AN INVESTIGATION OF NOVEL DNA BASED VACCINES FOR PROTECTION AGAINST BRUCELLOSIS

by

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ABSTRACT

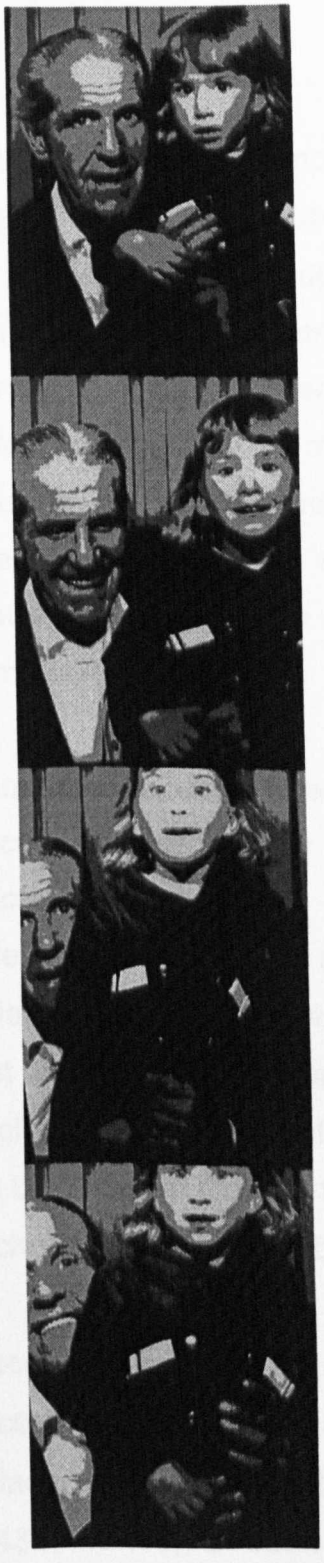
This thesis describes design, construction and evaluation in a BALB/c mouse model, for five novel *Brucella* specific DNA vaccines.

Brucellosis is worldwide zoonosis of economic significance that poses a significant threat to both animal and human health. Vaccination of livestock can be valuable for reducing transmission and facilitating control. Development of an efficacious non-living vaccine is therefore a valuable goal in brucellosis research.

Five candidate antigens were identified within the *Brucella melitensis* 16M genome. *In silico* selection was supported by confirming transcription of the selected genes from cultured *Brucella*, and evidence of candidate protein immunogenicity in *Brucella* infected sheep. Eukaryotic and prokaryotic expression plasmids were constructed for each candidate antigen. The protective efficacy of six DNA vaccine constructs was evaluated in a BALB/c model of brucellosis. From this evaluation, two protective antigens were identified for further study: Invasion protein B and the 25 kDa outer membrane protein. The DNA vaccines p-*omp25* and p-*ialB* were shown to have equivalent protective efficacy in the mouse model to that achieved through vaccination with the live vaccine strain Rev.1. DNA vaccine mediated protection was associated with production of specific antibodies and priming of both CD4+ and CD8+ IFN-γ secreting cells. High numbers of CD8+ cells were observed for the p-*omp25* vaccine, whereas CD4+ cells and antibodies were more prevalent following p-*ialB* vaccination. The vaccines were found to be most effective when three homologous booster vaccinations were used. Single dose vaccination afforded only modest levels of protection. Attempts to improve delivery of DNA vaccines through adsorption of DNA to cationic liposomes was partially successful in that there was a notable increase in specific humoral immune responses. However, these increases were not associated with increased cell mediated immunity or protective efficacy.

DEDICATION

For
My Granddad



Joseph Mullis (1911 – 1996)

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DECLARATION

I have read and understood the Schools definition of plagiarism and cheating given in the research degrees handbook. I declare that this thesis is my own work and that I have acknowledged all results and quotations from the published and unpublished works of other people.

NICOLA JANE COMMANDER

DATE:

SIGNATURE:

COMMONLY USED ABBREVIATIONS

ACDP Advisory Committee on Dangerous Pathogens

APC Antigen Presenting Cell

ATCC American Type Culture Collection

BHIB Brain Heart Infusion Broth

cELISA competitive ELISA

CFU Colony Forming Units

CL Containment Level

DC Dendritic cell

Defra Department of the Environment Food and Rural Affairs

DMEM Dulbecco's Modified Eagles Medium

DNA Deoxyribose Nucleic Acid

DTH Delayed Type Hypersensitivity

EC European Community

ELISA Enzyme Linked Immunosorbent Assay

FAO Food and Agriculture Organisation

GB Great Britain

GMM Genetically Modified Microorganism

HSE Health and Safety Executive

KO Knock out

iELISA Indirect ELISA

IFA Immunofluorescence assay

IFN-γ Interferon gamma

Ig Immunoglobulin

IL Interleukin

IPTG Isopropyl β thiogalactopyranoside

IU International Units

LPS Lipopolysaccharide

MHC Major Histocompatibility Complex

NBT NitroBlue Tetrazolium

OIE Office International de Epizooties (world organisation for animal health)

OBF Officially Brucellosis Free

PCR Polymerase Chain Reaction

PI Post Infection

OPS O-polysaccharide

Omp Outer membrane protein

rcf Relative centrifugal force

RNA Ribonucleic Acid

RT-PCR Reverse Transcriptase - Polymerase Chain Reaction

SAPO Specified Animal Pathogens Order

SDA Serum Dextrose Agar

SFC Spot Forming Cells

T_{cm} Central Memory T cell

T_{em} Effector memory T cell

TNF-α Tumour Necrosis Factor alpha

UN United Nations

WHO World Health Organisation

Chapter 1: INTRODUCTION

1.1 Brucella melitensis and brucellosis

Brucellosis is a worldwide zoonosis of considerable social and economic importance. It is considered by the Food and Agriculture Organisation (FAO) and World Health Organisation (WHO) to be the most widespread zoonoses in the world, and is on the Office International des Epizooties (OIE) List B, denoting diseases of significant socio-economic, public health and international trade importance. The causative agents of the disease are facultative intracellular bacteria of the monospecific genus *Brucella melitensis*. Despite extensive genetic homogeneity amongst strains, historical classification defines six separate species, *B. melitensis, B. abortus, B. suis, B. ovis, B. canis and B. neotomae*, and a multitude of phenotypically defined sub-species or biovars. For review, see Verger *et al,* (1987). This nomenclature remains common practice amongst researchers in the field, and has epidemiological and clinical relevance. *Brucella* infections can be found in a wide range of animal hosts. The six species display distinct animal host preferences and have variable virulence in man. *B. abortus* infections are mostly associated with the disease in cattle, *B. melitensis* with small ruminants and camelids, *B. suis* with pigs, hares, rodents and rangifers (depending on biovar), *B. ovis* with sheep and *B. canis* with dogs. *B. neotomae,* has only ever been reported in desert wood rats. Recently, strains of *Brucella* not belonging to the currently recognised species have been isolated from marine mammals (Jahans *et al,* 1997).

The history of brucellosis

Brucellosis was first described in the late 1800's and early 1900's. The earliest recorded work on brucellosis is largely attributed to the physician Sir David Bruce, after whom the genus was eventually named. Brucellosis was brought to the attention of the British Army Medical Corps when British soldiers stationed on the island of Malta became sick with a debilitating and severe undulant fever. Bruce was commissioned to head the Mediterranean Fever Commission

charged with investigating the disease. Bruce isolated the bacterium from the blood of a patient and named it *Micrococcus melitensis*. A local Maltese scientist, Theomisticles Zammit is credited with establishing the link between the undulant fevers in man and the drinking of unpasturised goats milk. Zammit was the first to isolate the bacterium from the milk of the goats. The 1904 report of the Mediterranean Fever Commission recommended a ban on the consumption of raw local goat milk and that sterilisation of the milk was required in order to protect the army.

The isolation of *Brucella abortus* came some years later, with the description of Bangs disease in humans associated with contagious abortion in cattle. The causative organism was originally termed *Bacillus abortus* (Bang, 1897). The discovery of similarities between the various abortifacient diseases and their causative organisms is attributed to Alice Evans (1918), and as these relationships became apparent the nomenclature was adapted to reflect the knowledge and the genus named in honour of Sir David Bruce (Meyer and Shaw, 1920). *Brucella suis* was first isolated by Traum in 1914, and identified by Huddleson in 1929. Subsequent isolates of other *Brucellae*; *B. canis* (Carmichael and Brunner, 1968), *B. ovis* (Buddle and Boyes, 1956) and *B. neotomae* (Stoener and Lackman, 1957), were named according to this pattern.

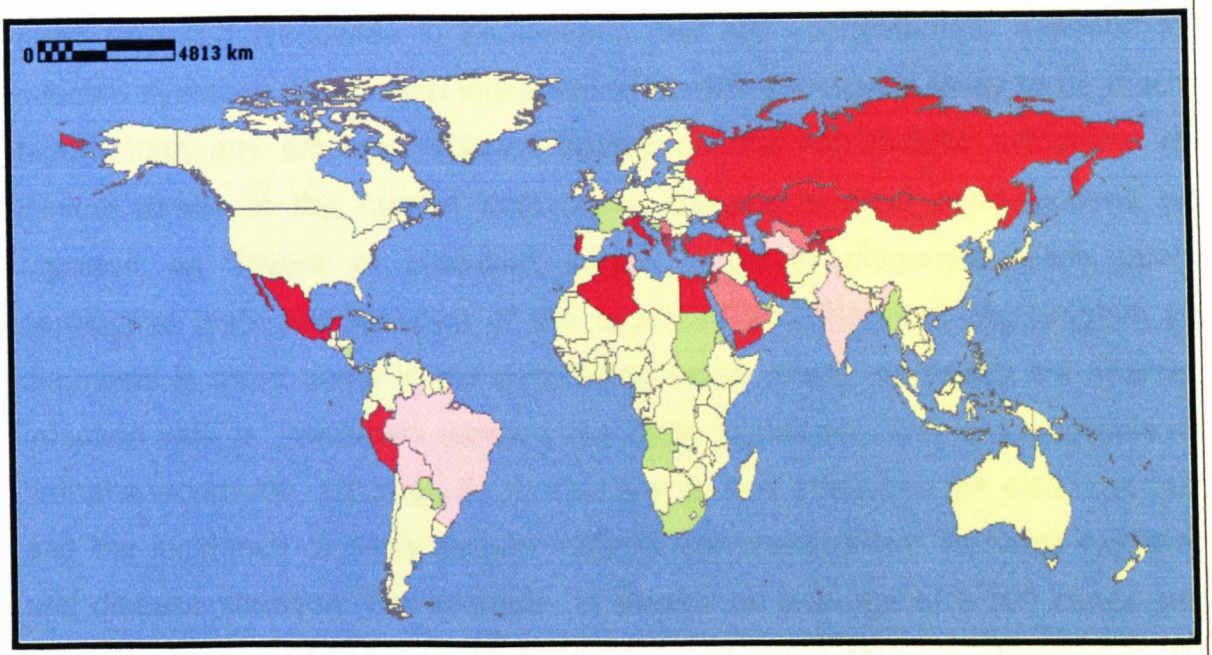
The bacteria of the *Brucella* genus are coccobacilliary in morphology. Under low power light microscopic examination the colonial morphology is smooth and refractory to light, giving a characteristic rainbow effect. As with other Gram-negative bacteria phase dissociation can occur and 'rough' variants or strains do occur. Rough colonies lack the refractive appearance of their smooth relatives. Both rough and smooth strains display characteristic Gram-negative cell wall properties and are relatively fastidious in laboratory culture. *B. melitensis* is most commonly isolated as smooth type morphology, and although rough strains of this species have been described, these are generally viewed as avirulent or attenuated.

1.2 Global epidemiology and impact of brucellosis

The principal economic and social impact of brucellosis is in the agricultural sector, where infections in cattle, pigs, sheep and goats cause enormous economic losses due to effects on animal health, and international trade in animals and their products. Zoonotic transmission of the disease means that it also poses a significant public health issue. Human brucellosis is most frequently associated with occupational exposure to the organism. Thus, farmers, veterinarians, laboratory workers, abattoir workers, and dairy industry technicians are the most at risk. In *B. melitensis* and *B. abortus* endemic areas the consumption of unpasteurised dairy products is the most common source of non-occupational exposure. Furthermore, *B. melitensis, B. abortus and B. suis* are listed as biological warfare or bioterrorism threat agents, indicating a further possibility for human infection following a deliberately orchestrated release of weaponised pathogen.

B. melitensis remains the most significant species in terms of zoonotic transmission and pathology in the human host, and the incidence of infection in endemic areas is reported to be rising. The most recent FAO report on brucellosis indicates the distribution of small ruminant (sheep and goat) disease to be widespread. Up to date epidemiological data regarding prevalence and incidence of the disease is obtainable from the FAO Global Livestock Production and Health Atlas (GLiPHA). Data for the period 1999-2003 is summarised in figure 1.1.

Figure 1.1: The global distribution and incidence of Ovine and Caprine brucellosis (excluding B. ovis) between 1999 and 2003.



Map legend

Livestock health cases

≥ 7,600
1,110 –7,600
400 – 1,110
100 – 400
≤ 100
No information avail

Number of outbreaks of caprine or ovine brucellosis reported between 1999 and 2003
Total # outbreaks (by country)

Italy	12,436
Portugal	7,754
Mexico	6,870
Algeria	2,718
Mongolia	1,624
FYR Macedonia	999
Greece	888
Saudi Arabia	712
Cyprus	340
Turkey	331
Yemen	203
Tajikistan	193
France	174
Serbia and Montenegro	141
Syrian Arab Republic	78
Kazakhstan	63
Lebanon	53
Israel	49
Brazil	46
Russian Federation	39

Data obtained from http://www.fao.org/ag/aga/glipha pertaining to the incidence of caprine and ovine brucellosis due to smooth strains of Brucella (Not including B. ovis infections).

Unfortunately, human infection remains the best sentinel for the prevalence of animal disease (http://www.who.int/inf-fs/en/fact173.html), and cases occur wherever the disease is endemic in livestock. High seroprevalence rates have been recorded worldwide in populations that are occupationally exposed to infected livestock. The actual incidence of human infection is likely to be much higher than any officially reported figures as the non-specific symptoms of clinical illness in the human host mean disease is often misdiagnosed or reported as 'fevers of unknown origin'. Accurate diagnosis relies upon serological tests and isolation of the organism and therefore the problem of diagnosis is more acute in the developing world where resources are scarce. Infection with *B. melitensis* remains the most significant form of brucellosis in humans worldwide, although *B. abortus* and *B. suis* infections are also reported and the incidence of each disease reflects the predominant livestock systems and disease situation. For example, in Mexico an average of 3,500 cases per year were recorded between 1990 and 2001 of which 98% are identified as *B. melitensis* from either caprine (93%) or bovine (5%) sources. In the same period for livestock up to 17% of cattle herds and 64% of goat herds were seropositive (Eduardo Luna-Martinez *et al,* 2002). In contrast, in Argentina where livestock agriculture is dominated by cattle farming, *B. abortus* is the most frequently reported human infection (Samartino *et al,* 2002). In the Near and Middle East regions *B. melitensis* biovar 3 is the most frequently reported human *Brucella* infection, but recently there has been a rise in the number of cases of human brucellosis associated with *Brucella* strains of bovine origin (*B. abortus*) (Refai *et al,* 2002). The reporting of both livestock and human brucellosis varies across continents, countries and regions making accurate comparisons of prevalence impossible. However, the incidence and impact of both human and animal brucellosis is also expected to be high in Sub-Saharan Africa (McDermott *et al,* 2002), the Indian Sub-Continent (Renukaradhyra *et al,* 2002., Bandara *et al,* 2002), and China (Dequi *et al,* 2002). Within the member states of the European Union many are now officially brucellosis free (OBF) for livestock. However, 2,857 cases of human brucellosis were recorded in 2002, the majority of which have acquired infection from ingestion of non-pasteurised dairy products whilst travelling in endemic areas (Godfroid and Kasbohrer, 2002). The prevalence and incidence of brucellosis is greater in some of the newest

member states of the European Union. In particular, *B. melitensis* infection remains a significant problem in the sheep and goat populations of the Balkan region (Taleski *et al*, 2002)

1.3 Brucellosis in livestock

In livestock, the principal symptom of infection with *Brucella* is abortion, and reduction or loss of fertility. Infections are acquired via contact, through ingestion, inhalation and direct sexual contact or artificial insemination. The risk of transmission within animal populations and to humans is greatest where abortion has occurred, as huge numbers of viable *Brucella* are shed when the foetus is expelled from the host. Ruminant brucellosis caused by the smooth strain varieties of *B. melitensis* (mainly in sheep and goats) and *B. abortus* (mainly in cattle), is characterised by late gestation abortion. Sheep are also affected by infection with the naturally rough species *B. ovis,* which mainly causes fertility problems in rams and is characterised by orchitis and epididymitis.

Establishing infection

Infection is acquired through direct contact. Potential hosts are exposed to the pathogen when it is shed in massive quantities with the secretions and tissues during an abortive episode. *Brucella* organisms can be ingested or inhaled, contaminate wounds or directly infiltrate mucus membranes. The pathogen crosses mucus membranes of the naso-pharynx, conjunctiva, vagina, etc., and is quickly engulfed by wandering phagocytic cells of the innate immune system. The mechanism by which *Brucella* spp., are able to penetrate the mucosa remains undefined. A local acute inflammatory reaction is elicited at the site of epithelial penetration, with the infiltration of a variety of cells to the area promoting phagocytosis of the *Brucella* (Enright, 1990). The process of phagocytosis is designed to lead to the destruction of the pathogen following fusion of the endosome with lysosomal vesicles containing destructive enzymes. However, as with other intracellular pathogens, it is estimated that up to 30% of virulent *Brucella* phagocytosed by naïve host cells manage to escape

destruction within the phagolysosome and survive to replicate within the parasitised host cell (Gorvel *et al,* 2002).

Survival, dissemination and persistence within the host

Intramacrophagic *Brucella* are distributed via lymphatic drainage to the local lymph nodes and subsequently throughout the body. In experimental infections the local lymph node draining the site of inoculation harbours greater numbers of *Brucella* for longer times than any other site in the body. In addition, experimental infection indicates a dose dependent dissemination of infection, with greater chances of finding *Brucella* in lymph nodes and tissues distal to the inoculation site when high dose inoculations are given. Infected lymph nodes become enlarged due to the infiltration of inflammatory cells (For review see Enright, 1990).

In the absence of protective immunity a failure to contain *Brucella* within the draining lymph nodes allows dissemination of infection throughout the host via haematogenous spread. In *Brucella abortus* experimental infection of cattle, infection via the conjunctival route (a mucosal surface) results in parotid lymph node colonisation between 2 – 4 days post inoculation (P.I.), splenic infection as early as 15 days P.I., and colonisation of distal lymph nodes, mammary tissue and the pregnant uterus at 22 – 29 days P.I. (Payne, 1959). A similar pattern of infection progression is anticipated for all ruminant hosts and strains of smooth *Brucella*, although the exact kinetics will vary depending upon host species and immune status and *Brucella* strain and infectious dose.

Brucella organisms are able to colonise many organs and tissues in the infected host but preferentially localise in the tissues of the reproductive tract and the lymph nodes. The *Brucella* tropism for the ruminant uterus and placental trophoblast cells in particular, is the ultimate cause of the abortive pathology in livestock.

The pathology of brucellosis in livestock

The principal symptom of brucellosis in ruminant livestock is abortion, but the mechanism of *Brucella* induced abortion remains poorly understood. Descriptions of clinical disease indicate that placentitis may ultimately cause a disruption in the homeostasis of the foetus, and endocrine imbalance in the mother leading to a failure of pregnancy. Damage to the placenta may result in an impaired ability to manufacture progesterone which is essential for the maintenance of pregnancy. Furthermore, infected trophoblasts have been shown to generate cortisol, which is normally only produced at parturition and may trigger premature delivery or abortion. There appears to be only a limited relationship between the severity of the placentitis and the abortive effect. The severity of placentitis in an individual *Brucella* infected ruminant host is highly variable, ranging from widespread destruction of the placenta and extending to foetal membranes, to small localised foci of damage. These observations suggest that in some cases placental disruption alone can account for the abortion, but for others placental damage is so slight it would not be expected to result in abortion or foetal distress. Therefore, additional factors may be involved in triggering the abortion.

Direct foetal infection may also play a role in initiating abortion. Experimental infection of the foetus results in increased levels of cortisol, associated with adrenocortical hyperplasia. The increase begins as early as 24 hours post infection, and the foetus is usually delivered dead between 5 – 7 days post infection. Exogenous administration of cortisol to the dam results in premature delivery within the same time frame, suggesting that the infection causes a stress response from the foetus and the elevated steroids are associated with the abortion. Ruminant foetuses are very susceptible to *Brucella* infection. Studies with sheep indicate widespread inflammatory lesions as early as 4 days P.I., and the presence of *Brucella* specific antibody by day 10 P.I. Neutrophil and macrophage numbers are increased, and these cell changes coincide with the elevation of foetal cortisol levels (Gorham 1983, cited by Enright 1990).

There is evidence to suggest that whilst some attenuated strains are capable of infecting the foetus, they do not result in premature delivery or abortion. The

outcome of brucellosis in the pregnant host is therefore dependent upon the virulence of the infecting organism and the pathology of the infection in both the placental tissue and the foetus.

In male animals acute clinical symptoms of *Brucella* infection are limited to orchitis and epididymitis. Often clinical symptoms are not apparent. Male sheep and goats are reputedly less susceptible to *B. melitensis* infection than females, and there is limited effect on reproductive ability in terms of male fertility. In contrast, *B. ovis* is largely considered an infection of male animals. *B. ovis* infections result in considerably reduced fertility of rams but are less frequently associated with abortions (For review see Blasco, 1990 or Stack *et al,* 2005).

1.4 Brucellosis in humans

From a veterinary perspective brucellosis is primarily associated with reproductive disorders. The abortive pathology of the infection in animals facilitates the spread of the disease, and association with infected animals facilitates the zoonotic acquisition of the disease. The most serious human cases of brucellosis are the result of infection with *B. melitensis*, *B. suis* or *B. abortus* respectively. *B. canis* infections have been recorded in humans (Bigler *et al,* 1977) but are uncommon. *B. neotomae* infection in humans has not been recorded and *B. ovis* is considered non pathogenic to man (Corbel, 1997., Madkour, 2001). Human brucellosis is predominantly characterised by a debilitating undulant fever, and described as a 'flu-like illness'. Symptoms such as depression, anorexia, and general malaise are also frequently cited. Evidence for abortion in infected humans is limited and circumstantial. Human to human transmission is uncommon.

The pathology of human brucellosis

Febrile episodes are characteristic of acute human brucellosis. As with other febrile illnesses, the physical effect on the body is considerable in terms of dehydration and general weakness and psychologically disturbing symptoms of confusion and hallucinations are also experienced. Chronic brucellosis is a more insidious disease. The list of *Brucella* induced conditions, from mild joint

pain to severe osteoarthritis, mild depression to complex psychoneuroses, indicates the long-term persistence of the pathogen to have serious effects on the general health and welfare of the infected individual (Corbel, 1990). Fortunately, brucellosis is considered treatable and chronic infections can be prevented, or at least limited, by the use of appropriate antibiotics. Combinational therapy with doxycycline and rifampin is the current recommended treatment regimen. Effective treatment requires immediate and long-term treatment and subsequent monitoring for relapse. Treatment is an expensive option, and in situations where disease is endemic the health care systems have limited resources for either accurate diagnosis or effective treatment. At present there is no vaccine suitable for brucellosis prophylaxis in humans.

1.5 Brucella intracellular infection

The placental trophoblasts are the cells most frequently associated with *Brucella* infection in the pregnant ruminant. In non-pregnant animals the organism is generally isolated from the lymph nodes, indicating that in the absence of stimuli from the gravid uterus the natural home of *Brucella* is within the macrophages of the host.

The mechanisms of *Brucella* invasion or internalisation have been extensively studied (Gorvel *et al,* 2002). It has been apparent for many years that there are differences in the initial host-pathogen interaction between smooth and rough strain *Brucella*. Smooth *Brucella* O-polysaccharide (OPS) is considered a major virulence factor (Moreno *et al,* 1981., Porte *et al,* 2003), although the naturally rough species *B. canis* and *B. ovis* which lack externally expressed OPS are also pathogenic. Notably, OPS defective smooth strain mutants and phase variants are attenuated *in vivo* (Allen *et al,* 1998., Ugalde *et al,* 2000), but this attenuation is not always apparent in macrophage survival studies *in vitro* and may be linked to the fact that smooth *Brucella* are generally more resistant to complement mediated lysis (Corbeil *et al,* 1988). The OPS of the smooth *Brucella* does not activate the alternative pathway for complement deposition

(Hoffman *et al,* 1983) and thus the triggering of the initial complement response is restricted to classical and lectin pathway activation.

Opsonised *Brucella* appear to gain entry to professional phagocytes via Fc receptor ligation, whereas non-opsonised organisms bind via fibronectin or lectin receptors (Ackermann *et al,* 1998., Campbell *et al,* 1994., Harmon *et al,* 1988). In macrophages the fate of the invading bacteria is linked to the mode of internalisation, with *Brucella* entry via Fc receptors resulting in more effective bacterial killing by the invaded macrophages (Gross *et al,* 2000). For non-professional phagocyte invasion there is indirect evidence for *Brucella* specific ligands and host receptor interactions in that not all cells in a monolayer epithelial cell culture are reported to be permissive to infection. Although *Brucella* specific putative adhesion and invasion associated genes have been identified in the *B. melitensis* 16M genome (DelVecchio *et al,* 2002), the expression of these protein effectors has not yet been confirmed. Furthermore, the BvrR/BvrS two-component system, which is involved in the generation of and integrity of the outer-membrane, affects the internalisation of *Brucella,* suggesting that membrane proteins and structures play an important role in internalisation. BvrR/BvrS null mutants, which are defective in the production of several outer-membrane proteins (Omps) and the LPS, appear to be incapable of internalisation and remains bound the host cell surface (Sola-Landa *et al,* 1998., Lopez-Goni *et al,* 2001). In addition to the previously identified effects on the expression of known Omps, it is possible that the BvrR/BvrS mutants are defective in the production of molecules directly associated with the invasion process. Recently, it has been shown that *Brucella* transiently interact with the host cell cholesterol-rich-microdomains called lipid rafts (Waterai *et al,* 2002., Kim *et al* 2002), and that these may be significant in subsequent trafficking to the replicative niche.

Following internalisation, the majority of *Brucella* that are ingested by professional phagocytes are rapidly destroyed within phagolysomes. Whilst trophoblast cells are able to phagocytose *Brucella*, they are less effective at destruction of invading cells. Consequently, in contrast to the phagocytic cells of the immune system that always contain a relatively small number of *Brucella*,

infected trophoblasts contain vast numbers of viable bacteria. In both cell types bacterial replication occurs in a membrane bound compartment. The intracellular trafficking of virulent *Brucella* in both macrophages and trophoblasts is diverted to avoid lysosomal fusion and establish a replicative niche in a membranous compartment resembling the endoplasmic reticulum (Pizzaro-Cerda *et al,* 1998, Gorvel *et al,* 2002). The benefits of localisation within an endoplasmic reticulum (ER) type compartment and the reasons for preferential replication of *Brucella* within placental trophoblasts are not yet completely understood. It has been postulated that the ER location affords access to complex nutrients required for bacterial replication (Kohler *et al,* 2002b) since phagosomes of macrophages are understood to be nutrient poor. Similarly, the preferential replication of *Brucella* within trophoblasts may be related to the ability of these infected cells to produce growth factors for the bacteria. Trophoblast cells are metabolically highly active, secreting a variety of proteins including hormones, and at least some of these products are known to stimulate the growth of *Brucella in vitro* (Meyer *et al,* 1976).

Many studies have been conducted to evaluate the mechanisms by which bacteria are able to create and maintain a stable replicative niche within host cells (Duclos *et al,* 2000). There is a clear correlation with the virulence of a *Brucella* strain and it's ability to resist intramacrophagic destruction. The specific mechanism allowing delay or inhibition of phagolysosomal fusion is not yet understood, but the initial association with surface receptors and lipid rafts is considered significant (Waterai *et al,* 2002 and 2004). Furthermore, there is some evidence for the involvement of a *Brucella* secreted soluble mediator (Frenchick *et al,* 1985), and mutations in the VirB type IV secretion system of virulent *Brucella* have been shown to have a detrimental effect on the ability of the organism to reach the replicative niche and persist intracellularly (O'Callaghan *et al,* 1999., Comerci *et al,* 2001). The effector molecule that is secreted by the *virB* system and mediates intracellular trafficking has yet to be identified.

Stationary phase physiology plays an important role in the ability of the *Brucella* to survive within the hostile intramacrophagic environment. This is most

effectively demonstrated through deletion of the *hfq* gene. *Hfq* disruption prevents the production of essential σ factors resulting in reduced expression of a number of genes associated with defence against acid and oxidative stress (*hdeA*, *sodC* and *ahpC*), and stationary phase maintenance metabolism, resulting in observed attenuation *in vivo* and *in vitro* (Roop *et al,* 2003). Moreover, purine biosynthesis mutants and amino acid auxotrophs are highly attenuated in cultured macrophages and exhibit reduced persistence *in vivo* (Crawford *et al,* 1996., Foulongne *et al,* 2001). Tn*5* insertional mutagenesis studies further indicate the importance of nutrient acquisition, also revealing attenuation of strains which are mutated in the genes associated with purine, pyrimidine and amino acid biosynthesis (Kohler *et al,* 2002). In addition to genes associated with survival in a nutrient poor environment mutagenesis studies most frequently reveal genes associated with the *virB* operon and the synthesis of the OPS as essential for the establishment and maintenance of intracellular infection (Allen *et al,* 1998., O'Callaghan *et al,* 1999). The recent sequencing of the *B. melitensis* genome has failed to reveal any further immediately obvious virulence determinants that could account for the *Brucella's* ability to persist with the host. Moreover, the *Brucella* genome lacks the major classical virulence determinants observed for other intracellular pathogens such as Salmonella (DelVecchio *et al,* 2002., Moriyon *et al,* 2002). Such observations have lead to the use of descriptors such as enigmatic and stealthy to describe *Brucella* (Letesson *et al,* 2002).

1.6 Control of brucellosis

Control of and prevention of brucellosis can be achieved through instigating rigorous standards of farm hygiene concurrent to the implementation of sanitary measures for the eradication of infected animals, and, or, medical interventions such as vaccination to prevent infection.

1.6.1 Sanitary control measures

Extensive and expensive control policies implemented by a number of developed countries have significantly reduced the incidence of *B. abortus*

disease in cattle. Great Britain (GB) was declared Officially Brucellosis Free (OBF) in 1985 (European Commission Decision 93/52), following a comprehensive eradication campaign, involving comprehensive surveillance and a test, slaughter and compensation programme. Reduced control measures still exist in order to monitor and protect this OBF status, involving regular compulsory testing and the imposition of movement restrictions and ultimately compulsory slaughter orders upon identified *B. abortus* reactor herds. The procedures were originally instigated under the 'Notifiable Diseases: *Brucella melitensis* Order of 1940, and later amended to the Specified Diseases Order (http://www.defra.gov.uk) and EC directive 91/88. Other EC nations have adopted similar strategies for control of bovine disease, and consequently northern continental Europe is now largely free of cattle brucellosis. Import and export regulations throughout the EC exist to prevent trade and movement of potentially infected livestock. Outside Europe, Australia and New Zealand are OBF. Whilst the USA and Canada claim to be largely free of bovine brucellosis in domestic cattle herds, sporadic outbreaks as a result of transmission from wildlife reservoir hosts have been recorded (Godfroid, 2002). *Brucella* infection of livestock other than cattle, is economically and historically a less significant problem. In the UK sporadic (non-endemic) occurance of *B. suis* infection in pigs has been controlled by application of similar measures to those used with cattle, and *B. ovis* and *B. melitensis* infection have never been recorded in the UK livestock population.

Whilst test and slaughter policies have been successful at eliminating or reducing the disease from developed countries, they are not universally applicable or effective in all situations. In regions where brucellosis has been effectively eradicated, obligatory animal testing and compulsory slaughter of reactors has been overseen by a comprehensive, well-resourced and centrally coordinated veterinary service with the ability to track and restrict animal movements and epidemiologically monitor disease outbreaks. Limited resources in the developing world, where brucellosis remains endemic, mean that this approach is not always feasible or suitable. Furthermore, certain animal husbandry practices including extensive shared grazing and transhumance can facilitate inter-herd spread and create difficulties for animal identification and

tracing. In addition to these problems the effectiveness of test and slaughter procedures is only optimal under conditions where the prevalence of disease is relatively low (≤ 1% of the herds) (Garin-Bastuji, 1995).

1.6.2 Vaccination and protective immunity

Following natural infection and abortion animals will either clear infection or become latent carriers depending upon the effectiveness of the adaptive immune response. It is commonly observed that previously infected animals are resistant to reinfection. Moreover, if subsequently exposed to the pathogen they are better able to control *Brucella* replication to prevent placentitis, abortion, and foetal infection, thus providing anecdotal evidence for the development of protective immunity.

The majority of information regarding protective immunity against brucellosis comes from studies with small animal models. In the mouse model of brucellosis the principal site of pathology is the spleen and the outcome of protective immunity or infection can be defined in terms of the number of *Brucella* in this organ. Mice are readily infected with *Brucella* but do not succumb to disease, and eventually clear the infection. There is ample evidence for a role for Th 1 responses, and particularly IFN-γ, in promoting this bacterial elimination. A number of studies have demonstrated the *in vitro* effects of IFN-γ depletion or supplementation on *Brucella* growth in macrophage cultures. For example IFN-γ activation of mouse macrophages directly inhibits the growth of *B. melitensis* 16M (Eze *et al,* 2000), whilst others have shown that supplementation of *Brucella* macrophage cultures (from murine, human or bovine hosts) with IFN-γ increases the ability of the macrophage to control *Brucella* replication at around 48 hours post-phagocytosis (Stevens *et al,* 1992., Jones and Winter 1992., Baldwin and Winter 1993., Jiang and Baldwin 1993). In the latter studies the effect of IFN-γ does not appear to be through the enhanced macrophage activation as it is known that even unactivated macrophages effectively kill up to 95% of invading *Brucella*, but rather the effect

is hypothesised to be due to an as yet undefined interference with establishment of a replicative niche within the phagocyte.

In vivo evidence of a role for IFN-γ in *Brucella* control has been shown in many studies. Neutralisation of murine IFN-γ through administration of monoclonal antibodies has been shown to result in increased splenic load of *Brucella* in BALB/c, CBA and C57BL/6 mouse strains (Zhan and Cheers, 1993., Fernandes *et al*, 1996), whereas administration of recombinant IFN-γ to infected BALB/c mice results in a reduction of the splenic load (Stevens *et al*, 1992). Furthermore, Murphy *et al* (2001a and b) showed that infection of virulent *B. abortus* 2308 in wild type BALB/c mice resulted in the characteristic chronic infection, but IFN-γ knock out (KO) BALB/c mice (genetically impaired in their ability to produce IFN-γ) were unable to control infection ultimately leading to death at 10.5 weeks post-infection. Thus, indicating that generation of IFN-γ is crucial to the control of infection in the BALB/c mouse.

Despite its essential role in *Brucella* control, IFN-γ is not thought to be solely responsible for anti-*Brucella* immunity, and this is especially evident in the BALB/c mouse. The BALB/c mouse strain is often cited as *Brucella* susceptible whilst C57BL/6 mice are referred to as relatively resistant (Monteraz and Winter, 1996). Studies by Murphy *et al* (2001b) indicated that infection of BALB/c and C57BL/6 results in different kinetics for IFN-γ production during the course of infection. Indeed, there was a "hiatus" of IFN-γ generation observed in the BALB/c mouse commencing after the initial replication phase and lasting through the plateau phase of the infection. Production of IFN-γ resumed at a time when bacterial clearance was beginning. Initially, these data were thought to explain the observed differences in *Brucella* susceptibility, with the innate deficiencies in BALB/c IFN-γ production facilitating bacterial persistence in this mouse strain. Incongruously, in their studies with IFN-γ KO strains Murphy *et al* (2001b) showed that BALB/c IFN-γ $^{-/-}$ mice outlived the C57BL/6 IFN-γ $^{-/-}$ mice, and were comparatively more able to control *Brucella* replication. The authors speculated that the BALB/c mice may be using some additional form of control

in the absence of IFN-γ. Obvious contenders for this role are other Th 1 type cytokines, such as TNF-α and IL-12.

