG antibody specific to glnH or pre-immune rabbit diluted 1:50 in blocking buffer was added into the pellet and the suspension was incubated for one hour as above. Unbound antibodies were then removed by washing the cells three times in washing buffer. The washed cells were resuspended in 200 µl of colloidal gold particles (10 nm) conjugated to goat anti-rabbit IgG (whole molecule) (Cat. No. G7402, Sigma Life Sciences) diluted 1:100 in blocking buffer and were incubated for one hour at room temperature with rotation. The cells were washed two times in ammonium acetate buffer (0.1 M, pH 7.0) and were resuspended in 200 µl of the same buffer. Ten microlitres of the bacterial suspension was placed on Formvar-coated grids and allowed to partially air dry. Samples were examined with an electron microscope (Philip CM10 Transmission Electron Microscope) at the Manawatu Microscopy and Imaging Centre, Massey University, Palmerston North.

5.3.6 Interferon-gamma (IFN-gamma) assay

Cell-mediated immune responses of sheep vaccinated with Neoparasec™ against purified glnH recombinant protein were tested by the IFN-gamma (Bovigam®) assay as described in Section 2.10. Each antigen was prepared as described in Section 2.10.1 and used at 15 µg (glnH), 12.5 µg (PPDA) and 20 µg (ConA) per well. Each blood sample and antigen was analysed in duplicate. The identification numbers of the sheep used for this study are shown in Section 5.3.9 (Table 5.1).

5.3.7 Western blot analysis

Approximately 1 µg of the purified recombinant protein was loaded on to each lane of NuPAGE® 12% Bis-Tris gel (Section 2.9.2) and the proteins were transferred onto PVDF membrane for Western blotting. Sera obtained from sheep vaccinated with Neoparasec™, naturally infected sheep and naturally infected deer as described in Section 5.3.9 were used for Western blot analysis (Section 2.9.3). Briefly, each strip was incubated with 10 ml of diluted sheep/deer sera at 4^{o}C overnight and with 10 ml of diluted peroxidase-labelled goat anti-sheep (Cat. No. A3415, Sigma)/rabbit anti-deer (Cat. No. 04-31-06, KPL, USA) whole IgG antibody for one hour at room temperature. Strips were subsequently washed and developed using BM Chemiluminescence Blotting Substrate according to the manufacturer's instructions, prior to exposure to radiographic film for 20 sec. Western blots were done twice with each sample.

5.3.8 Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA technique was developed to detect IgG antibodies against glnH recombinant protein. Checkerboard titration (Crowther, 1995) was carried out to determine optimal concentration of antigen and sera used in ELISA. Briefly, the optimum concentration of the antigen was determined by diluting the antigen two-fold against constant dilution of selected positive serum samples. The optimal dilution of sera was determined by titration of positive and negative sera in a two-fold series against previously optimized antigen. Flat bottom 96-well MaxiSorp microtitre plates (Cat. No. 439354,

Nunc Roskilde, Denmark) were coated with 100 μl containing 500 ng of antigen diluted in 0.1 M carbonate coating buffer pH 9.6 (0.06 M $NaHCO_3$, 0.04 M Na_2CO_3). The plates were incubated for two hours at 37°C. After incubation, the antigen-coated plates were washed four times with washing buffer (PBS [0.1 M; pH 7.4] containing 0.05% [v/v] Tween-20). The uncoated surfaces were then blocked (200 μl per well) with blocking buffer (PBS [pH 7.4] containing 0.05% [v/v] Tween-20 and 5% [w/v] skim milk) for one hour at 37°C. Following four washes with washing buffer, 100 μl of sera diluted 1:100 in blocking buffer were added to each well in duplicate. The plates were incubated at 4°C overnight, emptied and washed four times with washing buffer. Subsequently, 100 μl of secondary antibody was added to each well and incubated for one hour at 37°C. As sera were collected from sheep and deer, the secondary antibodies used in this assay were peroxidase-labelled goat anti-sheep whole IgG antibody at a dilution of 1:15,000 in blocking buffer and peroxidase-labelled rabbit anti deer diluted 1: 500 in blocking buffer, respectively. After further four washing cycles, 200 μl of chromogenic substrate solution of 3,3', 5,5'-tetramethylbenzidine (TMB) (Cat. No. T2885, Sigma-Aldrich, Inc) prepared according to the manufacturer's recommendations was added to each well. The plates were incubated for 30 min in the dark at room temperature and the resultant blue reaction product when reacted with peroxidase was stopped by the addition of 50 μl of 2 M H_2SO_4 per well. The extent of the colour development was measured at an absorbance of 450 nm ($OD_{450\ nm}$) on a Versamax™ micro plate reader (Molecular Devices, Sunnyvale, California). The dilution buffer control was used to calculate the background OD. The OD value of each sample was calculated by subtracting the background OD from the mean OD of duplicates.

The presence of anti-glnH antibodies in rabbit was determined using goat anti-rabbit IgG peroxidase conjugated antibody as a secondary antibody at 1:30,000.

5.3.9 Serum samples

5.3.9.1 Sheep sera

Sheep sera used in this study were obtained from two different sources. The first was a Neoparasec™ sheep vaccination trial carried out in 2008 under Massey University Animal Ethics Committee approval MUAEC # 07/13 dated 16/02/07. Thirteen Romney-cross wethers as listed in Table 5.1 were used for the study. At four months of age, seven were vaccinated as described in Section 4.3.6 and the remaining were kept as unvaccinated control animals.

Table 5.1 Sheep used in the 2008 vaccination trial.

Sheep tag number	
Neoparasec vaccinated	**Unvaccinated (control)**
270*	084
256	165
478	295
166	002
284	112
428	376
258	

*humanely euthanised because of a broken leg at 12 weeks post-vaccination.

The second source of serum samples were from naturally infected sheep. Thirty-seven sheep were brought in to the Veterinary Teaching Hospital, Massey University, Palmerston North with general poor condition and serum samples were taken. Five of these sheep (sheep number 3, 11, 19, 25 and 32) were later diagnosed as having paratuberculosis based on gross pathology of relevant tissue examined by pathologist at the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North.

5.3.9.2 Deer sera

Blood samples for sera collection were kindly provided by Lesley Stringer of the Epicentre, Massey University, Palmerston North. The blood was taken from paratuberculosis clinical weaner (under one year old) deer farmed in the South Island. The animals were classified as MAP infected based on positive mesenteric lymph node culture results. Negative control sera were obtained from weaner deer with no known history of paratuberculosis farmed at the Massey Deer Research Unit, Massey University, Palmerston North.

Table 5.2 Deer sera used in this study.

Deer tag number	
Naturally infected animals	**Uninfected animals**
2316	805
2305	819
311	861
772	881
679	824
668	825
1331	887
5212	876
5195	830
	817
	877

Blood samples from different animal species were taken from the jugular vein of each animal using non-heparinized 10 ml vacutainers (Cat No. 366430; Betckton Dickinson). Sera were then collected using the procedure described in Section 2.12.1.

5.3.10 Statistical analysis

Data pertaining to IFN-gamma responses to antigens (IFN-gamma $OD_{450\ nm}$) and serum antibody responses (ELISA $OD_{450\ nm}$) in the sheep vaccination trial were analysed using the PROC MIXED procedure in SAS® 9.1 (SAS Institute Inc., Cary, NC, USA). The linear mixed model (Littell *et al*., 1998) included the fixed effects of groups (Neoparasec vaccinated and unvaccinated control),

time (different time-points between 0 and 24 weeks post-vaccination) and their interaction, and random effect of animal within group. The covariance error structure for repeated measures over time points within animals was determined using Akaikes information criterion.

Statistical comparisons were also made to test the differences in antibody responses between naturally MAP infected sheep and uninfected controls and similarly between naturally MAP infected deer and uninfected controls. The linear mixed model for these comparisons included the fixed effects of group and random effect of animal within group.

Least square means and their standard error were obtained for each group, time-points and their interaction and were used for multiple comparisons between group effects.

5.4 Results

5.4.1 Alignments of the *glnH* gene among mycobacterial species

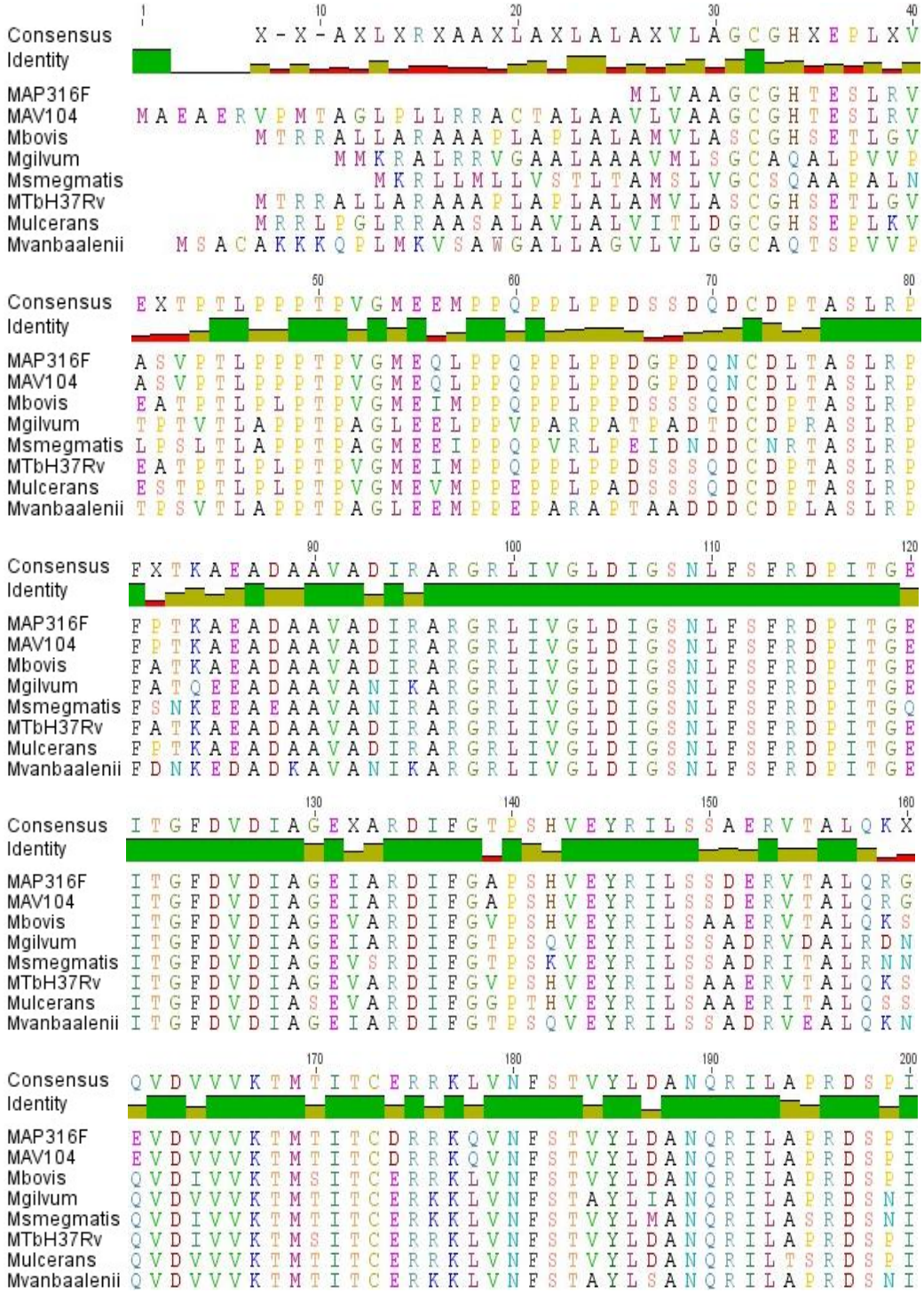
Pair-wise alignments of the *glnH* gene in MAP and other mycobacterial species (Appendix 2) showed DNA identity scores ranged from 78% to 99%. *GlnH* showed the highest identity score to the extracellular-solute binding protein of Mycobacterium *avium* subspecies *avium* (99% identity). Similar genes, which exist in *M. bovis*, *M. tuberculosis*, *M. ulcerans*, *M vanbaalenii*, *M. smegmatis* and *M. gilvum*, showed identity scores of 85.1%, 85.1%, 83.4%, 78.9%, 78.1% and 78%, respectively.

At the amino acid level (Figure 5.2), pair-wise alignments revealed 99.7% identity between glnH in MAP and the extracellular-solute binding protein of *M. avium* subspecies *avium*. Alignments of glnH in MAP and similar proteins present in other mycobacterial species revealed 85.8% identity between MAP and *M. bovis* and *M. tuberculosis*, 84.1% with *M. ulcerans*, 75.7% with *M. vanbaaleni*, 74.4% with *M. gilvum* and 72.5% with *M. smegmatis*. Multiple sequence alignment presented in Figure 5.2 showed 78.6% identity. The

figure also shows that the region between amino acids 1-75 is more variable than the region between amino acids 76–334.

5.4.2 Amino acids ID confirmation

To further confirm the peptide sequence of the recombinant glnH protein after expression in *E. coli* (Section 4.4.3), the protein was analysed by mass spectrometry. Amino acid sequence search of MALDI MS/MS data against the SWISS-PROT database identified the protein band as glnH (MAP strain K-10) with sequence coverage of about 31% (Figure 5.3).


Consensus
Identity
MAP316F
MAV104
Mbovis
Mgilvum
Msmegmatis
MTbH37Rv
Mulcerans
Mvanbaalenii

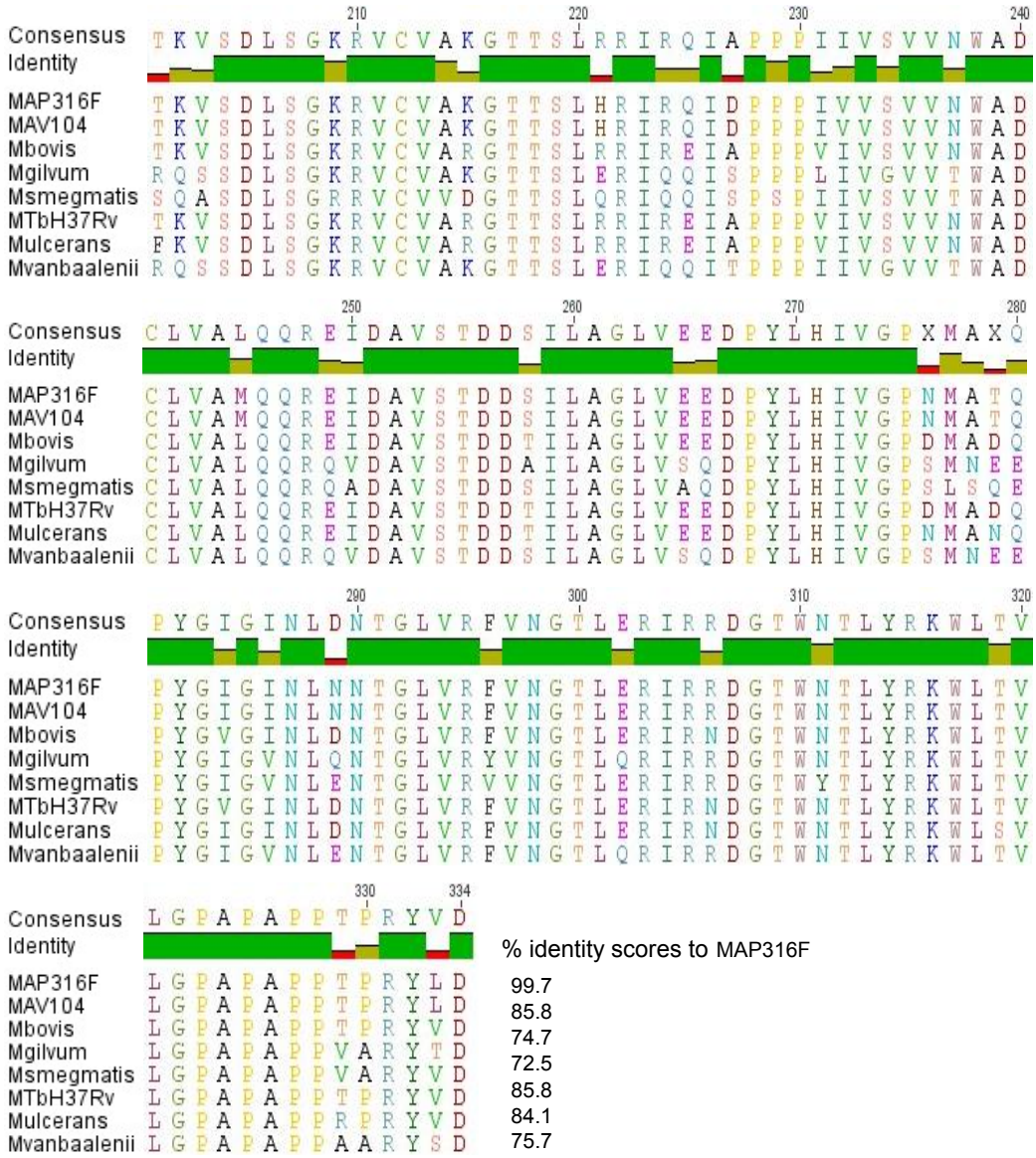


Figure 5.2 Multiple sequence alignment of MAP strain 316F glnH protein to other homologues mycobacterial glnH protein and the extracellular solute-binding protein of *M. avium* subspecies *avium*. Similarity is indicated in the identity colour chart. Residues conserved in all sequences are represented as green on the identity chart.

Match to: gi|41409992 Score: 583
GlnH [Mycobacterium avium subsp. paratuberculosis K-10]
Found in search of pps_p0_08_C_1207325601.txt

Nominal mass (Mr): 33581; Calculated pI value: 5.48
NCBI BLAST search of gi|41409992 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Mycobacterium avium subsp. paratuberculosis K-10
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|41398825 from Mycobacterium avium subsp. paratuberculosis K-10

Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 31%

Matched peptides shown in **Bold Red**

1	MLVAAGCGHT	ESLRVASVPT	LPPPTPVGME	QLPPQPPLPP	DGPDQNCDLT
51	ASLRPFPTKA	EADAAVADIR	ARGRLIVGLD	IGSNLFSFR**D**	**PITGEITGFD**
101	**VDIAGEIARD**	**IFGAPSHVEY**	**R**ILSSDERVT	ALQRGEVDVV	VKTMTITCDR
151	R**KQVNFSTVY**	**LDANQR**ILAP	RDSPITKVSD	LSGKRVCVAK	GTTSLHRIR**Q**
201	**IDPPPIVVSV**	**VNWADCLVAM**	**QQR**EIDAVST	DDSILAGLVE	EDPYLHIVGP
251	NMATQPYGIG	INLNNTGLVR	FVNGTLERIR	R**DGTWNTLYR**	**KWLTVLGPAP**
301	**APPTPR**YLD				

Figure 5.3 Mascot search result of the purified glnH analysed by mass spectrometry. Sequence coverage was obtained from the number of matched peptide found by MALDI MS/MS shown in red divided by the entire sequence of the protein.

5.4.3 Production and purification of polyclonal antibody raised to glnH

Sera collected from the rabbit immunised with purified recombinant glnH were assessed for antibody production using Western blot and ELISA. As shown in Figure 5.4, the glnH was recognised by the post-vaccination serum samples but not by the pre-immune serum on Western blot. ELISA results (Table 5.3) further confirmed that sera taken from rabbit on three occasions after immunization were shown to have notably higher OD values than pre-immune sera to the purified protein.

Table 5.3 Mean antibody response of pre-immune and immune rabbit sera to purified recombinant glnH protein in ELISA.

	Mean OD values (OD_{450nm}) ± standard deviation
Pre-immune sera	0.02295 ± 0.016829
10 days after first boost	2.47875 ± 0.070004
12 days after second boost	2.63640 ± 0.105712
Final bleed	2.80525 ± 0.124451

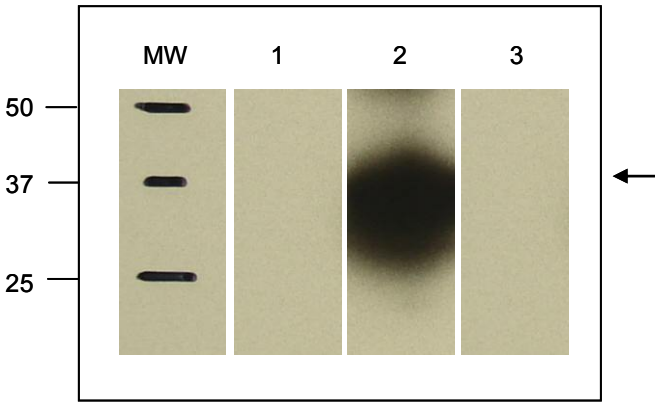


Figure 5.4 Western blot detection of rabbit antibody raised to the purified recombinant glnH protein. Blots of recombinant glnH were individually incubated with 1:500 dilution of rabbit sera. Goat anti-rabbit IgG peroxidase conjugated antibody was used at 1:40,000. Lane MW, Precision Plus Protein Standard Dual Color (kDa); lane 1, pre-immune rabbit sera; lane 2, immunized rabbit sera taken at 10 days after first boost; lane 3, negative control (no rabbit serum was added). Arrow indicates the position of recombinant glnH band.

Purification of IgG antibody from the rabbit serum using DEAE-Sepharose chromatography performed after an ammonium sulphate precipitation yielded approximately 30 fractions. Figure 5.5 shows the OD values ($OD_{280\ nm}$) of collected fractions. Fraction numbers 7 to 10 that gave a high absorbance reading at 280 nm were examined for purity by SDS-PAGE, pooled and used for the immunohistochemistry study.

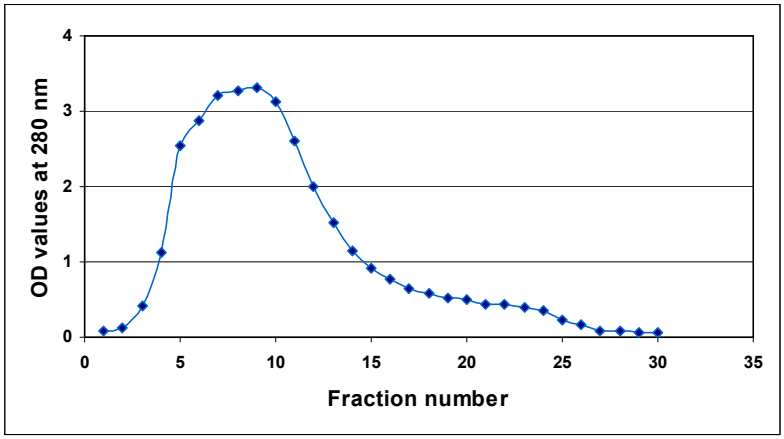


Figure 5.5 The distribution of purified IgG from rabbit serum at OD 280 nm.

5.4.4 Immunohistochemistry

To determine if the glnH protein is located on the surface of MAP strain 316F, the immunohistochemistry studies were performed using the purified polyclonal anti-glnH antibody described above. In the first instance, indirect immunofluorescence was performed using the anti-glnH polyclonal sera and FITC-conjugated anti-rabbit sera. A positive light green fluorescence signal was observed when MAP cells were probed with anti-glnH antibody (Figure 5.6a) whereas no signal was visible in the control (Figure 5.6b) suggesting that the glnH protein is located on the surface of MAP cells. However, it was not possible to ascertain the exact location of the protein on the bacterial cells due to the limited resolution of the fluorescence microscopy employed in this study. The attachment of the anti-glnH antibodies at the surface of MAP cells was further confirmed by immunogold labelling viewed by transmission electron microscopy. The study was repeated twice with consistent results. As depicted in Figure 5.7a, colloidal gold particles were observed on bacteria by use of antisera against purified recombinant glnH protein. In contrast, no gold particles could be detected in the control (Figure 5.7b). Taken together, these results demonstrated that the glnH can be recognised by the anti-glnH antibody on the intact MAPstrain 316F cell surface.

(a)

(b)

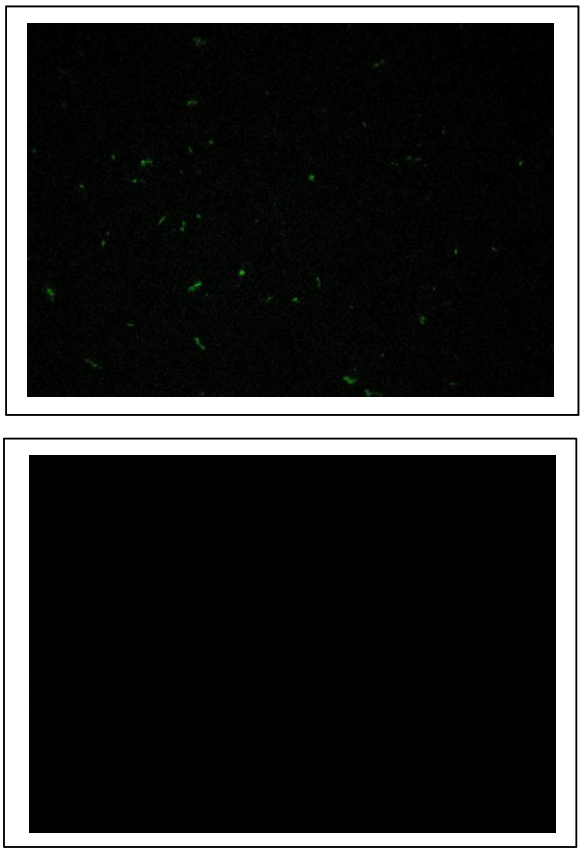


Figure 5.6 Immunofluorescence images of whole cells of MAP strain 316F. The whole cells were fixed with 4% PFA and probed with diluted (1:40) **(a)** polyclonal anti-glnH antibody or **(b)** pre-immune sera, and diluted (1:100) FITC-conjugated goat anti-rabbit IgG. Magnification x 400.

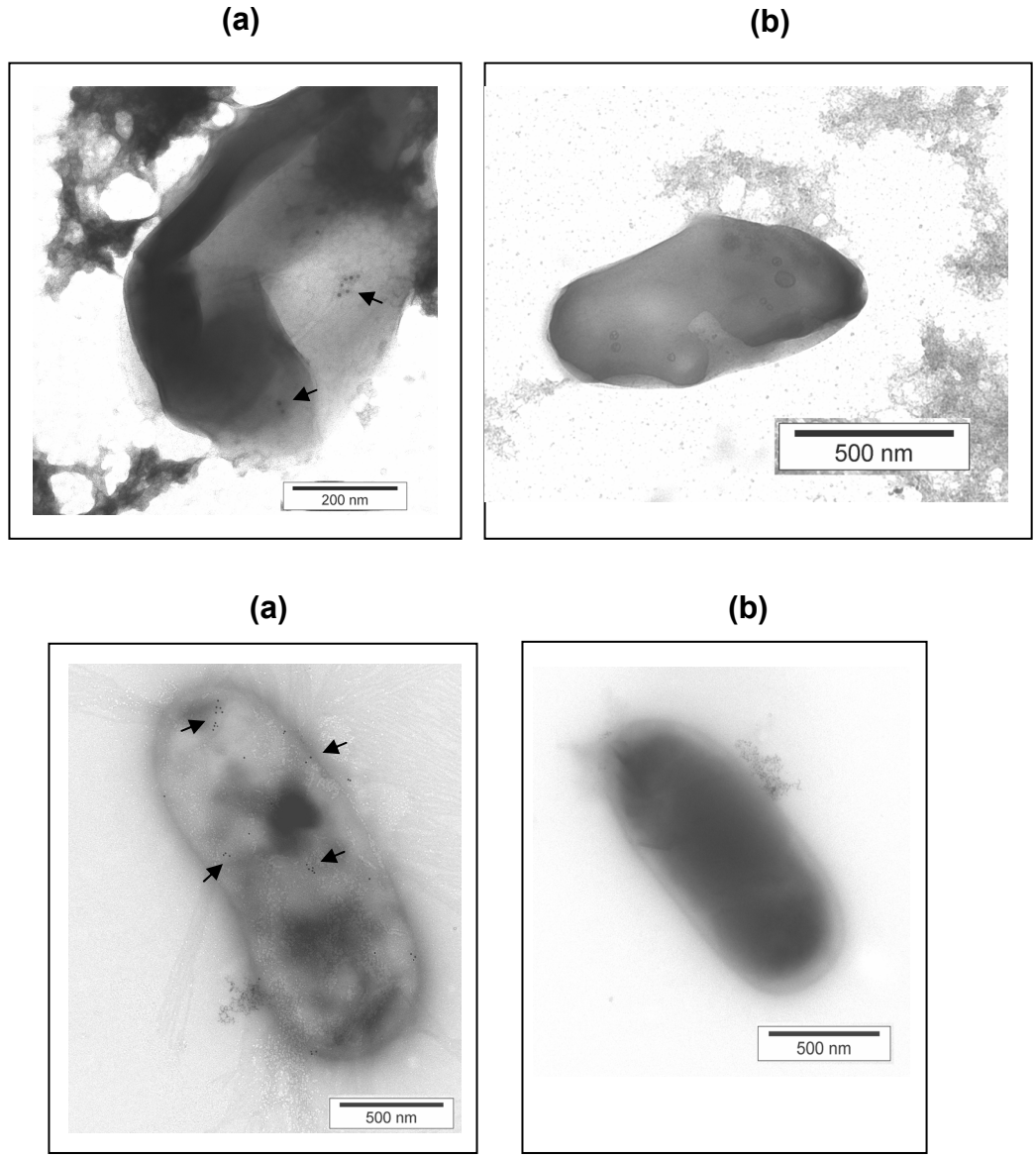
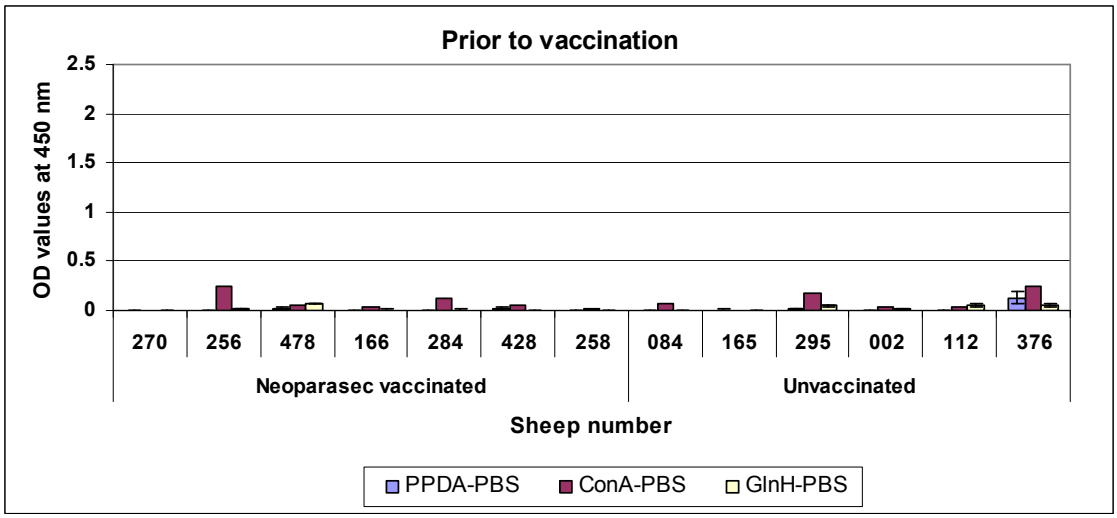


Figure 5.7 Transmission electron micrographs of whole cells of MAP strain 316F. The whole cells were successively probed with diluted (1:50) **(a)** polyclonal anti-glnH antibody or **(b)** pre-immune sera, and diluted (1:100) gold-conjugated secondary antibody. Arrows indicate gold particles.

5.4.5 Cell-mediated immune responses to purified recombinant glnH protein

To investigate if purified recombinant glnH could stimulate IFN-gamma responses in the peripheral blood mononuclear cells (PBMC's), whole blood collected from sheep experimentally vaccinated with Neoparasec™ and unvaccinated control sheep (Section 5.3.9.1) were used in the IFN-gamma assays. Results of individual animal and group responses at different time points are depicted in Figures 5.8-5.11. A summary of mean IFN-gamma responses between the vaccinated and unvaccinated groups is presented in Table 5.4. There was no significant difference ($p>0.05$) in the IFN-gamma responses to the glnH protein between the vaccinated and unvaccinated groups at time points 0, 4, 8 and 12 weeks post-vaccination indicating that vaccinated animals did not produce IFN-gamma following stimulation with the recombinant protein. The responses to PPDA did not differ significantly between the vaccinated and unvaccinated groups prior to vaccination ($p = 0.9328$) and 4 weeks after vaccination ($p = 0.3920$). Neoparasec™ vaccinated sheep gave a significant response ($p < 0.05$) to PPDA at 8 and 12 weeks post-vaccination. The mean difference in IFN-gamma production between the vaccinated and unvaccinated control groups was not significant ($p > 0.05$) at all time points following ConA stimulation.