Both IL-12 and TNF-α have been detected in assays involving specific antigen stimulation of splenocytes from infected BALB/c mice, but their contribution to brucellosis control in this model or in target species is complex and not completely understood. In particular their role in *Brucella* control during the IFN-γ hiatus is not clearly defined. The generation of IL-12 in response to *Brucella* infection has been largely attributed to innate mechanisms including the interaction of *Brucella* LPS with TLR-2 on macrophages and dendritic cells. Endogenous bioactive IL-12 (the p70 heterodimer) has been demonstrated *in vivo* as early as three days post-infection with both the vaccine strain *B. abortus* S19 (Zhan and Cheers, 1995) and virulent strain *B. abortus* 2308 (Fernandez-Lago *et al,* 1999). The overall effects of IL-12 appear to be similar to those described for IFN-γ. *In vivo* depletion of IL-12 in the *Brucella* infected BALB/c mouse results in an increased splenic load (Zhan and Cheers, 1995), whereas *in vivo* administration of IL-12 improves the control of bacterial replication (Sathiyaseelan *et al,* 2005). Despite this, direct evidence for enhanced production of IL-12 p40 or p70 during the IFN-γ hiatus is not seen in the BALB/c mouse infection model (Murphy *et al,* 2001b). In the Sathiyaseelan study the ability of IL-12 to reduce *Brucella* CFU in the infected BALB/c spleen was partially abrogated by treatment with anti-IFN-γ antibodies, indicating that the effects of these two cytokines are linked and possibly dependent upon one another.

TNF-α also plays a role in control of *Brucella*. *In vitro* studies showed that TNF-α presence in macrophage cultures enhanced the controlling effect of IFN-γ on *Brucella* growth and that in the absence of IFN-γ mediated activation, TNF-α was essential to promote the brucellacidal activities of macrophages (Jiang *et al*, 1993). Conversely, the *in vivo* inhibition of TNF-α using monoclonal antibodies causes an exacerbation of splenic infection of BALB/c mice (Murphy *et al,* 2001a). Furthermore, the work of Zhan *et al* (1993) supports the notion of an *in vivo* role for TNF-α through illustration that mice lacking TNF receptors

(TNF-$R^{-/-}$) also had increased numbers of *Brucella* in their spleens, and a marked deficit of IL-12 production. Despite the fact that all of these data indicate a role for TNF-α in control of brucellosis, TNF-α is not detected at elevated levels during the IFN-γ hiatus (Murphy *et al,* 2001b). Notably, in all of these studies the effects of TNF-α depletion or supplementation are only significant in the early stages of infection. Overall, research to date indicates that cytokine responses to *Brucella* infection are dominated by IFN-γ, and the action of this critical cytokine is influenced and augmented through IL-12 and TNF-α production.

Analysis of the relative contributions of CD4+ and CD8+ cells to protective immunity in the *Brucella* infected mouse has revealed conflicting results. Whilst there are plenty of studies indicating an essential role for CD8+ T cells in control of *Brucella* (Pavlov *et al,* 1982., Mielke *et al,* 1991., Splitter *et al,* 1996., Oliveira *et al,* 1998), the effect appears dependant upon the virulence of the infecting strain and the time post-challenge when the effect was observed. For example: CD8+ T cells contribute to the control of virulent *B. abortus* 2308 infections early on in infection but not at later times (Murphy *et al,* 2001), but they appear to be important in all stages of *B. abortus* strain 19 control. It is possible that undefined virulence factors expressed by *B. abortus* 2308, but absent in strain 19, permit this strain to avoid CD8+ T cell priming and thus do not trigger effective cytotoxic responses important for the elimination of infected cells. It is therefore proposed that CD8+ T cells are important in the early stages of infection, but CD4+ T cells are important for control of infection in the long term (Baldwin and Gorenka, 2004). Similar observations have been made with another abortifacient zoonosis: Chlamydia. Buendia *et al* (1999) described the rapid generation of CD8+ T cells by the vaccine strain of *Chlamydia pscittaci* and the preferential priming of CD4+ T cells from the virulent wild type strain during primary infection. This mirrors the observations of Baldwin *et al,* regarding *Brucella* infection. Importantly, adoptive transfer studies have revealed that polyclonal *Chlamydia* specific CD4+ cells are more important in protection against challenge than CD8+ cells and that CD8+ T cells do not play a role in protective adaptive immunity (For review see Morrison *et al,* 2003).

Although the majority of this information has been obtained from studies in mice, similar mechanisms of *Brucella* control are expected to be active in livestock and man (Wyckoff *et al,* 2002., Dornand *et al,* 2002).

A number of vaccines are currently licensed for use in livestock against the various important pathogenic species of *Brucella,* all of which are live attenuated strains of the organism. *B. abortus* S19, and *B. abortus* RB51 (For review see Cheville (2000)) vaccines, have been used for the protection of cattle against brucellosis. S19 was developed through laboratory culture and selection of altered phenotypic characteristics when compared to the parent strain *B. abortus* 544 (Buck, 1930). RB51 was similarly selected as a stable 'rough' mutant of *B. abortus* 2308 (Schurig *et al,* 1991). *B. suis* S2, a naturally occurring avirulent isolate of *B. suis* biovar 1, has been used extensively for the vaccination of pigs in China, where success rates are reportedly high (Xie *et al,* 1986). However, evaluation of this vaccine in other locations has resulted in extremely variable efficacy and its use has not been widely adopted (Bosseray *et al,* 1990). The vaccine *B. melitensis* Rev. 1 has been established as recommended for the protection of sheep and goats against *B. melitensis* (Alton 1987., Garin-Bastuji *et al,* 1998) and is intermittently used as part of the control strategy in affected Mediterranean countries. Rev. 1 was derived from a streptomycin dependant field isolate of *B. melitensis* following selection of altered phenotypic characteristics: loss of streptomycin dependency and becoming sensitive to penicillin (Elberg, 1957). There are no *Brucella* vaccines available for the prophylaxis of human infection.

The use of a live attenuated vaccine strain essentially involves the deliberate infection of the naïve animal with attenuated *Brucella* such that protective immune responses are elicited without concurrent pathology. The genetic mechanism underlying the attenuation of each of the vaccine strains is not fully understood. Phenotypically RB51 is distinct as the only rough strain licenced vaccine. The principal defect in this strain in OPS biosynthesis is contributed by a mutation in the *wboA* gene (Vermulapalli *et al,* 1999 and 2001). Whilst the lack of OPS undoubtedly contributes to the reduced virulence of this strain, a

simple disruption or deletion of *wboA* from *Brucella* spp, does not confer the levels of attenuation observed in RB51 (Winter *et al,* 1996). Furthermore, not all rough phase *Brucella* are avirulent (Moriyon *et al,* 2004), thus indicating that other mutations contribute to the attenuation of RB51.

The specific mechanism by which virulent *Brucella* causes abortion is unknown, and as such information regarding the loss or attenuation of abortogenic properties in vaccine strains is also limited. In the case of *B. abortus* S19, a gene deletion in the *ery* locus influencing the ability of the organism to metabolise erythritol is involved in, or at least linked to, the attenuation in ruminant systems. Erythritol is found in the ruminant placenta and a loss of ability to metabolise or detoxify this sugar reduces the capability of *Brucella* to colonise the placenta, thereby reducing its' potential to cause abortion in ruminant hosts (Sangari *et al,* 1994, 1996, 1998). However, S19 retains characteristics of reduced persistence in the BALB/c mouse model, where erythritol and abortive potential are not associated with pathology. Thus suggesting that this is not the sole facet of the S19 attenuation.

There have been few reported genomic differences between Rev.1 and virulent *B. melitensis* biovar 1, although proteomic analysis hints toward inefficient iron metabolism as a mechanism underlying Rev.1 attenuation (Eschenbrenner *et al,* 2002). Notably, Rev.1 is the only licensed vaccine providing appropriate levels of protective efficacy against *B. melitensis* in ruminants (Blasco *et al,* 2005., Garin-Bastuji *et al,* 2005).

In all cases the effective vaccine strains are disabled in either their ability to replicate within the host macrophages and establish chronic disease, or through being less able to evade the host attempts to clear infection. The S19, RB51, Rev.1 and S2 vaccines all exhibit reduced persistence compared to the respective parent strains during infection of the natural host and or reduced survival abilities in macrophage culture. This reduced persistence is also demonstrable *in vivo* using mouse models.

Although there have been attempts to produce non-living vaccines, these have not been as widely accepted or successful as the live strains. One of the most widely studied non-living vaccines was the *B. abortus* 45/20 vaccine. Various preparations of this vaccine existed ranging from simple heat killed bacterin preparations to semi-fractionated preparations formulated with incomplete Freunds adjuvant. Strain 45/20 was originally isolated from a cow and serially passaged through Guinea Pigs (20 passages) resulting in a naturally rough phase *B. abortus* strain. It was originally used as a live vaccine but there were problems associated with reversion to smooth morphology and virulence (McEwen, 1940). The adjuvanted bacterin vaccine was reported to be protective in cattle and did not directly cause abortion. However, yearly boosters were required to maintain immunity, and local reactogenicity at the inoculation site was found to be a considerable problem. Furthermore, insufficient standardisation in the production and evaluation methods for the killed preparations resulted in unacceptable levels of variability in efficacy between batches. Ultimately the use of the 45/20-adjuvant vaccine was abandoned in favour of the more consistent and longer-lived immunising properties of the live vaccines such as S19. Numerous other attempts to generate non-living *Brucella* vaccines, either using simple bacterins or fractionated antigen preparations, have concluded that these preparations are poorly immunogenic (Montaraz and Winter 1986., Nicoletti, 1990., Schurig *et al,* 2002).

Similarly, a number of recombinant protein based vaccines have been assessed but in each case these have been unable to promote comparable protective immunity to the live vaccines. A variety of delivery strategies have been employed for these recombinant antigens ranging from simple direct inoculation of the protein to formulation with adjuvants such as CpG ODN or delivery via vaccinia or *E.coli.* Table 1.1 lists some of the *Brucella* antigens that have been evaluated as recombinant vaccines.

Table 1.1: Brucella antigens studied as potential recombinant vaccines

Antigen	Details	Reference
Brucella O-chain LPS	Purified O-chain LPS	Cherwonogrodsky, 1993
B. abortus HSP60/ GroEL	Stress response proteins, e.g.: HSPs are immunodominant antigens in natural *Brucella* infections. Recombinant proteins evoke strong specific immune responses. Vaccinia expressing GroEL provide limited protection in the mouse model.	Teixeira-Gomes *et al*, 2000 Bagolu *et al*, 2000 Lin *et al*, 1996
B. abortus Cu/Zn Superoxide Dismutase	Vaccination with live *E.coli* expressing the *Brucella* Cu/Zn SOD antigen has been shown to protect mice against challenge with virulent *B. abortus*	Onate *et al*, 1999
Brucella spp. outer membrane proteins	Recombinant Omps used directly as vaccines are protective. The efficacy of vaccination is not consistent between different studies or different Omps. Passive transfer studies have shown that monoclonal antibodies raised against certain Omps are protective against challenge with rough strains in the mouse model.	Brooks-Worrell *et al*, 1992 Bowden *et al*, 1995, 1998, 2000 Guilloteau *et al*, 1999 Cloeckaert *et al*, 1991, 1995, 1996 Estein *et al*, 2003
B. abortus P39	P39 protein delivered with CpG adjuvant has a modest protective effect	Al-Mariri *et al*, 2001

Recently a novel non-living vaccine based upon antigenic extracts of rough strain *B. ovis* has been described (Murillo *et al*, 2001). The vaccine consists of hot saline extracted membrane antigens microencapsulated within poly-ε-caprolactone particles, and is able to protect rams against infection with *B. ovis*. However, at this present time there are not any non-living vaccines that are licensed for use against brucellosis, and none with reported efficacy against smooth strain *Brucella*.

1.6.3 Problems with currently licensed live vaccines for brucellosis

An effective live vaccine promotes the development of pathogen specific adaptive immune responses without the risks of pathology associated with the virulent strain. Ultimately, this procedure introduces immunological barriers that prevent either the introduction of infection to susceptible groups of animals, or prevents transmission within the group. Although the live vaccines for brucellosis are presently the most effective option for prophylaxis, there are a number of problems associated with the use of such vaccines. The problems are largely linked to the persistence of the vaccine strain within the host.

Residual virulence

All live *Brucella* vaccines persist within the host for a limited period. Persistence has long been associated with the ability to generate lasting protective immunity, but it remains a source of concern over possibilities for reversion to virulence and release into the environment.

With each of the vaccines the timing of vaccination is of paramount importance to ensure safe and efficacious use. *B. melitensis* Rev. 1 when used optimally in the intended host species (sheep and goats) confers adequate protection against virulent *B. melitensis* challenge. However, the vaccine retains abortogenic qualities, and the presence of persisting viable Rev. 1 organisms in female animals when they become pregnant, can result in abortion (Alton, 1975 and 1988., Blasco *et al,* 1985 and 1988). Similarly, *B. abortus* S19 and RB51 vaccines have been associated with abortion (Mingle *et al,* 1942., Palmer *et al,* 1996). Consequently, vaccination must be timed such that the *Brucella* will be eliminated from the host before pregnancy is initiated. Vaccine associated abortion can result in the shedding of large numbers of *Brucellae*, and can therefore transmit vaccine strain infections to other susceptible animals in the flock or herd, and contaminant the environment. A less frequently reported complication of vaccination is the development of immune-complex mediated arthropathies (Bracewell and Corbel 1980).

Reported pathogenicity of vaccine strains in alternate hosts

S19, RB51, S2 and Rev. 1, when use optimally in the preferred host, confer adequate protection against challenge with homologous species. However, despite the high levels of genetic homogeneity of the *Brucella* genus, no single brucellosis vaccine is capable of protection against challenge with all species of *Brucella*, or conferring protection against homologous challenge in all possible animal hosts. Moreover, administration of vaccines to inappropriate hosts can have devastating consequences. Clearly, more research into the host specifics of *Brucella* strain pathogenesis is required before accurate extrapolation of protective efficacy and immunogenicity between potential hosts can be made.

Significantly none of the currently available brucellosis vaccines are suitable for use in man, and indeed may remain virulent and lead to disease in the human host. From a public health perspective, one of the greatest issues surrounding the use of live *Brucella* in vaccination is that the organisms retain virulence and can be fully pathogenic to humans. Occupational exposure to Rev. 1 vaccine is a problem and isolated epidemics of human Rev. 1 infection have been documented (Blasco, 1993). Ironically, the Rev.1 vaccine was originally developed as a candidate vaccine for use in humans. Although the vaccine afforded good protection it was ultimately considered too virulent and it lead to active brucellosis when administered to human volunteers. The attempted use of the S19 *B. abortus* vaccine in humans was also reported to have caused unacceptable levels of active disease (Hoover *et al,* 2004). Recently, the first case of human RB51 associated brucellosis has been reported (Ashford *et al*, 2004).

Differential diagnosis of vaccinated and infected animals

Serodiagnosis of brucellosis is largely based upon the detection of an antibody response to the immunodominant LPS of the *Brucella* organism. Differentiation of animals producing antibodies in response to LPS from vaccine strains or virulent strains is not possible using these assays, resulting in vaccinated animals presenting as '*Brucella* positive' in serodiagnostic tests. The practical

virulence. Therefore, alternatives to live vaccines must be sought. The use of non-living antigen for vaccination eliminates the risk of vaccine acquired infection, and knowledge of the antigenic components of the vaccine may permit differential diagnosis. Additionally, sub-unit vaccines have practical advantages such as reproducibility and stability.

One of the major impediments to the development of sub-unit vaccines has been the paucity of information identifying potentially protective *Brucella* antigens. The knowledge of *Brucella* genomes was limited until recently. Allardet-Servent (1991) produced the first physical map of the *B. melitensis* 16M genome, indicating two chromosomes. Michaux-Charcharon (1997) completed mapping of the main type strains of *Brucella* showing similar genomic structure between *Brucella* species. Notably, *Brucella* spp. are highly genetically homogenous. Jumas-Bilak (1998), Michaux-Chacharon (1997) and Boschiroli (2001) proposed that the separate species originated from a single clonal ancestor following isolation in the favoured host (1993). Genomic differences that can be exploited for diagnosis and molecular epidemiology are limited. A transposable (Vermulapalli *et al,* 1999) insertion sequence (IS*711* / IS*6501*) (Halling *et al,* 1993, Ouahrani *et al,* 1993) is found in variable copy number in all *Brucella* spp., and shorter intergenic repeats also occur (Halling and Bricker 1994). None of the *Brucella* contain plasmids, although broad range plasmids can be maintained in *Brucella* (Verger 1991). Recently the genome sequences of the three most important *Brucella* pathogens: *B. melitensis* 16M (DelVecchio *et al,* 2001), *B. abortus* 544 (Sanchez *et al,* 2000, Halling *et al,* 2005) and *B. suis* 1330 (Paulsen *et al* 2002), have been completed. Comparison reveals considerable gene synteny between *Brucella* species (Halling *et al,* 2004), and related members of the α2-Proteobacteriacae (Paulsen *et al,* 2002). Notably, classical virulence factors were not identified, and the small differences in predicted gene content between species is not sufficient to account for the observed differences in pathogenicity and host preference. Post genomic analysis is expected to greatly assist the rational selection of potentially protective antigens for vaccine development. Furthermore, such information may be useful in identifying antigens that are difficult to characterise *in vivo*. For

instance the identification of specific host cell binding proteins, invasion associated proteins, and potential secreted antigens, which are practically difficult to investigate, may be facilitated by the availability of genomic, transcriptomic and proteomic data.

The lack of success so far seen with sub-unit vaccines is not just related to the selection of an appropriate antigen. A significant hurdle in vaccine development for any pathogen is the effective targeting of antigens to generate and sustain a protective immune response (Kauffman, 1999., Gurunathan, 1998). Much of the information pertaining to antigen presentation and effectors in *Brucella* protective immunity come from studies in small animal models. Immunity against brucellosis in all species appears to involve antigen specific T cell activation, CD4+ and CD8+ T cells, activation of macrophages by IFN-γ, and specific humoral responses. In particular, IFN-γ production and the presence of CD8+ cytotoxic T cells are considered essential to the clearance of virulent *Brucella* from the host (Murphy *et al,* 2001a & b., Splitter *et al,* 1997). The protective response is therefore described as essentially a Th1 style immune response. Hence, in order to develop an effective sub-unit vaccine an antigen evoking Th 1 style immune responses must be used. Additionally, the delivery route of the sub-unit vaccine is associated with effective presentation to the host immune system and is of paramount importance in generation of appropriate responses (Feltquate, 1998., Pachuk *et al,* 2000). The development of successful sub-unit vaccines requires both the selection of appropriate candidate antigens and the delivery of these antigens in a manner that will induce an appropriate and lasting protective response.

1.8 The potential of DNA based vaccination

Recent developments in vaccinology provide renewed promise for the development of effective sub-unit vaccines against intracellular pathogens. Genetic or DNA vaccines are plasmid-based DNA constructs engineered to enable expression of a chosen gene in a eukaryotic host. Vaccination with the plasmid construct results in the *in vivo* transfection of the host and the

expression of the gene of interest from the construct within the host cell. The immune response is directed against the expressed product of the gene of interest. The simplicity of the approach, ease of development and production of the constructs, and the potential for a prolonged delivery of antigen has ensured that DNA vaccination has received much attention.

DNA vaccination was born with the demonstration that intramuscular delivery of plasmid resulted in the *in vivo* production of the antigen within myocytes (Wolff *et al,* 1990), and that *in vivo* production of the encoding antigen could induce an immune response (Tang *et al,* 1992). Further work indicated that an influenza DNA vaccine was able to promote protective immunity against a virulent viral challenge in a mouse model (Ulmer *et al,* 1993).

One of the major advantages of DNA vaccination is the ability to prime cytotoxic T cells essential in the clearance of intracellular infections. This is largely achieved as a result of the endogenous production of the antigen within the transfected host cell, which results in antigen processing via MHC class I and subsequent presentation to CD8+ T cells. Antigen presentation following DNA vaccination has been the subject of much debate. Most DNA vaccine studies use the intramuscular route of inoculation where DNA is presumed to be directly transfected into the somatic cells such as myocytes and keratinocytes, or the professional antigen presenting cells (APCs) such as dendritic cells (DCs). Studies have shown that very little protein antigen (pg amounts) is produced in the host, and thus it is inferred that the CpG based adjuvant effect of the DNA vector itself, and the mechanism of antigen presentation contribute significantly to the development of the immune response. Direct transfection of professional APCs allows direct priming of CD8+ cells following endogenous processing and presentation of the antigen via MHC class I (Corr *et al,* 1996). Secreted antigen is taken up by phagocytosis and presented in the context of MHC class II to prime CD4+ T cells. However, direct transfection of APCs is not thought to be particularly efficient following i/m inoculation of naked plasmid DNA, and most of the detectable antigen expression occurs within the myocytes (Casares, S., 1997). Interestingly, myocytes are not efficient APCs. They are capable of presenting antigen in the context of MHC class I molecules but lack the

necessary co-stimulatory molecules that allow for effective T cell priming. Considering that DNA vaccination is an effective strategy for priming CD8+ T cells, and the effects of direct priming from the myocytes are limited, it is likely that the immune response is heavily dependent upon involvement of professional APCs. In addition to direct priming from either DC or myocytes it has been shown that antigen can be prepared and secreted from the transfected cell and taken up by professional APCs and presented as peptides to T cells in the context of MHC class I and class II. Peptides from exogenous sources do not normally access the MHC I presentation pathway, but generation of primed CD8+ cells has been demonstrated following uptake of antigen secreted from transfected cells or phagocytosis of transfected apoptotic or necrotic transfected cells (Albert *et al,* 1998., Fu *et al,* 1997). This route of CD8+ priming is termed cross-priming. There remains considerable uncertainty as to the relative contributions of direct and cross-presentation in the immune response to DNA vaccines. Some researchers propose that cross-presentation is the principal mechanism for CD8+ induction (Cho *et al,* 2001), but others consider that direct uptake and endogenous production of the protein within the APC providing direct access to the MHC class I presentation pathway is the main mechanism of CD8+ priming (Porgador *et al,* 1998).

In addition to the appropriate generation of CD4+ and CD8+ effector cells, DNA vaccines also have the advantage of sustained delivery of antigen. Theoretically the plasmid DNA may persist within the host cell for the life of that cell. In this respect the plasmids are similar to live viral or intracellular bacteria where persistence can be postulated to deliver antigen over a sustained period. The prolonged stimulation with antigen is a factor considered beneficial for the optimal development of memory immunity. Furthermore, the DNA vaccine approach is highly advantageous when linked with genome data permitting the selection and investigation of putative antigens without the need for culture, characterisation and genetic manipulation of the live bacteria.

1.8.1 DNA vaccines for intracellular pathogens

A large number of viral, bacterial and protozoan antigens have been assessed for protective efficacy against a variety of important pathogens. In addition to demonstrating direct protective efficacy these studies have also been valuable in identifying protective antigens from a number of organisms. Moreover, the results demonstrating variation due to vaccination schedule, dose, delivery route, or challenge dose and route, has generated important information regarding the development of protective immune responses (Bohm *et al,* 1998., Mor, 1998., Felgner, 1998., Gurunathan *et al,* 2000., Mollenkopf, 2001). DNA vaccination for protection against a complex intracellular bacterial pathogen was first described by Lowrie *et al,* (1994) using the *Mycobacterium leprae* 65 kDa heat shock protein, to provide protection against *M. tuberculosis* challenge.

Examples of DNA vaccines developed for protection against complex intracellular pathogens (bacterial, mycoplasma and protozoan) are provided in table 1.2.

Table 1.2: DNA vaccines against bacteria and parasites

Pathogen and study details	Reference
BACTERIAL DISEASES	
Tuberculosis	
Ag85 complex, as a DNA vaccine, is immunogenic and provides significant protection against virulent H27rV challenge in the guinea pig and mouse model.	*Lozes et al, (1997)*
MPT64, ESAT-6, evoke appropriate immune responses and provide limited protection against challenge both individually and in combination formulations.	*Morris et al, (2000)*
TB DNA vaccine antigens co-delivered with murine GM-CSF have enhanced cellular immune responses but limited protective efficacy.	*Kamath et al, (1999)*
Mycobacterium bovis specific antigens MPB70 and MPB83, as DNA vaccines are immunogenic and protective in the BALB/c mouse model, and elicit strong cellular responses from immunised cattle.	*Chambers et al, (2000)*
The use of TB specific antigens in a DNA vaccine, demonstrated to have therapeutic value during active TB infection.	*Lowrie et al, (1999)*
Leprosy	
The immunodominant 35 kDa protein as a DNA vaccine is protective. (Demonstrating protection achievable through a response to a single antigen).	*Martin et al, (2001)*
Chlamydiosis	
Chlamydophila abortus Dna-K antigen evokes specific immune responses and provides partial protection against challenge in the pregnant mouse model. Immune responses vary between pregnant and non-pregnant models and protection is predicted to be due to non-specific effects of the CpG motifs in the construct backbone.	*Hechard et al, (2004)*
Listeriosis	
DNA vaccine encoding Lysteriolysin-O is protective in mice.	*Cornell et al, (1999)*
Meningitis	
Generation of meninococcal group B vaccine candidates, through reverse vaccinology and DNA vaccination.	*Rappuoli et al, (2001)*
Mycoplasma diseases	
Use of the entire genome randomly fragmented and expressed in DNA vaccine plasmids protects against challenge (Expression library immunisation (ELI). The identity of the protective antigens or mediators of the protective effect remain unknown.	*Barry et al, (1995)*

Continued...

.....continued

Table 1.2 continued....

INTRACELLULAR PROTOZOAL PARASITES	
Leishmaniasis	
Protection against L. donovani through DNA vaccine encoding the A2 virulence is enhanced through inhibition of the host P53 response.	*Ghosh et al, (2001)*
DNA vaccine encoding LACK Ag protects mice against L. major challenge.	*Gurunathan et al, (1997)*
cDNA based ELI provides limited protective effect against L. major challenge in the mouse model.	*Piedrafita et al, (1999)*
Malaria	
DNA vaccines based upon merozoite surface protein 1, protects mice against P. yoelii sporozoite challenge.	*Becker et al, (1998)*
DNA vaccines against malaria for humans now in phase 3 clinical trials.	*Doolan et al, (1997)*

1.8.2 DNA vaccines for brucellosis

DNA vaccination has been investigated for *Brucella*. It was first reported in 1997 (Kurar *et al,* 1997), using the *B. abortus* ribosomal L7/L9 sub-unit as the expressed antigen in a simple pCMV based plasmid. The results of this first *Brucella* DNA vaccine, demonstrated development of a specific and appropriate immune response, but no significant protection against challenge. *Brucella* specific DNA vaccines have since been reported for *B. abortus* GroEL (LeClerq *et al,* 2001), *B. abortus* P39 and Bfr (Al-Mariri *et al,* 2001, 2002), *B. abortus* Lumazine synthetase (Velikovsky *et al,* 2002) and *B. abortus* glyceraldehyde-3-phosphate-dehydrogenase (Rosinha *et al,* 2002). Assessment of a *B. suis hsp62* based vaccine (A homologue of the candidate investigated by LeClerq *et al* 2002) for immunogenicity and preliminary efficacy in the BALB/c mouse model (Mansour, 2003) indicated a modest protective effect observed early post-challenge. In all reported investigations so far, DNA vaccines have evoked appropriate Th 1 biased immune responses against the specific antigens, and in some cases evidence of protection in a mouse model.

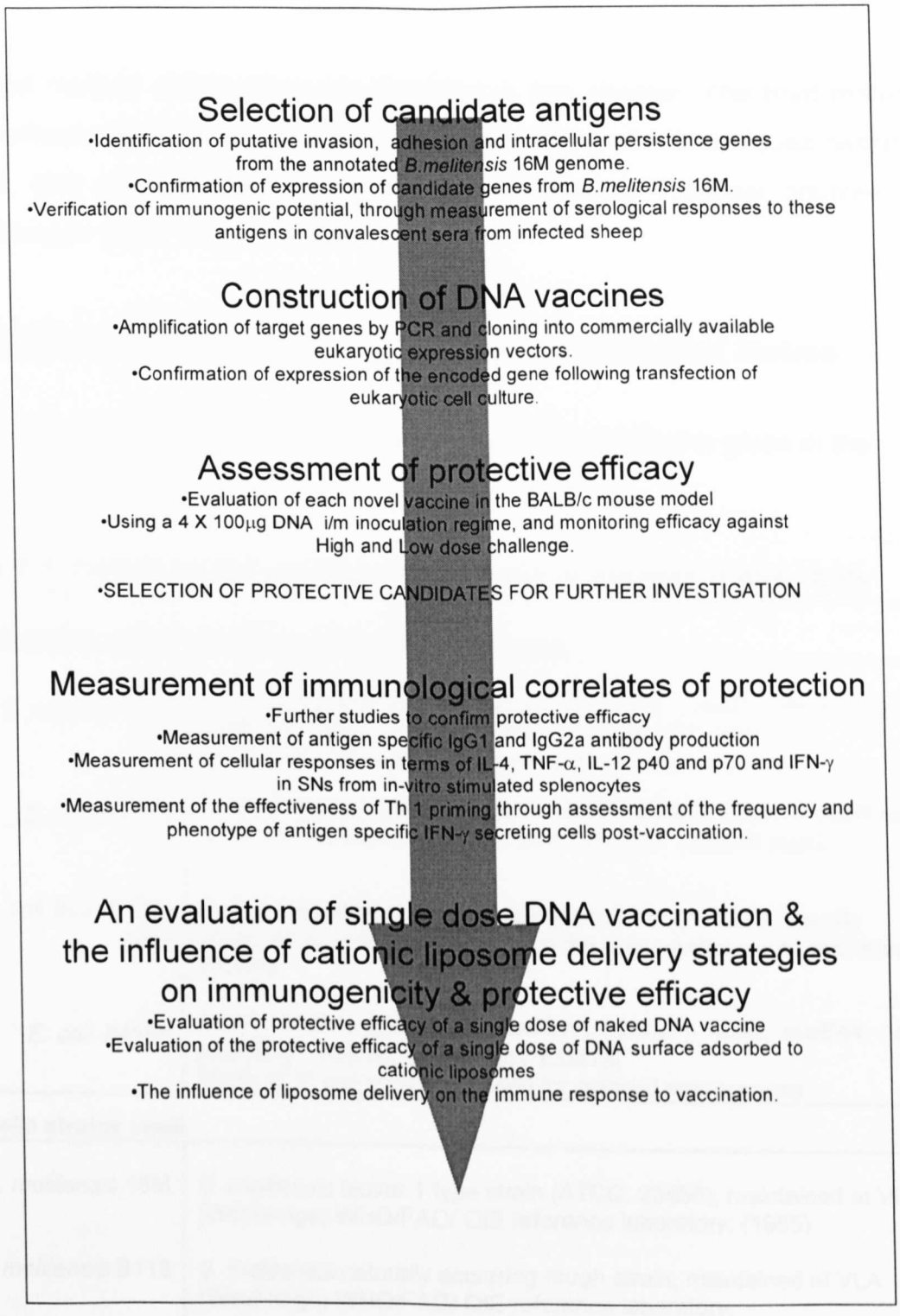
1.8.3 The future for DNA vaccines

The transfer of DNA vaccines from the laboratory setting to clinical and veterinary investigations has proven disappointing in a large number of studies, prompting a backlash of negative reactions regarding the overall concept of such vaccines. Nevertheless, in 2005, the first commercially available veterinary use DNA vaccine was approved by the United States regulatory authorities. The vaccine "West Nile Innovator DNA" manufactured by Fort Dodge Animal Health has recently been granted marketing authority for use in horses in the USA for protection against West Nile Virus, and is expected to be available to veterinary practices from 2006. Regulatory approval of the West Nile Virus vaccine sets an important precedent in the commercialisation of DNA based vaccines, and also in combat of the popular perception that these vaccines are ineffective in large animals. Significantly, a second DNA vaccine "Apex IHN" (Aqua Health, Novartis, Canada) to prevent infectious haematopoietic necrosis in farm raised Atlantic Salmon, has also passed the first stages of the regulatory process (Anon, 2005), signalling that the application of these vaccines for use in food animals is not unfeasible. These recent events signal the advancement of DNA vaccines from a theory, through the clinic, and now into the field. Undoubtedly, further DNA vaccines will follow.

1.9 The aims of this investigation

Brucellosis in livestock remains a considerable problem in many areas of the world, and thus remains a threat to public health. Previously, vaccination has proved to be a useful strategy for reducing the prevalence of diseases in livestock in endemic areas and consequently lowering the incidence of human disease. Currently available live vaccines used for protection of livestock against pathogenic *Brucella* infections have a number of undesirable characteristics, including problems associated with efficacy, interference with current statutory diagnostic assays, and residual pathogenicity. Furthermore none of these vaccines are applicable for use in humans, and accidental inoculation may lead to disease. Novel or improved, rationally designed vaccines are necessary in order to address these issues.

Figure 1.2: A flow chart summarising the investigations discussed in this thesis

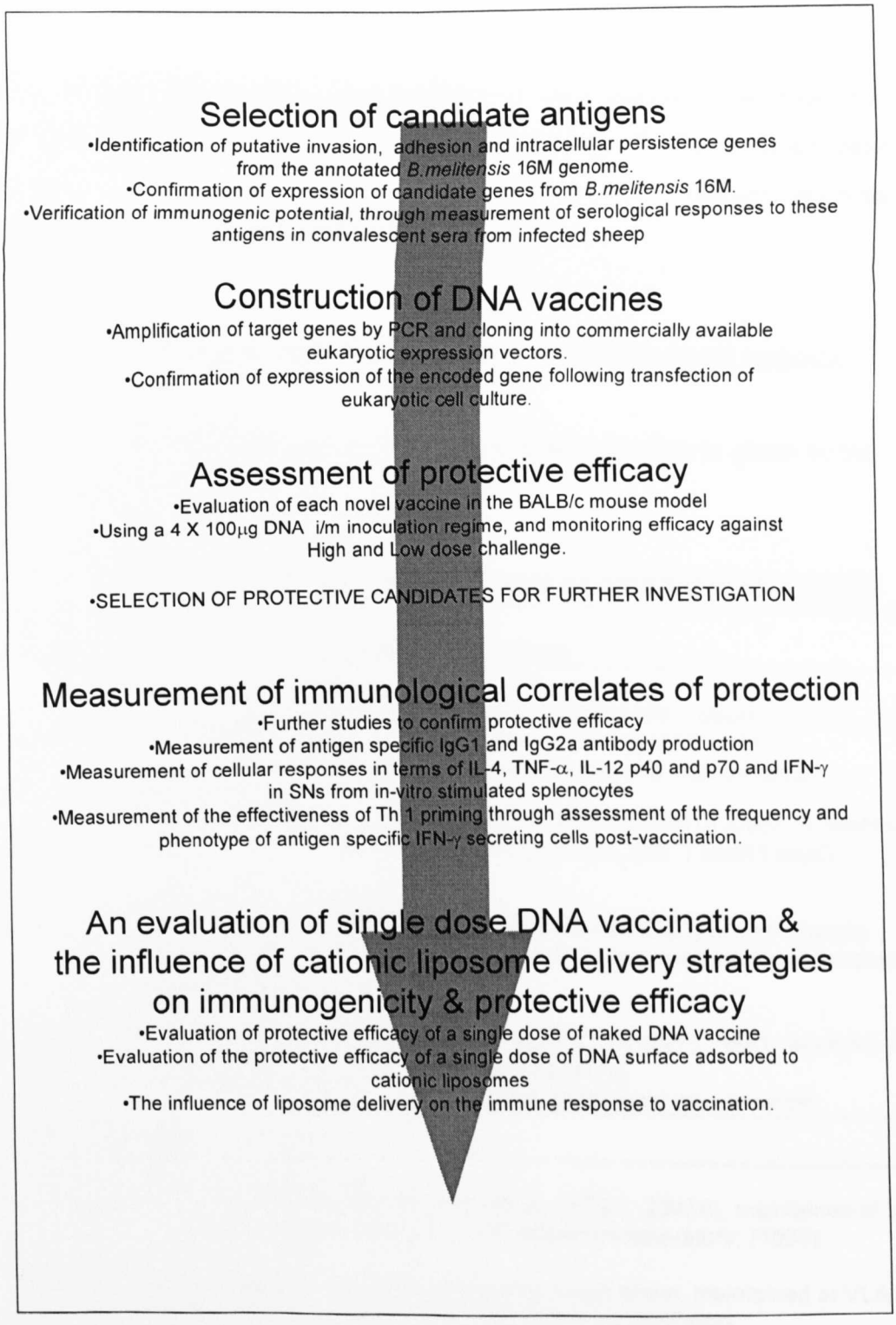


An efficacious sub-unit or non-living *Brucella* vaccine has the advantage of being non-infectious to man or animals, and with careful selection of the antigenic components it can be created to produce immune responses that are distinguishable from those evoked by virulent infection. DNA vaccination technology, the recent availability of genome data for *B. melitensis* 16M and an increasing immunological knowledge relating to effective vaccine antigen delivery and presentation, and *Brucella* specific immunity, provide a unique opportunity for the rational investigation of new sub-unit DNA vaccine formulation for the protection of livestock against brucellosis, and the subsequent protection of humans handling livestock.

The aim of the research presented in this thesis was to develop and evaluate novel DNA vaccines to protect livestock and humans against brucellosis.

It is hypothesised that DNA vaccination using constructs encoding proteins involved in the establishment of infection will provide protection through priming adaptive immune responses that act to inhibit *Brucella* invasion or reduce their ability to persist within host macrophages. The research makes timely use of the *Brucella* genome sequencing project and allied post genomic technologies. Genome data will be used to identify novel candidate antigens for which DNA vaccines will be produced and evaluated for protective effect in the BALB/c mouse model. Immunological correlates of protective immunity will be measured. Furthermore, since naked DNA vaccines are reported to be poor immunogens in livestock mechanisms to improve the immune response will also be investigated. The overall plan for this investigation is summarised in Figure 1.2.

Figure 1.2: A flow chart summarising the investigations discussed in this thesis



Chapter 2: MATERIALS AND METHODS

Detailed method descriptions are provided in this chapter. The brief materials and methods sections in subsequent chapters refer to the techniques described herein, and provides the context for these methods. Reagent sources and suppliers are listed in appendix 3.

2.1: Bacterial culture techniques and containment issues

A list of all bacterial strains used or created in these studies is given in the following table.

Table 2.1: Details of all bacterial strains used or created in this study

***E. coli* strains used in genetic modification procedures**	
E. coli TOP10F'	F' Φ80d*lacZ*ΔM15 Δ(*lacZ*YA-argF)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1 Strain of *E. coli* commonly used for plasmid maintenance
E. coli TOP10	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 (Δ*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G
E. coli BL21(DE3) pLys	F- *ompT hsd*SB (r_B-m_B-) *gal dcm rne*131 (DE3) *pLysS* (CamR) Strain of *E. coli* commonly used for the expression of recombinant proteins
E. coli JM109	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, (r_K-m_K+), *rel*A1, *sup*E44, Δ(*lac-pro*AB) [F', *tra*D36, *pro*AB, *lac*1^qZΔM15] Strain of *E. coli* commonly used for plasmid maintenance
***Brucella* strains used**	
B. melitensis 16M	*B. melitensis* biovar 1 type strain (ATCC: 23456), maintained at VLA (Weybridge) WHO/FAO/ OIE reference laboratory. (1955)
B. melitensis B115	*B. melitensis* naturally occurring rough strain, maintained at VLA (Weybridge) WHO/FAO/ OIE reference laboratory.
B. melitensis Rev.1 (Vetoquinol,)	*B. melitensis* biovar 1 vaccine strain, from seed stocks maintained by the Vaccine production facility at Vetoquinol©, France.

Continued.....

Table 2.1 continued...

GM *E. coli* based strains produced in this study

(a) Eukaryotic expression

p-omp25 (1) TOP10F'	TOP10F' *E. coli* transformed with the pCR3.1 plasmid with *omp25* gene insertion.
p-omp25 (2) JM109	JM109 *E. coli* transformed with the pTargeT plasmid with *omp25* gene insertion.
*p-ialB*TOP10F'	TOP10F' *E. coli* transformed with the pCR3.1 plasmid with *ialB* gene insertion.
p-frpB TOP10F'	TOP10F' *E. coli* transformed with the pCR3.1 plasmid with *frpB* gene insertion.
p-fliC TOP10F'	TOP10F' *E. coli* transformed with the pCR3.1 plasmid with *fliC* gene insertion.
p-acvB TOP10F'	TOP10F' *E. coli* transformed with the pCR3.1 plasmid with *acvB* gene insertion

(b) Prokaryotic expression

pGEX-Omp25 TOP10	TOP10 *E. coli* transformed with the pGEX-6P plasmid containing the omp25 gene insertion. Plasmid production /maintenance strain. GST fusion protein.
pGEX-Omp25 BL21	BL21 *E. coli* transformed with the pGEX-6P plasmid containing the *omp25* gene insertion. Recombinant protein production strain. GST fusion protein.
pGEX-iaiB TOP10	TOP10 *E. coli* transformed with the pGEX-6P plasmid containing the *ialB* gene insertion. Plasmid production /maintenance strain. GST fusion protein.
pGEX-IalB BL21	BL21 *E. coli* transformed with the pGEX-6P plasmid containing the *ialB* gene insertion. Recombinant protein production strain. GST fusion protein.
pGEX- acvB TOP10	TOP10 *E. coli* transformed with the pGEX-6P plasmid containing the *acvB* gene insertion. Plasmid production /maintenance strain. GST fusion protein.
pGEX-*acvB* BL21	BL21 *E. coli* transformed with the pGEX-6P plasmid containing the *omp25* gene insertion. Recombinant protein production strain. GST fusion protein.
pRSET- *frpB* TOP10	TOP10 *E. coli* transformed with the pRSET plasmid containing the *frpB* gene insertion. Plasmid production /maintenance strain. Histidine tagged fusion protein.
pRSET-frp*B* BL21	BL21 *E. coli* transformed with the pRSET plasmid containing the *frpB* gene insertion. Recombinant protein production strain. Histidine tagged fusion protein.
pRSET-fliC TOP10	TOP10 *E. coli* transformed with the pRSET plasmid containing the *fliC* gene insertion. Plasmid production /maintenance strain. Histidine tagged fusion protein.
pRSET-fliC BL21	BL21 *E. coli* transformed with the pRSET plasmid containing the *fliC* gene insertion. Recombinant protein production strain. Histidine tagged fusion protein.

Contained use of *Brucella spp.*

Brucella melitensis 16M and all related strains are ACDP category III zoonotic pathogens, and as such can only be handled within designated appropriate containment laboratories. Containment of the pathogen is also required under the Specified Animal Pathogens Order (SAPO) 1998. For this project all culture and manipulation of live *Brucella* spp., was performed within SAPO licensed containment level III laboratories at VLA Weybridge.

Brucella melitensis strains 16M, Rev.1 and B115 were used in this study for provision of antigens and inocula for *in vivo* and *in vitro* work. *B. melitensis* 16M lyophilised aliquots are maintained at the VLA Brucellosis reference centre. These stocks were generated in 1958 from the original 16M isolate listed as the *B. melitensis* biovar 1 type strain (ATCC 23456). Rev.1 seedlot strain was provided by Vetoquinol, courtesy of Dr Ariane Van Der Moer and Dr Thierry Masse. B115 was obtained from the lyophilised stocks in the VLA reference collection.

Culture of *Brucella melitensis* 16M on solid agar media for isolation of total RNA

B. melitensis 16M was resurrected from lyophilised stocks into a minimal volume of sterile water, and transferred immediately to serum dextrose agar (SDA) + 10% horse serum media. Plates were incubated at 37°C in a 10% CO_2 atmosphere incubator for 4 days. Bacterial growth was harvested directly into Tri-Reagent™ by adding 1 ml of Tri-reagent™ to each plate surface and gently scraping the bacterial lawn into the solution using a sterile bacterial loop. The suspension was transferred to a sterile 50 ml Falcon™ style centrifuge tube, vortexed for 10 minutes and frozen immediately at –80°C to facilitate cell lysis. The suspension was subjected to three cycles of freezing and thawing, before proceeding with RNA isolation.

Culture of *Brucella melitensis* 16M in Albimi broth for isolation of total RNA

B. melitensis 16M was resurrected from lyophilised stock and seeded to SDA +10% horse serum as described above, except that cultures were plated to provide single colonies. After 4 days growth on agar, a single colony was selected and transferred to a 1 ml aliquot of Albimi Broth Culture. The sample was thoroughly vortexed and then incubated for 24 hours at 37°C in 10% CO_2 atmosphere. The 1 ml suspension culture was then added to 9 ml of fresh Albimi solution in a sterile Falcon style tube, and incubated for a further 18 -24 hours under the same conditions. For bacterial harvest, the tubes were centrifuged at 4000 rcf for 15 minutes. Supernatant was discarded. The pellet was immediately resuspended into 1 ml of Tri-Reagent™, and subjected to freeze thaw cycles as described above.

Maintenance and culture of *B. melitensis* 16M, for use as the challenge strain in protective efficacy studies

Each time *B. melitensis* 16M was required as inocula for use *in vivo* in challenge studies, individual lyophilised stocks were reconstituted in a minimal volume of sterile water and plated on SDA as described above for culture of *B. melitensis* 16M for RNA isolation. Cultures were incubated at 37°C for 4 days in a 10% CO_2 supplemented atmosphere. Cultures were visually inspected on a daily basis for the presence of extraneous agents, or indications of phase dissociation. Cultures not observed to be 100% smooth phase *Brucella* were not used in inocula preparation. At the time of inocula preparation a representative sample of heat-killed inocula was analysed by 16S rRNA bacterial sequencing to confirm *Brucella* spp., identity.

Maintenance and culture of *B. melitensis* Rev. 1, for use as live vaccine controls in protective efficacy studies

B. melitensis Rev.1 was used as a positive control for successful vaccination throughout these studies . The strain was propagated on the recommended *Brucella* blood agar in order to generate lyophilised stocks. Enough aliquots were produced to last for the duration of the project. When required, a freeze-dried aliquot was reconstituted in a minimal volume of sterile water and seeded onto agar. Following four days growth at 37°C growth was visually assessed for purity and evidence of phase dissociation. As indicated for *B. melitensis* 16M cultures, only pure smooth strain cultures were used to prepare the inocula.

Preparation of viable *Brucella* suspensions for vaccine and challenge inocula used in protective efficacy studies

A standard procedure was followed in order to prepare a known CFU inocula of the vaccine control strain Rev.1 or the challenge strain 16M. *Brucella* were cultivated as described above and then harvested from the plate into 0.1M PBS. The opacity of the suspension was determined using the nephelometer device. The suspension was altered until a transmission value of 42 – 46 % was obtained. This value is known to equate to a *Brucella* concentration of approximately 1 X 10^{10} CFU / ml. Serial dilution from this stock solution was then used to generate a solution with an appropriate expected concentration, which was inoculated into mice as soon as possible after preparation in order to limit time during which there could be a natural decrease in viability.

For retrospective enumeration of the actual inoculation dose, remaining *Brucella* suspension was returned to the laboratory immediately after inoculation of mice was completed. The sample was further serially diluted to the anticipated dilution limit. Each of the dilutions, including the 'neat' suspension were plated onto SDA plates (100 µl per plate, in triplicate). Plates were incubated at 37°C for 7 days and CFU counted.

Recovery of *Brucella* from infected mouse tissues for bacterial enumeration in protective efficacy studies

Mice were sacrificed by cervical dislocation. Spleens were aseptically removed and immediately placed in 3 ml of 0.1 M PBS. Spleens were prepared in PBS using sterile scissors to disrupt the membranes and the barrel of a sterile 5 ml syringe to macerate the spleen by passing the material through a 100 μM mesh cell sieve. Sieving permitted *Brucella* and splenocytes to be separated from fibrous connective tissue. For enumeration of the *Brucella* the sieved material was serially diluted to 1 in 10,000 dilution. Each dilution was plated in 100 μl aliquots in triplicate onto SDA plates. Samples isolated from animals that had received Rev.1 vaccination were also plated onto both trypticase soya agar (TSA), and TSA + 5 I.U./ml penicillin. Plates were incubated at 37°C for 5 – 7 days. CFU were counted on all plates, and used to determine the number of *Brucella* recovered from the spleen of each mouse. Comparison of CFU recovered on TSA and TSA + penicillin was used to determine the relative percentage of Rev.1 from the total recovered *Brucella*.

Recovery and culture of *Brucella melitensis* 16M from infected mouse splenocytes for isolation of total RNA

Mice were sacrificed by cervical dislocation. Spleens were aseptically removed and processed to generate free suspension splenocytes as described above, except that suspensions were produced in DMEM media (Invitrogen Life Technologies, Paisley, UK) rather than PBS. DMEM media without antibiotics was used as streptomycin can inhibit the growth of 16M. Cultures were incubated overnight at 37°C, 5% CO_2, in a humid atmosphere incubator. Following the 24-hour incubation plates were centrifuged at 200 rcf to sediment the bacteria and the cells. Supernatant was discarded and the bacteria / cell suspension was resuspended into 0.5 ml of Tri-reagent™ per well. Suspensions were transferred to sterile Eppendorf tubes and subjected to freeze thaw cycles as described previously.

Sterility assessment of samples prepared in Tri-Reagent

Sterility validation exercises were performed to show that following suspension in Tri-Reagent™ and freeze thawing there were no remaining viable *Brucella* in the suspension. In order to determine that samples did not contain viable *Brucella*, an aliquot of each sample (not less that 10% of the total sample volume) was assessed. The sample was centrifuged to sediment bacteria and bacterial debris and the supernatant discarded. The pellet was washed twice by centrifugation at 4000 rcf for 10 minutes in 0.1 M PBS, in order to remove residual traces of the Tri-Reagent solution from the preparation. The sample was resuspended in 400 µl of 0.1 M PBS and plated in 100 µl volumes in triplicate onto SDA plates. Plates were incubated at 37°C, 10% CO_2 for 7 – 10 days, and assessed daily for the presence of viable bacteria. Samples, which were demonstrated to be sterile, were then classed as non-infectious and could be dealt with at containment level II rather than III. This was necessary because RNA isolation procedures at containment level III proved to be impractical, as the need for rapid and dexterous sample manipulation could not be accommodated under the strict containment conditions. Thus sterility of the sample was a prerequisite for this procedure.

Detection of any bacterial growth, including non-*Brucella* contaminants, resulted in the entire sample being discarded.

Culture and maintenance of *E. coli* strains.

E. coli host organisms were used for the maintenance of DNA vaccine plasmids, and for production of recombinant proteins from prokaryotic fusion-protein expression constructs. Plasmid maintenance strains were classified as Class I genetically modified microorganisms (GMMs) under the genetic modification contained use regulations (2001) Expression strains were designated as class II GMMs. All work with live *E. coli* strains (both maintenance and expression) was performed within designated containment

level II laboratories, and work with live bacteria was restricted to a class II safety cabinet to maintain isolation of the organisms.

The *E. coli* strains used and created in this project were maintained as frozen seed stocks at –80ºC. Broth cultures for each strain were produced as described below for the isolation of plasmids. *E. coli* was harvested from broth by centrifugation (4000 rcf, for 10 minutes, at 4°C (Heraeus MegaFuge 1.0R)) and resuspended in a minimal volume of Brain-Hearth Infusion Broth (BHIB) + 10% Glycerol. This suspension was transferred to sterile eppendorf tubes in 1 ml aliquots and frozen immediately at –80°C. At least 10 aliquots of each *E. coli* strain were produced.

Culture of *E. coli* for the isolation of plasmids

GMM *E. coli* containing the DNA vaccine plasmids were grown on LB agar and in Luria-Bertani (LB) broth containing ampicillin (at 100 μg/ml).

For solid agar cultures *E. coli* were seeded onto LB-Agar (100 μg/ml ampicillin), to generate single colonies. A single vial of frozen seed stock was removed from storage at –80°C, but not allowed to defrost completely. A sterile bacterial loop was used to scrape a sample of the cell stock from the frozen surface, which was streaked onto the agar plate. Plates were incubated overnight at 37°C.

For broth cultures, a 30 μl bacterial loop of frozen cell stock or a single colony from a plate culture was inoculated into 10 ml of cold LB Broth (100 μg/ml ampicillin). This culture was incubated at 37°C in a shaker incubator (225 rpm), for 12 - 16 hours. If larger volumes were required this culture was then transferred directly into a larger vessel containing the appropriate volume of pre-warmed LB broth plus ampicillin, and incubated for a further 24 hours.

Culture of *E. coli* for the expression of recombinant proteins

Culture of expression clones was essentially the same as for plasmid isolation, except that liquid culture used LB broth without antibiotic. Optimal expression conditions varied between individual *E. coli* strains. Generally for each procedure, the frozen seed stock of the GMM *E. coli* was plated to provide single colonies on LB agar containing selective antibiotic (either ampicillin at 100 μg/ml or kanamycin at 40 μg/ml). After 24 hours a single colony was transferred to a minimal volume (approximately 5 – 10 ml) of LB broth without antibiotics. After 24 hours the suspension culture was passaged by transfer of an aliquot to a larger volume of pre-warmed LB broth, generally a ratio (v/v) of 1: 10, culture : LB broth was used. Bacterial growth in suspension was monitored using a spectrophotometer in terms of the optical density of the suspension at 600 nm until the culture was determined to be ready for induction. Expression of the recombinant protein was induced through the addition of IPTG to the media. Culture supernatant or cell paste was harvested after optimal incubation and the presence of protein confirmed in the appropriate fraction through SDS-PAGE and western blotting procedures.

Large scale protein production (for Omp25-GST and IalB proteins) was sub-contracted to a commercial company. Protein details are provided in appendix 2.

Maintenance of Cos 7 cell cultures

Cos7 cells were routinely cultured in 75 ml Nunc tissue culture flasks in DMEM, supplemented with 200 mM L-Glutamine (Glutamax™), 100 μg/ml Antibiotic antimycotic solution, and 10% Foetal Bovine Serum. Cultures were incubated at 37°C in a 5% CO_2 atmosphere. Cells were passaged on a weekly basis. For transfection studies cells were cultured in 12 well Nunc tissue culture plates containing with sterile glass coverslips (18 mm diameter, no.2, BDH Lab supplies). Twenty-four hours prior to the transfection procedure the cells were

passaged and seeded to sterile 12 well plates at 1 X 10^5 cells/ml. Growth under standard incubation conditions resulted in cells at between 40 – 80% confluence at the time of transfection.

2.2 RNA isolation, reverse transcription and polymerase chain reaction procedures

Procedures were optimised for the amplification of specific *Brucella* genes from both genomic DNA and cDNA templates.

Isolation of total RNA from Tri-reagent™ suspensions

RNA isolation from material suspended in Tri-reagent™ was performed according to manufacturers instructions. The following modifications to these standard procedures were applied. For material harvested from SDA plates it was necessary to include an additional centrifugation step before the addition of chloroform to the samples. Samples were centrifuged at 10,000 rcf for 5 minutes in order to sediment any agar harvested with the bacteria. The supernatant, containing the released RNA in solution, was transferred to a clean sterile tube for processing. The agar and bacterial debris in the pellet were discarded. All other samples were processed as described in the manufacturers guidelines.

Briefly, each Tri-Reagent sample was distributed into 1 ml aliquots in sterile DNAse/Rnase free eppendorf tubes and 0.2 ml of chloroform (Molecular Biology Grade Reagent) was added to each tube. These samples were vortexed briefly and then incubated on ice for 5 minutes. Samples were centrifuged at 16,000 rcf (Heraeus Biofuge Fresco) at 4°C for 10 minutes, and the clear aqueous layer carefully transferred to a new sterile tube. The pink solvent layer and opaque interface were discarded. The RNA present in the aqueous phase was precipitated by addition of 0.5 ml of isopropanol (Molecular Biology Grade Reagent). Samples were briefly vortexed to ensure mixing, and incubated on ice for 20 minutes before centrifuging (16,000 rcf, for 20 minutes at 4°C ,

Hereaus Biofuge Fresco) to pellet the precipitated RNA. Precipitated RNA was washed twice in 75% ethanol by centrifugation (10 minutes, 10,000 rcf, 4°C, Hereaus Biofuge Fresco). The ethanol supernatant was carefully decanted and the pellets air-dried for 20 minutes, before resuspension in 20 µl of DEPC treated water (Ambion™). Samples were 'cleaned up' using the Ambion™ DNA-Free™ procedure to eliminate traces of genomic DNA.

Genomic DNA removal from total RNA samples

All samples were treated with the Ambion DNA-Free™ to remove traces of genomic DNA. Ambion™ DNA-Free™ procedures were conducted according to the manufacturers instructions. Briefly, 5 µl of DNAse1 was added to each 20 µl aliquot of RNA. Samples were incubated at 37°C in a water bath for 30 minutes. 10 µl of DNAse removal agent was added to each tubes and mixed using a Denley rotary cell mixer for 10 minutes. Samples were centrifuged (10,000 rcf, 4°C, for 1 minute, Hereaus Biofuge Fresco) to sediment the removal reagent. Supernatant was transferred to a clean labelled sterile tube, and used immediately in cDNA production or frozen at –80°C until use.

Reverse transcription of total RNA to generate cDNA

cDNA production was performed using the reagents supplied with the Ambion Retroscript™ RT-PCR kit. For each RNA sample, Oligo dT primers or Random decamers (Sourced from Ambion) were used to prime the reaction for reverse transcription. Oligo dT priming was used in reactions with template RNA obtained from Cos7 cells, whereas Random decamer priming was used for reactions using *Brucella* RNA as template.

For Cos 7 RNA reverse transcription , 2 µl of Oligo dT primer was added to 12 µl of the RNA sample. The sample was then heated to 82°C for 3 minutes to denature any secondary structures and open the RNA template for annealing of the primer. Incubation at 82°C was carried out in a Progene thermal cycler (Techne). The sample was immediately cooled to 4°C and remaining reagents for RT-PCR added on ice. 4 µl of 10 mM dNTP solution, 1 µl of M-MLV –RT

enzyme (or 5 IU), 1 μl (or 5 IU) of Superasin™ RNAse inhibitor and 2 μl of reverse transcription buffer (Retroscript™) was added to each tube. The tubes were heated to 42°C in the Techne™ Progene™ thermal cycler for 90 minutes, and then heated to 80°C for 10 minutes to denature the enzymes and stop the reaction, before cooling to 4°C. The cDNA was used immediately in specific PCR reactions or stored at –80°C in 5 μl aliquots until required.

Reverse transcription of *Brucella* total RNA was achieved using random decamer priming. The procedure was essentially the same as that described for Cos 7 RNA reverse transcription, except that only 5 μl of RNA solution was used as template in these reactions because quantities of this material were more limited. RNA was prepared for reverse transcription as follows: 5 μl of RNA was added to a sterile PCR tube, 5 μl of Nuclease-free water was added, along with 2 μl of random decamer™ primers. The sample was heated to 82°C for 2 minutes in order to destroy RNA secondary structure and anneal the random decamers™ to the RNA template. The rest of the procedure was identical to that described above for the Cos 7 RNA samples.

Polymerase Chain Reaction

PCRs were developed to amplify the selected candidate antigen genes. Reactions were devised to amplify the entire coding sequence of the selected gene, and were optimised for amplification of either *Brucella* genomic DNA as template for the generation of product for cloning, or for amplification of cDNA template obtained from either Cos 7 RNA or *Brucella* RNA. Additional PCRs were used in control reactions throughout these studies.

Primer design

PCR primers were designed to permit amplification of the entire ORF for each selected candidate, and to introduce novel restriction cleavage sites to either end of the product and a Kozac signal sequence (ribosomal binding site) to the 5' end of the product. Primer details are provided in the following table.

Table 2.2: Primers used for the amplification of Brucella specific genes

Target gene	Primer details
omp25	Forward primer: AAACTCGAGCCGCCACCATGCGCACTCTTAAGTCT
	Reverse primer: AAATCTAGAACCGGATGCCTGAAATCCTT
acvB	Forward primer: AAAAGCTTGCGCCACCATGAAGAAAGAACGCGTATTC
	Reverse primer: AAAGGATCCTCGCGCAGGGCGCGGCGGC
frpB	Forward primer: AAACTCGAGGCCACCATGAAGTTCACGAGAATGCTG
	Reverse primer: AAATCTAGATCAGAACTTGAAGGCCGTCTGGAAA
ialB	Forward primer: AAAGGATCCGCCACCATGAAAAATTATCGTG
	Reverse primer: AAAGAATTCTTACTTGGTCAATGCCTGAAT
fliC	Forward primer: AAACTCGAGGCCACCATGGCTAGCATTCTTACAAACT
	Reverse primer: AATCTAGATCCGGTTTATTAGCCGCGGAACAG
*omp25**	Forward primer: TGCGCAACTCTTAAGTCTCTT
	Reverse primer:TTAGAACTTGTAGCCGATGCC
*IF-1**	
	Forward primer: ATGGCGAAAGAAGAAGTCCT
	Reverse primer: ACTAGAACCTTGTCACCGGC (Eskra *et al*, 2001)

Restriction enzyme sites boxed
Kozac sequence underlined * primers used only for RT-PCR investigations (not cloning)

Primers were synthesised by Sigma Genosys, or Oswel laboratories UK.

PCR optimised for cloning

PCR reactions were optimised for the amplification of each candidate product. Reactions were set up using *B. melitensis* 16M genomic DNA as template, and using the conditions specified for the IS*711* *Brucella* specific PCR (Bricker *et al*, 1994). For each specific PCR the conditions such as $MgCl_2$ concentration, Glycerol concentration, primer concentration, annealing temperature and number of cycles per reaction were optimised from this starting point until a clean product was obtained. Genomic DNA, obtained by phenol-chloroform extraction procedure was kindly donated by the *Brucella* reference laboratory, VLA Weybridge. The concentration of template DNA in each reaction was adjusted to obtain optimal conditions. Concentration was estimated using spectophotometric readings of absorbance at 260 nm. All PCR's were conducted using a Progene thermal cycler (Techne). Optimised amplification conditions for each gene are described in the following table.

Table 2.3: Optimised PCR conditions for cloning, using genomic DNA from B. melitensis16M as template

Gene identity	PCR details
omp25	Total volume: 100 µl. Template: 20 µl *B. melitensis* 16M genomic DNA @ 200 µg/ml. Reaction conditions: Perkin Elmer PCR system: X1 PCR Buffer II, 1.5 mM $MgCl_2$, 1.0 mM dNTPs, 200 nM ea. Primer, 10% glycerol, 5U Taq Cycling conditions: • 1 [95°C X 5', 58°C X 2', 70°C X 3'] • 30 [95°C X 1', 58°C X 2', 70°C X 3'] • 1 [95°C X 1', 58°C X 2', 70°C X 12'] Product size: 750 bp
frpB	Total volume: 50 µl Template: 5 µl *B. melitensis* 16M genomic DNA @ 200 µg/ml Reaction conditions: Perkin Elmer PCR system: X1 PCR Buffer II, 3.0 mM $MgCl_2$, 2.5 mM dNTPs, 200 nM ea. Primer, 5U Taq Cycling conditions: • 1 [94°C X 5'] • 35 [95°C X 90", 56°C X 90", 72°C X 120"] • 1 [72°C X 12'] Product size: 2008 bp
ialB	Total volume: 50 µl Template: 5 µl *B. melitensis* 16M genomic DNA @ 200 µg/ml . Reaction conditions: Perkin Elmer PCR system: X1 PCR Buffer II, 10% glycerol, 3.0 mM $MgCl_2$, 2.5 mM dNTPs, 200 nM ea. Primer, 5U Taq Cycling conditions: • 1 [94°C X 5'] • 35 [95°C X 90", 56°C X 90", 72°C X 120"] • 1 [72°C X 12'] Product size: 546 bp
acvB/virJ	Total volume: 50 µl Template: 5 µl *B. melitensis* 16M genomic DNA @ 200 µg/ml Reaction conditions: Perkin Elmer PCR system: X1 PCR Buffer II, 12% Glycerol, 2.75 mM $MgCl_2$, 2.5 mM dNTPs, 200 nM ea. Primer, 5U Taq Cycling conditions: • 1 [94°C X 5'] • 35 [95°C X 90", 57°C X 90", 72°C X 120"] • 1 [72°C X 12'] Product size: 1435 bp
fliC	Total volume: 50 µl Template: 5 µl *B. melitensis* 16M genomic DNA @ 200 µg/ml Reaction conditions: Perkin Elmer PCR system: X1 PCR Buffer II, 8% Glycerol, 3.0 mM $MgCl_2$, 2.5 mM dNTPs, 200 nM ea. Primer, 5U Taq Cycling conditions: • 1 [94°C X 5'] • 35 [95°C X 90", 56°C X 90", 72°C X 120"] • 1 [72°C X 12'] Product size: 880 bp

PCR optimised for use with cDNA templates

For PCR from cDNA templates from both *Brucella* RNA and Cos 7 RNA, the Accuprime™ system was found to be more suitable than the reactions described for cloning. Additional control reactions were also devised for use in these studies. For each sample of cDNA assessed a control reaction using the corresponding RNA sample as template was used. This reaction was included in each assay to serve as a "no reverse transcription" control and verify the absence of contaminant DNA from the sample.

For reactions utilising cDNA template generated from *Brucella*, a specific PCR for a constitutively expressed gene (IF-1) was used to determine the presence of intact *Brucella* specific sequence in the template. This procedure was to act as a simple control procedure to determine the quality of the RNA and cDNA preparation. The IF-1 PCR was based upon that developed by Eskra *et al,* (1997).

Amplification procedures for the candidate genes (and the IF-1 control) were optimised using the Accuprime™ system reagents. Accuprime reagents provided a complete PCR mastermix, to which the primers at an appropriate concentration (0.2 nM), and the template cDNA samples were added. 2 –5 μl of template cDNA was used in each reaction. Accurate quantitation of the amount of cDNA in each template was not possible because of the small sample size, but visualisation of a 1 μl aliquot of cDNA on agarose gel electrophoresis and comparison of the intensity of ethidium bromide (EtBr) staining against a known plasmid concentration indicated the concentration of each preparation to be between 0.001 μg/μl and 0.01 μg/μl.

For each reaction 1 – 5 μl of template cDNA was used. Reactions were set up in 50 μl total volumes, with 25 μl of the Accuprime I ™ reagent, 1 μl of each primer at 0.2 nM concentration, and 19 – 22 μl of DEPC treated water. Each reaction involved an initial denaturation and enzyme activation step at 94°C for 5 minutes, followed by 20 cycles of denaturation at 94°C for 30 seconds, annealing at a specific temperature dependant upon the gene target for 30 seconds (specific annealing temperatures for these reactions are provided in

table 2.4), and extension at 72°C for 30 seconds. A final prolonged extension step for 5 minutes concluded the reaction.

Table 2.4: Annealing temperatures for specific Accuprime™ PCR reactions

Specific reaction	Annealing Temperature
IF-1	59°C
acvB	56°C
ialB	58.5°C
fliC	58.5°C
frpB	56°C
omp25	58.5°C

Agarose gel electrophoresis

Visualisation of PCR products, plasmids and RNA samples was performed by agarose gel electrophoresis in the presence of ethidium bromide (EtBr). Agarose gels were prepared as 0.8% - 1.2% agarose w/v in TAE (4.84 g/l Tris base, 1.14 ml/l Glacial acetic acid, 20 ml/l 0.5M EDTA, pH 8.0) buffer. For samples requiring isolation of the product from the gel, Low melting point (l.m.p) agarose was used. For all other applications general-purpose laboratory grade agarose was used. EtBr was added to the gels to give a final concentration of 0.05 μg/ml just prior to gel pouring. DNA samples were prepared in Sigma Blue-Orange loading buffer, at a ratio of 5:1 (DNA sample: loading buffer), and applied to gels. Samples were electrophoresed through the gels at 120 V until the required resolution was obtained. The size of PCR products (or other DNA sample bands) was estimated through comparison with standardised molecular ladders.

Gels were viewed under u.v light and images were captured using either a Polaroid camera or directly via the Alpha-Imager™ Gel Documentation system.

2.3: DNA cloning procedures

Extraction of PCR products from agarose gels

Following successful amplification by PCR, products were separated by electrophoresis through 0.8 - 2% low melting point agarose gel. Products were excised from the gel, and purified using the Qiagen Gel Extraction kit following the manufacturers instructions. Briefly, the area of the gel containing the required fragment was excised from the gel using sterile scalpel blades, and solubilised using proprietary buffer and heating at 50°C for 10 minutes. 3 mM Sodium acetate was added to the sample until the desired pH (approximation based upon colour change) was achieved. Samples were added to Qiagen Gel extraction columns and passed through the column matrix by centrifugation at 10,000 rcf for 1 minute (Hereaus Biofuge Pico). Fall through was discarded. The process was repeated until all sample had been passed through the column. The columns were washed twice with 750 µl of prepared buffer PE, by centrifugation. After the second wash, the columns were centrifuged again to remove all residual buffer, and the columns were placed above clean sterile collection tubes (Sterile, DNAse/Rnase free eppendorf tubes). 30 – 50 µl of Nuclease free™ water (Ambion) was added directly to the membrane surface of the column. The columns were incubated at RT for 5 minutes to allow the water to penetrate the column matrix, and then centrifuged at 10,000 rcf for 1 minute to elute the DNA from the column.

Purified product was visualised by agarose gel electrophoresis to confirm size and purity, and quantified by spectrophotometry at 260 nm.

Estimation of DNA concentration by spectrophotometry

The concentration of plasmid genomic DNA samples was estimated through measurement of the absorbance of a diluted preparation at 260 nm using a spectrophotometer. DNA concentration was determined as O.D @ 260 nm X 50 X dilution factor.

Ligation with plasmid vectors

Ligation of purified DNA product with appropriate vector was achieved using T4 ligase enzyme and optimised reaction buffers. DNA vaccine constructs were generated through direct cloning of the PCR amplicon into selected TA cloning vectors, capable of eukaryotic expression. Prokaryotic expression constructs were produced by sub-cloning these gene specific inserts from the DNA constructs into appropriately purified vectors. Ligation reactions were incubated at 14°C for 16 to 18 hours, before transformation into the appropriate cell line for plasmid propagation.

Transformation of DNA into *E. coli* host cells

- ***pCR3.1 based constructs***

TOP10F' One Shot™ competent cells (Invitrogen) were transformed with the pCR3.1™ based constructs. Transformations were conducted according to a standard basic protocol. Briefly, an aliquot of competent cells was defrosted on ice, 2 μl of the relevant ligation mix, and 2 μl of 2-mercaptoethanol was added to each vial of cells and incubated on ice for 30 minutes. The mixture was then heat shocked, by incubation in a water bath at 42°C for 45 seconds. After heat shock SOC media was added and the vial was incubated with vigorous shaking at 225 rpm at 37°C for 90 minutes. Finally, cells were plated onto selective media (LB based agar supplemented with an appropriate selection antibiotic (100 μg/ml of ampicillin or, 50 μg/ml of kanamycin. For each transformation the cell mix was plated in one 50 μl aliquot and one 150 μl aliquot, and incubated at 37°C overnight to allow growth of the recombinant colonies.

- ***Prokaryotic expression plasmids***

Prokaryotic expression constructs based on the pGEX or pRSET vectors were transformed into TOP10 *E. coli* (For maintenance) or BL21 *E. coli* (For expression) according to identical procedures to those described above.

- ***pTargeT constructs***

pTargeT based constructions were transformed into JM109 competent cells using a slightly modified transformation procedure. For these constructs 5 μl of ligation mix was used in transformation mixes, 2-ME was not added to the cells, and 400 μl of SOC media was added to each reaction mix. Incubation of cells in SOC media was performed at 37°C, with vigorous shaking at 225 rpm, for 120 minutes. Cells were plated onto appropriate selective media (100μl / plate), and incubated at 37°C overnight to allow the growth of recombinant colonies.

Plasmid isolation using Qiagen™ Plasmid Prep kits

Qiagen plasmid prep methods utilise a range of proprietary buffers to lyse *E. coli* host cells and release plasmid DNA into solution. The lysate from overnight (O/N) *E. coli* cultures was applied to Qiagen columns containing a matrix that binds plasmid DNA. Unwanted material was removed by washing with proprietary buffers and plasmid DNA was eluted using the recommended permitted volume of endotoxin free distilled water. To generate small volumes and quantities of plasmid, Qiagen mini-prep™ (3-10ml cultures, yielding up to a maximum of 20 μg of plasmid) or Midi-prep™ columns (10-25 ml cultures yielding up to a maximum of 250 μg of plasmid) were used. For larger volumes Maxi-prep™ (500 ml cultures, yielding up to 2.5 mg of plasmid) or Giga-Prep™ (2.5 L of culture, yielding up to 10 mg of plasmid) columns were used. In all cases buffers supplied with the Qiagen Endofree plasmid isolation kits were used, and manufacturers instructions were followed. Plasmid was eluted into endotoxin-free water.

Restriction endonuclease digestion

For analysis of recombinant plasmids, and generation of DNA fragments for sub-cloning, restriction endonuclease cleavage was performed. For analytical purposes assays were performed in 20 μl volumes. For sub-cloning, where purification of restricted product was required, 200 μl volumes were used. In all cases restrictions were performed according to the enzyme manufacturers guidelines. For 20 μl volume reactions 5 – 10 U of each enzyme was used in

each reaction, together with the optimal buffer system (as per manufacturers guidelines) and 5 μg of template plasmid. For larger volume digestions 40 – 100 U of each enzyme was used in each reaction. Restriction enzymes used include *Bam*H1, *Eco*R1, *Hin*d111, *Xho*1, *Xba*1, *Not*1, and *Sma*1. All digestions required incubation at 37°C in a water bath for 2 hours, denaturation of the enzyme by heating to 80°C for 5 minutes. Fragments were analysed immediately by agarose gel electrophoresis or used directly in sub-cloning procedures.

DNA Sequencing

Plasmid constructs selected based on their restriction profiles were sequenced to confirm the sequence fidelity of the product insert. Briefly, 20 μg of plasmid was sequenced by commercial sub-contracted service providers : Oswel Sequencing laboratories (University of Southampton, Southampton, UK). Sequencing of eukaryotic expression constructs was conducted starting from the T7 primer site on the 5' strand, across the insert (each reaction provides readable sequence for about 500 bp), and from the vector specific reverse primer site on the 3' strand. Where necessary primer walking reactions were employed to determine the sequence for the full length of the insert.

2.4: Determining expression of Brucella specific genes from DNA vaccine constructs

Each DNA vaccine construction was assessed for the ability to express the encoded *Brucella* gene. Since the DNA vaccine plasmids can only express their encoded products within eukaryotic cells, the plasmids were transfected into Cos 7 cells for this analysis.