(a)



(b)

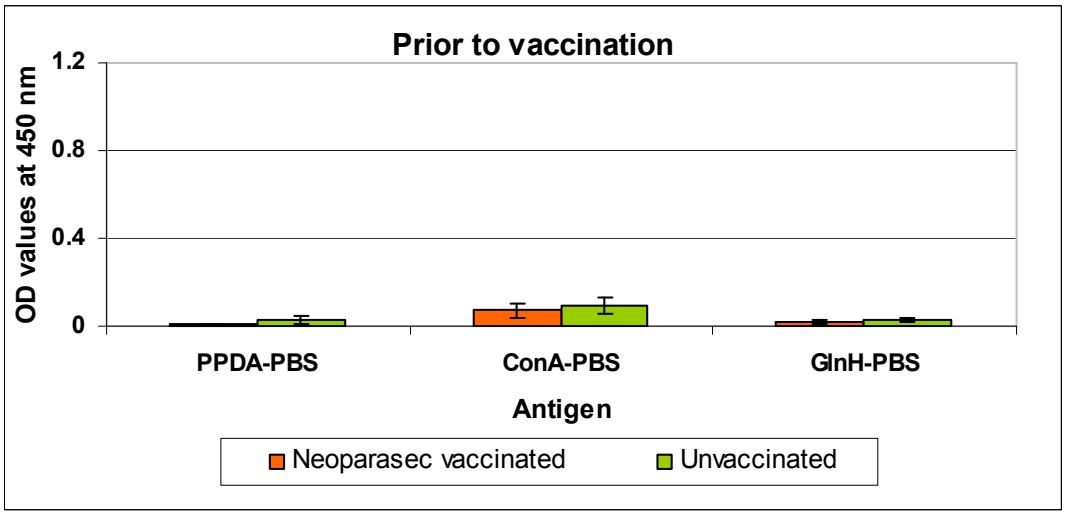
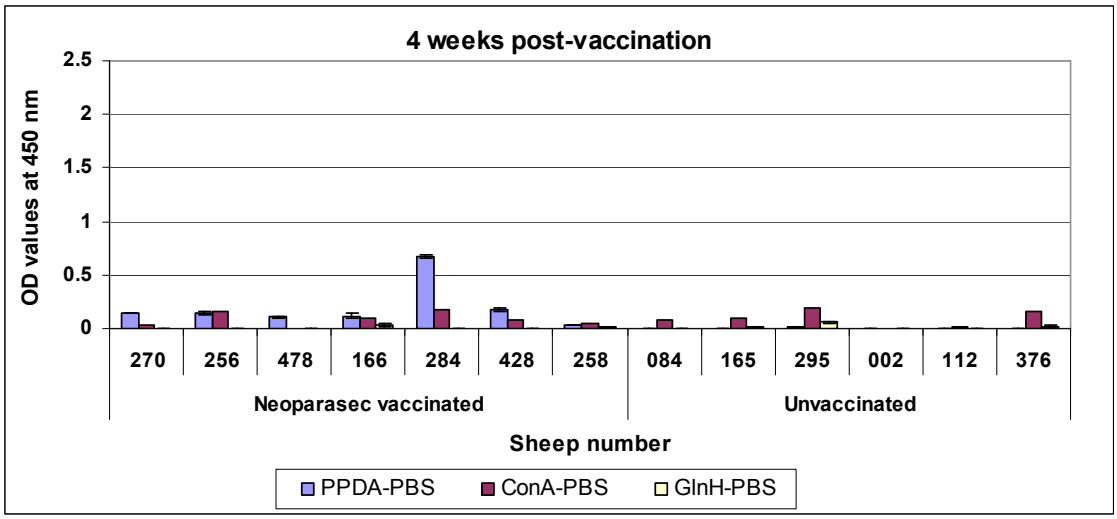


Figure 5.8 IFN-gamma production in Neoparasec™ vaccinated and unvaccinated sheep prior to vaccination (0 week). **(a)** Individual IFN-gamma responses in both vaccinated and unvaccinated. **(b)** Group IFN-gamma responses to each antigen. Whole blood was incubated in duplicate wells with 12.5 µg/ml PPDA, 20 µg/ml ConA which was used as a non specific T-cell activator to check cell viability, and 15 µg/ml purified recombinant glnH protein. PBS was also included as a negative control in duplicate wells. IFN-gamma results for each antigen were expressed as corrected absorbance at 450 nm that was defined as the absorbance at 450 nm of the antigen stimulated well minus the average absorbance at 450 nm of the PBS control wells for each animal. Raw data are presented in Appendix 3.

(a)



(b)

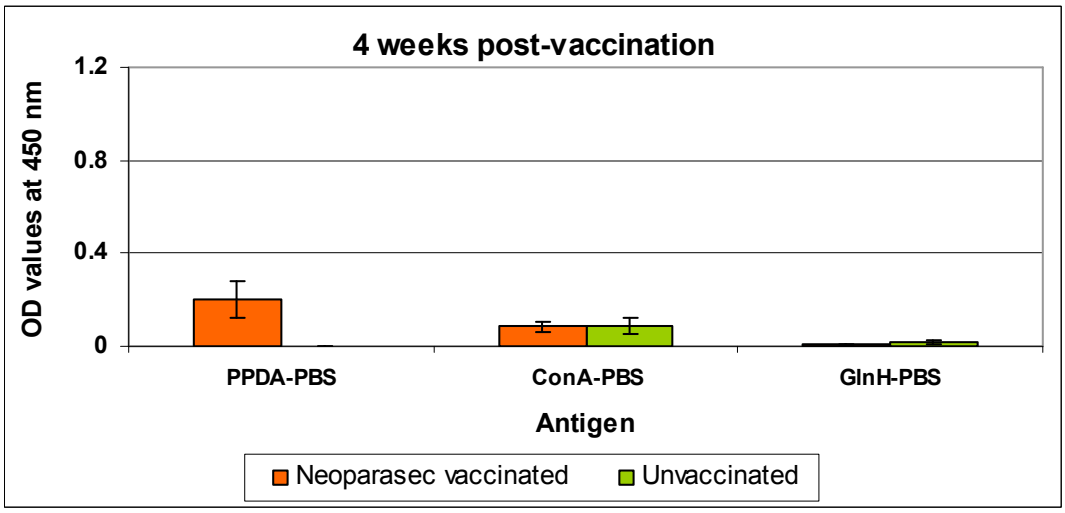
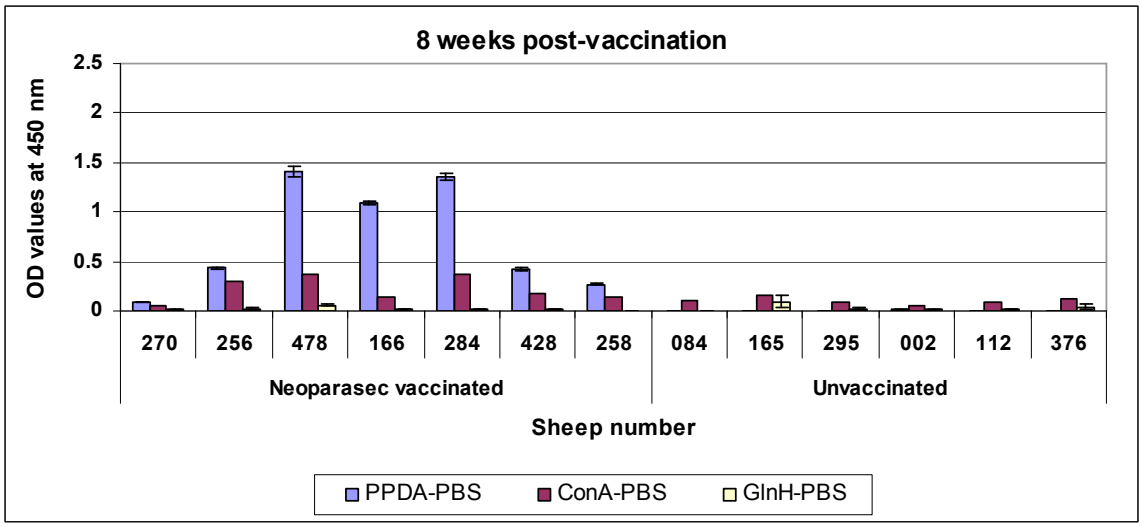


Figure 5.9 IFN-gamma production in Neoparasec™ vaccinated and unvaccinated sheep at 4 weeks post-vaccination. **(a)** Individual IFN-gamma responses in both vaccinated and unvaccinated. **(b)** Group IFN-gamma responses to each antigen. Whole blood was incubated in duplicate wells with 12.5 µg/ml PPDA, 20 µg/ml ConA and 15 µg/ml purified recombinant glnH protein. PBS was also included as a negative control in duplicate wells. IFN-gamma results for each antigen were expressed as corrected absorbance at 450 nm that was defined as the absorbance at 450 nm of the antigen stimulated well minus the average absorbance at 450 nm of the PBS control wells for each animal. Raw data are presented in Appendix 4.

(a)



(b)

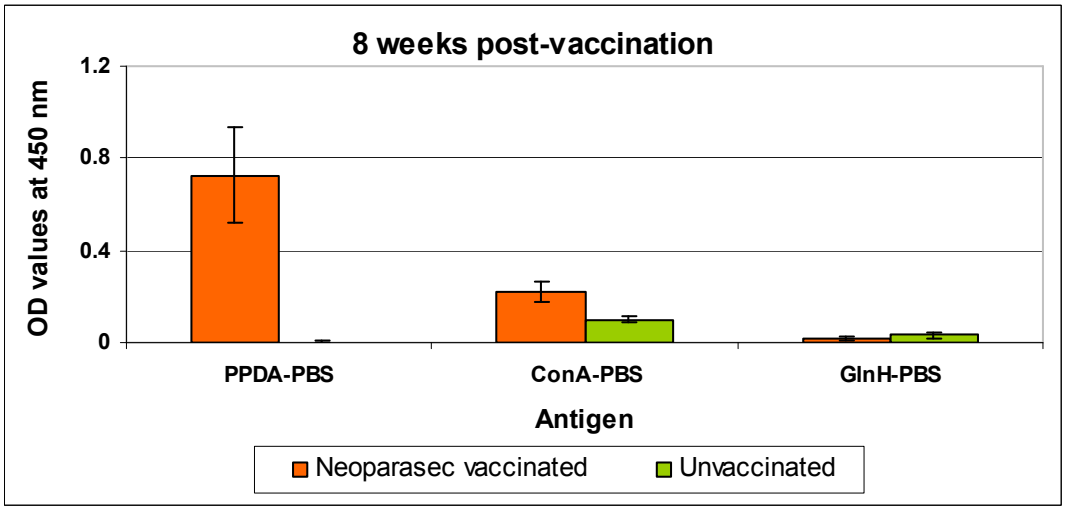
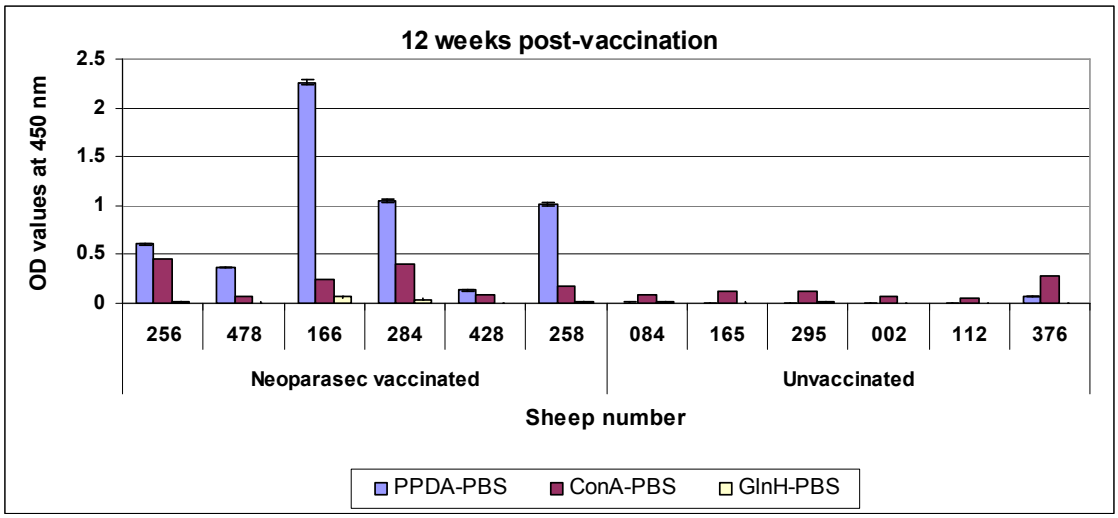


Figure 5.10 IFN-gamma production in Neoparasec™ vaccinated and unvaccinated sheep at 8 weeks post-vaccination. **(a)** Individual IFN-gamma responses in both vaccinated and unvaccinated. **(b)** Group IFN-gamma responses to each antigen. Whole blood was incubated in duplicate wells with 12.5 µg/ml PPDA, 20 µg/ml ConA and 15 µg/ml purified recombinant glnH protein. PBS was also included as a negative control in duplicate wells. IFN-gamma results for each antigen were expressed as corrected absorbance at 450 nm that was defined as the absorbance at 450 nm of the antigen stimulated well minus the average absorbance at 450 nm of the PBS control wells for each animal. Raw data are presented in Appendix 5.

(a)



(b)

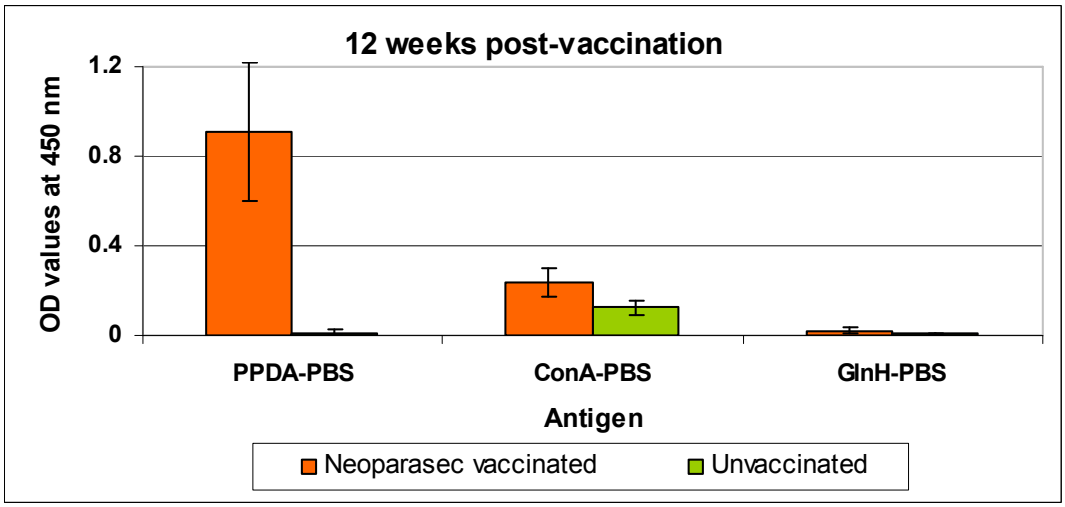


Figure 5.11 IFN-gamma production in Neoparasec™ vaccinated and unvaccinated sheep at 12 weeks post-vaccination. **(a)** Individual IFN-gamma responses in both vaccinated and unvaccinated. **(b)** Group IFN-gamma responses to each antigen. Whole blood was incubated in duplicate wells with 12.5 µg/ml PPDA, 20 µg/ml ConA and 15 µg/ml purified recombinant glnH protein. PBS was also included as a negative control in duplicate wells. IFN-gamma results for each antigen were expressed as corrected absorbance at 450 nm that was defined as the absorbance at 450 nm of the antigen stimulated well minus the average absorbance at 450 nm of the PBS control wells for each animal. Note that blood from sheep number 270 was not available at this time point (animal humanely euthanized because of a broken leg). Raw data are presented in Appendix 6.

Table 5.4 Mean IFN-gamma responses of Neoparasec™ vaccinated and unvaccinated controls sheep. Asterisk (*) indicates significant differences ($p < 0.05$) between vaccinated and unvaccinated groups.

Time/Antigens	Least square OD mean value (OD_{450nm}) ± standard error		p-value
	Neoparasec™ - vaccinated group	Unvaccinated group	
0 weeks (pre-vaccination)			
glnH	0.01482 ± 0.09402	0.02591 ± 0.01035	0.6446
PPDA	0.00693 ± 0.00364	0.02605 ± 0.02004	0.9328
ConA	0.07314 ± 0.03133	0.09170 ± 0.03888	0.8703
4 weeks			
glnH	0.00735 ± 0.00869	0.01465 ± 0.00873	0.7614
PPDA	0.19877 ± 0.07990	0.00243 ± 0.00183	0.3920
ConA	0.08358 ± 0.02487	0.08825 ± 0.03142	0.9672
8 weeks			
glnH	0.01990 ± 0.07115	0.03228 ± 0.01353	0.6069
PPDA	0.72518 ± 0.20623	0.00335 ± 0.00176	0.0024*
ConA	0.22146 ± 0.04675	0.10035 ± 0.01371	0.2886
12 weeks			
glnH	0.02235 ± 0.00965	0.00522 ± 0.00194	0.4899
PPDA	0.90611 ± 0.30770	0.01288 ± 0.01054	0.0008*
ConA	0.23640 ± 0.06452	0.12289 ± 0.03236	0.3440

5.4.6 Humoral immune responses to purified recombinant glnH protein

Reactivity of the purified recombinant protein glnH with serum samples was determined by Western blotting and ELISA. Sera were collected from experimentally vaccinated sheep and sheep naturally infected with MAP. In addition, sera were obtained from deer naturally infected with MAP. Raw data for individual antibody responses for both control and Neoparasec™ vaccinated sheep at different time points as well as for infected sheep and deer are presented in Appendices 7-9.

5.4.6.1 Western blot analysis

In the sheep experiment described in Section 5.3.9.1, seven sheep were vaccinated with Neoparasec™ and six were used as controls. Western blot analyses of vaccinated animals prior to vaccination and 4 to 24 weeks following vaccination are shown in Figure 5.12. No vaccinated animals reacted with the glnH protein prior to vaccination but all had detectable antibodies to glnH protein at 4 and 8 weeks post-vaccination on Western blots Animal number 478 gave the strongest band of antibody reactivity towards glnH protein in the Neoparasec™ vaccinated group, and this was maintained over 24 weeks post-vaccination. By 16 weeks post-vaccination, the intensity of the bands decreased although a faint band corresponding to molecular mass of the purified glnH protein could be observed from five out of six animals. Similar reactivity was seen from sera belonging to the vaccinated group at 20 and 24 weeks post-vaccination. Generally, antibody was not present in the unvaccinated control sera over the seven time points with exception for animal number 376 and 084 (Figure 5.13) that showed very low reactivity to the region of the protein at 16 and 20 weeks post-vaccination, respectively.

Sera from naturally infected sheep and deer reacted with the glnH protein with different intensities. Visible bands of reactivity against the glnH protein could be observed with sera from four of five naturally infected sheep while no bands were seen from uninfected control on the Western blots (Figure 5.14). Western blot analysis of deer sera depicted in Figure 5.15a shows that all the infected deer recognised the glnH protein with the bands intensity varying from high to low. Reactivity to the purified glnH protein was not detected in the uninfected control deer (Figure 5.15b).