Transfection of Cos 7 cells

Transfections were performed using Lipofectamine-plus™ reagent. The transfection procedure was adapted from the manufacturers guidelines, for

optimal performance in a 12 well plate (cells grown on coverslips) format. Briefly, endotoxin free plasmid DNA (purified by plasmid Giga--prep) was mixed with the Lipofectamine™ transfection agent in the presence of the Plus™ reagent. The mixture was incubated for 30 minutes at RT to allow formation of DNA/Lipofectamine complexes. Cell culture, in 12-well plates, at between 40 – 80% confluence was washed three times in serum and antibiotic free Modified Eagles Media (MEM). After washing, each well was replenished with 0.4 ml of serum and antibiotic free MEM to which 0.1 ml of the appropriate transfection mixture or control solution was added. Plates were returned to the 37°C, 5% CO_2 environment for 12 -18 hours, to promote uptake of the transfection particles. After uptake (12-18 hours) media was supplemented with serum and antibiotics, by the addition of 0.5 ml MEM, containing 20% Foetal Bovine Serum and X2 Antibiotic Antimycotic solution (Stock solution: 10,000 U/ml Penicillin, 100 mg/ml Streptomycin, 25 mg/ml Amphotericin B). Cultures were then further incubated at 37°C, 5% CO_2 for between 24 to 72 hours depending upon the assay to be performed.

Indirect immunofluoresence assay (IFA)

Detection of specific proteins produced from transfected cells was assessed by indirect immunofluorescence assay. For the Omp25 DNA vaccines, the protein was detected using specific monoclonal antibodies. Anti-Omp25 monoclonal preparations were kindly donated by Prof Jean-Jacques Letesson, FUNDP, Namur, Belgium. Specific antibodies were unavailable for the remainder of the candidates, and therefore antiserum from *B. melitensis* infected ruminants (Anti-*Brucella*) was used in this assessment.

At 72 hours post transfection cells were washed 3X with 0.1 M Phosphate Buffered Saline (PBS), pH 7.6, prior to fixing for 30 seconds in ice cold (-20°C) methanol. Cells were permeabilised by incubation in 0.01% Triton X-100 in 0.1 M PBS, pH 7.6, for 5 minutes at RT. Cells were then incubated with blocking solution for 2 hours at RT. Blocking solution for the monoclonal antibody based reactions consisted of 10% normal goat sera in 0.1 M PBS, pH 7.6. For the Anti-*Brucella* sera reactions, blocking buffer comprised 10% normal rabbit sera in

0.1 M PBS, pH 7.6. After blocking cells were rinsed briefly in PBS, and the appropriate primary antibody solution (either AF59 (anti-Omp25 Mab) at 1: 500 diluted in 0.1 M PBS or anti-*Brucella* at 1: 200 in 0.1 M PBS) was added to the cells and incubated for 1 hour at RT. Cells were washed 3 times by incubation in 0.1 M PBS, pH 7.6 for 5 minutes each time, before application of an anti-species Fluorecein Isothiocyanate (FITC) labelled secondary antibody. For detection of the AF59 reaction Goat versus Anti-mouse Ig (whole molecule) FITC conjugate (Sigma), at 1:500 dilution in 0.1 M PBS was used as the detection antibody. For detection of the Anti-*Brucella* reaction Rabbit anti-Goat Ig FITC conjugate (Sigma), at 1: 200 dilution in 0.1 M PBS was used. Secondary antibody incubations were carried out at RT in the dark, for 1 hour. Cells were washed, 3 times for 5 minutes as previously described, before coverslips were removed from wells and mounted onto clean labelled glass microscope slides (BDH), using the VectaShield mountant (VectaLabs, California).

Fluorescence was observed with a Nikon Eclipse E400 Microscope, fitted with u.v. light and filters for observance of FITC. Filters and conditions were adjusted to minimise background for the untransfected and vector transfected samples and maintained constant between each set of conditions.

2.5: The generation and preparation of DNA vaccines for in vivo studies and the production Brucella specific protein antigens

Production and quality control of DNA vaccines for use in vivo

DNA vaccine preparations for assessment *in vivo* were prepared in-house or by a commercial sub-contractor: PlasmidFactory™ Inc. The method of production used in each individual *in vivo* trial is indicated in each relevant chapter.

In-house production of stocks of DNA vaccines

Large-scale in-house preparation of DNA vaccines was achieved using Qiagen™ Giga-Prep and Mega-Prep Endo-Free™ kits. These procedures are described in section 2.3. Individual preparations of each vaccine were pooled as

batches, to ensure uniformity of sample throughout each *in vivo* study. Each batch was stored in appropriate volume aliquots at –20°C.

Commercial production of DNA vaccine stocks by PlasmidFactory

Twenty mg each of the plasmids encoding omp25 (pCR3.1-*omp25* and pTargeT-*omp25*), p-*ialB* plasmids and pcDNA3.1 plasmids were prepared under sub contract by Plasmid Factory™ GmbH & Co. KG. (Bielefeld, Germany). Plasmids were prepared to GMP standards, and were certified as greater than 99% closed circular supercoiled (CCC) formation, and virtually endotoxin free. Plasmids were supplied at 1 mg/ml and 2 mg/ml concentrations in 0.1M PBS and stored in appropriate volume aliquots (200 or 600 µl per tube) at –80°C until required.

Quality control of DNA vaccines

An aliquot of each batch of DNA vaccine was analysed by restriction endonuclease cleavage, and sequencing as described in section 2.3, in order to confirm identity prior to use *in vivo*. Additionally, the expression capability of the encoded gene from each batch of DNA vaccine was confirmed by detection of the presence of specific gene transcripts through RT-PCR performed on RNA template isolated from specifically transfected Cos 7 cells. This procedure was performed as described in section 2.2.

The presence and quantity of endotoxin (*E. coli* LPS) was determined from each batch of DNA using the Limulus Amoebacyte Lysate (LAL) test: E-Toxate™, and the pyrochrome test.

Production of protein antigens

Production of specific protein antigens for the selected vaccine candidates

Purification of recombinant Omp25-GST or Invasion protein B (IalB), for use in ELISA and cell mediated immunology applications were outsourced to a commercial protein production facility (Lionex GmBH, Braunschweig, Germany). Crude specific recombinant protein lysates for use in ELISA or western blotting applications were produced in house and are illustrated in appendix 2.

B. melitensis 16M antigen

B. melitensis 16M antigen for use in immunology procedures was produced from solid phase cultures grown on SDA as previously described. Cultures were harvested after 4 days growth, and cells were washed twice by centrifugation (4000 rcf, for 10 minutes at 4°C) and resuspended in a minimal volume of sterile water. The preparation was rendered non-viable through heating to 80°C in a water bath for 90 minutes. The sterility of the preparation was confirmed through culture as described previously.

Brucellergene™ antigen

Brucellergene™, a commercial preparation of cytosolic protein antigens from the *B. melitensis B115* strain, was used in these studies. The cytotoxic preservative merthiolate was removed from Brucellergene™ samples by dialysis against 0.1 M PBS at 4°C overnight using 10 kDa cut off dialysis cassettes (Pierce, UK). The content of Brucellergene™ was checked by SDS-PAGE before and after dialysis to confirm that protein components were not lost during dialysis. Dialysis was able to abrogate the toxicity of Brucellergene™ when applied to cultured mouse splenocytes. Dialysed Brucellergene™ was aliquoted and stored at –80°C until required.

Estimation of protein concentration

The concentration of total protein in each sample was estimated using the Bicinchoninic Acid Micro and Standard Assay (BCA). The assays were

performed according to manufacturers instructions, using a series of bovine serum albumen (BSA) dilutions to generate a standard protein concentration curve against which to compare test samples.

SDS-PAGE techniques

Protein electrophoresis was performed using Lamelli SDS-PAGE procedures, with either the Bio-Rad Protean II mini-gel system with in-house produced acrylamide gels, or the Pharmacia Multiphor Flat-bed electrophoresis system using Excel-Gel™ pre-prepared commercially available gels.

SDS-PAGE mini-gels were set up using the Bio-Rad Protean II mini-gel apparatus, with 1 mm gel spacers and 1 mm thick combs. Each gel was prepared as a 12.5% bis-acrylamide, 1.5 M Tris-HCl (pH 8.0) separating gel and 5% bis-acrylamide, 0.5 M Tris-HCl (pH 6.0) stacking gel. Alternatively 12.5% homogenous Excel-gels™ (Amersham) were used.

For comparison and estimation of approximate molecular weight, See-Blue-2 protein molecular weight marker (Invitrogen) or Rainbow marker (Amersham) was used. Between 5 and 10 μl was used in a single well.

Samples were prepared for electrophoresis by a 1:1 dilution of required concentration sample in SDS-PAGE sample buffer, and heating to 80°C for 20 minutes. The total quantity of sample loaded into a single sample well on a gel was between 1 μg and 20 μg depending upon the application.
Electrophoresis using the Protean II system was conducted at 200 V (constant voltage, variable current), for 25 – 45 minutes. The reaction was stopped at the point when the dye front began to run off the end of the gel. Electrophoresis using the Pharmacia Multiphor Flat-Bed system was performed at 600 V / 50 mA for approximately 80 minutes. The procedure was stopped as soon as the dye-front reached the anodic buffer strip.

Silver staining of Protean II and Multiphor II SDS-PAGE gels for visualisation of protein.

Silver staining for detection of total protein within SDS-PAGE gels was performed using the Amersham Pharmacia Biotech Protein Silver staining kit, according to manufacturers recommendations. Briefly, gels were removed from SDS-PAGE apparatus and in the case of the Multiphor Excel Gel system, rinsed free of mineral oil in sterile distilled water. Gels were incubated in silver stain fixative (40% methanol, 10% acetic acid solution) for a minimum of 1 hour at room temperature (RT) with gentle agitation (rotary shaker at 30 rpm). Gels were rinsed briefly, and then incubated in sensitiser solution for 30 minutes at RT, shaking at 30 rpm. Sensitiser solution was decanted and gels were washed 3 X 10 minutes in sterile distilled water. Silver staining solution (10% silver nitrate solution, 0.01% formaldehyde) was incubated with the gel for 25 minutes, 30 rpm, RT. Gels were rinsed first in sterile distilled water and then in developer solution. The development of bands was observed and judged by eye. When reaction was judged complete the gel was rinsed with sterile distilled water before incubating in a stopper solution (5% EDTA) for 1 hour. Gel pictures were recorded by scanning.

Coomassie Blue staining of Protean II and Multiphor II SDS-PAGE gels for visualisation of proteins

Bio-Rad Simply Blue Safe Stain Coomassie stain was used to stain gels for the visualisation of total protein load. Gels were rinsed in sterile water before incubation in the stain at RT for 2 – 18 hours. The gel was then repeatedly rinsed in sterile distilled water until the optimal staining effect was obtained. Gel pictures were recorded through scanning.

Electrophoretic transfer of protein from Protean II SDS-PAGE gels to nitrocellulose membrane

On completion of electrophoresis gels were placed immediately into a tray of X 1 CAPS (3-(cyclohexylamino)-1-propane sulphonic acid)) solution (10 mM CAPS, 10% methanol, pH 11.0) and incubated on a shaker platform for at least 15 minutes. CAPS pre-soaked blotting paper, cellulose sponges and 0.45 μM

Nitrocellulose membrane (Bio-Rad, UK), were assembled together with the gel for electrophoretic transfer using the Mini Protean II blotting tank. Blotting cassettes were submerged in X1 CAPS solution within the gel tanks. A sealed ice block was added to the tank to assist with cooling during the reaction. Current was applied at 200 mA (constant current, variable voltage) for 75 minutes.

Following transfer, membranes were removed from the apparatus and trimmed to remove molecular weight markers. The main membrane was placed in blocking buffer (4% dried skimmed milk powder (Marvel™), in TBS) for between 1 to 2 hours at RT, or overnight at 4°C, on a rocking platform. Following blocking, membranes were either used immediately or washed 2 X for 5 minutes, with TBS-Tw wash buffer, and dried between clean blotting paper before storing at –20°C until required.

2.6 Vaccination procedures

All *in vivo* investigations of vaccine immunogenicity and protective efficacy were performed using a murine model.

Mice used in the study

Female BALB/c mice were sourced from Charles River, UK., and age six to eight weeks old upon receipt at VLA. The mice were allowed time to acclimatise prior to commencing the study. Mice were approximately eight to ten weeks old at the time of the first procedure. Mice were housed within standard cages, in groups of ten or less, and given standard unmedicated diet and water *ad libitum*. Environmental enrichment was permitted.

Mice vaccinated or challenged with viable *Brucella* were housed in cages maintained within flexible film isolators at containment level III.

Vaccinations

Mice were not anaesthetised for vaccination. *B. melitensis* Rev.1 vaccine was delivered to mice as a single sub-cutaneous (s/c) inoculation of approximately 2

X 10^5 CFU per mouse, into the scruff of the neck. *B.melitensis* 16M challenge was delivered as a single intra-peritoneal (i/p) inoculation. Procedures were performed within a class I microbiological safety cabinet. 27G X 1½ fixed needle tuberculin syringes were used to deliver the inoculation. A 100 µl inoculation volume was used.

For DNA vaccines, vector control, liposome-DNA, and PBS sham control, inoculations were performed intramuscularly (i/m), into the hind quadriceps muscles of the mice. For i/m delivery 27G X 1 ½ fixed needle tuberculin syringes were sheathed with polyvinyl tubing (internal diameter 0.56 mm), to control the depth of muscle penetration and ensure i/m delivery. At each inoculation time mice received 50 µl of inocula in each hind quadriceps muscle.

Liposome production and complexing with DNA

Liposomes were prepared and donated to this study by Dr J. Brewer (Department of Immunology and Immunopathology, Western Infirmary, Glasgow). Lipid vesicles were prepared from 1-monopalmitoyl glycerol, cholesterol stearyl amine and cetyl trimethyl ammonium bromide (CTAB) in the molar ratio 5:4:1:1, by the methods described previously (Brewer, 1998). Plasmids were surface adsorbed to cationic lipid vesicles immediately prior to administration. Briefly, 2 ml plasmid DNA at 1 mg/ml was added dropwise to an equal volume of liposome preparation. Liposome: plasmid mixture was incubated for 30 minutes at RT using a Denley Rotary Cell Mixer, and then centrifuged at 2000 rcf (Hereaus Microfuge) to sediment the liposomes. 2 ml of SN was removed. Remaining plasmid: liposome suspension was mixed gently and used within 1 hour for the inoculation of mice. The 2 ml SN sample was retrospectively assessed for the presence of residual DNA by spectrophotometry and agarose gel electrophoresis.

Immunological sampling

Serum was obtained from mice by venous puncture of the tail vein.

Mice were sacrificed by cervical dislocation, spleens excised aseptically. Spleens were placed into sterile fluid: PBS, DMEM, or FF depending on the intended use.

2.7 Immunoassay procedures

A number of immunoassays were developed for use in this study. Optimised procedures are described in this chapter.

2.7.1: Serological assay procedures

ELISAs were developed for measurement of vaccine induced antibody responses from the test groups of BALB/c mice, and also from experimentally infected sheep and goats.

Collection and processing of serum samples from mice

Blood samples were collected from individual vaccinated and infected mice at various times for assessment of specific serological responses. Blood was collected following venous puncture of the tail. Each sample was incubated at RT for 2 – 4 hours and 4°C overnight, before centrifugation at 3000 rpm (Hereaus Biofuge Fresco) for 30 minutes. The serum was transferred to a fresh labelled eppendorf tube and incubated at 56°C for 30 minutes to inactivate complement. The total volume of serum collected from an individual mouse at any time during these studies ranged between 10 and 80 µl of serum. Samples were stored frozen at –20°C until required.

10ul of serum from each individual mouse in a group were combined to provide the pooled samples. Pooled samples were also stored at –20°C.

B. melitensis 16M whole antigen ELISA for detection of Brucella specific antibodies in mouse sera.

This ELISA was developed and optimised in-house (data not shown).

16M antigen was prepared as described in section 2.5, and coated to Maxisorp™ 96 well plates (Nunc). Antigen was diluted to 50 μg/ml in Carbonate Coating Buffer (CCB): 0.034 mM Sodium hydrogen carbonate, 0.016 mM sodium carbonate, pH 11.0, and applied to plates at 50 μl per well. Plates were incubated at 4°C overnight. Plates were washed twice with PBS-T wash buffer and 200 μl per well of blocking solution (4% skimmed milk powder (Marvel) in PBS-EDTA buffer) was added to the plates, which were incubated at RT for ≥ 2 hours. Test sera and the selected control samples were prepared for analysis by dilution 1% marvel PBS-EDTA diluent buffer. Normal mouse sera (Sigma) was used as a negative control in these ELISAs. Positive control serum was obtained from mice that had been infected with *B. melitensis* 16M in previous research projects at VLA Weybridge. The serum was collected from 10 *B. melitensis* 16M infected mice at day 84 post-infection and pooled for use as a positive control.

After blocking, plates were washed once in PBS-T wash buffer and the test serum dilutions and controls applied to the plate. Each individual dilution / sample was plated into sufficient wells (50 μl per well) to permit assay of relevant isotype specific responses in duplicate. Plates were incubated at 37°C for 90 ± 10 minutes. Plates were then washed five times with PBS-T. Isotype specific anti-mouse Ig enzyme conjugated reagents were used as detection antibodies. Rat anti-mouse IgM (μ chain specific) peroxidase conjugated antibody at 1 in 2000 in diluent buffer, Goat anti-mouse IgG1 peroxidase conjugated antibody (STAR81P, Serotec) at 1 in 1000 in diluent buffer, and Goat anti-mouse IgG2a peroxidase conjugated antibody (STAR82P, Serotec) at 1 in 1000 in diluent buffer were used. These antibody solutions were applied in 50 μl per well volumes and incubated at 37°C for 1 hour. Plates were washed 5 times with PBS-T and blotted dry onto absorbent matting. TMB (3`3`5`5` Tetramethylbenzidine) working solution was prepared from the stabilised Substrate and Chromogen solutions (Vetoquinol) and added to the plates (100

µl per well). Plates were incubated for 10 – 30 minutes in the dark for reaction development. Reaction development was judged by eye, and when the IgG2a positive control wells had achieved sufficient intensity of colour, the reaction was stopped by the addition of 100 µl per well of 0.1 M Citric acid solution. Plates were read within 1 hour of reaction completion using a Titertek Multiscan II to determine optical density at 450 nm.

OMP25-GST and GST ELISAs, for the detection of OMP25 specific antibodies in mouse sera.

This procedure is essentially identical to that of the 16M antigen ELISA, except for the initial antigen coating stages. In this ELISA recombinant fusion protein Omp25-GST prepared from the pGEX-*omp25* construct and purified under contract by Lionex GmBH., was used as the antigen. Antigen was prepared in Coating buffer to a concentration of 5 µg/ml (or 0.5 µg / well) and incubated at 4°C overnight for the initial coating step. The rest of the procedure was performed identically to the 16M ELISA.

Recombinant IalB ELISA for the detection of IalB specific antibodies in mouse sera.

This procedure is essentially identical to that of the 16M antigen ELISA, except for the initial antigen coating stages. In this ELISA recombinant fusion protein IalB prepared from the pGEX-IalB construct and purified under contract by Lionex GmBH., was used as the antigen. Antigen was prepared in Coating buffer to a concentration of 15 µg/ml (or 1.5 µg/well) and incubated at 4°C overnight for the initial coating step. The rest of the procedure was performed identically to the 16M ELISA.

SAT, iELISA and cELISA

Serodiagnostic assays routinely used in the Laboratory Testing Division (LTD), and the brucellosis reference facility of VLA Weybridge, (serum agglutination tests (SAT), indirect ELISA (iELISA) and competition ELISA (cELISA)) were used in these studies. For use with sheep or goat samples these techniques were performed by the staff of the *Brucella* reference laboratory at VLA Weybridge according to UKAS accredited standard operating procedures.

Brucella diagnostic serum agglutination tests (SAT), and competition ELISA (cELISA) were occasionally performed on pooled sera from groups of mice. In these instances the serum was used at the maximum possible concentration (Generally approximately 1 in 50). Again, standard referenced procedures were followed.

Brucellergene™ ELISA for the detection of Brucella specific antibodies in sheep or goat sera

An ELISA was developed in-house to assess the reactivity of serum samples from *Brucella* infected sheep and goats against protein antigens of *Brucella*. Brucellergene™, which is free from the serodominant LPS of smooth *Brucella* sp., was used in these studies was coated to Maxisorp plates at a concentration of 45 μg / ml or 2.25 μg per well in CCB. Plates were incubated overnight at 4°C. The assay was essentially identical to that described for assessment of mice samples with whole *Brucella* 16M antigens, except that test serum was diluted 1 in 200 in diluent buffer for analysis, and identification of separate isotype antibody responses was not performed. An Anti-sheep IgG Horse Radish Peroxidase conjugate was used at a dilution of 1 in 4000 in diluent buffer.

OMP25-GST and GST ELISAs, for the detection of OMP25 specific antibodies in sheep or goat sera.

ELISA plates were coated as described for the analysis of mice samples. The assay was performed identically to that described for analysis of sheep and goat samples in the Brucellergene™ antigen ELISA.

Invasion protein B specific ELISA for the detection of Invasion protein B specific antibodies in sheep or goat sera.

ELISA plates were coated as described for the analysis of mice samples. The assay was performed identically to that described for analysis of sheep and goat samples in the Brucellergene™ antigen ELISA.

Crude lysate ELISAs to determine reactivity of sheep and goat serum against recombinant Omp25, IalB, FrpB, AcvB and FliC antigens.

Purified recombinant preparations of the candidate vaccine proteins AcvB, FrpB and FliC were unavailable for use in this study. Therefore, ELISAs were developed using crude lysate antigens from recombinant *E. coli* cultures expressing these proteins.

Expression of the proteins was induced through optimised procedures (see appendix 1) and confirmed through SDS-PAGE and where possible western blotting against a sera reactive to the fusion moiety of the expressed protein. Total protein concentration of lysates was determined by BCA protein analysis methods.

Crude lysate was coated to polysorp plates at 50 μg/ ml overnight at 4°C, prior to blocking. The blocking step utilised the standard blocking buffer (4% marvel in PBS–EDTA) supplemented with rabbit anti-*E. coli* immunoglobulins (DakoCytomation, Denmark). The anti-*E. coli* serum was diluted 1 in 4000 in blocking buffer. The blocking stage was incubated at 25°C for 2 hours. Test sera (from sheep and goats) was added to the plates diluted 1 in 200 in diluent buffer and incubated for 1 hour at 37°C. Plates were washed six times with washing buffer prior to the application of the anti-sheep IgG HRP conjugate. The rest of the reaction was performed as described for the Brucellergene™ ELISA.

2.7.2: Cell mediated immunology

In addition to the measurement of antibodies, the cell mediated immune responses of vaccinated and infected BALB/c mice were also investigated. For these assessments mouse splenocytes were isolated and fractionated. Cytokine production in response to *Brucella* specific in-vitro stimulation was measured using commercially available cytokine ELISAs from Biosource and in house optimised ELISPOT procedures.

Spleen harvest and isolation of splenocytes for CMI investigations

Mice were sacrificed by cervical dislocation and spleens were aseptically removed. Spleens were placed immediately in pre-warmed (37°C) DMEM complete media (DMEM + Non essential amino acids, 1,000 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B, and 10% foetal bovine serum), in order to preserve viability. Spleens were diced using sterile surgical scissors and macerated with the barrel of a 5 ml Syringe through a 80 μM mesh cell sieve to release splenocytes into the media. Splenocyte suspensions were washed twice in FACSflow Sheath Fluid Buffer [FF] by centrifugation at 330 rcf (Hereaus MegaFuge) for 15 minutes at 4°C, and resuspended in a minimal volume (≤ 1ml) of FF buffer Viable cells were enumerated after suspension of 50 μl of cell suspension in 450 μl of white cell counting fluid (1% glacial acetic acid in D-PBS, with a trace of Gentian violet dye), using the Neubauer Haemocytometer. Cells were reconstituted to 5 X 10^6 / ml in DMEM complete for stimulation for ELISA or ELISPOT reactions or used in T cell subset depletion studies according to the protocol described below.

T cell sub-set depletion procedures

MidiMACS™ (Miltenyi Biotech) technology was used for the depletion of T cell subsets from the whole splenocyte preparations. Anti-mouse CD4+ (L3T4) magnetic beads were used to bind CD4+ expressing cells, and Anti-mouse CD8+ (Ly-2) magnetic beads were used to bind CD8+ expressing cells from the total splenocyte population. Cell concentrations were adjusted to a total of 1 X 10^9 / ml by dilution in FF buffer and 100 μl of bead suspension was added to each 1 ml of cell suspension. Magnetic beads and cells were incubated on ice for 30 minutes with occasional gentle mixing. MACS LS columns were equilibrated with 3 ml of ice cold FF Buffer, and positioned within the MidiMACS™ magnets. Bead treated cell suspensions were applied to the LS columns and the fall through fraction collected in a sterile Falcon Tube over ice. Columns were washed through with 3 X 3 ml of ice cold FF. Again the eluted fraction was collected. Total eluate was diluted in ice cold FF and washed by centrifugation (300 rcf, 15 minutes, 4°C, (Hereaus MegaFuge)), and resuspended in a minimal volume of ice cold FF. Cells were enumerated and

diluted to a final concentration of 5 x 10^6 / ml in DMEM Complete for use in stimulation assays.

Stimulation of splenocytes with specific antigens

Antigens and control mitogen solutions were prepared to appropriate concentrations in DMEM Complete media. The lectin Concanavalin A Type IV purified (Con A) was used as the non-specific mitogen throughout the study; Con A was prepared in DMEM complete to a concentration of 10 μg/ml. Specific antigens were prepared to the following concentrations, Brucellergene™: 80 μg/ml, Recombinant OMP25-GST: 20 μg/ml, Recombinant GST: 20 μg/ml. Recombinant Invasion protein B: 30 μg/ml. Heat killed *Brucella melitensis* 16M antigen: 50 μg/ml. Antigen preparations were added to designated wells in either 96 well pre-coated IFN-γ ELISPOT plates (100 μl volumes per well), or sterile 24 well tissue culture plates (500 μl volumes per well). An equivalent volume of cells at a concentration 5 X 10^6 /ml were added directly to the appropriate wells containing antigen. Thus, wells contained cells at a final concentration of 2.5 X 10^6 /ml in either DMEM complete or a DMEM complete solution of 5 μg/ml Con A, 40 μg/ml Brucellergene™, 10 μg/ml OMP25-GST, 10 μg/ml GST or 15 μg/ml Invasion protein B. Stimulation cultures were incubated at 37°C, 5% CO_2 for either 24 (ELISPOT) or 48 (SN ELISA) hours.

IFN-γ ELISPOT

For ELISPOT assays 96-well PVDF membrane filter plates were pre-coated with 1.5 μg/ml well of anti-mouse IFN-γ monoclonal (AN18) 18 to 24 hours prior to harvest of splenocytes. On the day of splenocyte harvest plates were washed 2 X with PBS and blocked with 200 μl per well with DMEM Complete medium for 2 – 4 hours at 37°C prior to addition of cells. Splenocytes were harvested and stimulated as described above. The ELISPOT plates / stimulation cultures were incubated for 24 ± 2 hours, loosely wrapped in aluminium foil, at 37°C in a 5% CO2 atmosphere incubator. Following incubation cells were aspirated from the filter plates using a multichannel pipette, and taking care not to damage the membrane. Plates were washed 4 X with PBS-T wash buffer, and blotted dry after the final wash. Anti-Mouse IFN-γ biotinylated antibody was prepared to a

concentration of 1 μg/ml in ELISPOT diluent Buffer (1% BSA in PBS), and 100 μl per well was applied to the plates. Plates were incubated at 25°C for 1 hour wrapped loosely in aluminium foil. Following incubation plates were washed 4 X with PBS-T wash buffer, and blotted dry after the final wash. Streptavidin Alkaline Phosphatase reagent was diluted 1: 1000 in ELISPOT diluent buffer and 100 μl per well added to the plates. This stage was incubated at 25°C, plates wrapped loosely in foil, for 1 hour. After incubation plates were washed 4 X with PBS-T wash buffer and 2 X with distilled water, and blotted dry onto absorbent towelling. BCIP-NBT fast tabs™ were used to prepare the substrate solution. 1 tablet was dissolved in 10 ml of pre-warmed distilled water, and filtered through a 0.2 μM syringe filter prior to use. Substrate solution was applied to wells at 100 μl per well, and plates were incubated in the dark at 25°C for between 10 to 30 minutes. Reactions were halted once the colour development in the Con A stimulated sample wells was considered to be complete. Rinsing the plates at least twice in distilled water stopped reactions. The under drain section of the plate was then removed and the plates incubated at 37°C overnight in order to dry the filters.

Since these assays involved splenocytes isolated from 16M or Rev.1 infected mice, the splenocyte cultures were potentially infected with live *Brucella* spp., and procedures had to be conducted in a containment level III facility. Plates were fumigated (using standard formaldehyde fumigation procedures validated for use against *Brucella* spp.), before being removed from the facility for reading. Plates were read using an AID ELISPOT plate reader.

Measurement of SN cytokine concentration by ELISA

For ELISA work, stimulations were performed as described above using 1 ml total volumes per reaction (500 μl of antigen preparation and 500 μl of cells) in 24-well plates. Plates were wrapped loosely in foil and incubated at 37°C, 5% CO_2. After 72 hours incubation, the plates were centrifuged to sediment cells and the SN dispensed in 200 μl aliquots into sterile 96 well plates. These samples were treated with sodium azide (addition to SN to final concentration 1%) in order to kill *Brucella* and frozen at –80°C until required for use in ELISA.

Commercially available cytokine detection ELISA reagents were used to measure the presence of the cytokines IL-4, TNF-α. IL-12 and IFN-γ in the SNs harvested from stimulated splenocytes. ELISAs were conducted according to manufacturers recommendations.

Samples were defrosted and applied to appropriate wells of the test plate. Recombinant mouse cytokines supplied with the kits were prepared in diluent buffer to generate a standard curve for quantitative analysis of the results. A series of these 'standards' were included in each assay. Cytokine concentration [pg/ml] in SNs was calculated through non-linear regression using GraphPad™ Prizm software, to generate a curve of the known concentrations [pg/ml] plotted against the O.D. values of the standards. The concentration of cytokine in each unknown sample was interpolated from its O.D. value. Where sample O.D.s were found to be out of range for the standard curve the sample was retested following dilution in the appropriate standard diluent buffer.

For IFN-γ and TNF-α analysis the SN from individual mouse splenocyte cultures were analysed in ELISA. Each sample was tested in duplicate. For other cytokines, SN was pooled for analysis, such that each sample consisted of the combined SN of three mice from a single vaccine group stimulated with a single antigen. Pooled samples were also tested in duplicate in the ELISAs.

Chapter 3: THE SELECTION OF CANDIDATE GENES FOR VACCINE DEVELOPMENT

3.1: Summary

Five genes were identified from the B. melitensis 16M genome database as potentially useful vaccine candidates. Selection was based primarily upon assigned putative functions based on gene similarity to related organisms. The selected candidates were omp25 (BMEI 1249), ialB (BMEI 1584), frpB (BMEII 0105), fliC (BMEII 0150) and acvB or virJ (BMEII 0681). With the exception of omp25, these candidates had not previously been studied in Brucella spp. Experiments were devised to demonstrate specific transcription of these target antigens from in vitro cultured Brucella and Brucella isolated ex vivo from infected BALB/c mice. Transcription from all targeted genes was determined in material isolated from ex-vivo cultured Brucella, and transcription of omp25 and ialB was verified in samples from in vitro cultured Brucella. These findings provide evidence that these target genes are active during Brucella infection, and thus imply that corresponding proteins may be generated under physiologically relevant conditions. Such proteins are anticipated to be accessible to the host immune response. The involvement of the proteins in the generation of Brucella specific humoral immune responses was also investigated. The presence of antibodies reactive to Brucella protein was demonstrated using antisera from a panel of experimentally infected goats.

Together these data were used to demonstrate that each in silico selected antigen is expressed during Brucella infection and to suggest their accessibility to the host immune system, thus supporting their selection for investigation as vaccine antigens.

3.2: Introduction

Selection of candidate antigens

The development of defined non-living vaccines requires the identification of protective antigens. The nature of protective immunity in brucellosis in natural host species is poorly understood and consequently there has been little definitive work to date on the identity of protective antigens. The recent availability of the genome data for *Brucella melitensis* 16M (DelVecchio *et al*, 2001), *Brucella suis* 1330 (Paulsen *et al*, 2003), and *Brucella abortus* strains (Sanchez *et al*, 2001., Halling *et al*, 2005), has dramatically improved the process of antigen selection.

There are two important phases in the establishment of brucellosis that can be targeted for vaccine development. Most obviously, intervention to prevent the establishment of infection would prevent both disease in the individual exposed animal and further transmission of the infectious agent. Vaccines promoting immune responses against antigens of *Brucella* that are important in the initial interaction have the potential to augment the destruction of *Brucella*, thereby limiting the potential for development of disease. Generation of specific antibodies to promote opsonic entry into macrophages, and to interfere with initial host : *Brucella* interaction may reduce the ability of the *Brucella* to establish infection. Furthermore, macrophage activation will increase bacterial killing efficiency and the generation of antigen specific cytotoxic T cells will promote clearance from infected cells. Direct experimental evidence for *Brucella* specific invasion or adhesion-associated ligands is presently unavailable. Although, this may be because these attributes have traditionally been difficult to study.

The second important target for vaccination is the mechanism responsible for *Brucella* survival and replication within the host cells. Targeting molecules associated with the subversion of host phagosomes and establishment of the intracellular replicative niche is anticipated to reduce the bacterial burden in the host. Furthermore, promotion of cytotoxic immune responses against the

antigens expressed at this stage of infection helps to eliminate established infections.

Immune responses against antigens involved in both the initial host-*Brucella* interaction and the maintenance of the intracellular infection are likely to be required in an effective vaccine. In this study, the aim was to select some of the proteins involved in these aspects of *Brucella* infection and target the immune responses toward these effectors thereby hampering establishment of infection. As a first step toward this aim five *B. melitensis* candidate antigens were selected. Surface exposed antigens and putative secreted antigens were sought as they were anticipated to have intimate contact with the host cells and to be exposed to immune effectors.

Access to the *B. melitensis* 16M genome data enabled the candidate antigens to be selected on the basis of predicted exposure of the protein to the host immune system and the predicted involvement of the gene or protein product with the establishment or maintenance of infection in the natural host. The search facility of the WIT/ERGO genome database maintained at the University of Scranton, Scranton, PA., USA, was used to identify candidates that had been annotated to indicate relevant putative functions or qualities.

Information on the many candidates matching these criteria, and their nearest homologues, was sought in the literature in order to provide supporting evidence for their potential role as protective antigens. Candidates that had been previously studied with relation to brucellosis immunity and vaccine development were eliminated from the shortlist (with the exception of Omp25), in order to give priority to novel antigens. Five candidates were chosen for further study. These are summarised in table 3.1, and discussed in further detail below.

Table 3.1: A summary of the selected candidate antigens

Candidates implicated in the initial host–pathogen contact	
ialB BMEI 1584	**Predicted function:** Invasion or adhesion associated **Predicted localisation:** Secreted **Relevant homologues:** *Bartonella bacilliformis ialB, Rickettsia prowesakii invB*
fliC BMEII 0150	**Predicted function:** Invasion or adhesion associated **Predicted localisation:** outer membrane **Relevant homologues:** *Mesorhizobium meliloti fliC, Burkholderia cepacia FliC.*
omp25 BMEI1249	**Predicted function:** Membrane structural protein. Highly conserved in *Brucella* spp., Immunomodulatory properties **Observed location:** outer membrane **Relevant homologues:** *Sinorhizibium meliloti RopB, E. coli OmpA*
Candidates implicated in the maintenance and establishment of the replicative niche	
virJ / acvB BMEII 0681	**Predicted function:** potential proteinaceous effector of the VirB secretion system. **Predicted location**: secreted Relevant homologues: *Agrobacterium tumefaciens acvB*
frpB BMEII 0105	**Predicted function:** involved in iron transport, acquisition, metabolism **Predicted location:** Outer membrane **Relevant homologues**: *Neisseria gonhorreae, Neisseria meningitidis frpB*

omp25

Of the five selected genes, only *Brucella omp25* has been previously investigated or characterised. The outer membrane proteins (Omps) of *Brucella* have been extensively studied (Cloeckaert, *et al* 1996, Bowden, *et al*, 2000), to elucidate their role in *Brucella* pathogenesis and protective immunity. The major Omps of *Brucella* were first identified in the SDS-I insoluble fraction of the cell wall, and noted to have protective activity in the mouse model (Dubray and Bezard 1992). The Group 2 Omps (*omp2a* and *omp2b*) have received much attention recently due to their relative genetic diversity which can be exploited

as typing tools or aids to strain identification (Cloeckaert *et al,* 1995). In contrast, the group 3 Omps consisting of the 25 kDa Omp (Omp25) and the 31 kDa Omp (Omp31), are relatively well conserved, sharing 34% sequence identity to each other and 43% similarity to the RopB protein of *Rhizobium leguminosarum*, another member of the α-2 proteobacteria (Cloeckaert, A., *et al,* 1999). However, the 31 kDa protein is underrepresented in *B. abortus* strains due to a short deletion at the N terminus, which has bearing on the development of conformational B cell epitopes, and may restrict it's value as a potential vaccine candidate.

The 25 kDa Omp is the most conserved Omp across the known species of *Brucella*, making it a good candidate as a vaccine for protection against challenge with all currently recognised brucellae. A number of studies have indicated Omp25 to have potential as a sub-unit vaccine, especially for protection against challenge with rough strains of *Brucella*. Passive transfer studies using monoclonal antibodies generated against hot saline extract (containing LPS and Omps) of *B. ovis*, found the antibodies able to confer protection against ram epididymitis caused by *B. ovis* infection (Bowden *et al,* 1995). Identification of the Omps in the hot saline extract led to the cloning of the *omp*25 gene and evaluation of an Omp25 expressing *E. coli* as a vaccine candidate (Bowden *et al,* 1998). As a vaccine, *E. coli* expressing Omp25, was able to protect mice against challenge with rough strains of *Brucella* but gave only limited protection against smooth strains. The variability of protection has been attributed to the variation in accessibility of the Omps between the smooth and rough strains: close association of Omp25 and LPS acts to stearically hinder binding of anti-Omp25 antibodies on smooth *Brucella*. This implies a limited range of protective activity for the Omp25 sub-unit vaccines, although the generation of cell mediated immunity against Omp25 has yet to be studied in detail and may not be as susceptible to stearic hindrance.

More recently, Omp25 has been implicated as an important virulence factor in the pathogenesis of human brucellosis. *B. suis* Omp25 has been shown to inhibit the production of TNF-α by stimulated human macrophages (Jubier-

Maurin *et al,* 2001), indicating a possible mechanism by which *Brucella* delays or modulates the host inflammatory response thereby promoting the establishment of infection. Blocking experiments using specific anti-Omp25 to counteract the inhibition of TNF-α indicate that the generation of appropriate immune responses against Omp25 might be a valid component of a vaccine.

Investigation of *B. melitensis omp*25 deletion mutant, BM25, has indicated reduced virulence *in vitro* (macrophage survival) and *in vivo* (decreased persistence in the mouse model, and lack of abortogenicity in the goat) compared to the wild type (WT). When used as a vaccine BM25 was able to generate protection equivalent to the recommended Rev1 vaccine, but with advantages of reduced potential for causing abortion or shedding to the environment (Edmonds, *et al* 2001, 2002a and b). Furthermore, mutant *Brucella* defective in the BvrR/BvrS two component regulatory systems are reduced in virulence and are known to be deficient in Omp25 expression (Guzman-Verri *et al,* 2001). Whilst BM25 and BvrR/BvrS studies do not support a role for Omp25 specific effectors in protective immunity, these studies indicate that Omp25 is important in *Brucella* pathogenesis and is surface exposed. Other studies have also shown that Omp25 is released from *Brucella* in blebs (Gamazo *et al,* 1989), thus making it accessible for interaction with the immune system. Together these data indicate that Omp25 is worth investigation as DNA vaccine candidate.

ialB

The genome database was searched for proteins associated with 'adhesion' or 'invasion' and a number of potential ORFs were indicated which displayed homology to functional proteins in related organisms. Among the ORFs in the *Brucella* genome were genes designated 'Invasion proteins A and B'. These genes share identity with genes in the pathogen *Bartonella bacilliformis*, the causative agent of a haemolytic anaemia known as Oroya fever. These genes, *ialA* and *ialB*, from *Bartonella* were shown to confer the invasive phenotype to a non-invasive *E. coli* strain and increase the adhesion of the recombinant *E. coli* to the host cells (Mitchell and Minnick 1995). The *Bartonella bacilliformis*

homologue of *ialB* has been shown to be essential for the invasion of host erythrocytes. Isogenic deletion mutants (ΔiaiB) of *B. bacilliformis* are defective in erythrocyte invasion. Similarity with ORFs located in the genome of *Rickettsia prowesakii* (*invA*) (Andersson *et al,* 1998) has also been indicated. These genes remain unstudied in *Brucella* and their role in establishment of infection has yet to be determined. Their inclusion in this project as potential vaccine candidates may provide additional insight into their role in *Brucella* pathogenesis. If their hypothesised role in invasion is correct, the generation of immune responses capable of blocking the invasion protein may contribute the control of disease through preventing establishment of infection. Therefore these genes are logical choices for investigation as vaccine candidates.

fliC

Flagellae are well-known surface exposed antigens and virulence factors involved in the initial host–pathogen interaction in other diseases. It was recently demonstrated that the *B. melitensis* genome contains sequences corresponding to flagella apparatus in other species. Since all known species of *Brucella* are non-motile organisms, the inclusion in the genome of all the elements required to produce functional flagella seems somewhat anomalous. In other non-motile organisms similar flagellin homologues have been described forming part of a type III secretion system (TTSS). Indeed, TTS systems are important in the pathogenicity of a number of bacteria including intracellular pathogens such as *Chlamydia* spp., and the surface expressed or secreted portions of these systems may serve as interesting vaccine candidates. The flagella associated genes present in the *Brucella* genome, indicate three flagellar loci, comprising 28 structural genes, but do not comprise a complete TTSS (DelVecchio *et al,* 2002, Letesson *et al,* 2003). The gene and predicted protein sequence similarities with related pathogens are greatest with the TTSS of *Rhizobium* spp., which are implicated in the symbiotic interaction between the bacteria and the host plant cells (Viprey *et al,* 1998). Recent reports have also indicated sequence similarities between the *Brucella* flagella and TTSS of *Burkholderia cepacia,* which has been implicated in this organism's ability to penetrate host epithelial barriers and thereby establish infection (Tomich *et al,*

2002). At the time of selection expression of flagellin associated genes in *Brucella* had yet to be conclusively demonstrated, but their presence in the genome was enigmatic and warranted further investigation. As explained previously, it is possible that interference in the host pathogen interaction through the generation of immune responses to inhibit or neutralise the components involved in the interaction, would be a positive contribution to vaccine development.

Thus, the candidates *omp25*, *ialB* and *fliC* were selected for investigation based on their anticipated surface location or secretion from the bacteria and their hypothesised role in the initial host pathogen interaction. In particular targeting putative ligands that participate in *Brucella* and host cell binding and possibly influence the uptake or outcome of the phagocytic process.

As well as targeting host cell binding, factors involved with the modulation of the host cell to create a bacteria favourable replicative niche were also pursued.

virJ

One of the mechanisms contributing to trafficking to the replicative niche is the Type IV Secretion System (TFSS) of the *Brucella virB* operon (O'Callaghan *et al,*. 1999, Boschiroli *et al,* 2002). The *Brucella virB* system is homologous to the archetypal *virB* system of the plant pathogen *Agrobacterium tumefaciens*. In *A. tumefaciens* the *virB* system is responsible for the transfer of T-DNA from across the bacterial cell wall into the host cell. The T-DNA integrates into the host chromosome and directs the production of phytohormones responsible for plant cell proliferation, and the generation of opines which are essential nutrients for the bacteria. Thus the *virB* system allows the bacteria to subvert the host cells natural processes and create a niche acceptable for the growth of the bacteria. In *Brucella virB* is believed to function in a similar manner, but through the delivery of proteinaceous effector molecules rather than DNA, in order to manipulate the environment of the host phagosome to create a suitable niche for replication. These effector molecules have yet to be identified. *VirB* mutants have been shown to be defective in their ability to survive within

macrophage culture (Comerci *et al*, 2001.), and exhibit much reduced virulence (determined by persistence) in the mouse model (Hong *et al*, 2000). *VirB* appears to be activated by the stimulus encountered within the host phagosomes, including vacuole acidification, but does not appear to be involved in the initial interaction of host and bacteria and is therefore not involved in invasion or adhesion (Boschiroli *et al*, 2002).

The original *B. melitensis* 16M genome data revealed the presence of a *virB*-associated gene with significant homology to the *acvB* gene of *A. tumefaciens*. The gene was named *acvB* in the original WIT/ERGO database but is now listed as *virJ* in the Entrez database (BMEII 0681). The *acvB* homologue in *A. tumefaciens* has been identified as an exported protein effector molecule with relevance to the tumorigenesis process (Kalogeraki and Winans 1995). The presence or relevance of *acvB* as an effector molecule in *Brucella* infections has yet to be determined. However, in the event that *acvB* is utilised for similar purposes by *Brucella* it would seem logical that generation of immune responses capable of blocking the action of *acvB (virJ)*, would impair the ability of *Brucella* to control it's environment and create a replicative niche. This possibility indicates the *acvB (virJ)* gene to have potential as a vaccine candidate.

frpB

Regulation of iron metabolism plays a major role in virulence in a number of bacteria (Hantke 2001). The ability to acquire, store and effectively utilise iron in the iron-limited conditions of the host environment contribute greatly to survival of bacteria. Iron is essential for many biological processes, including electron transport, enzyme function, and the deactivation of reactive oxygen species in the form of peroxides. The concentration of available iron in mammalian tissues is significantly below that required to support bacterial growth and therefore the bacteria have developed a number of mechanisms by which to acquire iron from host iron storage and transport molecules. A search of the genome data for ORFs associated with iron acquisition or transport revealed a number of proteins that had yet to be investigated in *Brucella* pathogenesis. Among these

proteins was the Iron regulated outer membrane protein gene designated *frpB*. This ORF was identified through similarity to the *frpB* gene of *Neisseria gonorrhoeae*. In both *N. gonorrhoeae* and *N. meningitidis frpB* encode a 70 kDa protein, which is both surface exposed and immunogenic (Beucher *et al,* 1995). The protein is reasonably well conserved amongst the *Neisseria*, and has been shown to be susceptible to attack by bactericidal antibodies, making it an appropriate choice for assessment as a vaccine candidate (Ala'Aldeen *et al,*, 1996). Its role in *Brucella* pathogenesis was undetermined, although in general iron metabolism is known to be important in the development of infection (Roop *et al,* 2004).

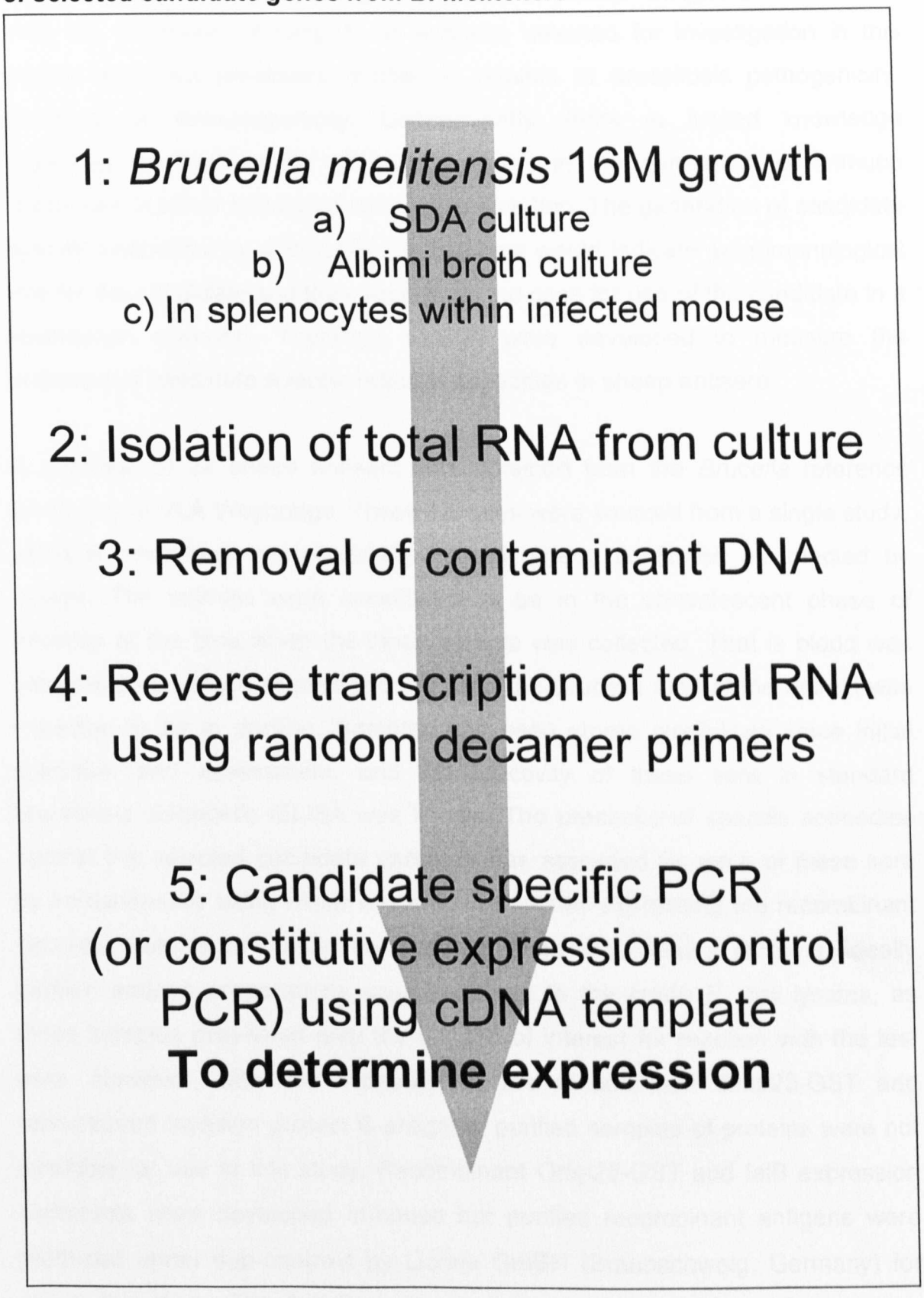
In summary, five genes were selected from the *B. melitensis* 16M genome database. These genes were *omp25*, *ialB*, *fliC*, *acvB (virJ)* and *frpB*. Each of these genes was ascribed a putative function related to *Brucella* intracellular survival or access to the host cell. With the exception of *omp25*, direct evidence for the function of these genes was not available. Therefore, the main aim of the work described in this chapter was to provide experimental evidence for a role for these candidates in *Brucella* pathogenesis, virulence and the development of protective immunity. Thus providing data supporting the choices made in the *in-silico* selection process.

3.3: Experimental procedures and aims

Part 1: Determining expression of candidate antigens from *Brucella melitensis* 16M.

B. melitensis strain 16M was grown under various culture conditions and assessed via candidate specific reverse transcriptase PCR (RT-PCR) for evidence of specific gene expression. The aim of this work was to demonstrate that it was possible for the selected genes to be expressed by the *Brucella* and therefore possible that such expression would produce accessible protein targets for attack by the acquired immune system of a vaccinated host. The experimental processes are summarised in Figure 3.1.

Figure 3.1: Experimental processes involved in determining transcription of selected candidate genes from B. melitensis



Part 2: Analysis of immunogenicity of candidate antigens

With the exception of Omp25, all antigens selected for investigation in this project were not previously studied in relation to brucellosis pathogenicity, virulence or immunogenicity. Consequently, there is limited knowledge regarding the involvement of such antigens in the generation of immune responses in either natural infection or vaccination. The generation of candidate specific antibodies by a *Brucella* infected host would indicate an immunological role for the candidate and thus strengthen the case for use of the candidate in a vaccination scenario. Therefore assays were developed to measure the presence of candidate specific reactive antibodies in sheep antisera.

A selection of 22 sheep antisera was obtained from the *Brucella* reference laboratory at VLA Weybridge. These samples were sourced from a single study, using *B. melitensis* experimentally infected sheep confirmed as infected by culture. The animals were considered to be in the convalescent phase of infection at the time when the blood sample was collected. That is blood was sampled post-partum or post-abortion, and the *Brucella* load of the animal was expected to be in decline. Samples had been stored lyophilised since initial collection and assessment, and the reactivity of these sera in standard brucellosis diagnostic iELISA was known. The presence of specific antibodies against the selected candidate vaccines was assessed for each of these sera by immunoassay using either crude *E. coli* lysates expressing the recombinant *Brucella* proteins or purified samples of these recombinant proteins. Ideally purified antigen preparations were preferred to the crude *E. coli* lysates, as these samples presented only the antigen of interest for reaction with the test sera. However, with the exception of the recombinant Omp25-GST and recombinant Invasion protein B antigens, purified samples of proteins were not available for use in this study. Recombinant Omp25-GST and IalB expression constructs were developed in-house but purified recombinant antigens were produced under sub-contract by Lionex GmBH (Braunschweig, Germany) for use in this study. The Anti-*Brucella* reactivity of samples was confirmed using the *Brucella* indirect ELISA (iELISA), and Brucellergene™ ELISAs.

3.4: Results

Expression of selected genes by *Brucella melitensis* 16M

Candidate antigen gene transcription was determined by specific RT-PCR following isolation of RNA from *B. melitensis* 16M cultured under different conditions.

The specific RT-PCR amplicons were resolved by agarose gel electrophoresis. Table 3.1 summaries the results from this study and Figure 3.2 shows the results from one of the replicate RT-PCR assays of cDNA isolated from *Brucella* infected splenocytes.

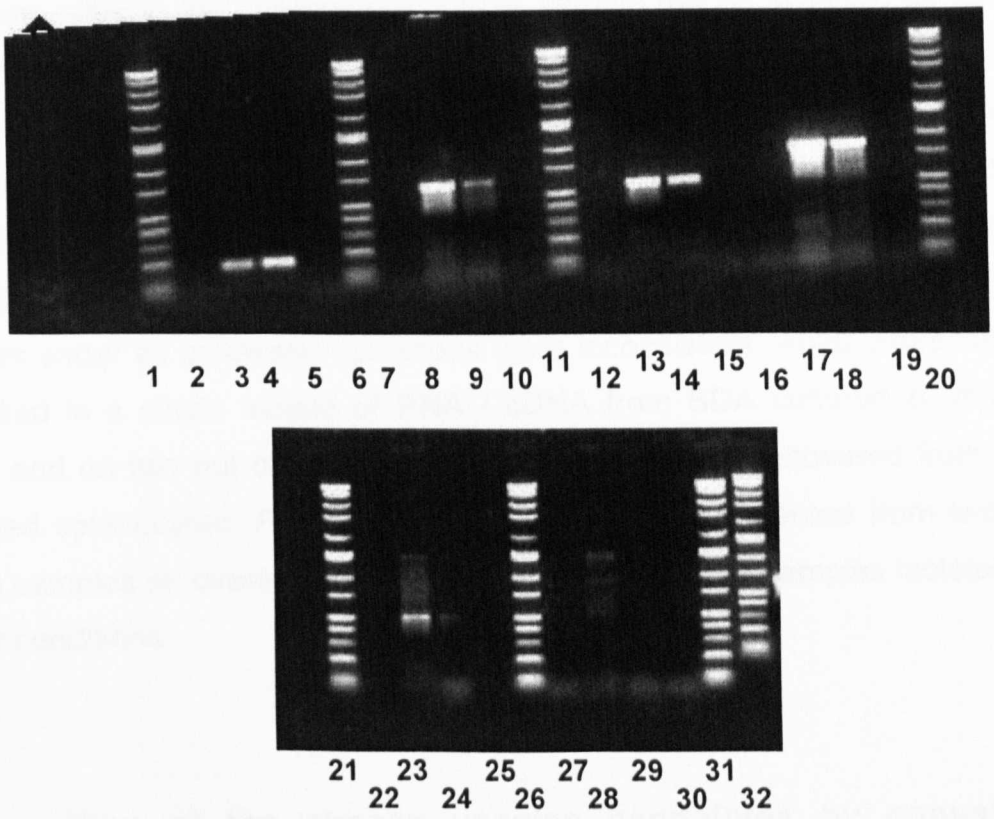
Table 3.1: A summary of the RT-PCR results demonstrating expression of the selected genes under different culture conditions

Culture conditions	Specific PCR reaction					
	IF-1	*omp25*	*ialB*	*flIC*	*acvB*	*frpB*
SDA plate culture	Pos	Pos	Pos	Neg	(Pos)	Pos
Albimi broth culture	Pos	Pos	Pos	Neg	Neg	Neg
Ex-vivo isolation from macrophages	Pos	Pos	Pos	(Pos)	(Pos)	Pos

Pos	Expression detected consistently in at least 3/3 assays
(Pos)	Expression detected, but inconsistently. Expression detected in at least 1/3 assessments
Neg	Expression never detected (0/3)

The data were collated from three replicate studies of each condition. A single representative example of the results from the infected splenocyte investigation is provided in figure 3.2.

Figure 3.2: Specific RT-PCR results obtained from B. melitensis 16M infected splenocytes in this study



PCR amplicons from specific PCR reactions using B. melitensis 16M infected splenocyte total RNA / cDNA template.
1: 1Kb ladder (Promega), 2 – 5: IF-1 reactions (164 bp product); 2: Negative control (water template), 3: 16M genomic DNA template, 4: cDNA template from infected splenocytes, 5: RNA template from infected splenocytes., 6: 1Kb ladder (Promega), 7- 10 omp25 reaction (642 bp product); 7: negative control, 8: genomic DNA, 9: cDNA, 10: RNA., 11: 1 Kb ladder (Promega), 12-15 ialB reaction (512 bp product), 12: water, 13: genomic DNA, 14: cDNA, 15: RNA., 16-20 fliC reaction(842 bp product), 16: water, 17: genomic DNA, 18: cDNA, 19: RNA, 20: 1 Kb ladder (Promega)., 21: 1Kb ladder (Promega), 22 –25: AcvB reaction (1413 bp product); 22: Negative control (water template), 23: 16M genomic DNA template, 24: cDNA template from infected splenocytes, 25: RNA template from infected splenocytes., 26: 1 Kb ladder (Promega), 27- 30 frpB reaction(1985 bp product); 27: negative control, 28: genomic DNA, 29: cDNA, 30: RNA., 31: 1 Kb ladder (Promega), 32: ladder (Promega)

Table 3.1 provides a summary of the expression data, determining consistent expression of genes *omp25* and *ialB*. Under all conditions investigated in this study, specific transcripts for these genes were detected from random decamer generated cDNA templates. Amplicons were not detected for equivalent reactions utilising the corresponding RNA sample (no RT Control) as template, indicating that the product was not generated through amplification of contaminating genomic DNA. Expression of the other candidate genes was determined on at least one occasion. The *frpB* gene was expressed in solid phase culture and from *ex vivo* macrophages, although expression was not detected from Albimi broth cultures. Data from both the *fliC* and *acvB* gene studies under all assessed conditions were inconsistent. *AcvB* expression was detected in a single isolate of RNA / cDNA from SDA cultured *B. melitensis* 16M, and on two out of three occasions from samples recovered from ex-vivo isolated splenocytes. *FliC* specific amplification was detected from two out of three samples recovered from splenocytes, but not from samples isolated under other conditions.

Recognition of the chosen vaccine candidates by convalescent sheep and goat sera in ELISA

ELISAs were developed to determine the presence of antibodies reacting with *Brucella* specific proteins. The reactivity of the sera samples in the internationally recognised *Brucella* diagnostic iELISA was compared to that in newly developed iELISAs. For the iELISA, data was interpreted through comparison of test sample OD's to the OD of the international standard Positive control sera. Samples with an OD > 10% of the positive control was considered positive in this assay. For the recombinant antigen based ELISAs a cut off (CO) was assigned to each individual assay based upon the mean + two standard deviations of the OD value of known negative serum in this assay.

Results from ELISAs based upon crude *E. coli* lysates or purified recombinant proteins (IalB and Omp25-GST) are summarised in Table 3.2. The table displays the ELISA result (positive or negative) for each of the known infected serum samples in each of the ELISAs. Crude lysate ELISA results are shown

for the FrpB, AcvB and FliC reactions. Purified recombinant protein results are given for the Omp25 and IalB reactions.

Table 3.2: Results from ELISAs to determine natural reactivity of Brucella infected sheep to the selected candidate antigens

	Purified recombinant antigens		Crude lysates from recombinant *E. coli*			Native antigens -S-LPS	+S-LPS
Sample ID	Omp25	iaiB	AcvB	FrpB	FliC	Bru	iELISA
3	+	+	+	-	-	+	+
4	+	+	-	+	+	+	+
5	+	+	+	+	+	+	+
6	+	+	+	-	-	+	+
7	+	-	+	+	+	+	+
10	+	+	-	+	-	+	+
12	+	+	-	-	-	+	+
14	+	+	-	-	+	+	+
16	+	+	-	-	+	+	+
18	+	+	+	-	-	+	+
24	+	-	-	-	+	+	+
25	+	-	+	-	+	+	+
26	+	+	-	-	-	+	+
27	+	-	-	+	-	+	+
29	+	+	+	-	+	+	+
30	+	-	-	+	+	+	+
33	+	+	+	-	+	+	+
34	-	+	-	-	+	-	+
35	+	+	+	+	+	+	+
36	+	+	-	+	-	+	+
38	-	-	-	+	-	-	+
39	+	+	+	+	+	+	+
	91%	***76%***	***45%***	***45%***	***59%***	***91%***	***100%***

The table indicates the result determined from the each serum sample assayed at 1/200 dilution in each ELISA test. The CO for defining positivity was determined from the mean OD value of five known negative sera in each of the individual ELISAs. Samples with a mean OD > CO are indicated +, samples determined as negative (mean OD < CO) are indicated –.
CO values for each ELISA are Omp25 = 0.652, IalB = 0.089, fliC = 0.340. acvB =0.51, frpB = 0.663.

These data indicate that 91% of the *Brucella* infected sheep produce antibodies specific to proteins present in the Brucellergene™ antigen or recombinant Omp25-GST preparation. A smaller percentage of these animals produce antibodies that react with the other recombinant antigens. These data indicate that the selected antigens are recognised and targeted by the sheep humoral immune system during a *Brucella* infection, although the results for AcvB, FrpB and FliC reactions require confirmation of specificity using purified recombinant protein antigens.

3.5: Discussion

The aim of the work described in this chapter was to select candidate antigens for development into DNA vaccines, based upon their expression during infection and their immunogenicity in the target species. Firstly, the transcription of the candidate antigen genes in the *Brucella* organism was verified through RT-PCR.

Detection of specific gene transcripts does not always equate to the generation of full-length protein product. However, for the purposes of this investigation, detection of specific transcripts in the RNA was considered evidence of the initiation of the expression process. The detection of transcripts at least indicates that the gene is active in *Brucella*. The data indicated that the *Brucella omp25*, *ialB* and *frpB* genes are transcribed by the organism under standard SDA culture conditions. Transcripts specific for *acvB* were detected intermittently from RNA isolated from SDA cultured *Brucella*. Liquid broth culture provided evidence of transcripts specific for the *omp25*, and *ialB* only. Whereas, transcripts from all the selected genes were identified in the RNA isolated from *Brucella* infected splenocytes on at least one occasion out of three. Thus it appears that the *omp25* and *ialB* genes were sufficiently important to the *Brucella* growth that they were expressed under all the conditions investigated. Contrastingly, the expression (or lack of expression) of *frpB, acvB* and *fliC* altered with varying culture or environmental conditions.

Although direct evidence of the protein expression was not sought in this investigation, direct evidence for expression of Omp25, FrpB and IalB have since become available through other studies. The presence of Omp25 in *Brucella* cell lysates has been verified in numerous studies, and the protein is known to be an important component of the outer membrane of the *Brucella* spp. The protein is readily detected in killed preparations of cultured *Brucella*. Data presented here indicate that Omp25 is expressed under all of the conditions investigated. Furthermore, because of its surface location this protein is immediately accessible to the host immune system upon infection.

The *ialB* gene transcripts were detected under all conditions investigated. The work of Wagner *et al,* (2002) supports these findings, through demonstration of the presence of the IalB protein in the proteome of SDA cultured *B. melitensis* 16M. The detection of transcription of *ialB* from *ex vivo* cultured splenocytes indicates that the gene may be necessary for the survival of the *Brucella* within the splenocyte, as well as its' hypothesised role in the initial host cell invasion. Notably, *ialB* expression is not ubiquitous to all *Brucella* spp. Genomic comparisons of the *B. melitensis* and *B. suis ialB* homologues indicates a frame shift mutation in the *B. suis* 1330 *ialB* gene which is expected to result in a truncated product (Paulsen *et al,* 2003). In the context of vaccine candidate selection, this suggests that a vaccine based upon the *ialB* gene may only be of relevance to the *B. melitensis* infections and would not be applicable for use against all species of *Brucella*.

Transcription of the *frpB* gene was detected in RNA isolated from SDA ex-vivo splenocyte cultures (See Chapter 3 for details), and Wagner *et al,* (2002) have confirmed the presence of FrpB in the proteome of SDA cultured *B. melitensis* 16M. Furthermore, Eschenbrenner *et al*, (2003), observed increased expression of the FrpB protein in the *B. melitensis* vaccine strain Rev.1 in comparison to the virulent type strain 16M. A role for the FrpB protein in the observed attenuation of the *B. melitensis* vaccine strain Rev.1 was therefore suggested. Eschenbrenner *et al,* postulated that the over expression of this, and other proteins, constituted energy inefficiency on the part of the Rev. 1 organism which contributed to its reduced virulence *in vitro* and possibly *in vivo*. An alternative hypothesis would be that over-expression of FrpB leads to a greater specific immune response, because of the increased availability of the antigen to prime the reaction, and that specific anti-FrpB responses actually contribute directly to protection. For instance, binding of specific antibodies to FrpB could hamper the iron acquisition function of the protein and therefore restrict the growth of the Rev.1 organism. In this study transcription of *frpB* was detected from SDA and ex-vivo splenocyte cultures but not from liquid broth. This implies that FrpB expression is not constitutive. Nevertheless the expression of the FrpB protein from *Brucella* cultured on SDA, and isolated ex-vivo from

splenocytes, suggests that this protein will be exposed to the host animal upon infection and thus accessible to the immune response.

Expression or transcription of the *fliC* and *acvB (virJ)* genes was not consistently determined in these studies. *AcvB (virJ)* specific transcripts were detected intermittently in the RNA isolated from both SDA and ex-vivo isolated splenocytes. At the time of writing, evidence for the expression of *Brucella* AcvB (VirJ) had not been reported. However, the *Agrobacterium tumefaciens acvB (virJ)* homologues had been investigated and were proposed to function as an effector or chaperone molecule transported through a VirB–like type IV secretion system, although AcvB *sec* dependent secretion into the supernatant (SN) of cultures was shown to occur independently of the VirB system (Chen *et al,* 2000). The VirB system of *Brucella* is essential for the establishment of the intracellular replicative niche (O'Callaghan *et al,* 1999., Sieira *et al,* 2000., Celli *et al,* 2003) but the effector molecules that are transported through this system are currently unknown. The potential involvement of the *acvB (virJ)* gene product in this process has been studied but remains undefined. Data presented here indicate that the gene is transcribed by *Brucella* although not constitutively. The conditions inducing transcription cannot be clearly defined from this work. As the *acvB / virJ* gene product is a possible effector of a TFSS, it's secretion from the *Brucella* into host cytosol or into the subverted phagolysosomal vacuole is anticipated to make it accessible for antigen presentation by infected cells and thus a target for the cell mediated immune responses of the host. Secretion of the antigen from the host cell may also make the protein available for antibody binding.

Expression of the *fliC* gene was not detected consistently in this study. *Ex vivo* splenocyte samples yielded the only evidence for expression of *fliC* in this study, suggesting that expression of this gene is induced by or limited to an intracellular location. Moreover, transcription from associated flagellar system genes (*flhB, fliP, fliR* and *fliF*) was not detected in studies with SDA and Albimi cultured *Brucella* (Abdullah *et al,* 2003) supporting a lack of expression of this gene under these culture conditions. More recently, intracellular *fliC* gene expression has been confirmed through reporter gene analysis (Fretin *et al,*

2005). In these studies expression of *fliC* and other associated flagella systems genes was detected transiently upon host cell invasion by the *Brucella*. Moreover, this group were able to demonstrate significant attenuation of a flagellar mutant *B. melitensis* strains, in both macrophage and BALB/c mouse models of infection (Delrue *et al,* 2005). These findings indicate a role for flagellin in the pathogenesis of *Brucella* infection, although the exact function of the flagellar system of *Brucella*, and indeed the particular role of the flagellin (FliC) antigen, remains undetermined.

Thus, as with *acvB*, the expression of flagellin (FliC) antigen appears to be restricted to certain conditions or induced by as yet undefined stimuli which occur either through contact with host cells or occur intracellularly. Nevertheless, evidence of transcription indicates that these proteins may play a role in the establishment or maintenance of infection.

Overall, these data indicate that all selected candidate genes were transcribed by *B. melitensis* 16M under certain conditions, thus supporting the notion that the protein products of these genes may be presented as antigens to the immune system of the host animal. Therefore, the results of this investigation support the selection of the putative antigens as vaccine targets.

Although evidence of gene expression is a useful indicator of the potential exposure of the protein to the immune system, exposure does not necessarily result in protective or even appropriate immune responses. For this reason, evidence of target antigen immunogenicity was sought through assessment of the serological responses of a panel of twenty-two sheep serum samples from experimental infection studies. For the Omp25-GST and IalB proteins, purified preparations of these antigens were prepared for use in these investigations. For the remaining antigens (FrpB, AcvB and FliC), antigen preparations were derived from recombinant *E. coli* cell lysates expressing the recombinant *Brucella* fusion proteins (FrpB-His, AcvB-GST, and FliC-His). Overall, over 40% of the sera from *Brucella* infected sheep were detected as positive in candidate specific ELISA. Thus providing supporting evidence that the selected candidate genes are not only transcribed, but also translated and recognised by the host

immune system. However, more data from more stringent ELISAs using highly purified recombinant proteins would be required to confirm these results and to determine whether these antigens have diagnostic potential.

In conclusion, antigens were selected from the *B. melitensis* 16M genome data based upon amino acid similarity to virulence factors and immunologically significant antigens in other important pathogens. The work described in this chapter indicated that these genes (*omp25, ialB, acvB, fliC,* and *frpB*) are transcribed by the *Brucella* organisms, and therefore the protein products of these genes are potentially available to the immune system of the host and may be useful targets for vaccination. Furthermore, the demonstration of a specific antibody response from greater than 40% of *Brucella* infected sheep against these proteins suggests that these proteins (Omp25, Invasion protein B (IalB), AcvB, flagellin (FliC) and FrpB) may have immunological significance in a natural infection.

Chapter 4: THE PRODUCTION AND IN VITRO EVALUATION OF DNA VACCINES BASED UPON THE *BRUCELLA* GENES *omp25, ialB, frpB, acvB,* AND *fliC*

4.1: Summary

The candidate antigens selected from the B. melitensis 16M genome database, and supported by the laboratory studies described in chapter 3, were produced as DNA vaccines. Their production and characterisation is described in this chapter. In total, six novel vaccines based upon the five selected antigens were produced. The expression of the encoded Brucella gene from each construction was confirmed by RT-PCR, providing indirect evidence that the vaccines were expression competent. Furthermore, direct evidence of specific protein production was obtained for pCR3.1-omp25 and pTargeT-omp25 vaccines from monoclonal antibody based detection of the protein in transfected Cos 7 cells by immunofluoresence assay. Similar studies, using anti-Brucella sera suggested the presence of Brucella specific proteins from Cos7 cells transfected with pCR3.1-ialB, pCR3.1-frpB and pCR3.1-acvB.

4.2: Introduction

Plasmid vectors suitable for use in DNA vaccination are commercially available. In the most basic form each vector consists of a plasmid capable of replication in *E. coli* K12 derived host strains (ColEI origin of replication), a selectable marker such as antibiotic resistance to facilitate selection of recombinants, and the required elements to allow expression of the inserted coding sequence from eukaryotic cells. A eukaryotic promoter (most commonly the Human Cytomegalovirus Early Intermediate promoter (pCMV), or the promoter from Simian Virus 40 (SV40)) drives the transcription of the inserted DNA sequence within mammalian cells, and the polyadenylation signal (most commonly Bovine Growth Hormone Polyadenylation signal (BGHPolyA)), which generates the

poly A tail to the transcribed mRNA and thereby promotes stability of the transcript. A number of modifications to this basic design can be made through inclusion of additional sequences. The inclusion of a Kozac or eukaryotic ribosome binding site signal (Kozac, 1988) has been shown to increase the level of expression of the inserted sequence *in vivo*.

The use of DNA provides opportunities for manipulation and engineering of a rationally defined vaccine. Techniques for manipulation of DNA are well understood and make it possible to modify antigen genes and vectors for different purposes. DNA vaccine plasmids can be engineered to direct and bias immune responses to the encoded antigen by, for example, modifying antigen expression and enforcing certain routes of antigen presentation. The backbone of the plasmids may be engineered to contain immunostimulatory or adjuvant DNA sequences such as CpG motifs and expression enhancing sequences such as intron elements, in order to augment the immune response to the encoded antigen (Kreig, 2000., Klinman, 2000., Heeg and Zimmermann, 2000). Specific immunological molecules such as cytokines can be incorporated into DNA vaccine constructs either with the antigens of interest or into a separate plasmid. Administering both the cytokine and specific antigen constructs serves to modify and direct immune responses (Pan *et al,* 1999).