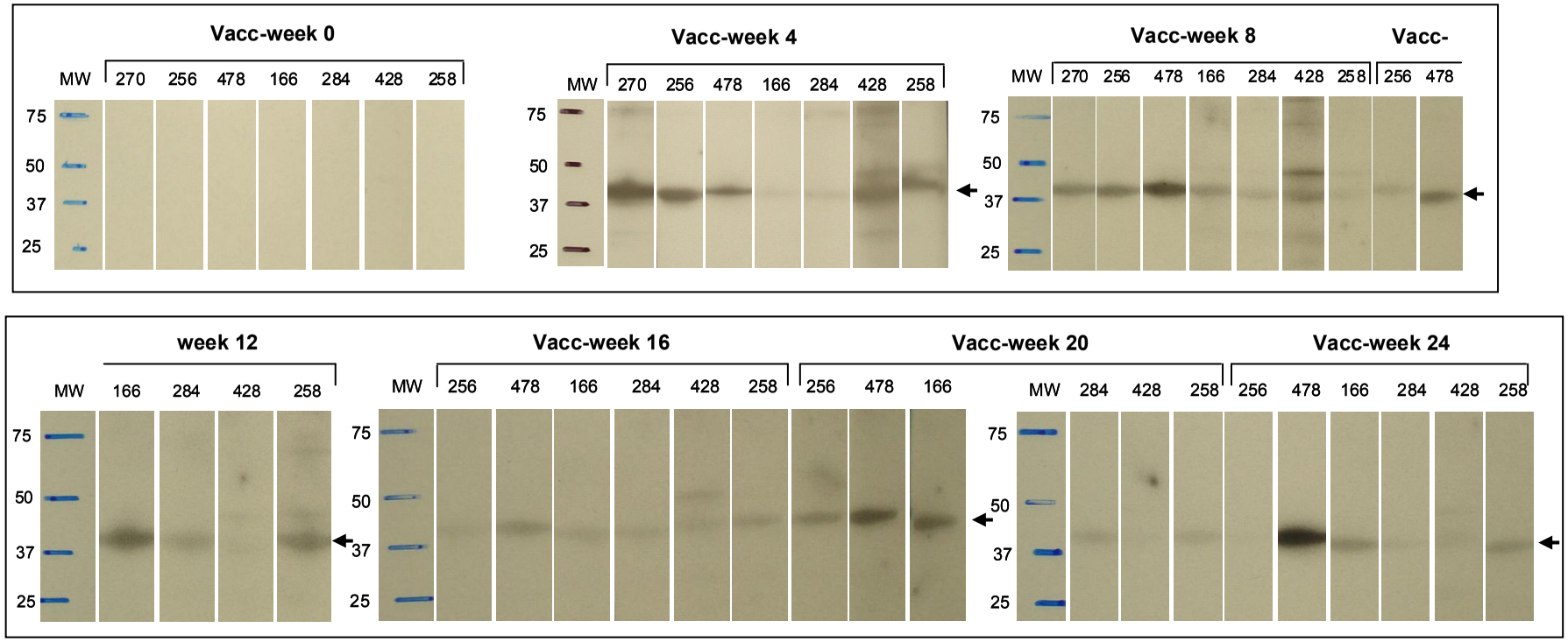


Figure 5.12 Immunodetection of serum IgG antibodies against purified recombinant glnH protein in the Neoparasec™ vaccinated sheep prior to vaccination (week 0) and 4 to 24 weeks post-vaccination. Blots of 1 µg recombinant glnH were individually incubated with a 1:100 dilution of serum from sheep before and 4 to 24 weeks following vaccination. Reactivity was detected using a 1:20,000 dilution of goat anti-sheep IgG peroxidase conjugated antibody. The number on the left side indicates the molecular weight (MW) of the protein in kDa. Arrows indicate the position of recombinant glnH bands. Note that serum from sheep number 270 was not available at 12 – 24 weeks post-vaccination (animal humanely euthanised because of a broken leg)

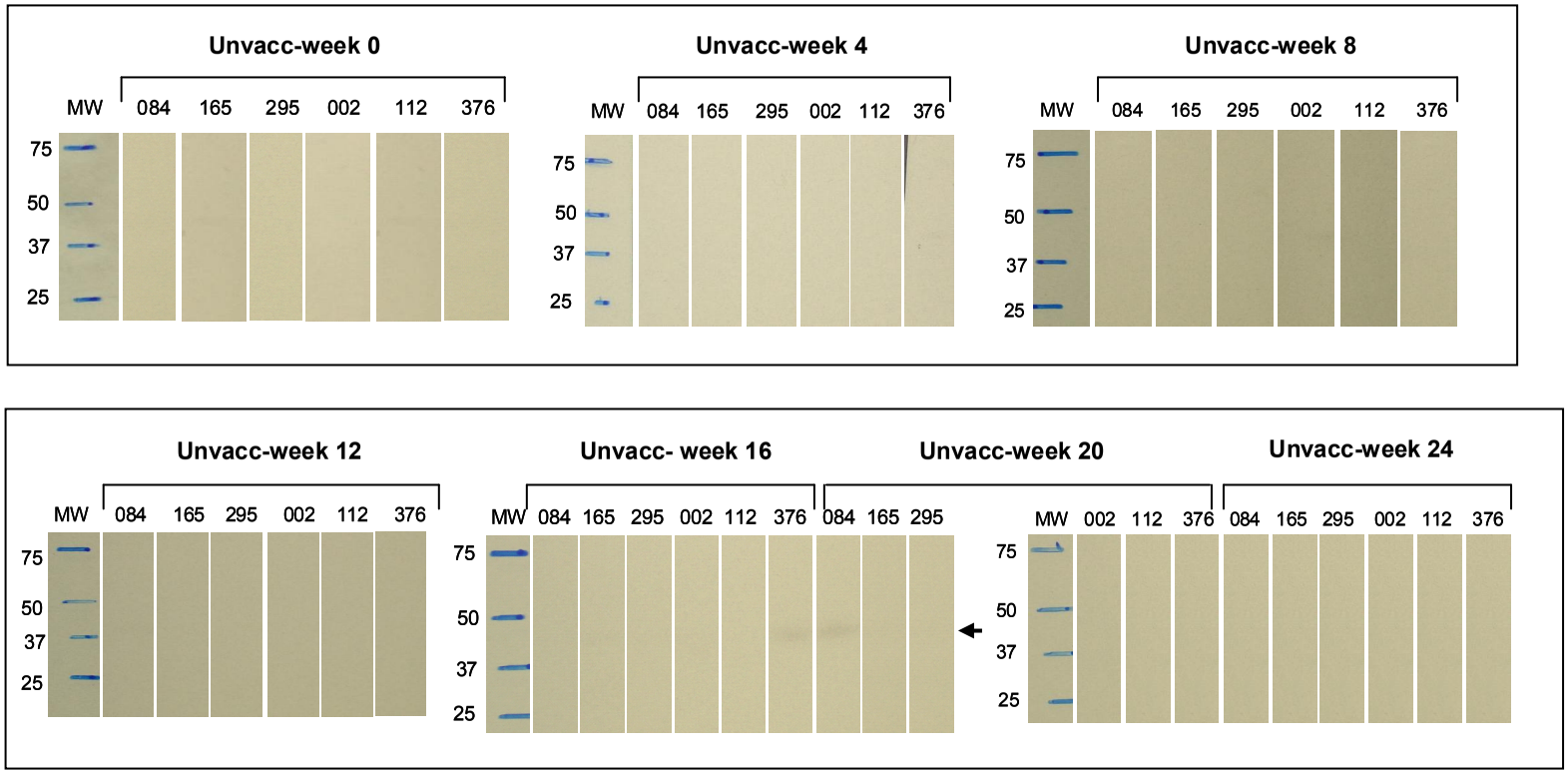


Figure 5.13 Immunodetection of serum IgG antibodies against purified recombinant glnH protein in the unvaccinated control sheep. Blots of 1 µg recombinant glnH were individually incubated with a 1:100 dilution of serum from unvaccinated control sheep prior to vaccination and 4 to 24 weeks post-vaccination. Reactivity was detected using a 1:20,000 dilution of goat anti-sheep IgG peroxidase conjugated antibody. The number on the left side indicates the molecular weight of the protein (MW) in kDa. Arrows indicate the position of recombinant glnH bands.

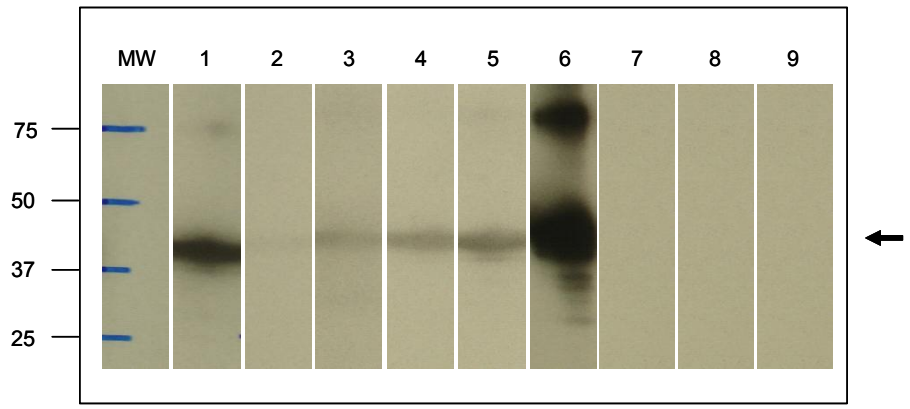


Figure 5.14 Immunodetection of serum IgG antibodies against recombinant glnH using sera from sheep naturally infected with MAP and control animals. Blots were individually incubated with 1:100 dilution of sera or with 1:1000 dilution of anti-histidine x 6 conjugated monoclonal antibody. Goat anti-sheep IgG peroxidase conjugated antibody was used at 1:20,000. Lane MW, Precision Plus Protein Standards Dual color marker (kDa); lane 1 to 5, infected sheep number 3, 11, 19, 25 and 32 respectively; lane 6, positive control (probed with anti-histidine antibody); lane 7 to 9, uninfected sheep number 1, 17 and 20 respectively. Arrow indicates the position of recombinant glnH bands.

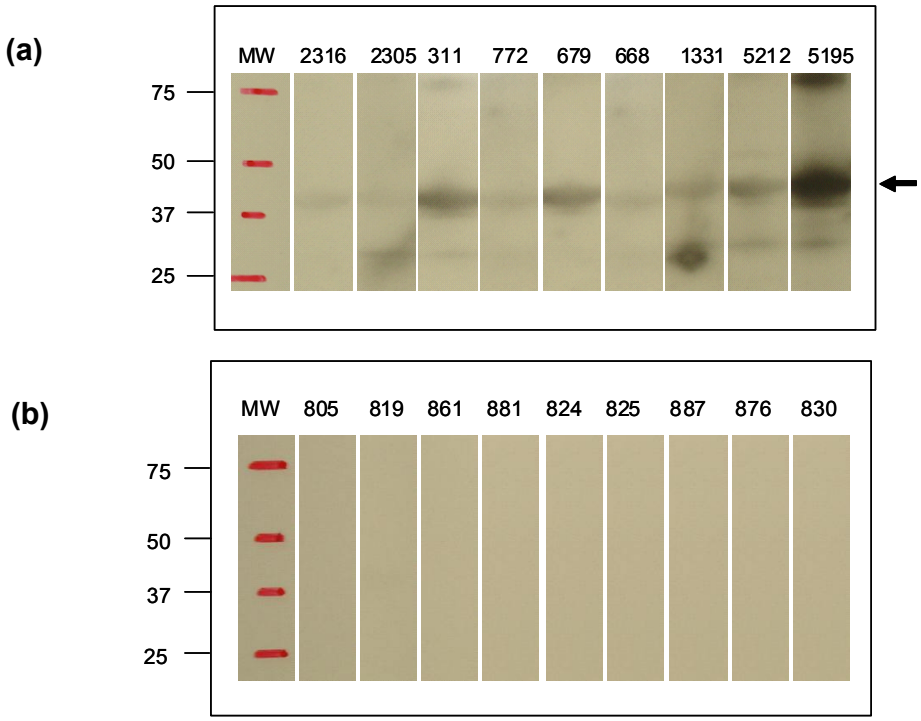


Figure 5.15 Immunodetection of serum IgG antibodies against recombinant glnH using sera from deer naturally infected with MAP and control animals. Blots were individually incubated with either **(a)** sera from naturally infected deer or **(b)** uninfected control deer at 1:100. Rabbit anti-deer IgG peroxidase conjugated antibody was used at 1:500. The number on the left side indicates the molecular weight (MW) of the protein in kDa. Arrow indicates the position of recombinant glnH bands.

5.4.6.2 ELISA

An ELISA was developed to assess antibody responses of sheep and deer to the purified recombinant glnH protein. Prior to ELISA assay, the antigen concentration and serum dilution were optimized using checkerboard titrations. The results indicated 500 ng of coating antigen, and a 1:100 dilution of sheep sera were optimal for use (data not shown). Antibody responses of sheep vaccinated with Neoparasec™ and unvaccinated controls are depicted in Figure 5.16. A summary of group antibody responses prior to vaccination (0 week) and from 4 to 24 weeks is shown in Table 5.5. There was no significant

difference (p = 0.8033) in antibody response between the vaccinated and the unvaccinated group prior to vaccination. It can be seen from the figure that individual antibody response from five of the sheep (270, 256, 478, 284 and 428) belonging to the vaccinated group generally peaked at 4 weeks post-vaccination, and were in the OD range of 0.57-0.78. Sheep number 258 exhibited an OD value of 0.6 at 4 weeks post-vaccination. Its OD value decreased to 0.4 by 8 weeks post-vaccination and increased again reaching the OD value of 0.8 at 12 weeks following vaccination. Sheep number 166 had the maximum OD value ($OD_{450\ nm}$ = 0.98) at 12 weeks post-vaccination. Antibody responses in the unvaccinated sheep were fairly constant and remained persistently low in comparison to the vaccinated animals during the time points of sera collection. The vaccinated group had significantly ($p < 0.05$) higher antibody responses than those of the unvaccinated group throughout the experimental period.

Table 5.5 Mean antibody responses of Neoparasec™ vaccinated and unvaccinated controls sheep. Asterik (*) indicates significant differences ($p < 0.05$) between vaccinated and unvaccinated groups.

Time of serum collection (week)	Least square OD mean ($OD_{450\ nm}$) ± standard error		p-value
	Neoparasec™ vaccinated	Unvaccinated controls	
0	0.056300 ± 0.015000	0.044383 ± 0.007111	0.8033
4	0.666357 ± 0.034090	0.053308 ± 0.009506	0.0001*
8	0.600707 ± 0.054216	0.068325 ± 0.024858	0.0001*
12	0.653450 ± 0.084854	0.073858 ± 0.012768	0.0001*
16	0.574333 ± 0.064850	0.060258 ± 0.012173	0.0001*
20	0.565142 ± 0.053381	0.073350 ± 0.014830	0.0001*
24	0.449650 ± 0.050942	0.072992 ± 0.013030	0.0001*

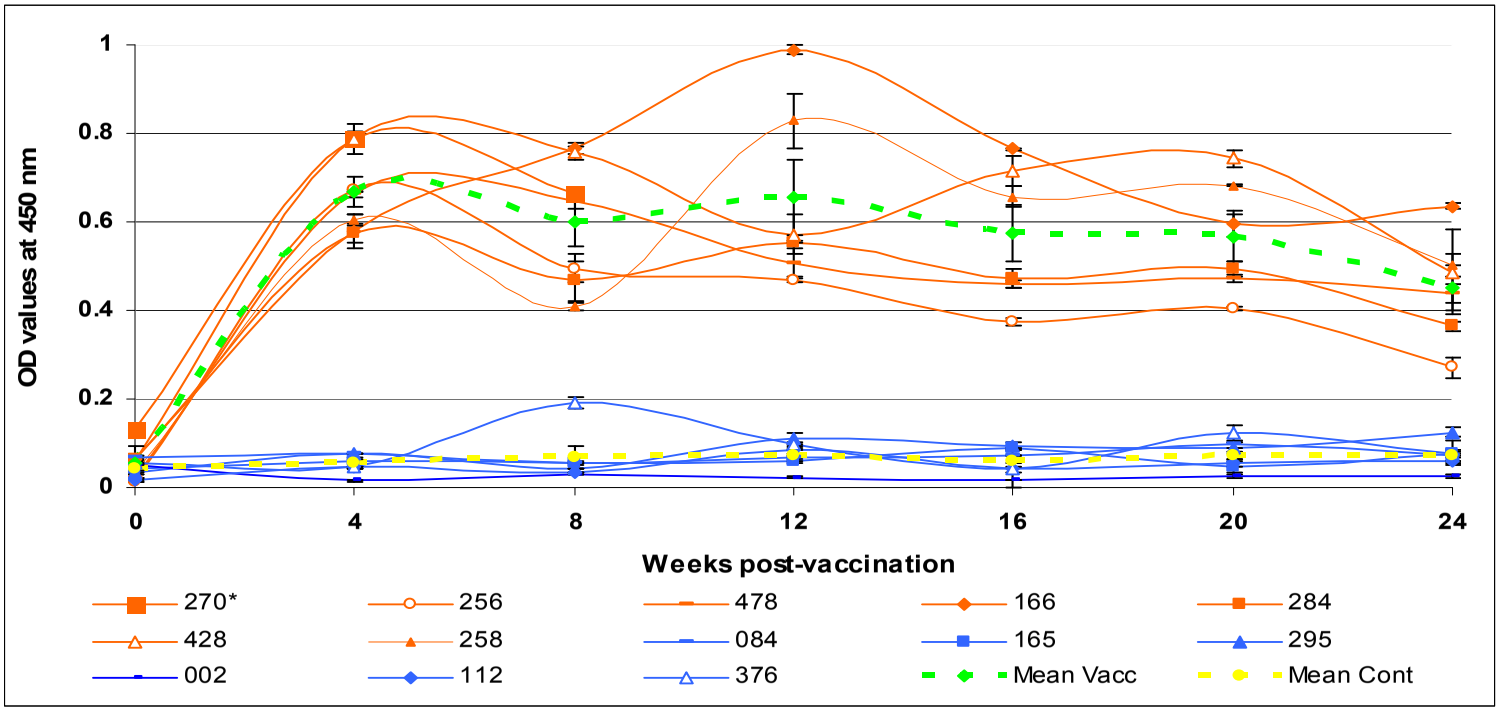


Figure 5.16 Antibody responses in Neoparasec™ vaccinated and unvaccinated sheep at different time points post-vaccination. The purified recombinant glnH protein (500 ng) was used as a coating antigen. Sheep serum was used at 1:100. Goat anti-sheep IgG peroxidase conjugated antibody was used at 1:15,000. Each OD value ($OD_{450\ nm}$) represents the mean value of two determinations minus background OD. Orange lines with different legend key represent individual sheep in Neoparasec™ vaccinated group while the blue lines represent sheep in the unvaccinated control group. Interrupted green line represents mean of vaccinated group whereas the interrupted yellow line represents mean of control group. Note that data on antibody responses for sheep number 270 indicated with an asterisk on a legend are available only up to 8 weeks post-vaccination. Raw data are shown in Appendix 7

Antibody responses of sheep naturally infected with MAP (Figure 5.17) showed that that the infected animals (n = 5) had an OD in the range of 0.69-1.21. Of the 34 uninfected sheep sera tested, 32 showed OD values between 0.03 and 0.12. The remaining uninfected sheep, sample numbers 15 and 12, had OD values of 0.689 and 0.474 respectively. The group mean responses showed that the infected sheep had significantly ($p < 0.05$) higher antibody levels than the uninfected sheep (Table 5.6)

Figure 5.18 shows the antibody responses of deer having natural MAP infection. All the infected deer (n = 9) had OD values above 2.0 whereas the the uninfected controls (n = 11) had OD values ranged from 0.11-0.80. The group mean antibody responses differed significantly ($p < 0.05$) between naturally infected and uninfected control group (Table 5.6).

Table 5.6 Group mean antibody responses of sheep and deer naturally infected with MAP. Asterisk (*) indicates significant differences ($p < 0.05$) between the infected and the uninfected groups.

Source of sera	Least square OD mean ($OD_{450\ nm}$) ± standard error		p-value
	Infected	Uninfected	
Sheep	0.9102 ± 0.0906	0.1096 ± 0.0217	0.0001*
Deer	2.6409 ± 0.0646	0.3121 ± 0.0585	0.0001*

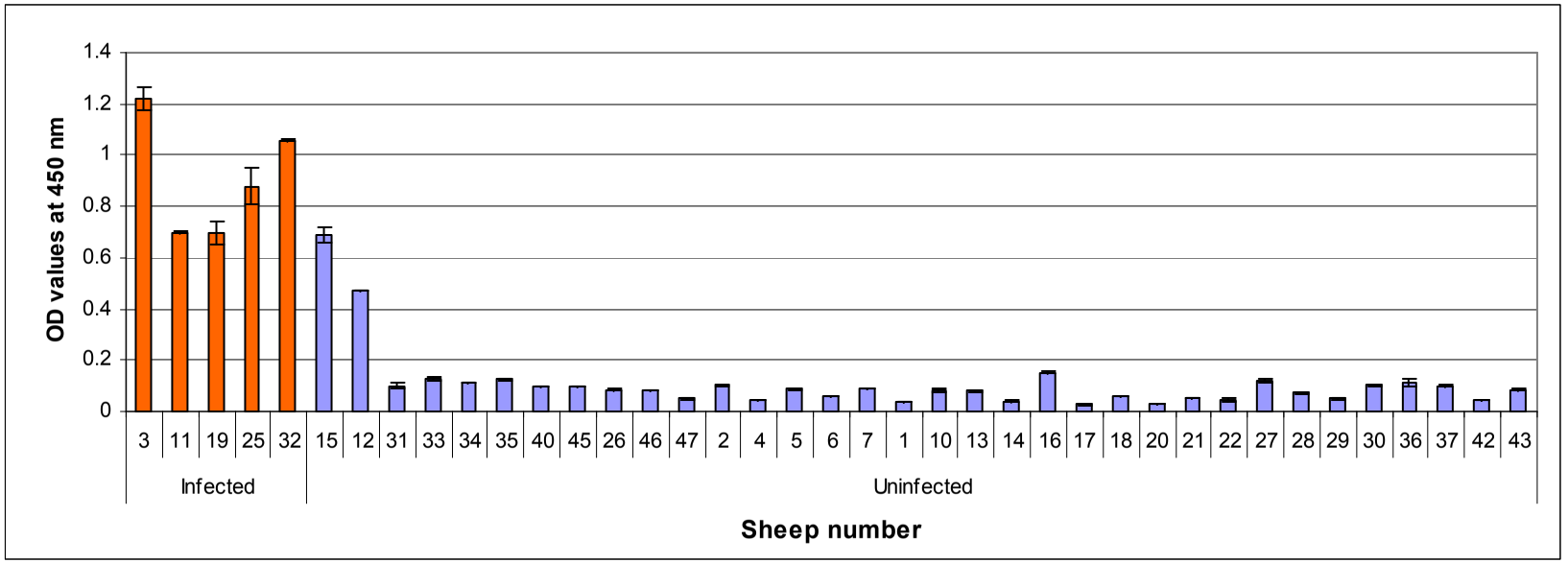


Figure 5.17 Antibody responses in sheep having natural MAP infection and uninfected controls. The purified recombinant glnH protein (500 ng) was used as a coating antigen. Sheep serum was used at 1:100. Goat anti-sheep IgG peroxidase conjugated antibody was used at 1:15,000. Each OD value ($OD_{450\ nm}$) represents the mean value of two determinations minus background OD. Raw data are shown in Appendix 8.

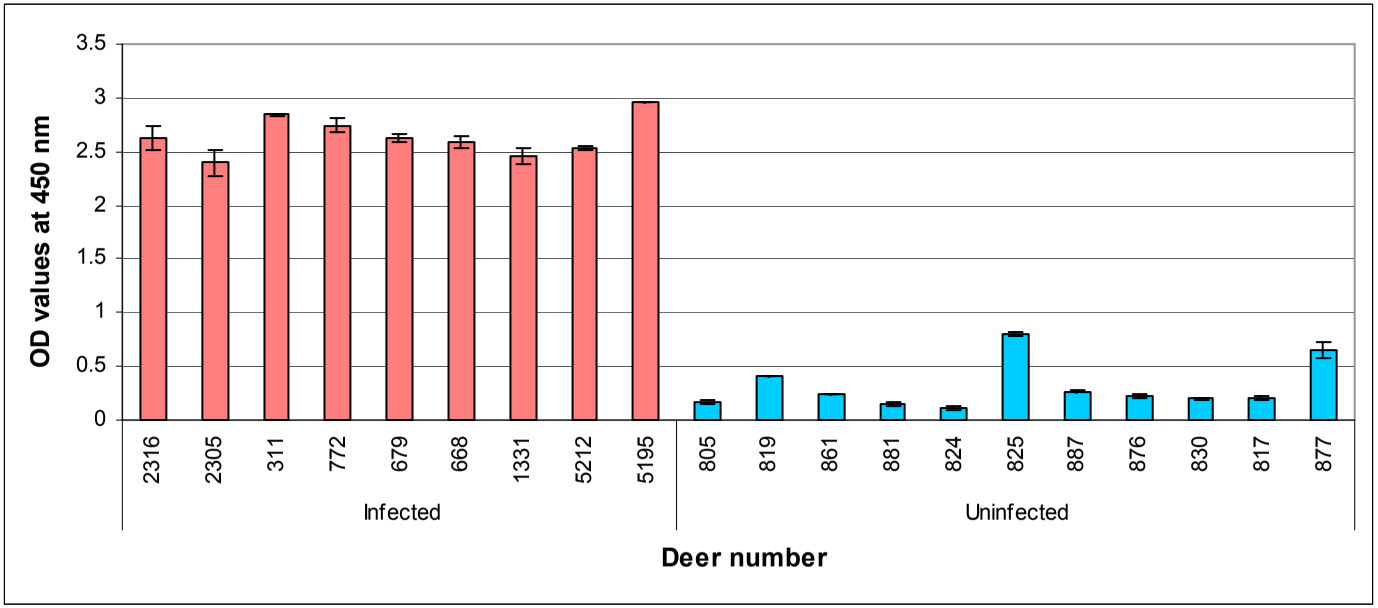


Figure 5.18 Antibody responses in deer having natural MAP infection and uninfected controls. The purified recombinant glnH protein (500 ng) was used as a coating antigen. Deer serum was used at 1:100. Rabbit anti-deer IgG peroxidase conjugated antibody was used at 1:500. Each OD value ($OD_{450\ nm}$) represents the mean value of two determinations minus background OD. Raw data are shown in Appendix 9.

5.5 Discussion

One approach towards understanding the pathogenic mechanism of MAP and the immune responses against these bacteria is to identify those MAP proteins that are recognised by the immune cells regulating the host's immune response. Several research groups have identified immunologically important MAP antigens including those associated with the cell wall or secreted antigens (Dupont *et al*., 2005; Basagoudanavar *et al*., 2006; Cho *et al*., 2006; Shin *et al*., 2006; Bannantine *et al*., 2007; Leroy *et al*., 2007; Kathaperumal *et al*., 2008; Pradenas *et al*., 2009; Santema *et al*., 2009; Newton *et al*., 2009). In the present study, the glnH protein that was recognised in Western blots by sheep experimentally vaccinated with the live attenuated MAP strain 316F (Neoparasec™) (Chapter 4) was further characterised.

Comparison of amino acid sequences carried out in this study indicated that similar proteins to glnH in MAP are present in a number of mycobacterial species, which include *M. tuberculosis*, *M. bovis*, *M. gilvum*, *M. smegmatis*, *M. ulcerans* and *M. vanbaalenii*. BlastP search of the glnH against *M. avium* subspecies *avium* suggested that the glnH exhibits significant amino acids sequence identity (99.7%) to the extracellular solute-binding protein, family protein 3, which is thought to be involved in the active transport of specific amino acids across the cytoplasmic membrane. As seen in Figure 5.2, the first 25 amino acids of the extracellular solute binding protein of *M. avium* subspecies *avium* are absent in the glnH of MAP, while the remainder (except amino acid 26, methionine instead of valine) are the same. Kyte-Doolittle analysis suggested that this region (amino acids 1-25) is hydrophilic and BlastP analysis gave no similarity to any annotated proteins of MAP strain K-10.

The high similarity observed between MAP and *M. avium* subspecies *avium* is not surprising as genetically they are reported to be nearly identical (Turenne *et al*., 2007). Several studies have shown a high degree of sequence identity between MAP and *M. avium* subspecies *avium* at the protein level. For instance,

the gene encoding the three Ag85 components from MAP, an immunodominant Th1-type antigen was reported to be highly similar (99% identity), at the protein level, with *M. avium* subspecies *avium* (Dheenadhayalan *et al*., 2002). A study of Mullerad and others (2003) also showed that the hypothetical thiol peroxidase (the 16.7 kDa protein) of MAP was very similar to the thiol peroxidase of *M. avium* subspecies *avium* strain 101.

The fact that the sequences of the glnH and the extracellular solute-binding protein are identical may suggest that cross-reactivity could occur and may have implications for use of the glnH protein as a diagnostic tool. However it is possible that animals exposed to *M. avium* subspecies *avium* may not produce significant levels of antibody that reacts with glnH, because the gene for the extracellular solute-binding protein in *M. avium* subspecies *avium* may not be expressed or it may only be expressed at very low levels. Further studies are required to determine potential cross-reactions in animals exposed to *M. avi*um subspecies *avium*.

Antigens that are produced in greater amount in MAP with respect to other mycobacteria might be useful differential diagnostic reagents. Alkyl hydroperoxide reductase C (AhpC) of MAP, which differs in only four amino acids in *M. avium* subspecies *avium* and shares 90% identity with *M. tuberculosis*, was shown to be highly expressed only in MAP cultures (Olsen *et al*., 2000). Antisera raised to MAP AhpC reacted with extracts from all MAP isolates tested but did not react with *M. avium* subspecies *avium* or *M. tuberculosis*. Elsaghier and co-workers (1992) reported that specifically elevated antibody responses to AhpC were found in MAP-infected mice but not in *M. avium* subspecies *avium*-infected mice suggesting that the protein was also expressed *in vivo* by MAP. The study of Cho and others (2007) showed that *in vitro* expression of the MAP hypothetical protein (MAP2168c), shown in ELISA using rabbit antisera raised to MAPJTC303 culture filtrate, was significantly higher in MAP than in *M. avium*

subspecies *avium*. Investigation of the expression of the glnH of MAP, *in vitro* and *in vivo*, would be of interest.

Histochemical and immunology procedures combined with recent advances in microscopy have enabled researchers to study surface localisation of target proteins. These techniques have been employed for localisation of surface immunogenic proteins in a number of bacteria including the group B Streptococcus (Rioux *et al*., 2001), the 35 kDa major membrane protein of MAP (Bannantine *et al*., 2003), the mycobacterial PE_PGRS of *M. bovis* and *M. tuberculosis* (Brennan *et al*., 2001) and a putative protein p27 (Rv2108) of *M. tuberculosis* (Le Moigne *et al*., 2005). In immuno-staining procedures, it is widely known that antibodies are an important tool for demonstrating both the presence and the subcellular/surface localisation of an antigen. In addition, it is very important to establish that immunoglobulins have been specifically raised in response to immunisation by the antigen. In this study, antibodies against the recombinant glnH protein have been successfully produced and purified from a rabbit and shown that they recognised epitopes on the surface of the MAP strain 316F cells (Figures 5.5 and 5.6).

Protective immunity for resistance to mycobacterial diseases is dependent on activation of the cellular immune response with the production of the Th1 cytokine gamma interferon (Cunningham *et al*., 2000; Flynn & Chan, 2001). As a measure of cellular immune responses, whole blood containing PBMC's from the vaccinated sheep was stimulated with the recombinant glnH protein. Results from this study revealed that there was no significant IFN-gamma production in Neoparasec™ vaccinated sheep in response to stimulation with the recombinant glnH protein (Table 5.4) suggesting that the protein was not a stimulator of proliferative T-cell responses. It has been reported that the immunogenicity of proteins can also be affected by post-translation modification especially glycosylation and acylation when expressed in *E.coli* (Samanich *et al*., 2000). This was observed in a study carried out by Temmerman and colleagues (2004)

who reported that the heparin-binding hemaglutinin of *M. tuberculosis* did not induce IFN-gamma secretion when expressed in *E. coli*. Similarly, Huntley and co-workers (2005) reported that the 19 kDa protein of MAP expressed in *E. coli* was not as strong stimulator of proliferative T-cell responses as has been reported for its counterpart in *M. tuberculosis*. Although not demonstrated by the studies described here, it is possible that post-translation modification differences may account for the absence of IFN- gamma responses.

Following phagocytosis of MAP bacteria by macrophages, two main immunological responses can occur; the Th1-type response (cellular immunity), which is involved in defence against the intracellular pathogen or the Th2-type response (humoral immunity), which promotes strong antibody responses (Stabel, 2007). In this study, antibody response in sheep and deer to the purified recombinant glnH was determined using Western blot analysis and ELISA. Western blots using the glnH protein resulted in prominent bands with different intensities in all the vaccinated sheep at 4 and 8 weeks post-vaccination (Figure 5.12) and in at least 5 of 6 sheep at a later time points. Further investigation to determine if antibody to the glnH was present in animals with naturally acquired MAP infection also showed that four of five naturally infected sheep had detectable antibody to the glnH protein on Western blots (Figure 5.14). This study also showed that nine serum samples taken from deer having natural MAP infection reacted with the glnH protein (Figure 5.15). The reactivity of sera particularly from naturally infected animals suggests that the glnH protein possesses immunoreactive epitopes that are expressed *in vivo* during the development of paratuberculosis in sheep and deer.

Faecal culture and ELISA are the most widely used tests for diagnosis of paratuberculosis (Pradenas *et al*., 2009). The ELISA developed in this study using the glnH recombinant protein as the solid-phase antigen in the assay further confirmed the immunogenicity of the glnH protein. Neoparasec™ vaccinated sheep showed significantly higher responses, with a peak reached at

4 weeks post-vaccination, to the glnH compared to unvaccinated sheep (Figure Figure 5.16, Table 5.5). Similarly, both naturally infected sheep and deer exhibited significantly higher antibody responses in comparison to uninfected animals (Table 5.6). Interestingly, deer sera showed higher responses to the glnH protein compared to sheep sera as it determined by the OD value. Koets and co-workers (2001) reported that antibody responses may vary depending on the properties of the antigen as well as the stage of disease. It has been reported that the process from MAP infection to death in deer progresses much earlier than in most animals, with animals dying as early as eight months of age (Mackintosh *et al*., 2004b). As the sera in this study were obtained from MAP infected young deer, this might indicate that the disease has progressed beyond the sub clinical stage. It would be interesting to monitor the antibody response to glnH during the onset and development of paratuberculosis from the preclinical through the clinical stage.