A defined DNA vaccine is also readily identifiable, enabling vaccinates to be easily differentiated from naturally infected hosts, due to the inclusion or exclusion of specific diagnostic or marker antigens. In addition to the practicalities of construction and purification of the vaccine, data have indicated DNA vaccination to induce Th 1 biased immune responses. This bias is related to the non-specific stimulus effect of the CpG motifs in the plasmid DNA, and the endogenous production and presentation of the encoded antigen. Th 1 based immune responses are essential for the clearance of intracellular pathogens and the generation of inappropriate immune responses (non – Th 1) is one contributing factor to the previous lack of success with sub-unit or dead whole bacterial vaccines. In addition, the immune stimulus from conventional sub-unit antigens is short lived and booster immunisations are required in order to assure the development of the necessary memory response. In contrast,

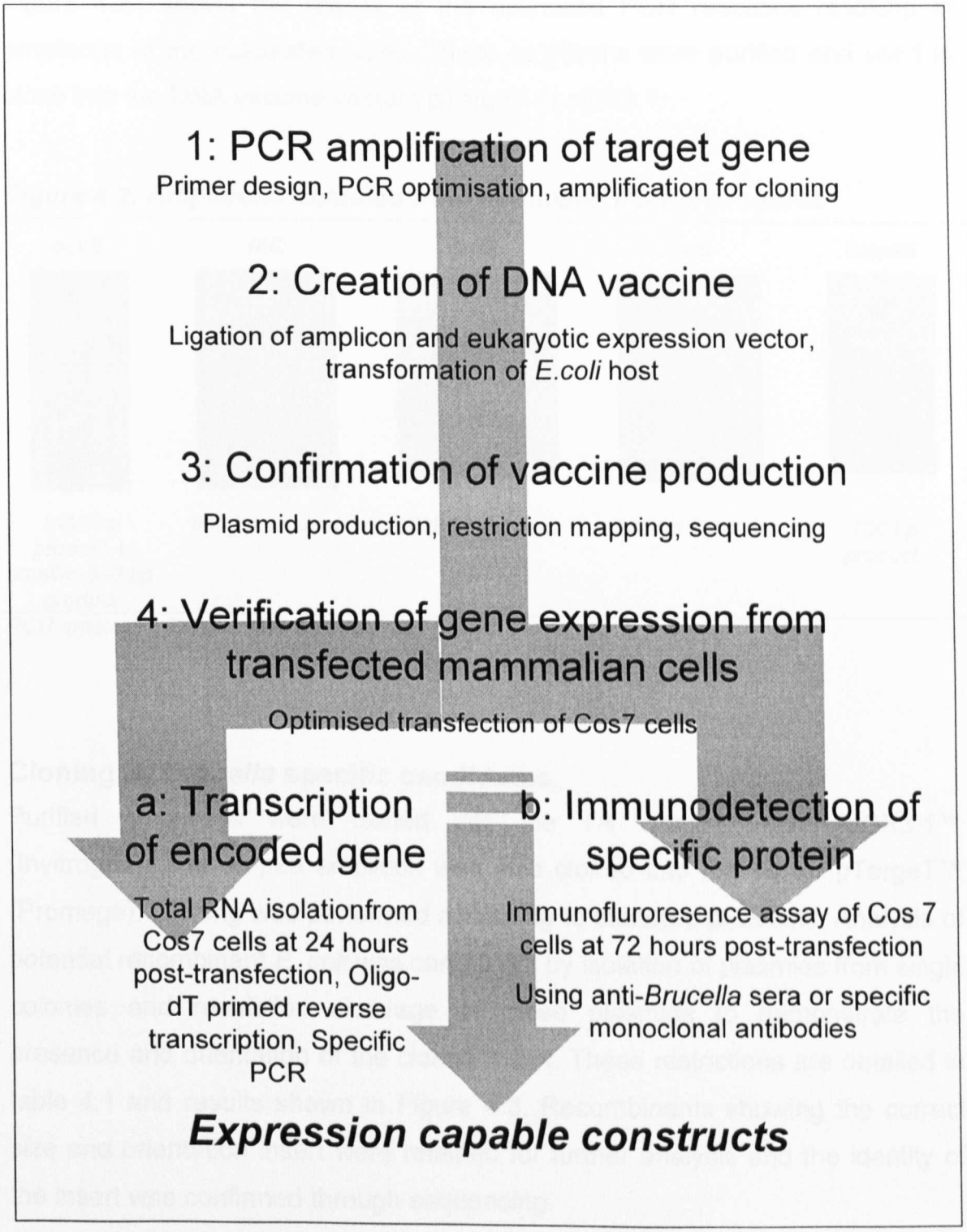
antigen presentation from DNA vaccines can be prolonged, with the plasmids within host cells capable of producing the antigens over a significant period. Additionally, DNA vaccination induces specific responses that are not directed against the plasmid vector itself, therefore making repeat administration of the same vector possible. Other advantages include the reported application of DNA vaccines in neonatal animals (Bot and Bona, 2002) and the ease with which vaccine constructs can be co-delivered or formulated to produce multi-partite vaccines. A number of review articles are available discussing the relative merits of DNA vaccination (Gurunathan *et al,* 2000., Ulmer, 1996., Davis and Whalen, 1995)

Ultimately the vaccine vector needs to be simple, well characterised and safe to use in clinical applications. A number of commercially available plasmid vectors have already been used as the basis of DNA vaccines undergoing clinical trials. Among the most widely cited commercially available DNA vaccine vectors are the pCMV based vaccines, including pcDNA3.1™ and it's derivatives produced by Invitrogen. pCR3.1™ (Invitrogen) is a TA cloning vector based upon the pcDNA3.1™ vector. pTargeT™ is a TA cloning vector similar in construction to pcDNA3.1™ but with the inclusion of an intron element immediately upstream to the insertion site. These TA cloning vectors were chosen for use in this study, as they permit rapid direct cloning of PCR products, are competent for mammalian expression, and are among the simplest of the eukaryotic expression vectors.

4.3: Experimental procedures and aims

The experimental procedures for design and construction of the DNA vaccines, and evaluation of expression are summarised in figure 4.a. Details of the materials and methods used for these experiments are described in chapter 2.
The eukaryotic expression vectors pTargeT™ (Promega) and pCR3.1 (Invitrogen) were used to generate DNA vaccines. Basic plasmid maps and sequence details are provided in appendix 1.

Figure 4.1: A summary of the experimental procedures used to create and evaluate DNA vaccines.

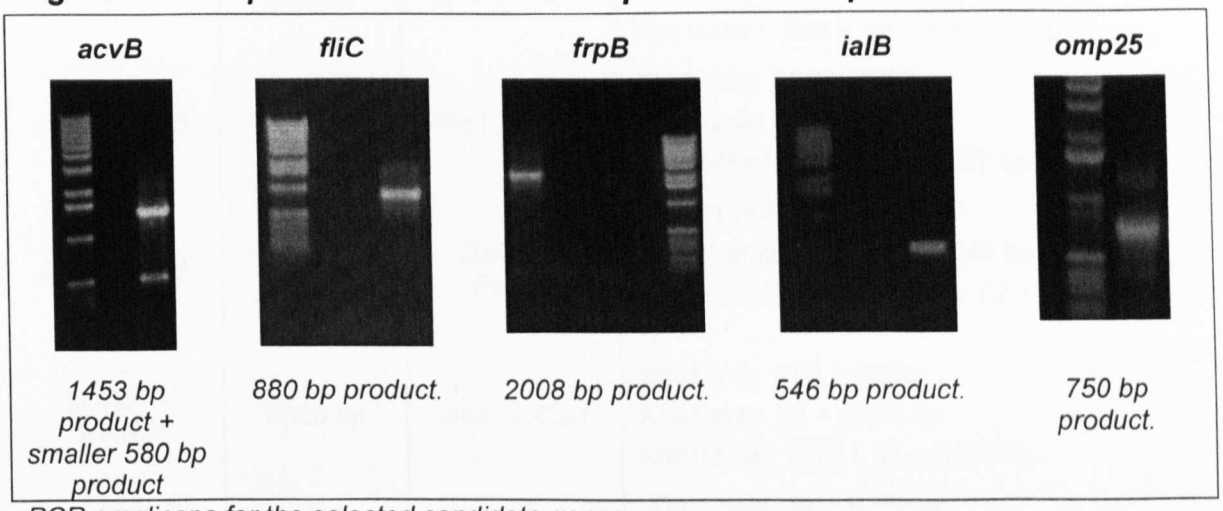


4.4: Results

Amplification of *Brucella* specific genes

Figure 4.2., shows the results of the optimised PCR reactions resulting in amplicons of the calculated sizes. These amplicons were purified and used to clone into the DNA vaccine vectors pTargeT or pCR3.1.

Figure 4.2: Amplicons obtained from optimised PCR procedures



PCR amplicons for the selected candidate genes.

Cloning of *Brucella* specific candidates

Purified amplicons were cloned into the TA cloning vector pCR3.1™ (Invitrogen). The omp25 amplicon was also cloned into the vector pTargeT™ (Promega). Cloning was performed according to standard protocols. Analysis of potential recombinant *E. coli* was carried out by isolation of plasmids from single colonies and restriction cleavage of these plasmids to demonstrate the presence and orientation of the cloned insert. These restrictions are detailed in table 4.1 and results shown in Figure 4.3. Recombinants showing the correct size and orientation insert were retained for further analysis and the identity of the insert was confirmed through sequencing.

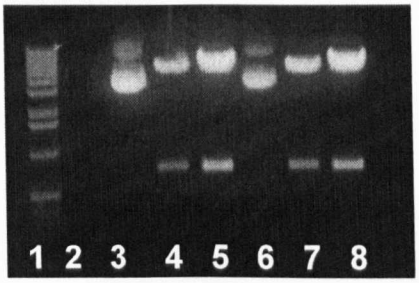
Table 4.1: Expected restrictions of the DNA vaccine plasmids

Vaccine construct	Plasmid size	Restriction enzymes introduced to sequence ends	Expected restriction fragments Boxed *Brucella* specific insert
pCR3.1-acvB	6494 bp	*Hin*dIII / *Bam*H1	*Hin*dIII only: 53 + 6441 bp *Bam*H1only:1459 + 5034 bp *Hin*dIII/*Bam*H1: **1442** + 35 + 18 + 4999 bp
pCR3.1-fliC	5939 bp	*Xho*1 / *Xba*1	*Xho*1 only: 915 + 5024 bp *Xba*1 only: 12 + 5927 bp *Xho*1/*Xba*1: **869** + 46 + 6 + 5018 bp
pCR3.1-frpB	7076 bp	*Xho*1 / *Eco*R1	*Xho*1 only: 2043 5033 bp *Xba1* only: 7076 bp *Xho*1/*Xba1*: **2043** + 6 + 5027 bp
pCR3.1-iaiB	5605 bp	*Bam*H1 / *Eco*R1	*Bam*H1 only: 36 + 5569 bp *Eco*R1 only: 547 + 14 + 5044 bp *Bam*H1/*Eco*R1: **534** + 13 + 23 + 14 + 5021 bp
pCR3.1-omp25	5809 bp	*Xho*1 / *Xba*1	*Xho*1 only: 784 + 5025 *Xba*1 only: 53 + 5756 bp *Xho*1/*Xba*1: 737 + 53 + 5019 bp
pTargeT-omp25	6420 bp	*Xho*1 / *Xba*1	*Xho*1 only: 18 + 6402 bp *Xba*1 only: 6420 bp *Xho*1/*Xba*1: 737 + 12 + 5653bp

Full plasmid maps and sequences are provided in appendix 1.

Figure 4.3: Restriction enzyme cleavage results for each DNA vaccine construct, to illustrate the presence and size of the inserted DNA

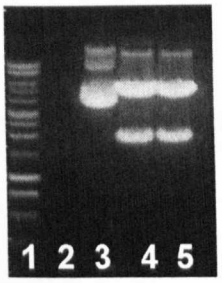
pCR3.1-*omp25* and pTargeT-*omp25*



1: 1kB marker (Life technologies), 3: unrestricted pCR3.1-omp25, 4: Xho1 restricted pCR3.1-omp25, 5: Xba1+ Xho1 restricted pCR3.1-omp25., 6: Unrestricted pTargeT-omp25., 7: Xho1/Xba1 restricted pTargeT-omp25, 8: Xba1 + Xho1 restricted pTargeT-omp25.

(737 bp insert)

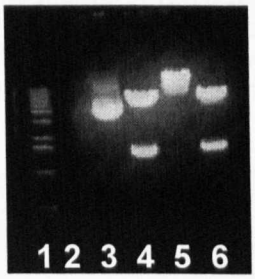
pCR3.1-*frpB*



1: 1 kB ladder (Promega), 3: Unrestricted pCR3.1-frpB, 4: Xho 1 restricted pCR3.1-frpB, 5: Xho1 + Xba1 restricted pCR3.1-frpB

(2043 bp insert)

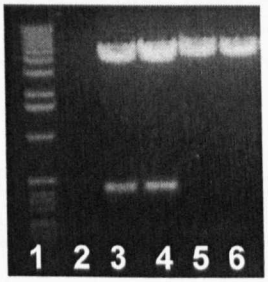
pCR3.1-*acvB*



1: 1kB marker (Life technologies), 3: unrestricted pCR3.1-acvB, 4: Hind111 + BamH1 restricted pCR3.1-acvB, 5: HindIII restricted pCR3.1-acvB. 6: BamH1 restrictedpCR3.1-acvB

(1442 bp insert)

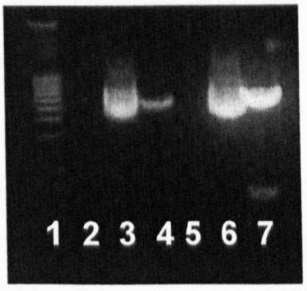
pCR3.1- *ialB*



1: 1kB marker (Life technologies), 3: EcoR1 restricted pCR3.1- ialB, 4: EcoR1 and BamH1 restricted pCR3.1- ialB, 5: BamH1 restricted pCR3.1- ialB, 6: unrestricted pCR3.1- ialB.

(534 bp insert)

pCR3.1-*fliC*



1: 1 kB ladder (Promega), 3: Unrestricted pCR3.1-fliC, 4: Xba1 restricted pCR3.1-fliC, 6: Unrestricted pCR3.1-fliC, 7: Xho1 + Xba1 restricted pCR3.1-frpB

(869 bp insert)

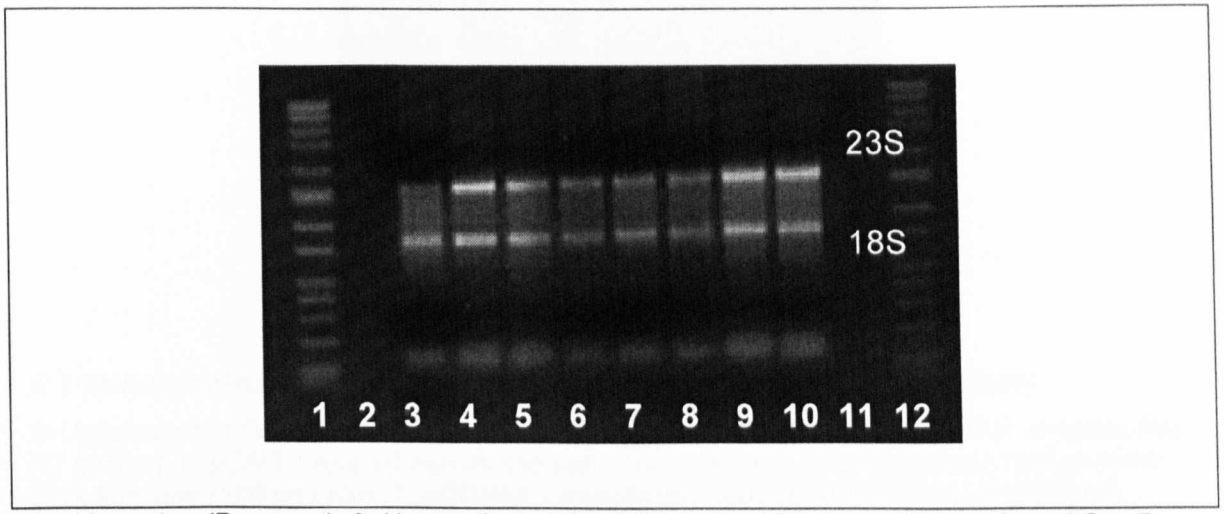
Each construction contains an appropriately sized and orientated insert

Expression of inserted sequences from DNA vaccine constructs

Candidate gene expression from the DNA vaccine plasmids was assessed following transfection of Cos 7 cell cultures with the plasmid. Expression was determined through RT-PCR following isolation of total RNA from the transfected cell cultures. The successful isolation of RNA was confirmed

through standard agarose electrophoresis to show the presence of the major 18S and 23S ribosomal RNA subunits in the preparation. RNA samples are shown in figure 4.4.

Figure 4.4: RNA isolated from Cos 7 cells, at 48 hours post-transfection.

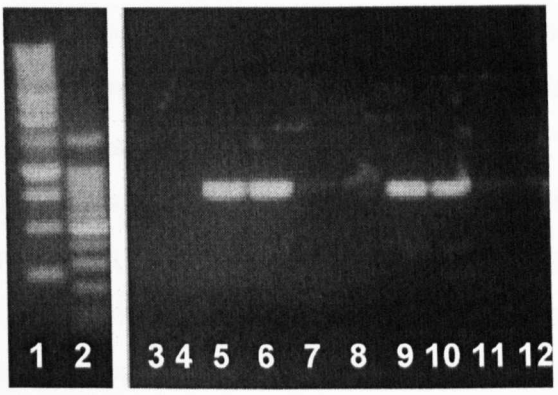


1: 1kb marker (Promega), 3: Untransfected Cos7 RNA, 4: pCR3.1-omp25 transfected Cos 7 RNA, 5: pTargeT-omp25 Cos 7 transfected RNA, 6: pCR3.1- ialB transfected Cos7 RNA, 7: pCR3.1-acvB transfected Cos7 RNA, 8: pCR3.1-fliC transfected Cos7 RNA, 9: pCR3.1-frpB transfected Cos7 RNA, 10: pcDNA3.1 transfected Cos7 RNA.

The picture shows the initial total RNA isolation samples from the Cos 7 cells. 23S and 18S ribosomal RNA bands are clearly visible at approximately (1.8 and 1.2 Kbp) respectively. The faint band visible at 300 bp is the 5S ribosomal RNA. The smearing visible in each lane could be due to either the presence of multiple mRNAs of different size or to contaminating fragments of genomic DNA. The genomic DNA removal step (DNAFree™ protocol) was used on this preparation to remove genomic DNA contamination. Samples of cleaned RNA were used immediately to generate cDNA, and the cDNA used as template in the *Brucella* specific RT-PCR. The results of the candidate specific PCR are shown in figure 4.5.

Figure 4.5: Candidate specific RT-PCR reactions for the transfected and control Cos 7 cells

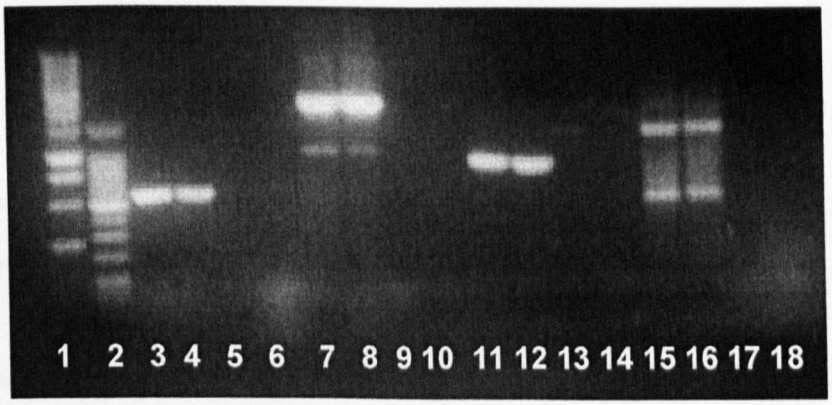
omp25 PCR reactions



1: 1 Kb ladder (Promega), 2: 100 bp ladder (Promega), 3 - 12: omp25 PCR results

3: Untransfected Cos7 cDNA template, 4: pCR3.1-omp25 transfected Cos7 RNA template (No RT control), 5: pCR3.1-omp25 transfected cell cDNA template, 6: B. melitensis 16M genomic DNA template (100 pg / rxn), 7: pCDNA3.1 transfected Cos7 cDNA template, 8: pTargeT-omp25 transfected Cos7 RNA template (No RT control), 9: pTargeT-omp25 transfected cell cDNA template, 10: B. melitensis 16M genomic DNA template (50 pg / rxn), 11 & 12: Sterile water template (Negative control)

ialB, frpB, fliC and _acvB_ PCR reactions



1: 1 Kb ladder (Promega), 2: 100 bp ladder (Promega), 3-6: ialB reactions, 7-10: frpB reactions, 11-14: fliC reactions. 15-18: acvB reactions

3: B. melitensis 16M genomic DNA template (100 pg / rxn), 4: pCR3.1- ialB transfected Cos7 cDNA template 5: pCR3.1- ialB transfected Cos7 RNA template (No RT control), 6: Sterile water template (Negative control), 7: B. melitensis 16M genomic DNA template (100 pg / rxn), 8: pCR3.1-frpB transfected Cos7 cDNA template 9: pCR3.1-frpB transfected Cos7 RNA template (No RT control), 10: Sterile water template (Negative control), 11: B. melitensis 16M genomic DNA template (100 pg / rxn), 12: pCR3.1-fliC transfected Cos7 cDNA template 13: pCR3.1-fliC transfected Cos7 RNA template (No RT control), 14: Sterile water template (Negative control), 15: B. melitensis 16M genomic DNA template (100 pg / rxn), 16: pCR3.1-acvB transfected Cos7 cDNA template 17: pCR3.1-acvB transfected Cos7 RNA template (No RT control), 18: Sterile water template (Negative control)

Figure 4.5, shows the summarised results from the Cos 7 expression data analyses. For each cell sample transfected with a particular vaccine construct the data shows positive PCR from the control samples using *B. melitensis* 16M genomic DNA as template, and cDNA isolated from the appropriate transfected cells demonstrating the presence of the specific DNA vaccine encoded transcript (mRNA). Reactions using appropriate RNA (the 'No RT' control) from each transfection isolation showed negative (no band) results, indicating the absence of the plasmid DNA from the RNA isolations. This is important as any contamination of the RNA sample by the plasmid DNA could be carried over into the cDNA sample to provide an alternative source of template for the PCR, and therefore generate a false positive result. Control reactions using untransfected or pcDNA3.1 transfected Cos 7 RNA and cDNA samples as templates were also negative, as were specific PCRs of heterologous transfected cells (For example: PCR for *omp25* conducted on cDNA samples derived from pCR3.1-*fliC* transfected Cos 7 cells) (data not shown). This confirms the specificity of the PCR for the plasmid encoded *Brucella* sequences.

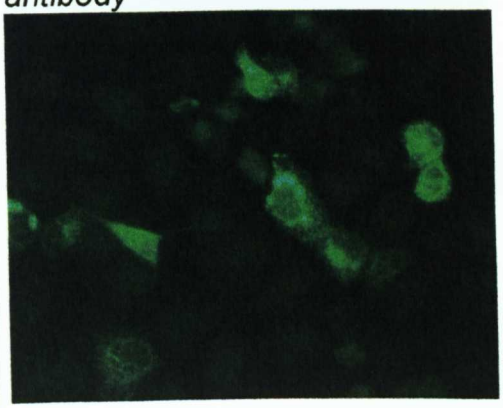
Detection of *Brucella* specific proteins by Immunofluorescence assay

Immunofluorescence assay was used to determine the production of the *Brucella* specific protein from the transfected Cos 7 cells. For the *omp25* transfected cells a monoclonal antibody specific for the Omp25 protein was used in these assays. The monoclonal antibodies AF59 (Anti-Omp25) and A43 (anti-P39) were kindly donated by Prof Jean-Jacques Letesson (Namur, Belgium). For other candidates specific sera was unavailable and therefore anti-*Brucella* polyclonal sera was used.

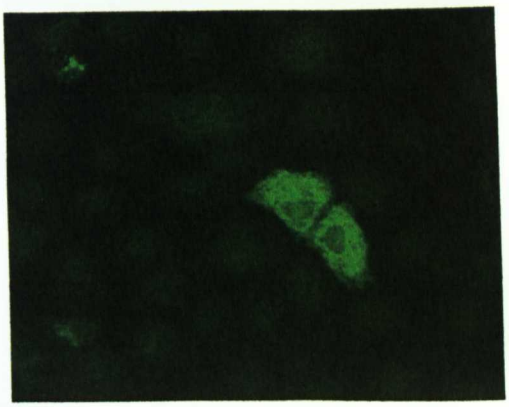
The findings of these studies are summarised in figures 4.6.1 to 4.6.6.

Figure 4.6.1: IFA results for pTarget-omp25 and pCR3.1-omp25 transfected cells and controls, using monoclonal antibody (AF59) to detect Omp25.

(a) *p-TargeT-omp25 transfected cells, detected using AF59 antibody and Anti-mouse Ig FITC labelled detection antibody*



(b) pCR3.1-omp25 transfected cells detected using AF59 antibody and Anti-mouse IgFITC labelled detection antibody



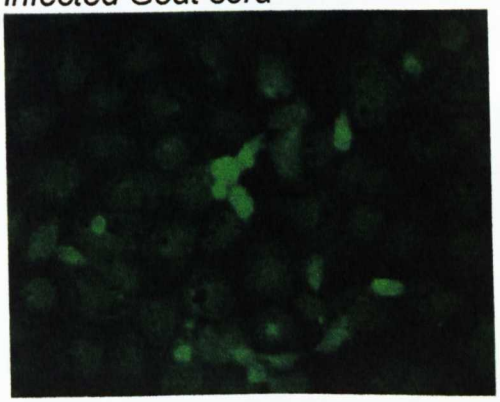
(c) pcDNA3.1 transfected cells, detected using AF59 antibody and Anti-mouse Ig FITC labelled detection antibody

(d) pCDNA3.1 transfected Cos7 cells strained with A43 antibody and Anti-mouse Ig FITC labelled detection antibody

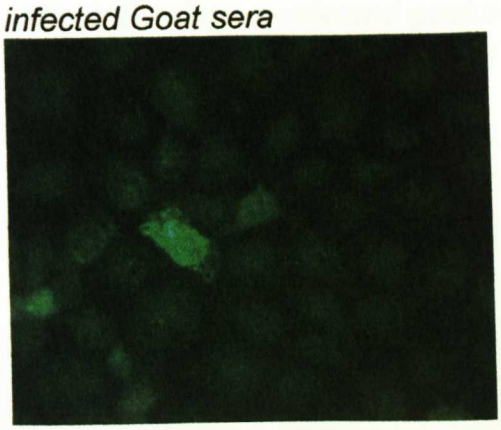
Figure 4.6.1(a - d) shows FITC labelled Omp25 in the cells of the pCR3.1-omp25 and pTargeT-omp25 transfected cells (Images (a) and (b)), and the absence of any labelling in pcDNA3.1 transfected cells using equivalent antibody reactions (c). Picture (d) indicates the lack of reaction seen when non-specific monoclonal antibodies are used as the detection reagent. Together these data indicate the presence of the Omp25 protein in the transfected Cos7 cells and therefore demonstrate that expression of the Brucella specific protein from the DNA vaccine can occur within eukaryotic cells in vitro. Approximately 2 to 3 positive (labelled) cells were detected in each field of view for cells transfected with either construct, suggesting equivalent levels of expression of the antigen from both pCR3.1 and pTargeT vectors.

Figure 4.6.2: IFA results for pTarget-omp25 and pCR3.1-omp25 transfected cells and controls, using B. melitensis infected goat sera to detect OMP25.

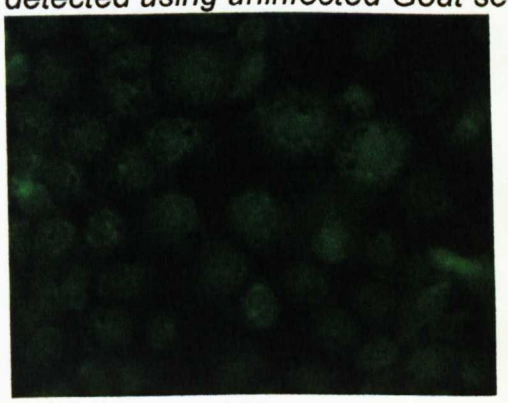
(e) pTargeT-omp25 transfected cells, detected using Brucella melitensis infected Goat sera



(f) pCR3.1-omp25 transfected cells, detected using Brucella melitensis infected Goat sera



(g) pTargeT-omp25 transfected cells, detected using uninfected Goat sera



(h) pCR3.1-omp25 transfected cells, detected using un-infected Goat sera

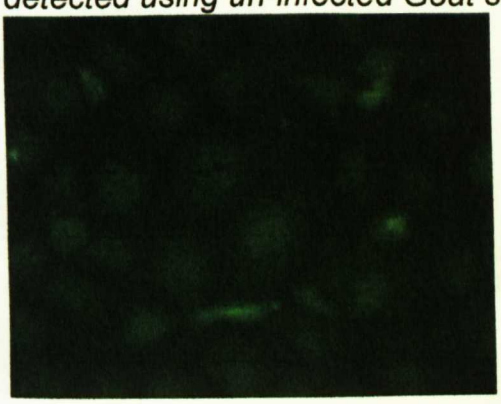
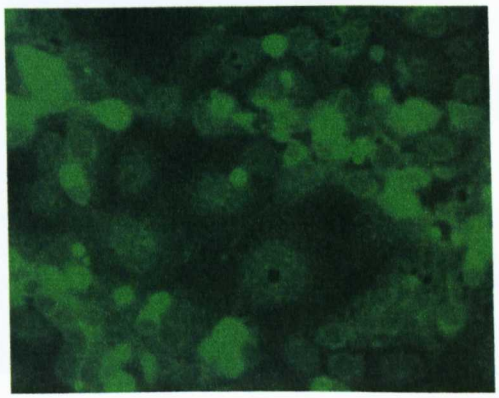


Figure 4.6.2 (e – h) demonstrated that expression of Omp25 could be detected from the cells that were transfected with the omp25 based constructs, using the monoclonal anti-Omp25 reagent. Figure 4.6.2 (e – h) illustrates that detection is also possible using the serum of an infected goat (anti-Brucella sera) as the detection antibody, although the sensitivity of this particular detection system is less than when using the specific monoclonal reagent. In transfected cell samples at 48 hours post transfection between 1 and 2 clearly labelled cells were detected in an average field of view (at X 400 magnification), corroborating the data from the monoclonal antibody investigations, but strong background reactions were observed reducing the sensitivity of the reaction in comparison to that of the monoclonal study. Nevertheless in optimised assays protein was not detected in the untransfected or control cells indicating that the detected protein was a specific product of the transfected plasmids. The optimised anti-Brucella sera assay was then used to assess the cells transfected with the other DNA vaccines.

Figure 4.6.3: IFA results for pCR3.1- ialB transfected cells and controls, using B. melitensis infected goat sera to detect the presence of Brucella specific protein (Invasion protein B)

(a) *pCR3.1-IalB transfected cells, detected using Brucella melitensis infected goat sera and anti-sheep/goat FITC labelled detection antibody*

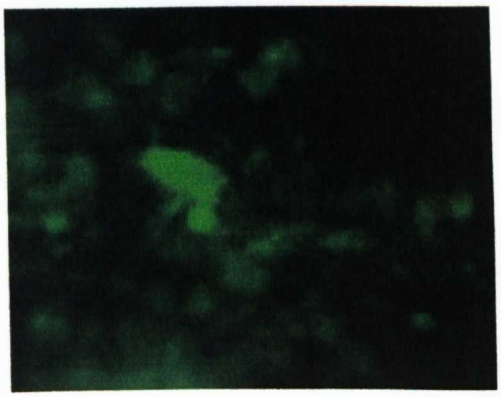


(b) pCR3.1-IalB transfected cells detected using un-infected goat sera and anti-sheep/goat FITC labelled detection antibody

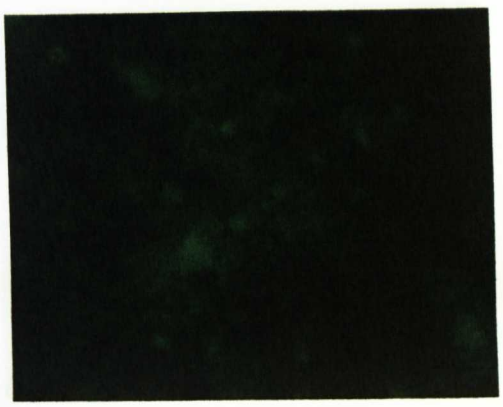
Figure 4.6.3 demonstrates clear staining of transfected cells, achieved through binding of the Brucella specific antisera and IalB protein expressed by the pCR3.1- ialB transfected cells. The background staining in this reaction is particularly high, and a clear cellular location of the proteins cannot be determined. The stain seems localised to the periphery of the cells or within cellular vesicles. Staining of this nature was restricted to cells transfected with the pCR3.1-IalB plasmid, and was not witnessed for identically processed untransfected cells or cells transfected with other expression capable plasmids (data not shown). Furthermore staining of this kind was not seen for the transfected cells with the negative control sera (Image (b)). This effect can therefore be attributed to the presence of the pCR3.1- ialB plasmid, and expression of the encoded protein product.

Figure 4.6.4: IFA results for pCR3.1-acvB transfected cells and controls, using B. melitensis infected goat sera to detect the presence of Brucella specific protein (AcvB)

(a) *pCR3.1-acvb transfected cells, detected using Brucella melitensis infected goat sera and anti-sheep/goat FITC labelled detection antibody*



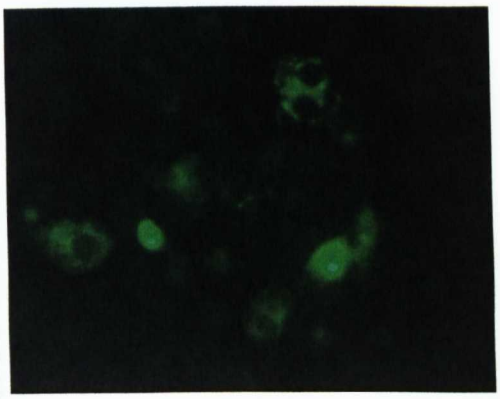
(b) pCR3.1-acvb transfected cells detected using un-infected goat sera and anti-sheep/goat FITC labelled detection antibody



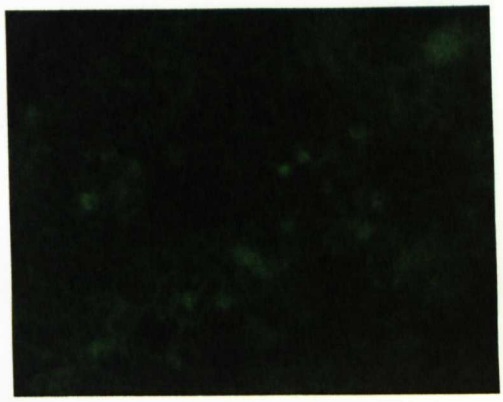
Results for the transfection and IFA of the pCR3.1-acvB construct (Figure 4.6.4) show a single brightly labelled cell within a field of view containing a number of diffusely stained cells. Staining distinct from the background was scarce but appeared specific to the pCR3.1-acvB transfected cell preparations. Brightly labelled cells were detected at a rate of one in every 5 –6 viewing fields (i.e.: 0.2 per field of view). This result reflects either a low transfection efficiency for this particular plasmid, a reduced level of expression compared to the other pCR3.1 based vaccines, or a low level of antibodies specific to the AcvB protein present in the B. melitensis detection sera leading to a low sensitivity assay.

Figure 4.6.5: IFA results for pCR3.1-frpB transfected cells and controls, using B. melitensis infected goat sera to detect the presence of Brucella specific protein (FrpB)

(a) *pCR3.1-frpB transfected cells, detected using Brucella melitensis infected goat sera and anti-sheep/goat FITC labelled detection antibody*



(b) pCR3.1-frpB transfected cells detected using un-infected goat sera and anti-sheep/goat FITC labelled detection antibody

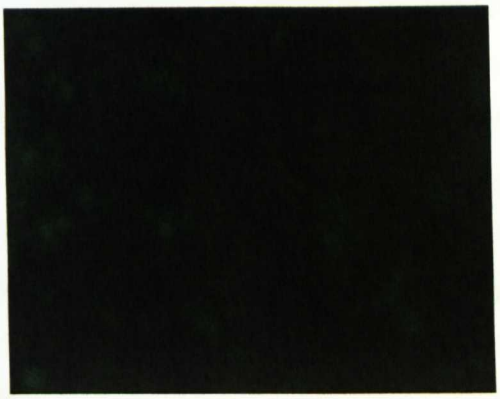


For the pCR3.1-frpB transfected cells specific staining was detected, indicating the presence of a Brucella sera reactive protein, thought to be the FrpB protein, in the cell preparation (image (a)). Strong staining was evident in the pCR3.1-frpB transfected cell cultures but absent from the untransfected cells stained with the same antibody (data not shown), or transfected cells stained with a non-Brucella reactive sheep sera (image (b)).