In summary, the results presented in this study showed that serum antibodies from experimentally vaccinated animals as well as naturally infected animals reacted to the recombinant glnH protein. This suggests that further studies are warranted to investigate its usefulness as an immunodiagnostic reagent for MAP infection.

CHAPTER 6

General discussion and summary

Paratuberculosis, a chronic enteritis of ruminants, is responsible for substantial worldwide economic losses in livestock and dairy industries. Control programmes have been initiated, to stop the spread of paratuberculosis, but several factors including the slow multiplication rate of the micro-organism, the long survival time of MAP in the environment, ineffectiveness of currently available vaccines and the large proportion of subclinically infected animals have limited the success of controlling this disease (Roupie *et al*., 2008). An important step towards the design of improved vaccines and diagnostic reagents that can assist paratuberculosis control is the identification and characterisation of the MAP components involved in the host immune response. The aim of the work presented in this thesis was to identify MAP exported proteins that may be immunogenic, as determined by their ability to elicit IFN-gamma secretion or antibody production in vaccinated or infected animals. This was achieved by the use of alkaline phosphatase (AP) fusion technology, which allows genes to be identified on the basis of the encoded protein being exported from the cytoplasm.

In Chapter 3 the construction of MAP strain 316F PhoA fusion clones is described that led to the identification of a number of genes encoding exported proteins including lipoproteins, proteins involved in a number of cellular processes and hypothetical proteins of unknown function. Analyses of the deduced amino acid sequences revealed that 13 out of 17 fusion proteins producing active alkaline phosphatase had signal peptides that may direct the secretion process. This suggested that the the PhoA technology identified the proteins exported by the general export pathway. The results are in agreement with the study of Gomez and colleagues (2000), which indicates that many secreted proteins of *M. tuberculosis* are secreted via the general export pathway.

Apart from fusion proteins that had signal peptides, a number of recombinant plasmids contained a very short sequence of amino acids fused to PhoA that were capable of directing export in *M. smegmatis* were also found in this study. This leads to a false positive result demonstrating a limitation associated with this technology. A further limitation is that the strategy identifies, in most instances, only a part of the gene sequence which then requires locating the full length gene for further characterisation. Construction of a bacteriophage lambda genomic library and/or combination of proteomic and genomic analyses is required to facilitate the identification of the full gene sequence. However, this study suggests that in spite of these limitations, *phoA* gene fusion technology is a useful and rapid technology to identify novel exported proteins.

Three fusion proteins, glnH (MAP3894c), subI (MAP2213c) and a hypothetical protein (MAP3273c), identified through screening PhoA positive blue colonies and database searches, were chosen for further studies. The proteins were selected for a preliminary study aimed at evaluating their capacity to induce a humoral antibody response. All proteins were cloned and expressed in *E. coli*. The results presented in Chapter 4 confirmed that the expression vector allowed heterologous expression of recombinant protein in *E. coli.* Moreover, the inclusion of a polyhistidine tag together with the cobalt affinity chromatography purification system resulted in reasonably pure recombinant protein, as evidenced by SDS-PAGE analysis. If required, further purification of these recombinant proteins could be achieved by size exclusion column chromatography.

Although the proteins were successfully produced and purified from *E. coli*, they were all found to be in the insoluble fractions of the bacterial cell pellet. Such solubility problems are often encountered when mycobacterial proteins are expressed in *E. coli*. Bashiri and others (2007), who reported the statistical data from 134 laboratories joining the tuberculosis structural genomics consortium, indicated that while 80% of cloned *M. tuberculosis* ORFs were expressed

successfully in *E. coli*, only 32% of these were obtained in the soluble form. The authors suggested that the use of an alternative expression host, such as *M. smegmatis* that is more closely related to the organism from which the target protein comes, may be one alternative approach to overcome insolubility. However, it is more difficult to obtain large quantities of recombinant protein in *M. smegmatis* because commercially available high yield expression systems are currently not available for this organism.

A second approach is to solubilise and refold the target protein from insoluble inclusion bodies. Khan and co-workers (1998) used a high pH (pH 12) Tris-HCl buffer in the presence of low concentrations of denaturants, such as urea, for solubilisation of ovine growth hormone expressed in *E. coli*. They found that more than 90% of the protein from inclusion bodies could be solubilised by this method, which also enhanced the overall yield of the bioactive protein. In this study, solubilisation of target proteins from *E. coli* inclusion bodies, with yield ranging around 2.82-8.7 mg of purified protein from 500 ml of induced culture, was achieved by using a high concentration of urea. Despite problems associated with insolubility, a number of immunogenic MAP proteins including P22 (Ridgen *et al*., 2006) have been produced in *E. coli*.

Preliminary immunological screening of glnH, subI and the hypothetical protein (MAP3273c) using sera from Neoparasec™ vaccinated sheep and from control pre-vaccinated animals suggested that glnH was immunogenic as shown by Western blots. The blots showed that sera from five out of eight vaccinated sheep (62.5%) strongly recognised the glnH protein compared with sera from one out of eight (12.5%) control pre-vaccinated animals. Although sera from vaccinated sheep also reacted with the hypothetical protein (MAP3273) and subI, cross-reactive antibodies could also be detected in the sera taken prior to vaccination. Further studies, with larger numbers of animals, would be required to establish the statistical significance of this observation.

The glnH protein is responsible for the first step in the active transport of L-glutamine across the cytoplasmic membrane. The protein was found on the surface of its native host, MAP strain 316F, as shown by immuno-histochemistry studies (Chapter 5). Database searches and multiple sequence alignment conducted in this study indicated that similar proteins are present in a number of mycobacterial species, suggesting that immunological cross-reaction may occur if this protein was used as a diagnostic reagent. Although there is a high amino acid sequence similarity between glnH in MAP and the extracelular solute-binding protein, family protein 3, in *M. avium* subspecies *avium*, it is not known whether *M. avium* subspecies *avium*-exposed animals develop antibodies to glnH. Clearly, this topic would require further research. With the availability of anti-glnH antibody generated in this study, it would be of interest to undertake immunogold electron microscopy on *M. avium* subspecies *avium* cells.

Another relevant approach to evaluate the specificity of glnH would be to screen herds or flocks assessed to be free of MAP infection. If cross-reactions were found to occur, it may be possible to use epitope mapping of glnH to produce peptide antigens to improve specificity. In serodiagnostic assays, pre-adsorption of serum with crude fractions from environmental mycobacteria, such as *M. phlei*, can diminish cross-reacting antibodies. A pre-adsorption step with *M. phlei* has proven to increase the specificity of ELISA by removal of non-specific antibodies from test sera (Shin *et al*., 2008; Gumber *et al*., 2009; Santema *et al*., 2009). However, minimal changes in antibody binding patterns to culture filtrate proteins, due to serum adsorption with *M. phlei*, have been observed in Western blots (Cho & Collins, 2006; Pradenas *et al*., 2009).

The disease pattern of paratuberculosis is linked to the immune status of the host. Both humoral and cellular responses may play a role in the host during MAP infection (Basagoudanavar *et al*., 2006), but the latter are predominant in the early stages of infection (Stabel, 2000; Coussens, 2001). The important characteristics of antigens that elicit protective immunity is not known, but those

proteins that are capable of inducing high levels of IFN-gamma secretion have generally been selected for inclusion in novel mycobacterial vaccine preparations. Results of the IFN-gamma assays presented in Chapter 5 suggest that Neoparasec™ vaccinated sheep did not elicit an IFN-gamma response to *E. coli* produced glnH recombinant protein. If time permitted, a study on the biological activity of glnH produced in *M. smegmatis* would have been worthwhile because the *E. coli* produced protein may be missing critical modifications, which are crucial for its activity. Furthermore, it might be worthwhile to investigate the IFN-gamma responses in both experimentally and naturally infected animals using more sensitive techniques. A recent study has shown an improved method, known as the enzyme-linked immunospot (ELISPOT) (Begg *et al*., 2009), for the detection of IFN-gamma in paratuberculosis. The method detects the number of IFN-gamma producing cells rather than the total amount of IFN-gamma as is measured by traditional cytokine ELISA assays, such as Bovigam® assay. Moreover, this method has been reported to be 10-200 times more sensitive in the detection of cytokines than traditional methods (Tanguay & Killion, 1994). Using both experimentally immunised and naturally exposed sheep, Begg and colleagues (2009) in their study indicated that the ELISPOT may provide a method as sensitive as or more than the traditional IFN-gamma ELISAs.

Chapter 5 describes the antibody responses to the glnH protein. Western blot analyses showed that in the 2008 Neoparasec™ vaccination trial, 7 out of 7 sera collected 8 weeks post-vaccination reacted with the glnH protein compared to 0 out of 7 pre-vaccination sera (Figure 5.12). If these data regarding reactivity to the glnH is considered along with the 2007 Neoparasec™ vaccination trial data (Table 4.3, Chapter 4), the combined results show that a total of 12 out of 15 (80%) had antibodies to the glnH 8 weeks post-vaccination compared with only 2 out of 15 (13%) animals prior to vaccination. This suggests that the glnH is an immunologically active component of the Neoparasec™ vaccine. Western blot analyses also confirmed that antibodies to this protein were present in naturally infected sheep and deer.

Although Western blot analysis is valuable for research purposes, it is impractical for large- scale field studies. In addition, Western blots are not easily quantitated. An ELISA assay developed in this study showed that there was a significant ($p < 0.05$) difference in antibody responses to the glnH protein, between the vaccinated and the unvaccinated sheep. Similarly, both naturally infected sheep and deer showed significantly ($p < 0.05$) higher antibody responses to glnH protein as compared to uninfected animals. There was also good agreement between the ELISA and Western blots particularly in naturally infected sample sera. For example, the infected sheep number 3 and the infected deer number 5195 showing the highest OD value in the ELISA also showed the strongest band intensity in Western blot analyses. This may suggest that the ELISA developed is as sensitive as the Western blots. Collins and others (2005) stated that ELISA offers the best testing option, for large-scale serological testing, for the support of paratuberculosis control programmes due to its convenience of sample collection, large capacity, rapid laboratory turnaround time and low cost. Although a very limited number of animals were included, this study has confirmed the immunogenicity of the glnH protein and suggests that the protein may be worth investigating as a serodiagnostic reagent. Clearly, further studies with more animals are needed. As natural MAP infection in most species is characterised by considerable variation in pathology and immune responsiveness between animals (Begg & Whittington, 2008), it is also necessary to determine if antibody responses to glnH are present at all stages of infection.

Several promising MAP species-specific seroantigens have been identified (Bannantine *et al*., 2004b; Shin *et al*., 2006; Leroy *et al*., 2007; 2009). The use of a specific purified individual protein as the solid phase antigen in paratuberculosis ELISA may be attractive but such assays have suffered from low diagnostic sensitivity (Huntley *et al*., 2005). Therefore, the most efficient diagnostic test would be based on a combination of purified antigens. Several studies in *M. tuberculosis* have suggested that the pool of two or more purified

antigens can be useful for the development of an efficient serodiagnostic test (Lyashchenko *et al*., 2000; Julian *et al*., 2004). Leroy and others (2007) evaluated the antibody response of seven positive reference sera that originated from naturally infected cows against three different MAP secreted proteins, MAP1693c, MAP4308c and MAP2677c, either individual or in combination in ELISA. Their results showed that only three or five animals were detected positive in ELISA using a single antigen while the three antigens tested as a single pool was detected by all positive sera used. In this respect, the glnH protein identified in this study may be worth considering for inclusion in a cocktail of antigens to develop a high efficiency ELISA-based serological test for paratuberculosis. Other sequences representing potentially novel exported protein genes isolated from the MAP PhoA fusion clones described in Chapter 3 may also be evaluated as potential vaccine candidates or diagnostic antigens.

In summary, the studies presented in this thesis have contributed to the general body of information concerning the immunogenic proteins of MAP vaccine strain 316F. The genetic approach taken to search for immunogenic proteins by selecting for exported proteins using PhoA technology has resulted in the identification and characterisation of an immunogenic glutamine binding protein (glnH), a protein that has not previously been studied in MAP.

Appendix 1 Predicted export signal for MAP fusions protein.

Clone	Amino acid sequence[a]	Export signal[b]
Mptb271	MDIRTAARWRPVLALVLTAGVVAGCHGGASDA↓VGGTGPADARTSITLVAYSVPEPGWSKIIPAFNASDEGKGIQVVTSYG ASGDQSRGVVDGKPADVVNFSVEPDIARLVKAGKVAKDWNTDATKGIRIRTVPDSYTQVASWTEPEPFC	33
Mptb371	VRGWTPTTEGNWRIVAIKILALVGSLRSASINRQIAELASA↓VAGEDVVVTVFEGLGELPFYNEEIRIRTVPDSYTQVASWTEPEPFC	41
Mptb229	MTAGLPLLRRACTALAAVLVAA↓GCGHTESLRVASVPTLPPPTPVGMEQLPPQPPLPPDGPDQNCDLTASLRPFPTKAEADAAVADIRA RGRLIVGLDIGSNLFSFRDPITGEITGFDVDIAGEIARDIFGAPSHVEYRIRIRTVPDSYTQVASWTEPEPFC	23
Mptb131	MNPMTVQAEREVAMANASTSRFDGDLDA↓QSPAADLVRVYLNGIGKTALLNAAGEVELAKRIEAGLYAEHLLETRKRLGENRKRDLEA VVRDGQAARRHLLEANLRLVVSLAKRYTGRGMPLLDLIQEGNLGLIRAMEKFDYTKGFKFSTYATWWIRIRTVPDSYTQVASWTEPEPFC	28
Mptb385	LIRYGGIVALPQLTDEQRAAA↓LEKAAAARRARAELKDRLKRGGTNLSQVLKDAETDEVLGKMKVSALLAELPKVGKVKAQEIMTELEI RIRTVPDSYTQVASWTEPEPFC	21
Mptb368	VIVAGARTPIGKLMGSLKDFSASDLGAIAIAGALEKA↓DVPADLVEYVIRIRTVPDSYTQVASWTEPEPFC	37
Mptb416	MRACYAGSLNCRGVAYG↓SPMPAVEFATLDDDIRIRTVPDSYTQVASWTEPEPFC	17
Mptb419	MTIQTVMIVWSGRLTA↓KEKRVAPINSPVSGTDEALTRRGLRHALDKTTDLAERELRVPLHYYRDPKIRIRTVPDSYTQVASWTEPEPFC	16
Mptb409	VTLGTERERRLTGWLRGRLPDA↓DDVRVEGIRIRTVPDSYTQVASWTEPEPFC	22
Mptb376	MIRASRTGLSRGDRFCQPAISPPRA↓GPRDVLVSHPVLPERHRKVTTPKTFADLGVPARIVDALTARGITSPFPIQAETLPDTLAGRDV LGRGKTGSGKTLAFSIPLVGRLSTGNRRPARPTGLVLAPTRELATQITATLEPLAAACGLRVSTIFGGVSQHRQVTALKAGVDIVV ACPGRLEDLMRQRLIRIRTVPDSYTQVASWTEPEPFC	25
Mptb305	LGPRRALTGEPLPALLPATAAAQRYG↓AIGTDHVAVIRQFFDQLPEAVDVETCEHAERQLAAQATQFRPDQLSKLARRLMDCLNPDGRY SDEDRARARGLVLGNQQADGMSRLSGWLTPEARASWEAVLAKLAALGMCNPDDDAPVVDGPPPEAAAQRDSRSAAQRNHD GLNAALRALLAGGKLGQHNGLPASIRIRTVPDSYTQVASWTEPEPFC	26
Mptb259	MTHYTPLL VALPLAGA↓AILLFGGRRTDRWGHWLGCATAVAAFVVGVGLLDELLGRPADQRAIRIRTVPDSYTQVASWTEPEPFC	16
Mptb257	MNTGFPDPETVRTALALASR A↓PSVHNTQPWRWRIRIRTVPDSYTQVASWTEPEPFC	21

Appendix 1 Continued ……..