Figure 4.6.6: IFA results for pCR3.1-fliC transfected cells and controls, using B. melitensis infected goat sera to detect the presence of Brucella specific protein (Flagellin)

(a) *pCR3.1-fliC transfected cells, detected using Brucella melitensis infected goat sera and anti-sheep/goat FITC labelled detection antibody*

(b) pCR3.1-fliC transfected cells detected using un-infected goat sera and anti-sheep/goat FITC labelled detection antibody



For the fliC transfected cells, specific staining was not detected using the anti-Brucella sera (Image (a)). This suggests that either the cell preparation does not contain Brucella specific proteins, or that proteins are present but specific anti-flagellin reactive antibodies are absent from the Anti-Brucella sera. Therefore these data do not preclude the possibility of flagellin production from the transfected cells, but merely indicate that the Anti-Brucella IFA is unsuitable for use with this particular construct.

Overall, IFA data demonstrates Brucella specific protein production from each of the candidate vaccines (with the exception of FliC production from pCR3.1-*fliC*) following transfection of Cos7 monolayers with the plasmid constructs.

4.5: Discussion

The data discussed in this chapter describes the development of the vaccine constructs. Six DNA vaccines were constructed based upon five selected candidate antigens. Expression was assessed for each of these candidates by specific RT-PCR and IFA. These studies were necessary to confirm that the eukaryotic expression plasmids were functional prior to use in *in vivo* studies.

RT-PCR was used to demonstrate the presence of candidate specific mRNA transcripts from Cos 7 cells at 24 - 48 hours post transfection. Detection of specific mRNA transcripts indicated that the expression process had been initiated, and that the plasmid constructions were functional in this cell culture

setting. Although this result does not confirm expression of the actual encoded protein, it does indicate that all of the constructs are able to generate relatively stable specific mRNA signals within a eukaryotic cell host. Translation of the mRNA species into protein is then under control of the host cell protein synthesis apparatus.

Detection of the actual protein was considerably more difficult to achieve as specific immunological reagents for the detection of the novel candidates were unavailable with the exception of Omp25. For Omp25, a monoclonal antibody (AF59) was donated by Prof. J.J.Letesson (FUNDP, Namur, Belgium), facilitating accurate detection of the protein from transfected Cos 7 cells. Using this specific reagent it was possible to confirm expression from pCR3.1-*omp25* transfected cells. These *omp25* transfected cells were then used to optimise an assay in which sera from *Brucella* infected goats (anti-*Brucella* sera) was used to detect protein expression in IFA. The *B. melitensis* infected goat sera, which is currently standardised for use as the positive control reagent for the diagnostic *Brucella* iELISA, was shown to contain antibodies that react with a protein of 22- 26 kDa molecular weight in native *B. melitensis* 16M antigen preparation in western blot, and also with the recombinant Omp25-GST protein (data not shown), thereby indicating the suitability of this serum for detection of the Omp25 protein in transfected cells. An IFA was then optimised that was able to confirm the production of a *Brucella* specific protein by the pCR3.1-*omp25* and pTargeT-*omp25* transfected Cos 7 cells, although the reaction was not as distinct as that seen with the monoclonal antibody reagents. Heavy non-specific background staining of cells was encountered when using sheep and goat sera (both *Brucella* infected and the uninfected control) at high concentrations in the assay resulting in poor sensitivity. Consequently, the final sensitivity of the Anti-*Brucella* sera IFA for the detection of Omp25 was reduced in comparison to the monoclonal antibody reaction. However, with both assays the presence of the FITC labelled protein within transfected Cos 7 cells was clearly visible, supporting the data from the RT-PCR study by confirming production of the protein from the DNA vaccines.

The conditions optimised for assessment of Omp25 expression using Anti-*Brucella* sera were used to assess expression from the other candidate antigens by IFA. The suitability of the sera for detection of the putative invasion protein (IalB) was demonstrated using this sera in immunoblotting (data not shown). The presence of *Brucella* specific proteins was demonstrated in Cos7 cells transfected with pCR3.1-*ialB* following reaction with *Brucella* infected goat antisera. Staining of the transfected Cos 7 cells showed brightly labelled vesicles within cells against a diffusely stained background. Repeated analysis indicated that this effect was specific and reproducible for this construct. One possible explanation for this effect is that the expressed protein is packaged within vesicles for export or secretion from the cells. The strongest staining can be seen within the vesicles and the associated with cell membranes, where concentrated protein is suspected to be present. The diffuse staining surrounding the cells is attributed to the release of soluble protein from the cells, which remains associated with the membrane surfaces. Support for the possibility of protein secretion was obtained during the development of purification techniques for the recombinant IalB-GST and IalB-His fusion proteins. It was determined that the protein was exported from the *E. coli* host cell, and that the removal of the fusion tag and secretion of the IalB protein was mediated through the *ialB* gene (see appendix 1 for details). From this data it appears that this secretion mechanism is also active within the eukaryotic host cells. Further studies that were not considered within the scope of this thesis would be necessary to confirm this hypothesis. For the purposes of this investigation, the demonstration of the specific RT-PCR result from this pCR3.1-*ialB* construct, and the demonstration of a specific but non-quantifiable protein staining effect in IFA was considered sufficient to verify the expression capabilities of the vaccine.

Transcription of *acvB* in pCR3.1-acvB transfected cells was conclusively demonstrated by RT-PCR studies. However, the lack of specific anti-AcvB reagents made the detection of the protein difficult. The anti-*Brucella* IFA showed weak specific staining of a relatively low number of cells per slide. The limited number of specifically stained cells may be attributed to a poor transfection or expression capacity for this construct, or also influenced by the

limited sensitivity of the IFA. Anti-*Brucella* sera was found to react poorly with recombinant AcvB protein in crude *E. coli* lysates (data not shown), and thus a low concentration of AcvB specific immunoglobulins was expected to be present in the sera resulting in a low sensitivity IFA.

For the pCR3.1-*frpB* construct, RT-PCR results indicated specific transcription of the *Brucella frpB* sequence. IFA data supported the conclusion that the FrpB protein was expressed from the DNA vaccine by showing specific staining of transfected cells. Under the conditions used in this assay expression was detected readily and reproducibly.

Evidence of expression from the pCR3.1-*fliC* constructs was only demonstrable through RT-PCR. IFA was attempted on cells transfected with this construct but failed to detect any proteins reacting with the *Brucella* specific serum, indicating either the absence of the protein in the Cos7 cell samples, or the absence of Flagellin reactive antibodies in the *Brucella* specific antisera. The lack of anti-flagellin antibodies in *Brucella* antisera was corroborated by studies with recombinant FliC protein, whereby a convincing reaction was not detected when the anti-*Brucella* sera was tested for reactivity against recombinant Flagellin-His protein by western blotting (data not shown). These data suggest that the detection of the flagellin protein from the Cos7 cells using Anti-*Brucella* sera is unlikely if not impossible. Thus, the RT-PCR data indicate functional transcription of the *fliC* gene from transfected mammalian cells, but the presence of the corresponding protein could not be confirmed using the reagents available for IFA.

In summary, these data indicated that each DNA vaccine construct was capable to generating *Brucella* gene specific transcripts from appropriately transfected mammalian cells. IFA was able to provide direct evidence of *Brucella* specific protein production from five of the six DNA vaccine plasmids.

Production of the *Brucella* specific protein by vaccine transfected cells is an essential first step in the DNA vaccination process. A number of authors have endorsed the use of plasmids that promote high levels of antigen expression in

order to generate greater magnitude responses (for review see Garmory *et al*, 2003). The efficiency and quantity of protein production will undoubtedly influence the development of the specific immune response, and thus high-level expression *in vitro* may be indicative of a good vaccine candidate. However, since the specificity and sensitivity of the anti-*Brucella* sera IFA was optimised with respect to one antigen (Omp25), in this investigation, it is not possible to accurately compare the efficiency of protein expression between constructs using the techniques described herein. Hence, it is difficult to determine if any of the constructs are superior or inferior in the expression of protein. This work demonstrates only that candidate antigen expression is possible, and thus indicates that vaccines are able to produce the *Brucella* proteins thereby exposing them for interaction with the host.

It should be noted that a number of further factors beyond the scope of this investigation have been shown as important for the efficient generation of proteins from DNA vaccine constructs. These include a requirement for codon optimisation and the influence of varying post-translational modifications between the eukaryotic host cells and the original bacterial systems. During the construct design, the *Brucella* gene sequences were assessed to confirm the absence of eukaryotic termination signals in the gene sequence, but codon optimisation and identification and elimination of potential glycosylation motifs was not undertaken. This does not appear to have prohibited transcription in this setting, although it is possible that due to sub-optimal gene sequences, the production of the protein is affected during the mRNA processing, or translation, and truncated or exotic forms of the protein may be produced. Furthermore, the folding of the encoded polypeptide sequence into the quaternary structure of the mature protein may have been aberrant, or post-translational modifications that do not normally take place in *Brucella* may have occurred, rendering the protein conformationally dissimilar to the native *Brucella* proteins. Such sub-optimal processing could result in the generation of proteins or protein fragments that cannot be detected by antibodies raised against conformational epitopes of native *Brucella* proteins. With this in mind it should be noted that the absence of staining (e.g.: from *fliC* transfected cells), or comparatively weak staining (e.g.: from *acvB* transfected cells) with anti-*Brucella* sera, might not necessarily

indicate poorer levels of protein expression. On the other hand, generation of proteins, which are structurally distinct from native proteins, may limit the potential of the vaccine to evoke immune responses against potentially important conformational epitopes. The possible generation of antigenically restricted responses and the effect that such restriction may have on the development of protective immunity cannot be judged from these *in vitro* investigations, but may become important in subsequent *in vivo* investigations.

In summary six novel DNA vaccines, comprising five candidate antigens were produced. Each construct was checked to determine the identity of the *Brucella* gene through sequencing across the plasmid insertion site. The ability to express the encoded *Brucella* protein was verified through specific RT-PCR for all vaccines, and supported by IFA studies for five out of six of the vaccines. These studies provided essential evidence to show that the vaccine plasmids were appropriate for further study.

Chapter 5: ASSESSMENT OF THE PROTECTIVE ACTIVITY OF THE NOVEL DNA VACCINES IN THE MURINE BRUCELLOSIS INFECTION MODEL

5.1: Summary

Following construction of six novel DNA vaccines, analysis of their protective efficacy was performed. A laboratory animal model of protection was selected and refined to permit an initial assessment of protective efficacy. A BALB/c mouse model, involving challenge with approximately 1 x 10^4 CFU B. melitensis strain 16M with assessment of Brucella numbers in the spleen at day 15 ± 1 post-challenge was determined to be optimal.

Immunogenicity of the vaccines was determined through an ELISA based upon B. melitensis 16M antigen. Specific antibodies were detected following p-omp25 [1], p-omp25 [2] p-ialB, or p-acvB DNA vaccination. Protective efficacy was determined by comparison of the recovery of viable Brucella from the spleens of the vaccinated and control groups. Data indicated that the vaccines based upon the putative invasion protein B (IalB) antigen and the 25 kDa outer membrane protein (Omp25) were able to impart significant protective activity. Other candidates were not significantly protective.

5.2: Introduction

The protective effect of a *Brucella* vaccine can defined in terms of either the relative number of vaccinated animals protected from developing disease, or the ability of those animals to control the bacterial replication and persistence *in vivo*, following challenge. The method of choice will depend upon the pathogen, presentation of clinical disease symptoms, and the host system under investigation.

Brucellosis in livestock is a disease associated with abortion. The *Brucella* organisms colonise the reproductive tract of the infected animal and the associated lymphoid tissues. Clinical indications of infection in non-pregnant animals are not detectable. Thus determining vaccine potency and efficacy in the ruminant host ultimately requires the use of an abortive challenge model, in which pregnant vaccinates are challenged with virulent *Brucella* and the protection is correlated with the effects on normal full-term delivery and survival of the foetuses. Since all significant *Brucella* spp., are ACDP category III pathogens, and work in the UK is also restricted by DEFRA SAPO (1998) regulations, such studies require containment level III laboratories and suitable large animal accommodation facilities. As such these are difficult and expensive to perform and there are ethical considerations regarding the use of pregnant animals and late term foetuses in experimental settings. In addition, the gestation period for a sheep is 147 days and the abortive nature of brucellosis is not seen until the final trimester of the pregnancy, making any study a protracted one. Therefore, for both practical and ethical reasons small animal models have been developed that allow faster evaluation of vaccine candidates.

Development of animal models that accurately reflect any disease in a separate and dissimilar host is a difficult task. With brucellosis the broad host specificity (with restricted preferences), generalised non-specific symptomology and lack of overt clinical symptoms further complicate the issue. A number of laboratory animal models of brucellosis have been described in the literature: different models are useful for different purposes.

The rabbit, rat, hamster and gerbil are widely used laboratory animal species, but their use in brucellosis research has been limited. Rats (*Rattus spp.*) are considered relatively resistant to *Brucella* infection, Golden or Syrian Hamsters *(Mesocricetus auratus)* are susceptible and although they have been used in the past they are not recommended as models because of a high degree of variation in individual animal susceptibility. The rabbit (*Oryctolagus cuniculus*) is only partially susceptible to *Brucella* infection, and variation between individuals is significant. Pregnant rabbits, rabbits in a state of pseudopregnancy or

progesterone treated rabbits show an increased susceptibility to *Brucella* infection, but no evidence of uterine infection. Only very limited data is available on the use of gerbils (*Meriones unguiculatus)* in brucellosis research. (Garcia-Carillo, 1990).

Guinea pigs (*Cavia porcellus*) were widely used in early brucellosis work, and are considered to be among the most susceptible of the laboratory animals to *Brucella* infection. They are readily infected by all common routes of inoculation with low infectious doses of all the *Brucella* species except *B. neotomae* and the severity of the disease is correlated with an obvious retardation in body weight gain. Macroscopic and microscopic lesions are detected in the spleens of infected animals, and a splenic weight index (weight of spleen / body weight of animal) is considered a useful correlate for pathology. *Brucella* can be found in the semen of experimentally infected guinea pigs and experimental infection of pregnant females results in congenital transmission and abortion. Neonatal transmission of the infection has also been reported in this species. The *Brucella* colonisation of the reproductive system and abortive pathology observed in guinea pigs makes this a particularly suitable laboratory animal for the study of pathogenesis. However, a relative paucity of immunological reagents specific for the guinea pig means that any assessment of cell mediated protective immunity in this model is restricted to aspects such as lymphocyte proliferation and delayed type hypersensitivity reactions. The most frequent use of the guinea pig in brucellosis research has been in the standardisation or potency testing of the DTH antigen, Brucellin (Brucellergene™, Synbiotics Corps, France). Data regarding the specifics of the cell-mediated response in guinea pigs to *Brucella* antigens, infection, or vaccination are limited.

The mouse (*Mus musculus*) is the most widely used of all laboratory animals and is the small animal model of choice for present day brucellosis research. The availability of specific immunological reagents for mouse work and the accumulated wealth of knowledge regarding mouse immunology and genetics afford considerable practical advantages over other model hosts. In the mouse, infection with *Brucella* causes a transient bacteraemia. Upon initial infection

most of the bacteria are destroyed via innate immune mechanisms. Those that survive invade and subvert the host phagocytes, and are trafficked to the draining lymph nodes and the spleen of the infected animals. There are reports of *Brucella* spp., colonising the reproductive organs of the mouse, although the presence of the organism does not cause either abortion or foetal distress (Bosseray, 1980). Since mice do not suffer abortive disease, murine brucellosis is characterised by a systemic infection without overt clinical signs. Splenomegaly is often the only clinical indication of infection. The lack of abortive pathology in mice means that the number of organisms in the spleen at intervals post-infection is more readily used as a read-out of pathology and immunity. Enumeration of *Brucella* retrieved from the spleens of mice following infection indicates there is a brief lag phase in growth after inoculation followed by a period of multiplication during the first two weeks. The bacterial numbers are maximal after 10 – 15 days, and then decline in a manner corresponding to the chronic or latent phase of infection in other hosts. The time to clearance varies depending upon the virulence and dose of the infecting *Brucella* organisms and the immune status of the host animal. Infected mice are resistant to reinfection when challenged. Although the infection may be chronic or protracted, it is not lethal and the mouse eventually clears the infection.

Plommet and Bosseray conducted many studies with the mouse model relating to vaccine development and assessment. In 1984 they proposed a standard method for the titration of novel vaccines against a reference vaccine using the mouse model. Over the years, modifications have been made to the procedures and standardisation between laboratories is rudimentary, but use of the mouse model for the assessment and comparison of vaccine potency is well documented. The procedures are described in the OIE manual of vaccines and standards (OIE, 2000). Assessment of the vaccine strains *B. melitensis* Rev. 1 and *B. abortus* S19, are described. In both cases vaccine strains are administered as a sub-cutaneous (s/c) inoculation of approximately 2 x 10^5 CFU per mouse at 30 – 45 days prior to challenge with a virulent strain. The challenge dose, a virulent *B. abortus* strain such as 2308 or 544, is given intraperitoneally as a dose of ~ 1 X 10^6 CFU and protective efficacy is estimated through enumeration of the viable challenge strain *Brucella* in the spleens of the

mice. In the case of the Rev.1 vaccination, heterologous strain challenge is considered acceptable for the quality assurance of the vaccine in the animal model, although it should be noted that optimal protection against heterologous strain challenge does not occur in the natural host (Schurig *et al,* 2002).

Overall, the mouse is a useful infection model because it allows the virulence of the live vaccine strains to be estimated and permits assessment of the impact of immunity on the progress of the disease. There is a correlation between the persistence of a *Brucella* strain in the mouse and it's virulence in the natural host: attenuated strains are eliminated more rapidly in either host. Protective immunity is most clearly demonstrated as a reduction in the number of viable *Brucella* retrieved from the spleens of immune mice compared to non-immune mice at 15 days post-challenge. The ability of vaccines to promote clearance of *Brucella* in a murine model corresponds well to its protective efficacy in target species.

Numerous laboratory strains of mouse exist, each with particular assets for use in vaccine research. Importantly, there is variation in susceptibility between different laboratory strains of mice. The C57/BL-6 mouse is considered relatively resistant to brucellosis in comparison to the BALB/c mouse. (Baldwin and Parent 2002). In the investigations described in this thesis, the BALB/c mouse was selected as the animal model of choice. Previous investigations using this mouse model at VLA Weybridge (N.Commander, unpublished observations) had suggested that protection mediated by DNA vaccination studies were not reproducibly efficacious against heterologous strain challenge, and therefore a *B. melitensis* 16M challenge was considered most appropriate for the assessment of vaccines based upon putative antigens in the *B. melitensis* 16M genome. Rev.1 vaccination was selected as a positive control for effective immunity as this is the currently recommended gold standard vaccine for protection of small ruminants against *B. melitensis*.

The aim of these investigations was to refine and standardise the BALB/c mouse model for analysis of anti-*Brucella* DNA vaccines and to utilise the model to identify the most promising DNA vaccine candidates for further analysis.

5.3: Experimental procedures and aims

The DNA vaccines developed in Chapter 4 were assessed for protective efficacy in a BALB/c mouse model of brucellosis.

Two separate studies were conducted to estimate the protective efficacy of the six novel DNA vaccines against challenge with virulent *Brucella melitensis*, in the BALB/c mouse model. The first study involved the challenge of vaccinated mice with a relatively high dose (~1 X 10^6 CFU per mouse) of *B. melitensis* 16M, and the second study involved challenge with a lower dose of this strain (~1 X 10^4 CFU per mouse). The high dose challenge value was selected based upon the experimental regime used in previous *B. melitensis* and *B. suis* challenge studies in our laboratory (Mansour, E.A., 2003). The low dose challenge dose was selected based upon recommendations for the determination of residual virulence and immunogenicity of the Rev.1 control vaccine cited in the O.I.E. manual of laboratory standards and diagnostics (O.I.E., 2004). The general vaccination, challenge and sampling schedule for both studies is provided in figure 5.a. A summary of the vaccines and controls used in these studies is provided in Table 5.1.

The schedule of four 100 μl intramuscular (i/m) DNA vaccinations given at three week intervals was adapted from that described by Chambers *et al*, 2000. A non-vaccinated group of mice (PBS inoculated) was used as a non-protected control. Two groups of mice were inoculated with the *B. melitensis* Rev.1 vaccine strain: Group Rev.1 (V1) received inoculation with 2 X 10^5 CFU viable Rev.1 coincident to the time of the first DNA vaccination. The purpose of this group was to permit monitoring of the development of the *Brucella* specific immune response in comparison to that observed from DNA vaccinated mice. Group Rev.1 (V4) was inoculated at the time of the fourth DNA vaccination in accordance with the recommended protocols for measuring vaccine efficacy (O.I.E., 2004). All groups of mice were challenged at 30 days post-vaccination. Protective efficacy was measured in terms of the number of *Brucella* per spleen harvested from mice at 15 ± 1 days post-challenge.

To assess the immunogenicity of the vaccines serum was collected from all mice at three week intervals. 16M antigen ELISA was used to determine *Brucella* specific antibody in the sera of the mice. Specific IgM, IgG1, and IgG2a isotype responses were assessed at week two, five, eight and eleven of the trials.

The general outline for the experimental schedule is provided in figure 5.1.

Figure 5.1: General outline of the experimental schedule for the vaccine efficacy trials

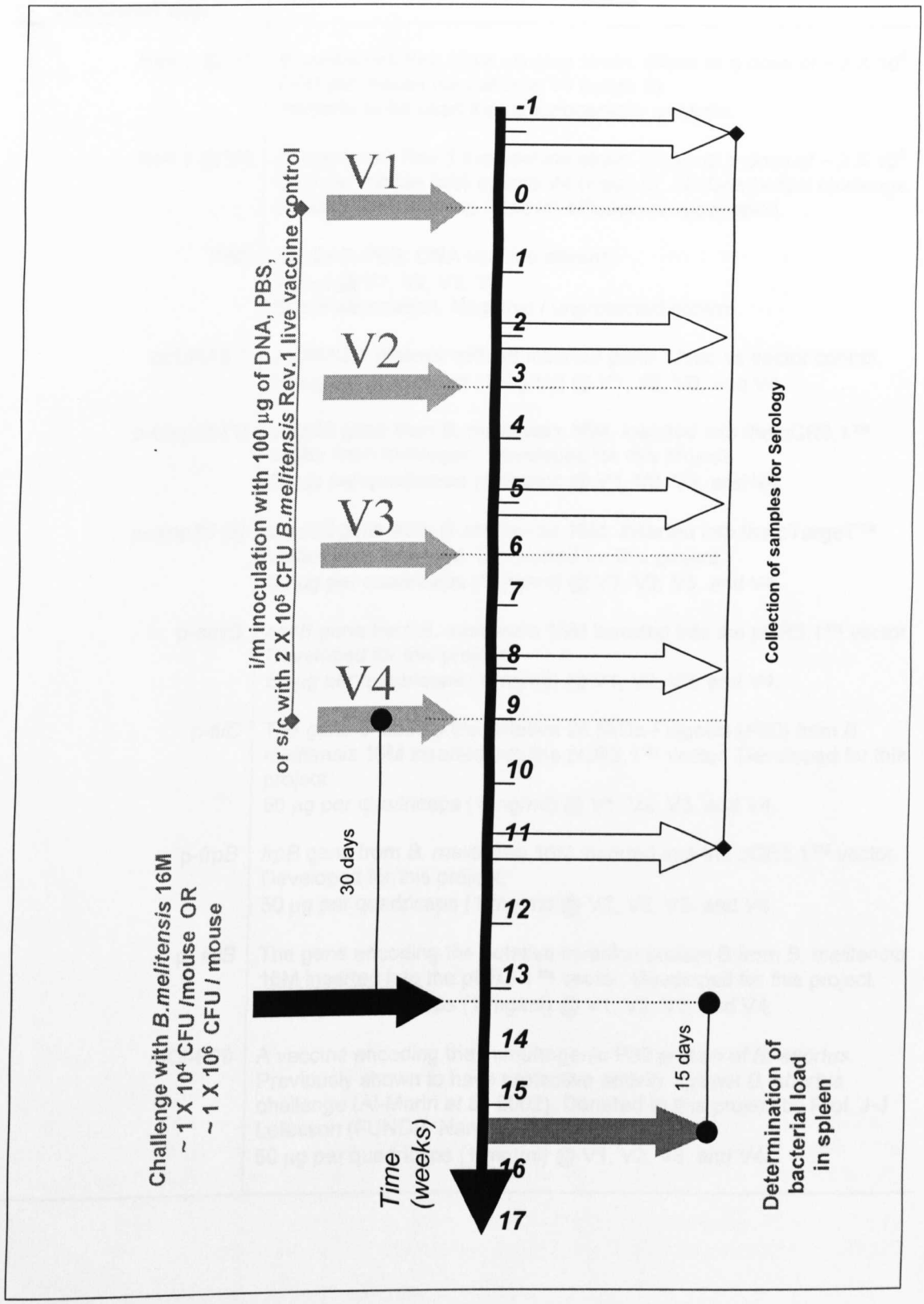


Table 5.1: Vaccines and controls used in this study

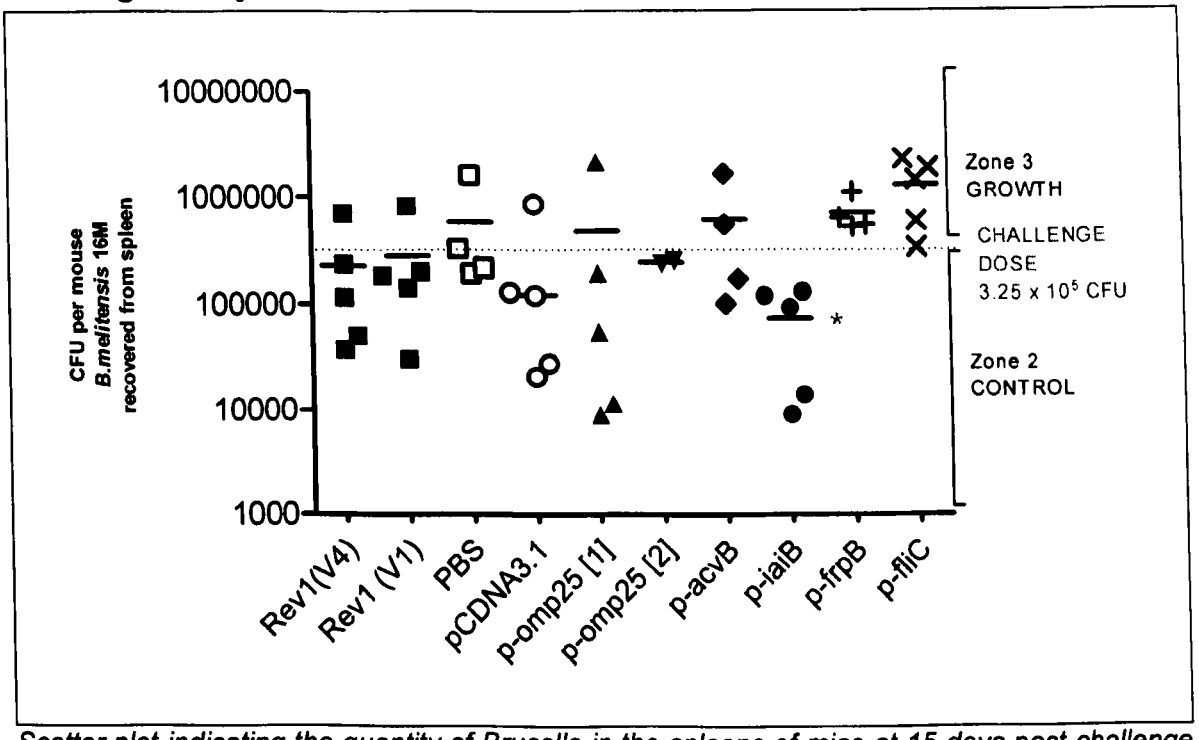
Group identity: Inoculated with...	Details
Rev.1 @ V1	*B. melitensis* Rev.1 live vaccine strain. Given at a dose of ~ 2 X 10^5 CFU per mouse (s/c) at time V1 (week 0). Primarily to be used for immunogenicity analysis.
Rev.1 @ V4	*B. melitensis* Rev.1 live vaccine strain. Given at a dose of ~ 2 X 10^5 CFU per mouse (s/c) at time V4 (week 9): 30 days before challenge. Positive control group / Known efficacious vaccination.
PBS	Sterile D-PBS: DNA vaccine diluent. 100 µl @ V1, V2, V3, V4. Sham vaccination. Negative / unprotected control.
pcDNA3.1	pcDNA3.1 plasmid without inserted gene. Used as vector control. 50 µg per quadriceps (1 mg/ml) @ V1, V2, V3, and V4.
p-*omp25 [1]*	*omp25* gene from *B. melitensis* 16M, inserted into the pCR3.1™ vector from Invitrogen. Developed for this project. 50 µg per quadriceps (1 mg/ml) @ V1, V2, V3, and V4.
p-*omp25 [2]*	*omp25* gene from *B. melitensis* 16M, inserted into the pTargeT™ vector from Promega. Developed for this project. 50 µg per quadriceps (1 mg/ml) @ V1, V2, V3, and V4.
p-*acvB*	*acvB* gene from *B. melitensis* 16M inserted into the pCR3.1™ vector. Developed for this project. 50 µg per quadriceps (1 mg/ml) @ V1, V2, V3, and V4.
p-*fliC*	The gene encoding the putative 28.5kDa Flagellin (*FliC*) from *B. melitensis* 16M inserted into the pCR3.1™ vector. Developed for this project. 50 µg per quadriceps (1 mg/ml) @ V1, V2, V3, and V4.
p-*frpB*	*frpB* gene from *B. melitensis* 16M inserted into the pCR3.1™ vector. Developed for this project. 50 µg per quadriceps (1 mg/ml) @ V1, V2, V3, and V4.
p- *ialB*	The gene encoding the putative invasion protein B from *B. melitensis* 16M inserted into the pCR3.1™ vector. Developed for this project. 50 µg per quadriceps (1 mg/ml) @ V1, V2, V3, and V4.
pCI-P39	A vaccine encoding the immunogenic P39 protein of *B. abortus*. Previously shown to have protective activity against *B. abortus* challenge (Al-Mariri *et al*, 2002). Donated to this project by Prof. J-J Letesson (FUNDP, Namur, Belgium) 50 µg per quadriceps (1 mg/ml) @ V1, V2, V3, and V4.

5.4: Results

Protective activity of vaccines

The protective activity of the vaccines was measured through the enumeration and comparison of viable *Brucella* recovered from the spleens of challenged mice at 15 days post-challenge. Two studies were performed using identical vaccines and control strains, but two different challenge doses: The data from the 1 X 10^6 and 1 X 10^4 challenge dose studies are summarised in figures 5.2 and 5.3.

Figure 5.2: Scatter plot showing the number of viable B. melitensis strain 16M recovered from mice at 15 ± 1 days post-challenge, in the high dose challenge study.



Scatter plot indicating the quantity of Brucella in the spleens of mice at 15 days post-challenge. Data points for individual Brucella load per mouse with a line to depict the median value. Actual vaccination doses: Rev.1 (V1) ~ 1.48 X10^5 CFU /mouse, Rev.1 (V4) ~ 1.14 X 10^5 CFU/ mouse. Challenge dose for all mice ~ 3.25 X 10^5 CFU B. melitensis16M /mouse. Zone 2: Brucella load < challenge inocula, indicating some control of bacterial replication. Zone 3: Greater Brucella load than the challenge inocula indicating active replication of the Brucella in the mouse (Not protected). Five mice per group were used in the study.
*Mann-Whitney U Test was used to compare the CFU /mouse recovered from PBS (non immune) mice and vaccinated mice. * indicates a statistically significant reduction in Brucella splenic load.*
The detection limit of the culture technique is 10 CFU per spleen.

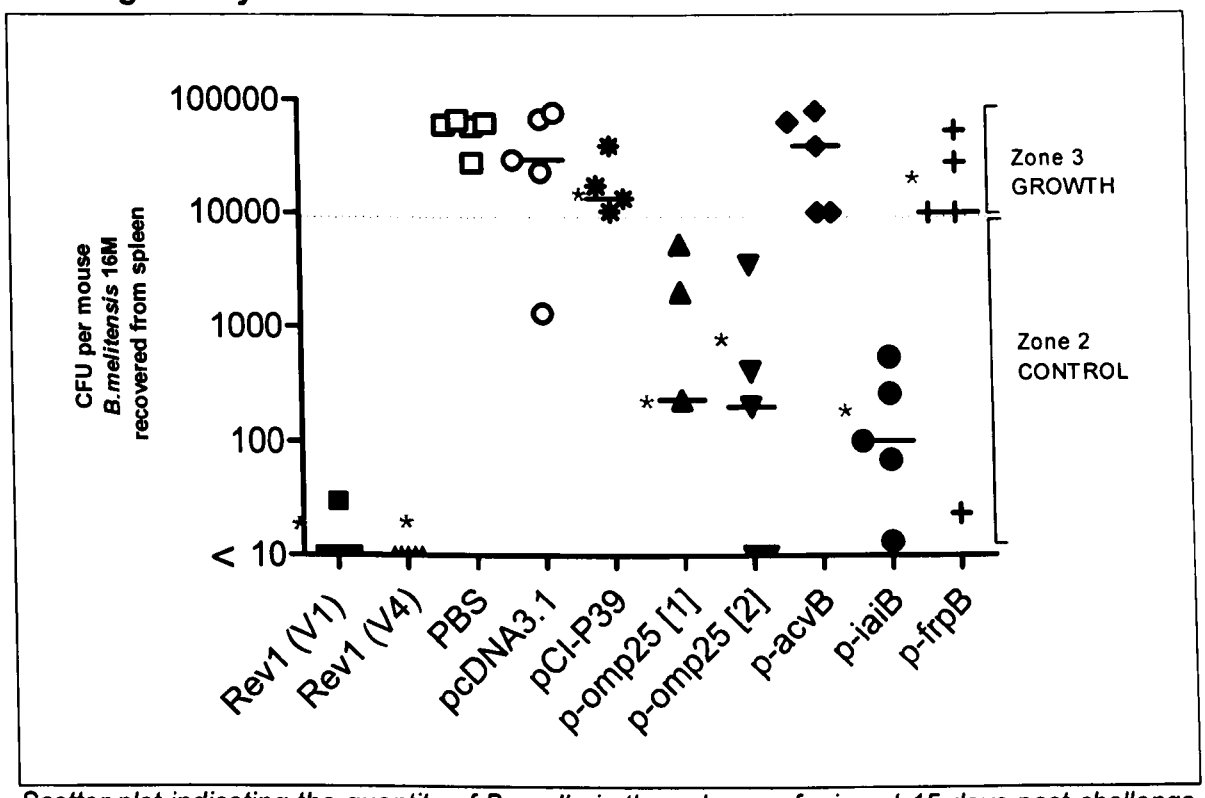
Figure 5.2., shows the number of viable *B. melitensis* 16M recovered from vaccinated mice following challenge with approximately 10^6 CFU *B. melitensis*

16M. Statistical interpretation of results using the Mann-Whitney U test to compare each vaccine group against the PBS (unprotected) inoculated controls, indicated that only one of the vaccines p-*ialB*, was able to contribute a statistically significant reduction in the *Brucella* load of challenged mice ($p = 0.0159$). The mean (± standard deviation) log *Brucella* CFU per spleen in these mice was 4.66 ± 0.49. Direct comparison of this value with the unprotected PBS controls indicates a protective effect of 0.93 protection units (Protection units = Mean Log CFU per spleen of PBS mice – Mean Log CFU of vaccinated mice). Although some mice in other groups had *Brucella* counts lower than the challenge dose, these were not statistically significant reductions compared to the PBS group ($p = 0.2857$ [Rev.1 (V4)], $p = 0.1905$ [Rev.1 (V1)], $p = 0.1111$ [pcDNA3.1], $p = 0.1905$ [p-*omp25* [1]], $p = 0.8875$ [p-*acvB*]). Statistical comparisons were not possible from the p-*omp25* [2] group because there were not enough data points from this group as only two mice survived to this point in the investigation. Importantly, significant protection is not evident in either of the Rev.1 vaccinated groups in this study. Furthermore, statistical significance is not achieved when ANOVA with Dunnetts post-test (comparing each group against PBS) is applied to the study.

It is important to note that during the course of this investigation (prior to challenge) a substantial number of mice were euthanised due to ill health. During this time, thorough investigations were conducted and evidence of extraneous infectious agents was not obtained in the affected mice. Furthermore, mice appeared to be in good health at the time of sacrifice and examination at necropsy did not reveal any macroscopic lesions in the mouse organs. A similar problem in the second experiment led to the conclusion that the cause of the morbidity was related to a mechanical injury inflicted by faulty apparatus used during the blood sampling procedure. Practices were immediately modified following this finding and no such problems have since been encountered.

The second investigation also used BALB/c mice and an identical vaccination and challenge schedule. However, for this study the challenge dose was reduced to approximately 1×10^4 CFU.

Figure 5.3: Scatter plot showing the number of viable B. melitensis strain 16M recovered from mice at 15 ± 1 days post-challenge. Low dose challenge study.