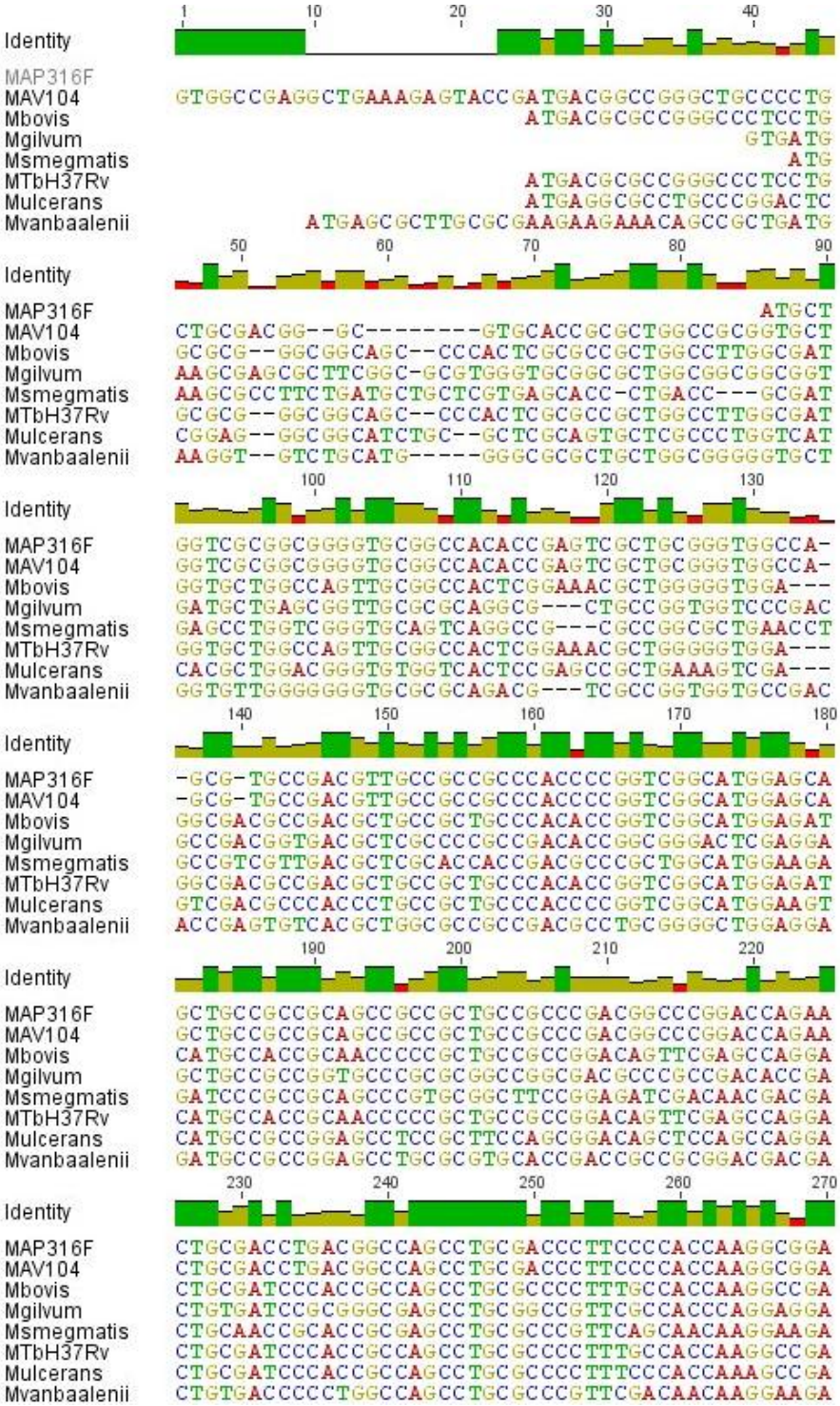
Mptb241	MTVPESLDEFARTDLLLDALAQRRPVPRGQVEDPDDPDFQMLTTLLEDWRDNLRWPPASALVTPEEAVNALRAQLAERRRGHRGLAVV GSVAATLMLLSGFGAMVVEARPGSTLYGLHAMFFDQPRVNEKDQVMLAAKADLAKVAESIRIRTVPDSYTQVASWTEPEPFC	TMS (1) 84-106
Mptb357	VAQSPYARLSRSELAVLVPELLLIRIRTVPDSYTQVASWTEPEPFC	0
Mptb356	MVSLLVHAVLGFSVIAWIRIRTVPDSYTQVASWTEPEPFC	0
Mptb206	LTLSGATAHAENQPAPPSPTPPPLAPKCLSFDFGPPAHWNYLPCGWTTDGKRWIRIRTVPDSYTQVASWTEPEPFC	0
Mptb327	GRPNRPPRPAARSAAPDLPVFRIRIRTVPDSYTQVASWTEPEPFC	N/A
Mptb284	WIRIRTVPDSYTQVASWTEPEPFC	N/A
Mptb354	TDGMLEQIRIRTVPDSYTQVASWTEPEPFC	N/A
Mptb315	AAAPSEIRIRTVPDSYTQVASWTEPEPFC	N/A
Mptb 380	GRPMPAPKIRIRTVPDSYTQVASWTEPEPFC	N/A

[a]PhoA linker (underlined), for inner membrane proteins cytoplasmic loops (red), transmembrane segments (light green), peripasmic loops (blue).

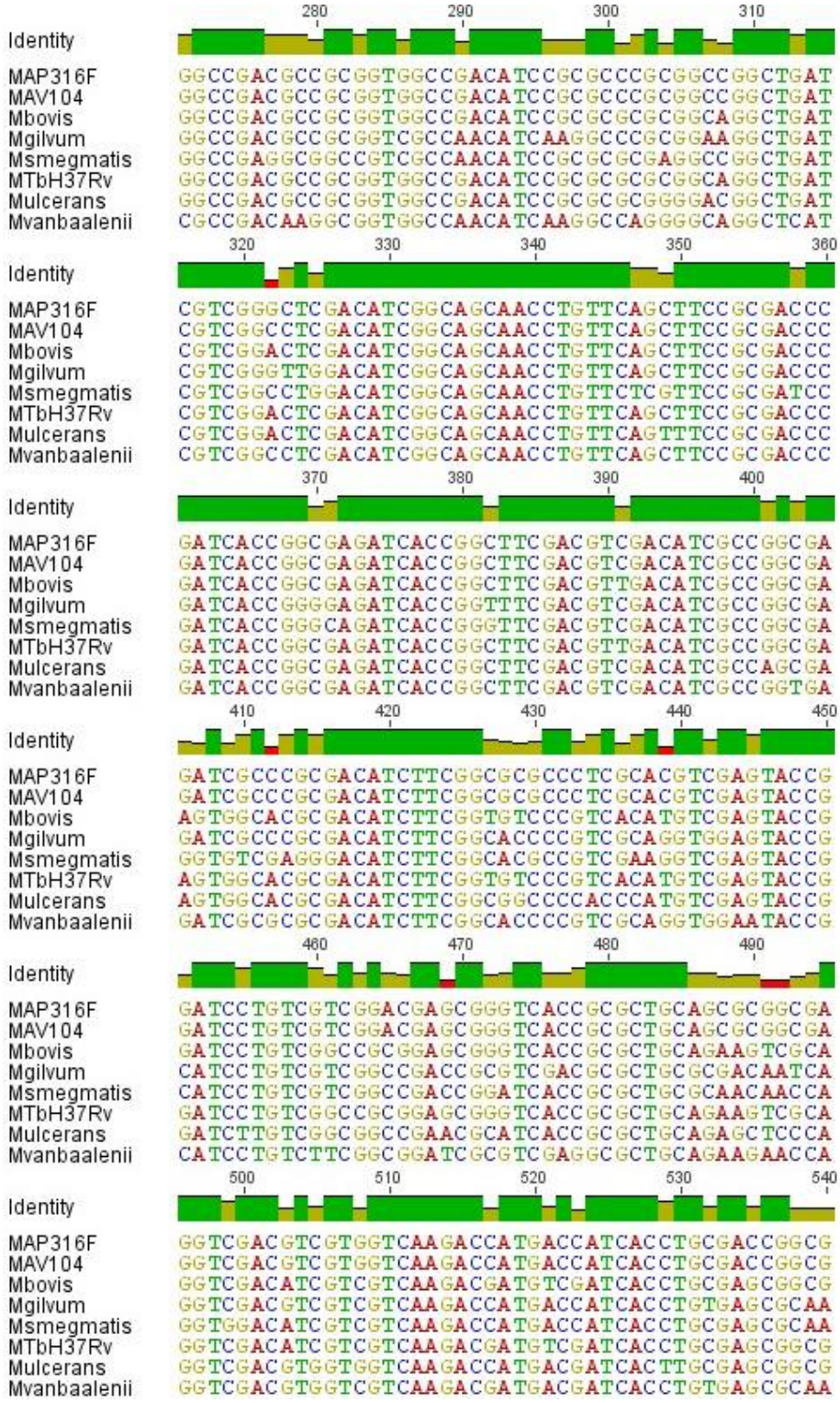
[b]Export signal, and their predicted cleavage sites of the fusion proteins were predicted based on HMM method (SignalP 3.0). Cleavage sites of fusions proteins are indicated with a red arrow. TMS, transmembrane segments were predicted using TMHMM v2.0. The number and location of predicted TMSs are shown in parentheses.

N/A, not applicable

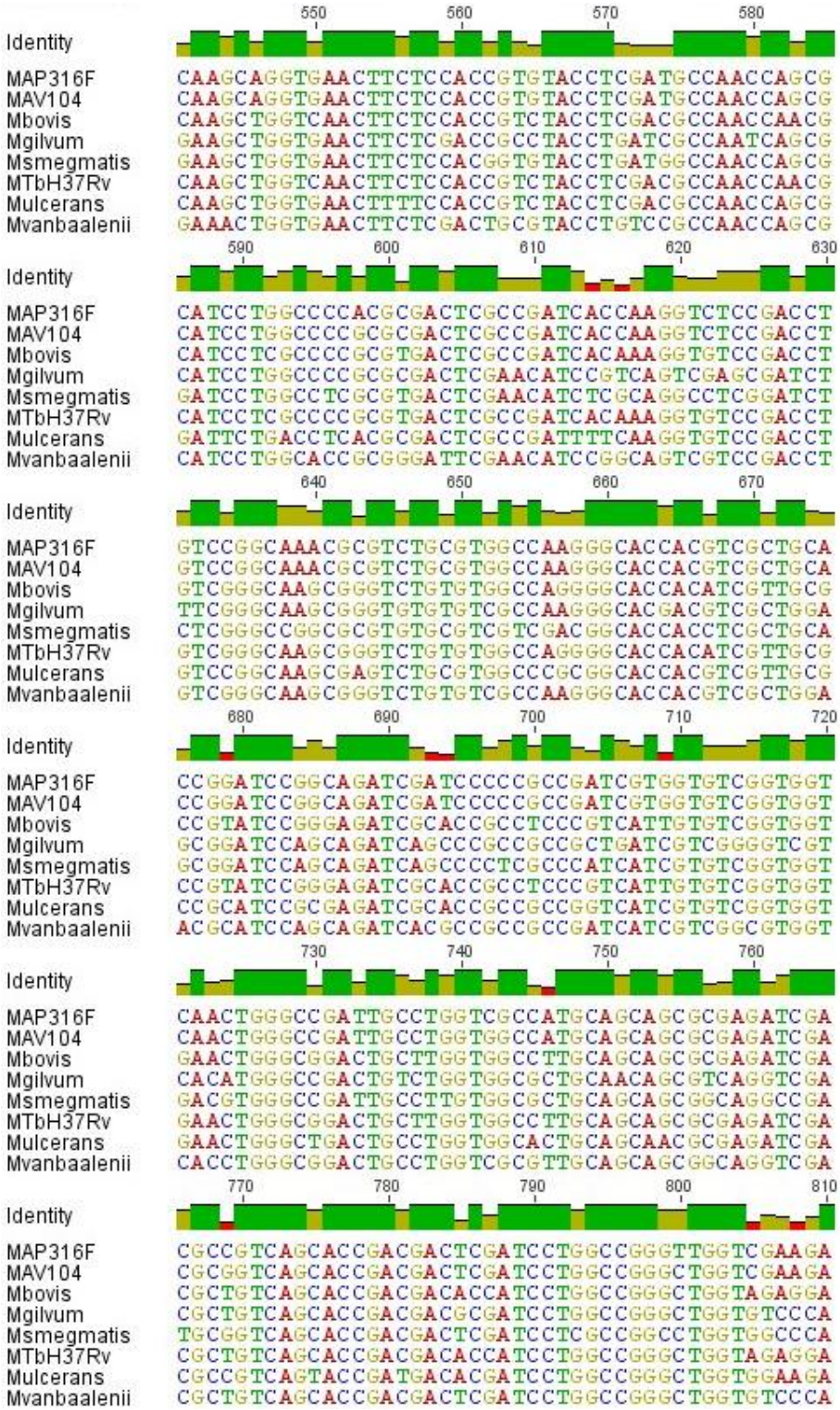
Appendix 2 Nucleotides alignment of the *glnH* gene in MAP and other mycobacterial species.



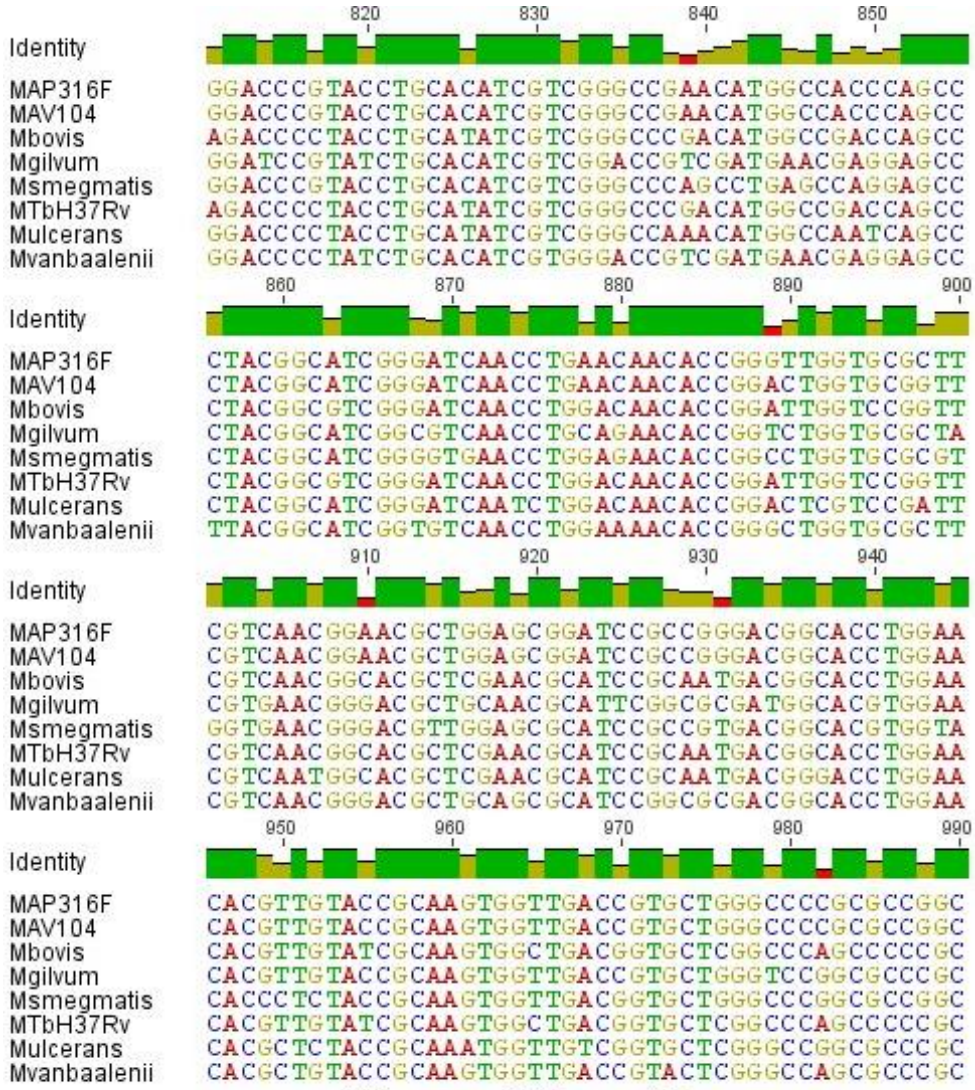
Appendix 2 Continued …….



Appendix 2 Continued ……..



Appendix 2 Continued …….



% identity scores to MAP316F

MAP316F	99
MAV104	85.1
Mbovis	78
Mgilvum	78.1
Msmegmatis	85.1
MTbH37Rv	83.4
Mulcerans	78.9
Mvanbaalenii	

Appendix 3 Raw data IFN-gamma assay Figure 5.8 (prior to vaccination).

Sheep number	OD 450 nm									
	PBS (1)	PBS (2)	ConA	PPDA (1)	PPDA (2)	glnH (1)	glnH (2)	PPDAavg - PBSavg	ConA - PBSavg	glnHavg - PBSavg
Vaccinated										
270	0.0744	0.0843	0.0711	0.0738	0.0671	0.0723	0.0703	0.00	0	0
256	0.0775	0.0978	0.3302	0.0804	0.0783	0.1078	0.1026	0.00	0.24255	0.01755
478	0.0637	0.0647	0.1153	0.0638	0.1038	0.1326	0.1349	0.02	0.0511	0.06955
166	0.0735	0.0714	0.1019	0.0687	0.0681	0.0698	0.0815	0.00	0.02945	0.004525
284	0.0731	0.067	0.1843	0.0771	0.0714	0.0792	0.0778	0.00	0.11425	0.00845
428	0.0735	0.0735	0.1273	0.0795	0.1112	0.0779	0.0764	0.02	0.0538	0.00365
258	0.0659	0.0634	0.0855	0.07	0.0607	0.0639	0.063	0.00	0.02085	0
Control										
084	0.0788	0.0703	0.1466	0.0652	0.0648	0.0738	0.0647	0.00	0.07205	0
165	0.2171	0.2295	0.2271	0.2329	0.213	0.2164	0.2209	0.00	0.0038	0
295	0.11	0.1204	0.2815	0.138	0.128	0.1645	0.155	0.02	0.1663	0.04455
002	0.0629	0.0642	0.0923	0.0696	0.0673	0.0712	0.0742	0.00	0.02875	0.00915
112	0.0642	0.122	0.1252	0.0984	0.0945	0.1214	0.166	0.00	0.0321	0.0506
376	0.0726	0.0672	0.3171	0.1352	0.2556	0.1091	0.133	0.13	0.2472	0.05115

Appendix 4 Raw data IFN-gamma assay Figure 5.9 (4 weeks post-vaccination).

Sheep number	OD 450 nm									
	PBS (1)	**PBS (2)**	**ConA**	**PPDA (1)**	**PPDA (2)**	**glnH (1)**	**glnH (2)**	**PPDAavg - PBSavg**	**ConA - PBSavg**	**glnHavg - PBSavg**
Vaccinated										
270	0.1069	0.0915	0.1243	0.2474	0.2358	0.0848	0.0882	0.1424	0.0251	0
256	0.0475	0.0486	0.2126	0.1813	0.21	0.0498	0.0522	0.1476	0.16455	0.00295
478	0.07	0.0847	0.0726	0.1913	0.1742	0.0589	0.0846	0.1054	0	0.003625
166	0.0499	0.0806	0.1551	0.2039	0.1664	0.0811	0.1077	0.1199	0.08985	0.02915
284	0.0799	0.0615	0.2428	0.7205	0.7552	0.0745	0.0778	0.66715	0.1721	0.00545
428	0.0681	0.1256	0.1808	0.2833	0.2625	0.0676	0.0639	0.17605	0.08395	0
258	0.0718	0.0701	0.1205	0.1028	0.1049	0.0775	0.085	0.0329	0.04955	0.0103
Control										
084	0.0475	0.0491	0.1273	0.0495	0.0497	0.0516	0.0473	0.0013	0.079	0.00165
165	0.0462	0.0762	0.1533	0.0485	0.0648	0.0746	0.0722	0.0018	0.0921	0.0122
295	0.0783	0.0815	0.2689	0.087	0.0957	0.1385	0.1338	0.01145	0.189	0.05625
002	0.1579	0.0726	0.079	0.0794	0.073	0.0591	0.0492	0	0	0
112	0.049	0.0509	0.0586	0.0463	0.0501	0.0509	0.0526	0	0.00865	0.0018
376	0.1208	0.0655	0.2539	0.0739	0.0715	0.0963	0.122	0	0.16075	0.016

Appendix 5 Raw data IFN-gamma assay Figure 5.10 (8 weeks post-vaccination).

Sheep number	OD 450 nm									
	PBS (1)	**PBS (2)**	**ConA**	**PPDA (1)**	**PPDA (2)**	**glnH (1)**	**glnH (2)**	**PPDAavg - PBSavg**	**ConA - PBSavg**	**glnHavg - PBSavg**
Vaccinated										
270	0.061	0.0673	0.1218	0.1503	0.1535	0.0748	0.0719	0.08775	0.05765	0.0092
256	0.0589	0.0937	0.3672	0.5243	0.4991	0.1044	0.0888	0.4354	0.2909	0.0203
478	0.0817	0.0845	0.4585	1.5491	1.4346	0.1456	0.1419	1.40875	0.3754	0.06065
166	0.0664	0.0748	0.2049	1.1721	1.1386	0.0844	0.0874	1.08475	0.1343	0.0153
284	0.0648	0.0678	0.4342	1.4624	1.3931	0.0804	0.0905	1.36145	0.3679	0.01915
428	0.0536	0.055	0.2297	0.4649	0.4954	0.0635	0.0651	0.42585	0.1754	0.01
258	0.0833	0.0803	0.2305	0.3611	0.3472	0.0883	0.0847	0.27235	0.1487	0.0047
Control										
084	0.0605	0.0567	0.159	0.0597	0.0586	0.0637	0.0647	0.00055	0.1004	0.0056
165	0.0596	0.0617	0.215	0.0583	0.049	0.0873	0.2245	0	0.15435	0.09525
295	0.0604	0.0548	0.1407	0.0592	0.0652	0.0685	0.0876	0.0046	0.0831	0.02045
002	0.056	0.0531	0.1116	0.0551	0.0764	0.0794	0.0547	0.0112	0.05705	0.0125
112	0.053	0.0654	0.1465	0.0668	0.0544	0.0828	0.0718	0.0038	0.0873	0.0181
376	0.0825	0.0611	0.1917	0.0714	0.0663	0.0924	0.1347	0	0.1199	0.04175

Appendix 6 Raw data IFN-gamma assay Figure 5.11 (12 weeks post-vaccination).

Sheep number	OD 450 nm									
	PBS (1)	**PBS (2)**	**ConA**	**PPDA (1)**	**PPDA (2)**	**glnH (1)**	**glnH (2)**	**PPDAavg - PBSavg**	**ConA - PBSavg**	**glnHavg - PBSavg**
Vaccinated										
256	0.0607	0.0492	0.5022	0.6478	0.6731	0.069	0.0706	0.6055	0.44725	0.01485
478	0.0838	0.0749	0.1551	0.4424	0.4506	0.0731	0.0922	0.36715	0.07575	0.006425
166	0.067	0.0615	0.3089	2.3485	2.3047	0.1238	0.13	2.26235	0.24465	0.06265
284	0.0533	0.0561	0.4535	1.0925	1.124	0.0989	0.0868	1.05355	0.3988	0.03815
428	0.0536	0.0649	0.1415	0.2071	0.19	0.0541	0.0535	0.1393	0.08225	0
258	0.0705	0.0701	0.24	1.097	1.0613	0.0803	0.0835	1.00885	0.1697	0.0116
Control										
084	0.053	0.0585	0.1519	0.0578	0.0689	0.0742	0.0539	0.0076	0.09615	0.009225
165	0.0621	0.0491	0.1745	0.0557	0.0518	0.0615	0.0662	0	0.1189	0.00825
295	0.0606	0.0575	0.1843	0.0604	0.0521	0.0668	0.0726	0.000675	0.12525	0.01065
002	0.0489	0.0499	0.12	0.053	0.0533	0.0495	0.0558	0.00375	0.0706	0.00325
112	0.0908	0.0466	0.1208	0.0524	0.0542	0.0633	0.0516	0	0.0521	0
376	0.2243	0.2782	0.5256	0.3182	0.3148	0.1293	0.1099	0.06525	0.27435	0

Appendix 7 Raw data ELISA assays Figure 5.16.

Sheep Number	OD 450 nm – prior to vaccination (week 0)				
	glnH (1)	glnH (2)	glnH(1) - bufferavg	glnH(2) - bufferavg	glnHavg
Vaccinated					
166	0.114	0.1091	0.06725	0.06235	0.0648
256	0.0613	0.061	0.01455	0.01425	0.0144
258	0.0773	0.0879	0.03055	0.04115	0.03585
270	0.185	0.176	0.13825	0.12925	0.13375
284	0.1148	0.104	0.06805	0.05725	0.06265
428	0.108	0.1069	0.06125	0.06015	0.0607
478	0.0691	0.0683	0.02235	0.02155	0.02195
Unvaccinated					
002	0.0999	0.0986	0.05315	0.05185	0.0525
084	0.1419	0.0852	0.09515	0.03845	0.0668
112	0.0662	0.063	0.01945	0.01625	0.01785
165	0.0887	0.0889	0.04195	0.04215	0.04205
295	0.0768	0.082	0.03005	0.03525	0.03265
376	0.1001	0.1023	0.05335	0.05555	0.05445

Sheep Number	OD 450 nm – 4 weeks post-vaccination				
	glnH (1)	glnH (2)	glnH(1) - bufferavg	glnH(2) - bufferavg	glnHavg
Vaccinated					
166	0.6644	0.5883	0.61765	0.54155	0.5796
256	0.721	0.7133	0.67425	0.66655	0.6704
258	0.6385	0.6618	0.59175	0.61505	0.6034
270	0.8664	0.8	0.81965	0.75325	0.78645
284	0.6002	0.6444	0.55345	0.59765	0.57555
428	0.8166	0.8514	0.76985	0.80465	0.78725
478	0.7033	0.7139	0.65655	0.66715	0.66185
Unvaccinated					
002	0.0631	0.0613	0.01635	0.01455	0.01545
084	0.1214	0.1254	0.07465	0.07865	0.07665
112	0.094	0.0907	0.04725	0.04395	0.0456
165	0.1022	0.1128	0.05545	0.06605	0.06075
295	0.1223	0.1245	0.07555	0.07775	0.07665
376	0.0913	0.0917	0.04455	0.04495	0.04475

Appendix 7 Continued ……

Sheep Number	OD 450 nm – 8 weeks post-vaccination				
	glnH (1)	glnH (2)	glnH(1) - bufferavg	glnH(2) - bufferavg	glnHavg
Vaccinated					
166	0.8247	0.798	0.77795	0.75125	0.7646
256	0.5107	0.573	0.46395	0.52625	0.4951
258	0.4462	0.4657	0.39945	0.41895	0.4092
270	0.7103	0.7134	0.66355	0.66665	0.6651
284	0.5578	0.468	0.51105	0.42125	0.46615
428	0.7892	0.8171	0.74245	0.77035	0.7564
478	0.7121	0.6782	0.66535	0.63145	0.6484
Unvaccinated					
002	0.0764	0.0756	0.02965	0.02885	0.02925
084	0.1078	0.0962	0.06105	0.04945	0.05525
112	0.081	0.0827	0.03425	0.03595	0.0351
165	0.1034	0.1032	0.05665	0.05645	0.05655
295	0.089	0.0908	0.04225	0.04405	0.04315
376	0.25	0.2248	0.20325	0.17805	0.19065

Sheep Number	OD 450 nm – 12 weeks post-vaccination				
	glnH (1)	glnH (2)	glnH(1) - bufferavg	glnH(2) - bufferavg	glnHavg
Vaccinated					
166	1.0257	1.0465	0.97895	0.99975	0.98935
256	0.5188	0.512	0.47205	0.46525	0.46865
258	0.8147	0.9382	0.76795	0.89145	0.8297
284	0.6023	0.5984	0.55555	0.55165	0.5536
428	0.5741	0.6638	0.52735	0.61705	0.5722
478	0.5859	0.522	0.53915	0.47525	0.5072
Unvaccinated					
002	0.0715	0.0668	0.02475	0.02005	0.0224
084	0.1173	0.1119	0.07055	0.06515	0.06785
112	0.1351	0.1253	0.08835	0.07855	0.08345
165	0.1131	0.1029	0.06635	0.05615	0.06125
295	0.1707	0.1469	0.12395	0.10015	0.11205
376	0.141	0.1448	0.09425	0.09805	0.09615

Appendix 7 Continued ……

Sheep Number	OD 450 nm – 16 weeks post-vaccination				
	glnH (1)	glnH (2)	glnH(1) - bufferavg	glnH(2) - bufferavg	glnHavg
Vaccinated					
166	0.8138	0.8104	0.76705	0.76365	0.76535
256	0.4283	0.413	0.38155	0.36625	0.3739
258	0.6827	0.7256	0.63595	0.67885	0.6574
284	0.5415	0.499	0.49475	0.45225	0.4735
428	0.795	0.7276	0.74825	0.68085	0.71455
478	0.4996	0.5165	0.45285	0.46975	0.4613
Unvaccinated					
002	0.0657	0.0642	0.01895	0.01745	0.0182
084	0.118	0.121	0.07125	0.07425	0.07275
112	0.1004	0.0804	0.05365	0.03365	0.04365
165	0.1368	0.1376	0.09005	0.09085	0.09045
295	0.1486	0.1305	0.10185	0.08375	0.0928
376	0.1327	0.0482	0.08595	0.00145	0.0437

Sheep Number	OD 450 nm – 20 weeks post-vaccination				
	glnH (1)	glnH (2)	glnH(1) - bufferavg	glnH(2) - bufferavg	glnHavg
Vaccinated					
166	0.6146	0.6702	0.56785	0.62345	0.59565
256	0.4568	0.447	0.41005	0.40025	0.40515
258	0.7311	0.7264	0.68435	0.67965	0.682
284	0.5568	0.5234	0.51005	0.47665	0.49335
428	0.7708	0.8079	0.72405	0.76115	0.7426
478	0.5287	0.509	0.48195	0.46225	0.4721
Unvaccinated					
002	0.0791	0.0667	0.03235	0.01995	0.02615
084	0.1524	0.1363	0.10565	0.08955	0.0976
112	0.1126	0.0944	0.06585	0.04765	0.05675
165	0.1103	0.0746	0.06355	0.02785	0.0457
295	0.1505	0.1258	0.10375	0.07905	0.0914
376	0.1499	0.1886	0.10315	0.14185	0.1225

Appendix 7 Continued ……

Sheep Number	OD 450 nm – 24 weeks post-vaccination				
	glnH (1)	**glnH (2)**	**glnH(1) - bufferavg**	**glnH(2) - bufferavg**	**glnHavg**
Vaccinated					
166	0.6752	0.6888	0.62845	0.64205	0.63525
256	0.34	0.295	0.29325	0.24825	0.27075
258	0.5743	0.5223	0.52755	0.47555	0.50155
284	0.4217	0.4018	0.37495	0.35505	0.365
428	0.4383	0.6296	0.39155	0.58285	0.4872
478	0.4634	0.5064	0.41665	0.45965	0.43815
Unvaccinated					
002	0.0688	0.0785	0.02205	0.03175	0.0269
084	0.1244	0.1156	0.07765	0.06885	0.07325
112	0.1081	0.1022	0.06135	0.05545	0.0584
165	0.128	0.1181	0.08125	0.07135	0.0763
295	0.182	0.1618	0.13525	0.11505	0.12515
376	0.1536	0.0958	0.10685	0.04905	0.07795

Appendix 8 Raw data ELISA assay Figure 5.17.

Sheep Number	OD 450 nm				
	glnH (1)	**glnH (2)**	**glnH(1) - bufferavg**	**glnH(2) - bufferavg**	**glnHavg**
Infected					
3	1.31	1.2198	1.26325	1.17305	1.21815
11	0.7412	0.7506	0.69445	0.70385	0.69915
19	0.6997	0.7908	0.65295	0.74405	0.6985
25	0.9959	0.8523	0.94915	0.80555	0.87735
32	1.1077	1.101	1.06095	1.05425	1.0576
Uninfected					
15	0.705	0.7683	0.65825	0.72155	0.6899
12	0.522	0.52	0.47525	0.47325	0.47425
31	0.1368	0.1571	0.09005	0.11035	0.1002
33	0.1788	0.1695	0.13205	0.12275	0.1274
34	0.1626	0.1561	0.11585	0.10935	0.1126
35	0.1734	0.1699	0.12665	0.12315	0.1249
40	0.1477	0.1446	0.10095	0.09785	0.0994
45	0.1468	0.1426	0.10005	0.09585	0.09795
26	0.1278	0.1373	0.08105	0.09055	0.0858
46	0.1263	0.1322	0.07955	0.08545	0.0825
47	0.0938	0.1012	0.04705	0.05445	0.05075
2	0.146	0.1507	0.09925	0.10395	0.1016
4	0.093	0.0937	0.04625	0.04695	0.0466
5	0.1351	0.1328	0.08835	0.08605	0.0872
6	0.1075	0.1053	0.06075	0.05855	0.05965
7	0.1367	0.1366	0.08995	0.08985	0.0899
1	0.0856	0.0875	0.03885	0.04075	0.0398
10	0.1355	0.124	0.08875	0.07725	0.083
13	0.1274	0.1244	0.08065	0.07765	0.07915
14	0.0844	0.0891	0.03765	0.04235	0.04
16	0.2005	0.1938	0.15375	0.14705	0.1504
17	0.0757	0.0723	0.02895	0.02555	0.02725
18	0.1103	0.1082	0.06355	0.06145	0.0625
20	0.0766	0.0776	0.02985	0.03085	0.03035
21	0.1024	0.0956	0.05565	0.04885	0.05225
22	0.0839	0.0989	0.03715	0.05215	0.04465
27	0.174	0.1613	0.12725	0.11455	0.1209
28	0.1166	0.1212	0.06985	0.07445	0.07215
29	0.0993	0.0954	0.05255	0.04865	0.0506
30	0.1477	0.1517	0.10095	0.10495	0.10295
36	0.1725	0.1404	0.12575	0.09365	0.1097
37	0.1486	0.1422	0.10185	0.09545	0.09865
42	0.0914	0.0931	0.04465	0.04635	0.0455
43	0.1313	0.1338	0.08455	0.08705	0.0858

Appendix 9 Raw data ELISA assay Figure 5.18.

Deer Number	OD 450 nm				
	glnH (1)	glnH (2)	glnH(1) - bufferavg	glnH(2) - bufferavg	glnHavg
Infected					
2316	2.5671	2.7913	2.52035	2.74455	2.63245
2305	2.3097	2.5687	2.26295	2.52195	2.39245
311	2.8823	2.8927	2.83555	2.84595	2.84075
772	2.8493	2.7285	2.80255	2.68175	2.74215
679	2.6304	2.7148	2.58365	2.66805	2.62585
668	2.6876	2.5737	2.64085	2.52695	2.5839
1331	2.4261	2.5777	2.37935	2.53095	2.45515
5212	2.5616	2.6013	2.51485	2.55455	2.5347
5195	3.0081	3.0073	2.96135	2.96055	2.96095
Uninfected					
805	0.2	0.2237	0.15325	0.17695	0.1651
819	0.4634	0.4568	0.41665	0.41005	0.41335
861	0.2898	0.2832	0.24305	0.23645	0.23975
881	0.1775	0.209	0.13075	0.16225	0.1465
824	0.1479	0.1743	0.10115	0.12755	0.11435
825	0.8237	0.8745	0.77695	0.82775	0.80235
887	0.3191	0.3126	0.27235	0.26585	0.2691
876	0.2974	0.2571	0.25065	0.21035	0.2305
830	0.2419	0.2449	0.19515	0.19815	0.19665
817	0.2704	0.2392	0.22365	0.19245	0.20805
877	0.6247	0.7641	0.57795	0.71735	0.64765

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