Scatter plot indicating the quantity of Brucella in the spleens of mice at 15 days post-challenge. Data shown as individual CFU per mouse with a line to depict the median value per group. Actual vaccination doses: Rev.1 (V1) ~ 0.82 X10^5 CFU /mouse, Rev.1 (V4) ~ 0.97 X 10^5 CFU/ mouse. Challenge dose for all mice ~ 0.87 X 10^5 CFU B. melitensis16M /mouse. Zone 2: Brucella load < challenge inocula, indicating some control of bacterial replication. Zone 3: Greater Brucella load than the challenge inocula indicating active replication of the Brucella in the mouse (Not protected). Five mice per group were examined.
*Mann-Whitney U test was used to compare the Brucella CFU/ mouse from the PBS (not protected) mice against the Brucella CFU /mouse of the vaccinated mice. * indicates statistical significance ($p < 0.05$). Notably $p < 0.01$ for p-ialB, p-omp25 [1] and [2], and Rev.1 [V1] and [V4] only.*

Figure 5.3 shows the number of viable *B. melitensis* 16M recovered from vaccinated mice challenged with approximately 10^4 CFU *B. melitensis* 16M.

Mann-Whitney U Test to compare the CFU /mouse recovered from PBS (non-immune) mice and vaccinated mice, indicated a statistically significant reduction in *Brucella* load in Rev.1 (V1) and (V4) vaccinated mice ($p = 0.0079$) and in mice receiving vaccination with p-*omp25* [1], p-*omp25* [2] and p- *ialB* ($p= 0.0079$). The data clearly illustrates that the Rev.1 vaccinated mice are controlling the *Brucella* infection, as there is clear significant reduction in the bacterial load compared to the non-immune groups (PBS and pcDNA3.1 inoculated groups). Rev.1 (V1) and Rev.1 (V4) vaccinated mice control *B.*

melitensis 16M replication such that this organism is not present in large numbers in the spleens of challenged mice. Rev.1 (V1) group mice consistently yield *Brucella* loads of around 10 CFU per spleen (Mean Log (± standard deviation) CFU per spleen: 1.10 ± 0.19), which forms the lower detection limit of this assay. Viable *Brucellae* were not recovered from Rev.1 (V4) vaccinated mice suggesting a reduction of *Brucella* load to below detectable levels. This protective effect can be expressed as 3.63 protection units, and > 3.7 protection units for Rev.1 (V1) and Rev.1 (V4) respectively. For the non-immune groups (pcDNA3.1 and PBS inoculated) the number of *Brucella* isolated from the spleen on Day 15 ± 1 post-challenge appears in excess of the actual challenge dose. These data points therefore fall within Zone 3 of the graph, representing uncontrolled *Brucella* replication in the naïve host.

p-*omp25* [1], p-*omp25* [2] and p-*ialB* vaccines compare favourably with the Rev.1 control vaccines in terms of protective efficacy. p-*omp25* [1] vaccinated mice contained a mean (± standard deviation) Log *Brucella* CFU per spleen of 2.14 ± 1.3, which was equivalent to 2.58 protection units in this experiment. p-*omp25* [2] vaccinated mice contained 2.10 ± 0.97 *Brucella* CFU per spleen, equivalent to 2.63 protection units, and p- *ialB* vaccination resulted in 2.02 ± 0.54 mean (± standard deviation) Log CFU per spleen or 2.7 protection units.

It is logical to assume that data points showing *Brucella* counts within zone 3 (above the level of the *Brucella* challenge dose) reflect that fact that mice have been unable to adequately control the replication of the challenge dose. However, a statistically significant difference (reduction in bacterial load) was determined comparing the *Brucella* load of the pCI-P39 and p-*frpB* groups to the PBS controls ($p = 0.0159$ and $p = 0.0317$ respectively), despite the fact that these data points are within Zone 3.

The *B. abortus* specific pCI-P39 vaccine was donated to this study. This vaccine had been previously investigated for protective efficacy against *B. abortus* challenge. In this study the vaccine was unable to adequately restrict growth of the *B. melitensis* 16M challenge dose (All data points in Zone 3), but was found to result in a significantly lower ($p = 0.0159$) *Brucella* load than the

non-immune (PBS inoculated) mice in this study. Thus indicating a possible weak protective effect from the vaccine toward control of *B. melitensis* 16M.

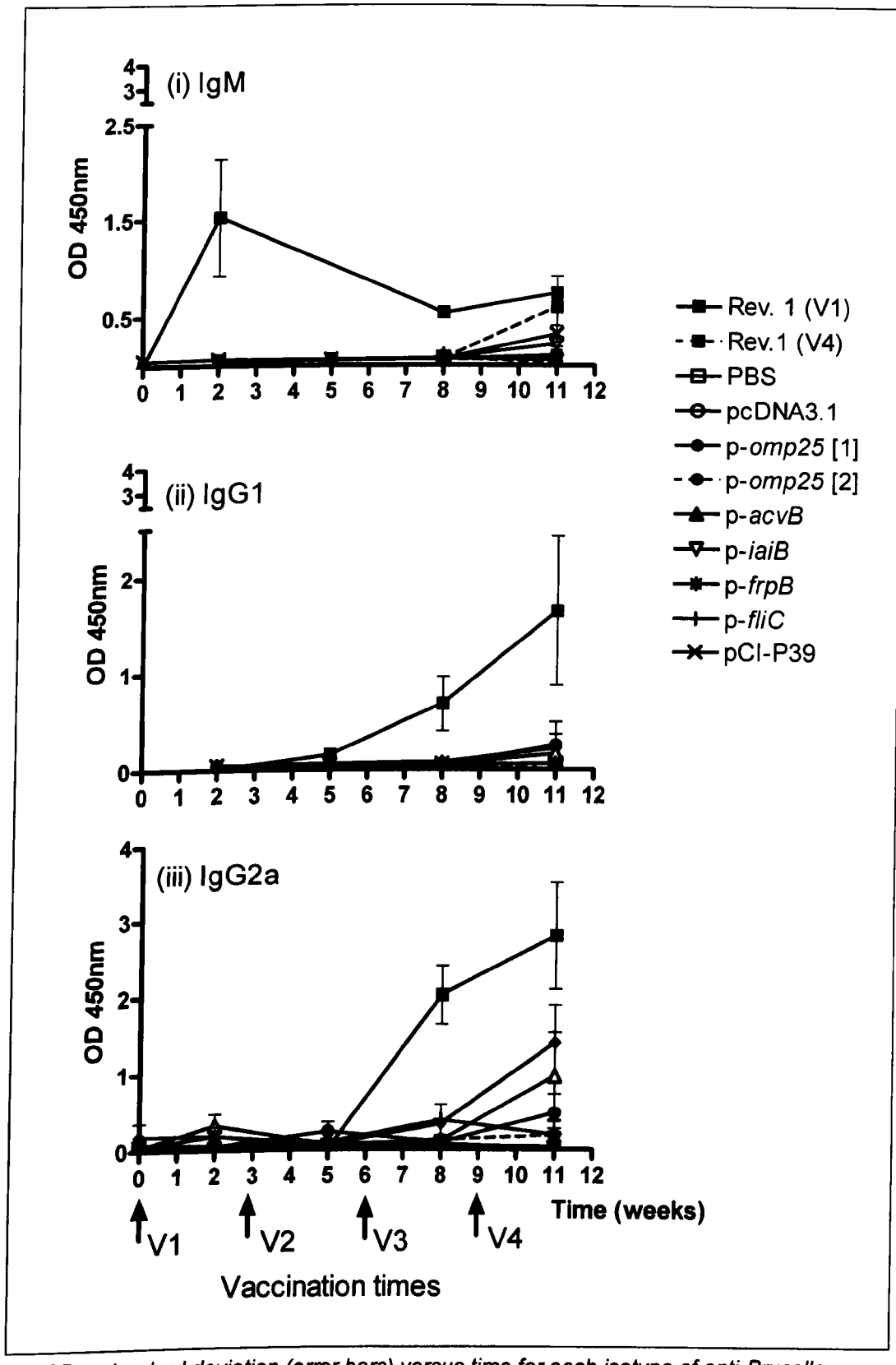
Analysis of the p-*fliC* group was not performed in this study, as the mice were euthanised prior to challenge. This was because a mouse in this group developed an illness similar to that witnessed in the high dose challenge study, and therefore to reduce the risk of transmission of any infectious agents all mice in the group were destroyed. Pathological analysis indicated no obvious cause of illness.

Serological analysis of mouse response to vaccination.

The serological response of the mice to vaccination was determined from samples collected in the low dose challenge study using the 16M antigen ELISA. Samples were considered positive when the recorded OD was greater than the mean + 2 X standard deviations of the negative control serum. Although serum was tested at dilutions of 1 / 100, 1/ 500 and 1/ 1000 (antisera / diluent), positive reactions were only detected from the 1/ 100 dilutions, and therefore only data obtained with this dilution are presented herein.

Brucella specific antibodies were detected from mice that were vaccinated with the DNA constructs p-*acvB*, p-*ialB*, p-*omp25* [1] and p-*omp25* [2]. The response of most DNA vaccinated mice was undetectable until the eleventh week of the study, corresponding to two weeks after the final vaccination. The kinetics of the *Brucella* specific antibody response are summarised in figure 5.4., which illustrates the mean IgM, IgG1, and IgG2a *Brucella* specific ELISA responses (OD).

Figure 5.4: The anti-Brucella response induced by vaccination against time



Mean OD ± standard deviation (error bars) versus time for each isotype of anti-Brucella response. The times at which mice received inoculation with the DNA vaccines (or controls) are marked as V1, V2, V3, and V4. Note Rev.1 (V1) animals were vaccinated at V1 only. Rev.1 (V4) mice were vaccinated at V4 only.

Substantial and specific IgM responses were only detected from the Rev.1 (V1) group. As expected these responses were maximal at week two and declined with time. Substantial specific IgM responses were not determined from the other groups until week eleven and then these were of lower magnitude than the corresponding Rev.1 response.

The greatest magnitude and earliest detectable IgG1 *Brucella* specific response was also detected in the Rev.1 (V1) group: specific IgG1 was evident at week five of the study, and continued to increase through to the final sampling time. At the end of the investigation, corresponding to week eleven of the study or two weeks after the fourth DNA inoculation, IgG1 antibodies were also detected from the mice inoculated with p-*acvB* or p- *ialB*.

IgG2a *Brucella* specific antibodies were detected in the sera of Rev.1 (V1) inoculated mice from week eight of the study. Detectable levels of specific IgG2a antibody were found in three of the DNA vaccinated groups of mice investigated in this study at week eleven of the study (after the fourth inoculation with DNA). The strongest mean responses were seen from the p-*acvB* vaccinated group, where all mice were determined to be seropositive at week eleven. The second highest mean OD readings were obtained from the p-*ialB* vaccinated mice. Again all animals were determined to be seropositive at week eleven of the study, although the magnitude of response was variable between individuals. The strongest reaction in this group achieved an OD value of 3.22, and the weakest 0.122. Three out of five of the p-*omp25* [1], and four out of five p-*omp25* [2] vaccinated mice were determined to be IgG2a seropositive when measured at week eleven. Notably, the difference between the p-omp25 [1] and p-omp25 [2] group responses was not considered statistically significant ($p > 0.05$) (Mann-Whitney U test, comparing mean OD from 1/100 serum dilution assay).

The proportion of seropositive mice per group at week eleven of the study is summarised in table 5.2.

Table 5.2: The percentage of mice in each group determined to be seropositive at two-weeks post vaccination (week 11).

Vaccine group	*IgM*	*IgG1*	*IgG2a*
Rev.1 (V1)	100	100	100
Rev.1 (V4)	100	0	0
PBS	0	20	0
pcDNA3.1	1	0	20
pCI-P39	80	0	20
p-*omp25* [1]	20	20	60
p-*omp25* [2]	20	0	80
p- ialB	80	80	100
p-*acvB*	20	40	100
p-*frpB*	40	0	0
p-*fliC*	20	0	20
ASSAY C/O	***0.250***	***0.151***	***0.089***

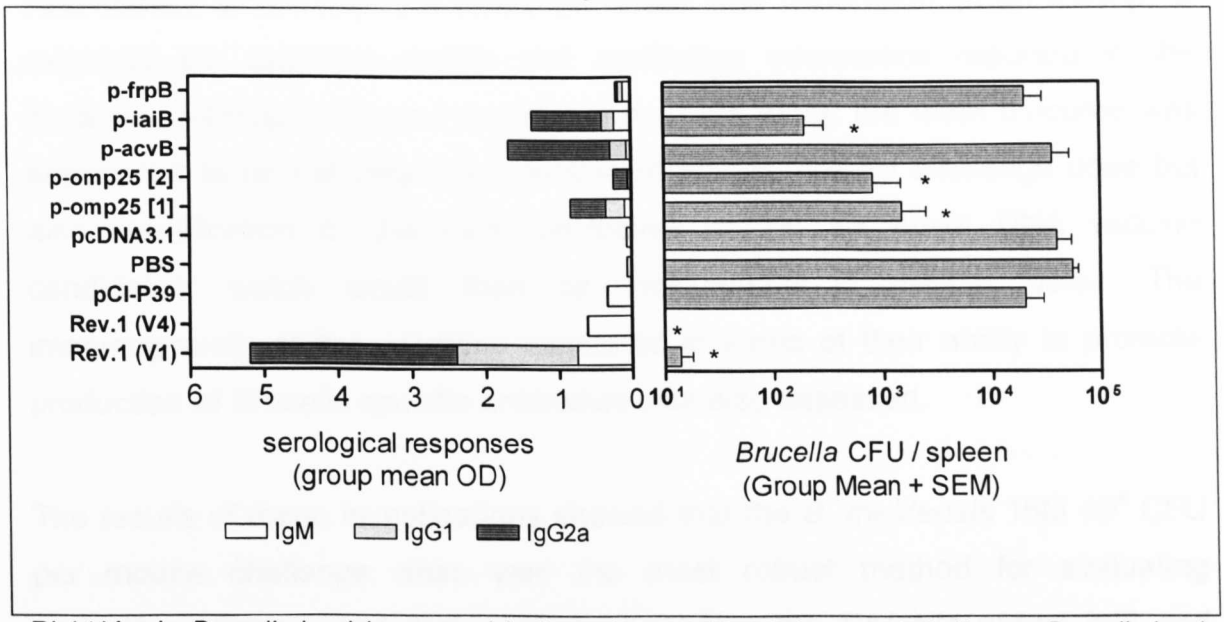
Cut-off (C/O) OD determined as mean + 2X standard deviations Negative control serum

Neither SAT nor cELISA methods showed any of the DNA vaccinated animals to be positive. Weak positive cELISA responses were detected from the Rev.1 vaccinated mice, but not from any other samples.

The relationship between antibody production and protective efficacy

There was no overall correlation between the magnitude of serological responses in ELISA (measured as OD from sera diluted 1:100) and the *Brucella* load recovered from the spleens of these mice. These data are summarised in figure 5.5.

Figure 5.5: Anti-Brucella serological responses at 2 weeks prior to challenge compared with Brucella splenic load post-challenge.



Right X axis: Brucella load (per group) in the spleen at 15 ± 1 days post challenge. Brucella load is displayed as mean of the group with error bars depicting standard error of the mean. Left X axis: OD in ELISA. IgM, IgG1 and IgG2a mean OD per group is indicated on the chart.

The graph indicates that there is not a direct relationship between the generation of anti-*Brucella* immunoglobulins and the protective effect of vaccination. Relatively strong IgG2a responses are demonstrable in the sera of p- *ialB*, p-*acvB* and p-*omp25* [1] vaccinated mice, as well as Rev.1 (V1) mice. However, whilst a protective effect is observed from p-*omp25*, p-*ialB* and Rev.1 vaccination, the p-*acvB* vaccinated mice do not appear to be protected despite the presence of antibody. Conversely, strong protection is evident in mice vaccinated with Rev.1 at time V4, although *Brucella* specific IgG1 and IgG2a are not evident at this time.

5.5: Discussion

In this investigation, a mouse model of brucellosis was used to determine whether any of the novel vaccines were able to protect mice against virulent *B. melitensis* 16M challenge. One of the principal aims of the experiment was to optimise the BALB/c mouse model to show the best distinction between protected and unprotected mice. Previous work in this laboratory had produced inconsistent results with a standard challenge of approximately 1 X 10^6 CFU per mouse, *B. melitensis* 16M used to measure the efficacy of Rev.1 or DNA

(pcDNA-62) vaccination (Commander and Spencer, unpublished observations). Assessment of two different challenge doses was undertaken in an attempt to reconcile the previous results and conflicting information reported in the literature. Although this was a major aim of the study the main outcome was anticipated to be not only the selection of an appropriate challenge dose but also identification of the most protective of the six novel DNA vaccine candidates, which would then be investigated in greater detail. The immunogenicity of the vaccines measured in terms of their ability to promote production of *Brucella* specific antibodies was also assessed.

The results of these investigations showed that the *B. melitensis* 16M 10^4 CFU per mouse challenge dose was the most robust method for evaluating protective efficacy, and that vaccines encoding the *Brucella* genes *ialB* and *omp25* were the most capable of promoting protection. The aim of this study was therefore fulfilled in clarifying the challenge model and allowing selection of two protective antigens for further study. In addition, the assessment of the specific humoral response indicated that the p-*ialB*, p-*acvB*, p-*omp25* [1] and p-*omp25* [2] vaccines were able to promote IgG2a dominated *Brucella* specific antibody in over 60% of vaccinated mice in each group after four inoculations, proving that *in vitro* expression of the encoded antigen had occurred, and that the antigen was presented appropriately for the generation of Th 1 type immune responses.

High challenge dose (3.25 X 10^5 CFU per mouse) was not found to be suitably robust for use in small-scale efficacy trials. The difference between control protected mice, i.e.: those that have been vaccinated with Rev.1 and the unprotected mice is unclear. Furthermore, statistical analysis (Bartlett's test for equal variance) reveals considerable variation ($p < 0.001$) between groups making data interpretation on this small number of samples difficult. This finding differs from other protection models using Rev.1 or S19 as control vaccines where alternative virulent challenge strains of *Brucella*, such as *B. abortus* 2308, 544 or *B. suis* 1330 at doses of approximately 1 X 10^6 CFU per mouse are frequently used without problem (OI.E, 2000, Bosseray and Plommet 1984),

and previous investigations in this laboratory that indicated that Rev.1 could protect against this challenge dose of *B. suis* 1330 or *B. melitensis* 16M (Mansour, 2003). In contrast, Bosseray and Plommet (1982) have previously noted a relative increase in virulence for a *B. melitensis* strain (H38) which was manifest as an increased initial growth rate of the bacteria following infection and a longer persistence of the strain in the model host than is observed for other *Brucella* strains. Although 16M (not H38) was used in the current study, a relative increase in the initial replication of *B. melitensis* strains compared to *B. abortus* or *B. suis* strains in the mouse model may explain the failure to observe the expected protective effect at 15 days post-challenge in this study.

An alternative hypothesis to explain the failure of the Rev.1 vaccine to protect these mice against this challenge is the possibility of sub-optimal vaccine preparation. The live Rev.1 vaccine is notorious for instability, with phase dissociation being a common source of increased attenuation leading to vaccine failure (Grillo *et al,* 2000., Bosseray, 1991). In this study Rev.1 cultures were examined carefully to ensure a preparation of entirely smooth phase colonies was used to produce the inocula and it is therefore unlikely that a shift to rough phase phenotype contributed to any vaccine failings. The Rev.1 organism was not recovered from the mice during the post-challenge bacterial enumeration studies, and whilst this suggests a rapid clearance of the vaccine from the host, it is not considered sufficiently rapid to be indicative of a hyper-attenuated state. Moreover, the detection of antibodies in the *Brucella* diagnostic cELISA (data not shown) indicated that the Rev.1 (V1) vaccinates were exposed to the *Brucella* smooth OPS and the vaccine was sufficiently immunogenic and persistent within the mouse to promote a detectable and suitably avid IgG response. Despite the failure of the Rev.1 vaccine to provide protection in the high dose challenge study, and the significant variation between groups, results from this trial indicated a statistically significant reduction in *Brucella* load of the p- *ialB* mice compared to the PBS inoculated mice (Mann-Whitney test, $p = 0.0159$). This is a promising result indicating a potent protective effect (0.93 protection units) from the p- *ialB* vaccine.

The data generated in the low dose (10^4 CFU per mouse) challenge study was more informative. Importantly this combination vaccine and challenge doses was suitable for the measurement of difference in splenic *Brucella* load between mice that had received the positive control vaccination Rev.1, and mice that had been sham immunised with PBS. Rev.1 vaccination resulted in demonstrable reduction of *Brucella* load in the spleens of mice at 15 days post challenge. Moreover, in this particular study Rev.1 vaccination given at either V1 or V4 was able to protect mice against virulent challenge. Neither the vaccine strain nor *B. melitensis* 16M challenge strain were recovered from the spleens of the Rev.1 (V4) mice at 15 ± 1 days post-challenge. This implies that Rev. 1 organisms were processed and eliminated relatively rapidly following vaccination and that the subsequent viable *Brucella* challenge was rapidly dealt with through induction of adaptive immune responses.

The persistence of a live vaccine strain such as Rev.1 is often correlated with its potential for generating appropriate immune responses. From many replicated analyses of Rev.1 infection kinetics in CD-1 mice, Bosseray (1995) surmised that the average time for clearance (RT50) of a protective dose of Rev.1 (~2 X 10^5 CFU per mouse) from infected mice was approximately 7.6 ± 1.3 weeks (Between 45 and 60 days). Rev.1 batches with shorter RT50 times were often sub-optimal in terms of protection against virulent challenge in the model and in the target species. Thus the absence of Rev.1 recovery from the spleens of Rev.1 (V4) group challenged mice at 45 days post-vaccination in this experiment represents a relatively rapid clearance of the vaccine strain, but is just within the expected and acceptable time frame for an effective batch of vaccine.

Since the detection limits of the bacterial culture technique are restricted to 10 CFU per spleen, this absence of *Brucella* recovery does not necessarily indicate sterile immunity but does provide a quantifiable measure of the protective effect of a licensed vaccine (Rev.1), that can be directly compared with the novel candidate vaccines. Moreover, protective immunity was clearly demonstrated in terms of a reduction in the number of viable *Brucella* recovered from Rev.1 vaccinates in comparison to the PBS inoculated controls. Thus, confirming in

this particular experiment the vaccine and challenge inocula had performed as expected and the protocol was proven an effective method suitable for the evaluation of novel candidates. Furthermore, the measurable success of Rev.1 against the 10^4 challenge dose compared to failure against the ~10^5 challenge dose indicated that the lower dose challenge model should be adopted in the subsequent studies.

Both groups of Rev.1 vaccinated mice (Rev.1 (V1) and Rev.1 (V4)) were considered protected following challenge with 10^4 *B. melitensis* 16M (3.62 and ≥ 3.72 protection units respectively) , although using splenic load as a quantitative correlate of protection indicated that the protective effect of the V4 vaccination was greater than that of the V1 vaccination. This suggests that the protective effect of Rev.1 vaccination in the BALB/c mouse reduces slightly over time. Evidence of a decline in protective efficacy of Rev.1 was not recorded in the studies of Bosseray and Plommet (1990) when using similar methods with CD-1 mice and a *B. melitensis* strain H38-S challenge administered at either 45 or 150 days post-vaccination. However, for assessment of immunogenicity of vaccine seed-lot strains an interval of between 30 and 45 days between vaccination and challenge of mice is recommended in order to standardise the model (O.I.E, 2004), and many years of experience of the model in this laboratory have shown that the results from challenge studies conducted at greater than 40 days post-vaccination are subject to greater variability (Commander and Spencer, unpublished observations). Interestingly, a reduction of protective effect over time has also been observed in target species. Sheep were shown to have a protection rate of approximately 80% when Rev.1 vaccinates were challenged during the third trimester of their first pregnancy post-vaccination, but only 62% if vaccinates were not challenged until a comparable gestation period of their second pregnancy (Verger *et al,* 1995). This observation suggests that the declining protective efficacy is a real effect but that this is difficult to measure or standardise in the mouse model. Consequently, for the purposes of these investigations where the Rev.1 vaccination is included as a positive or protected control, the data obtained from the Rev.1 (V4) group, but not necessarily the Rev.1 (V1) group, should be considered the protected control.

Statistical analyses (Mann-Whitney U test) were used to evaluate the *Brucella* load in the spleens of vaccinated mice in comparison to those of the PBS sham vaccinated mice. Overall, the DNA vaccines p-*omp25* [1], p-*omp25* [2], p-*frpB*, pCI-P39 and p-*ialB* showed a significant reduction in bacterial load compared to that of the PBS inoculated controls. The protective effect of these vaccines (p-*omp25* [1], p-*omp25* [2], p-*frpB*, pCI-P39 and p-*ialB)* was 2.57, 2.63 1.01, 1.13 and 2.70 protection units respectively. This data confirms the observations regarding a possible protective effect from p-*ialB* that were made in the high dose challenge study, and furthermore indicates that some of the other plasmids are also promising DNA vaccine candidates. Control of the splenic *Brucella* infection was most consistently observed for mice in groups vaccinated with p-*ialB* or *p-omp25* vaccines. There was not a sizeable reduction of *Brucella* load observed for the p-*frpB* and pCI-P39 vaccinated mice, although the effect was considered statistically significant. Vaccination with p-*acvB* did not result any measurable protective effect, and although p-*fliC* protective efficacy was not measured in this particular investigation, a repeat investigation at this challenge dose (with only p-*fliC*, PBS and Rev.1 study groups) failed to indicate protective activity from this candidate (data not shown).

In support of the protection data, antibody responses against *B. melitensis* 16M antigen were measured from Rev.1 vaccinated mice and from DNA vaccinated mice. *Brucella* specific antibodies were measured at the final time point from 100% of the mice receiving vaccination with Rev.1 (V1) and *p- ialB* and p-*acvB*, clearly demonstrating that these vaccines were consistently able to evoke *Brucella* specific adaptive immune responses. On average 70% of mice vaccinated with either of the p-*omp25* based constructs were IgG2a seropositive ELISA at this time, indicating only a slightly less robust immunogenicity compared to the p-*acvB* and p-*ialB* vaccines. However, only two mice vaccinated with p-*frpB* produced measurable levels of IgM specific antibody, and IgG1 and IgG2a were not detected from these mice suggesting that this vaccine was a weak immunogen. Only one p-*fliC* vaccinated mouse was shown to produce 16M reactive IgG2a antibodies at week 11 of the study.

The kinetics of the serological response to Rev.1 vaccination followed the expected course. IgM was generated first and IgG1 and IgG2a became evident at week five of the study. As the response developed the IgG2a isotype of antibody became dominant. The magnitude of antibody response measured in ELISA from the Rev.1 mice was significantly greater than that for the DNA vaccines, and although this may reflect improved immunogenicity on the part of the live vaccine it is also attributable to the measurement of a more comprehensive polyclonal antibody response against the multi-component 16M antigen ELISA than would be expected from the monospecifically responsive DNA vaccinated mice. In the case of the DNA vaccines, a specific response can only be directed and measured against the encoded protein. Interestingly, the data shows that whilst IgG1 and IgG2a antibodies are not apparent at week five and maximal at week 11, protective efficacy is greater following challenge at week four-five (30 days) post-vaccination. Thus supporting the claim that antibodies are less significant than other aspects of the immune response for protection against brucellosis.

Although the ELISA data are of limited value in comparing the immune response of the various groups of DNA vaccinated mice, the data provides important supplementary evidence that can be used to justify or eliminate candidate vaccines for further study. For instance, a lack of demonstrable antibody from p-*frpB* vaccinated mice consolidates the decision to eliminate this weakly protective candidate from further investigations. The p-*frpB* vaccine provided a slight but statistically significant reduction of bacterial load. However, the effect appeared to be largely the result of a single mouse in the group with a very low *Brucella* load and was therefore considered more likely to have been caused through an error in the challenge inoculation procedures than vaccine mediated protection. Moreover, the ELISA results indicate that a detectable specific immune response is not elicited, suggesting that either the vaccine is a weak immunogen, or the assay is unsuitable for the detection of FrpB specific responses. Investigations described in chapter 3 showed that approximately 45% of *Brucella* infected sheep contain antibodies that react with the crude lysate of BL21 *E. coli* expressing the *Brucella* FrpB protein suggesting that the native FrpB protein is immunogenic. Furthermore, *Brucella* FrpB was known to

be present in 16M antigen preparations (Wagner *et al,* 2002 and chapter 3) indicating that the 16M based ELISA was theoretically suitable for the measurement of anti-FrpB responses. The possibility of p-*frpB* being a non-functional or non-expressing construct was considered unlikely since the structure and sequence were confirmed as appropriate for expression, and transcription from Cos7 cells was demonstrated *in vitro*. However, these results reflect *in vitro* conditions and do not confirm effective transfection, transcription and translation *in vivo*. It is possible that either *in vivo* transfection efficiencies or protein expression levels from the construct were too low to generate adequate immune stimulation. Alternatively, protein production from the *in vivo* transfected mouse cell could have produced a protein that was structurally dissimilar to native FrpB and therefore presented inappropriate epitopes to the immune system. The exact reasons for the relative lack of response remain undefined. However, overall these antibody and protection data suggest that p-*frpB* is not useful for further investigation as a *Brucella* vaccine. In contrast, the homologous FrpB proteins of *Neisseria spp.* have been shown to be immunogenic and contribute to vaccine efficacy (Beucher *et al,* 1995). Moreover, Roop *et al,* are currently investigating the virulence of *B. abortus* 2308 mutant strains in which the BMEII 0105 gene (*frpB*, or alternatively named *bhuA*) has been deleted. These mutants are reported to have a reduced ability to utilise heme as an iron source. Experiments to confirm the role of the *bhuA* in iron transport are reported to be underway (Roop *et al,* 2004). It is possible that whilst the *frpB* DNA vaccine does not appear to be protective in these studies, *frpB* or *bhuA* mutants may prove useful as attenuated live strains.

The protective efficacy of p-*fliC* vaccination was not completed in the low dose study because of ill health in the mice of this group. Humoral immune responses were measured from these animals throughout the experiment up until the time of euthanasia, but conclusive evidence of *Brucella* specific antibodies was not detected. It must be noted however that the RT-PCR expression discussed in chapter 3 suggested that the *Brucella* flagellin (FliC) protein was not expressed during SDA based culture, and consequently the absence of this specific protein from the 16M ELISA antigen would make the detection of a FliC specific response impossible in this assay. Due to the

problems associated with this assay, a separate protection experiment was set up at a later time to assess the p-*fliC* vaccine, but a protective effect was not observed in this study (data not shown). For this separate study specific antibody responses were evaluated by ELISA using FliC-His BL21 *E. coli* cell lysate as antigen: FliC specific responses were observed from p-*fliC* vaccinated mice but there was no evidence of FliC reactive antibodies in control Rev.1 vaccinated samples (data not shown). Thus, immunogenicity and protective efficacy data from repeated p-*fliC* vaccine analysis suggested that this was not a suitable candidate for further study. As suggested for the frpB candidate, recent evidence suggests that flagellar based mutants may be useful as attenuated live strain vaccines (Fretin *et al,* 2005, Delrue *et al,* 2005).

The p-*acvB* vaccine produced some interesting results. Whilst the ELISA results indicate that AcvB is strongly immunogenic with 100% of the vaccinated mice producing measurable *Brucella* specific IgG1 and IgG2a following the full course of vaccinations, the protection study shows that this response does not contribute to a control of infection. A role for AcvB in *Brucella* infection has yet to be defined. The AcvB (VirJ) proteins of *A. tumefaciens* are located in the periplasm and believed to function as chaperones binding the T-DNA during transport through the TFSS. Moreover, Δ*acvB (virJ)* mutants are attenuated in *A. tumefaciens* (Wirawan *et al,* 2003). A similar role for *Brucella* AcvB (VirJ) protein as an effector of the VirB system cannot be ruled out, although Tn5 and STM screens have not indicated this gene to be essential in *Brucella* invasion or intracellular replication (Foulongne *et al.,* 2000, Min *et al,* 2000., Lestrate *et al,* 2003). Similarly, this failure of AcvB specific immunoglobulin to promote a measurable reduction in splenic *Brucella* load suggests that this protein is either inaccessible to the immune system or not an essential facet of *Brucella* virulence. In conclusion, AcvB is not a protective antigen, and therefore is not worthy of further investigation as a component of a sub-unit vaccine.

For the p-*omp25* vaccines *Brucella* specific IgG2a antibodies and protection were evident. This is in keeping with the results of other studies regarding the antigenicity of the *Brucella* Omp25 protein, but represents the first report of Omp25 specific immune effectors generating protection against smooth strain

B. melitensis 16M challenge. In passive transfer studies Cloeckaert *et al* (1991) assessed the potential of Omp25 specific monoclonal antibodies to protect mice against smooth and rough strain *Brucella* challenge. The antibodies were able to provide limited protection against challenge with rough strain *Brucella* species, such as *B. ovis*, but were inefficacious against challenge with smooth strains such as *B. melitensis* or *B. abortus*. Similarly, analyses of the Omp25 protein have indicated that this may be a useful protective antigen against rough strain *Brucella* challenge, but have been unable to demonstrate equivalent protective activity against the more virulent smooth strains. Bowden *et al* (1998) assessed the immunogenicity and protective efficacy of a strain of *E. coli* engineered to produce the *B. melitensis* Omp25 on its surface. This approach was able to generate anti-Omp25 antibodies principally of the IgG2a isotype, and demonstrable protection of BALB/c mice against challenge with rough *B. melitensis* strains B115, Smooth-rough *B. melitensis* EP, and notably the smooth *B. melitensis* strain H38 (H38-S). However, protection against challenge with smooth strain *B. melitensis* strain 16M was not achieved. Notably, the protective effect reported in the Bowden studies was small with less than a one log difference in the splenic load of protected and unprotected mice. In the investigation described here, p-*omp25* [1] and p-*omp25* [2] vaccination caused a mean 2 log and 1.5 log reduction of splenic load respectively (2.57 and 2.63 protection units), indicating that DNA based delivery of the Omp25 antigen is potentially a superior strategy.

Interestingly, protective efficacy and immunogenicity measured for p-*omp25* [1] and p-*omp25* [2] was not significantly different (Mann Whitney U test: $p > 0.05$). Thus suggesting that the presence of the intron element, considered to promote greater *in vivo* antigen expression, does not affect the success of the vaccination procedure. Further study of *omp25* based DNA vaccines are required to confirm their protective potential and elucidate the cell-mediated immune responses evoked by vaccination.

The p-*ialB* vaccine showed the most consistent protective activity against *B. melitensis* 16M challenge in both the high and low dose challenge studies, and specific antibodies of the IgG2a isotype were detected in the sera of all p-*ialB*

vaccinated mice suggesting the antigen is a good immunogen. Furthermore, results described in chapter 3 suggest that this antigen is expressed by *Brucella* under all conditions investigated and elicits production of specific antibody during natural infection. Together these data suggest that IalB may be an important protective antigen. As a previously unstudied antigen with demonstrable immunogenicity and protective efficacy against high and low dose challenge (0.93 and 2.70 protection units respectively) the IalB protein and p-*ialB* vaccine warrants further investigation.

Finally, it is widely accepted that protective immunity in brucellosis relies upon the generation of Th 1 immunity. Results generated so far have confirmed the antibody production from protected DNA vaccinated mice (p-*ialB* and p-*omp25* groups) was biased toward generation of IgG2a, thereby implying the involvement of the crucial Th 1 effector IFN-γ in promoting the antibody class switch. Since IFN-γ is known to be essential for the clearance of *Brucella* spp., the generation of IgG2a antibodies is an indicator that the vaccines are evoking appropriate Th 1 biased specific immune responses necessary for protection. However, unfortunately, an overall direct correlation between the production of *Brucella* reactive IgG2a and protective efficacy could not be demonstrated in this experiment. In particular, the lack of protective efficacy in the face of detectable anti-*Brucella* responses from p-*acvB* vaccinates indicates that production of this particular IgG2a does not result in protection. Conversely, the kinetics of the antibody response evoked by vaccination with Rev.1 indicate that detectable IgG2a is not present in the Rev.1 (V4) mice at the time of challenge, and therefore cannot be considered essential for the protective effect observed in these mice. Together these findings indicate that whilst Th 1 biased immune responses are undoubtedly important in protection against brucellosis, the measurement of IgG2a production by simple anti-*Brucella* ELISA is not sufficient to fully illustrate the complex relationship between immune effectors and a reduction in bacterial load in this model. It is therefore concluded that a more direct assessment of the cell mediated immune effectors would be required in order to characterise protective immune responses elicited by the p-*ialB* and p-*omp25* vaccines.

In conclusion, these investigations to assess the immunogenicity and protective efficacy of the six novel DNA vaccines (five candidate antigens) indicated that the p-*ialB* and the two *omp25* based plasmids were the most promising vaccine candidates. These candidate vaccines provided demonstrable protective efficacy and convincing evidence of induction of *Brucella* specific immune responses. Another aim of this work was to determine the optimal challenge dose to be used in the BALB/c model for determining protective efficacy. A challenge dose of approximately 1 X 10^4 CFU per mouse given 30 days post vaccination, and assessment of bacterial load at day 15 ± 1 post-challenge generated the most reliable data. This low dose challenge model and vaccines p-*omp25* [1] and [2], and p-*ialB* were thereby selected for use in all subsequent studies.

Chapter 6: FURTHER STUDIES ON THE IMMUNOGENICITY AND PROTECTIVE EFFICACY OF DNA VACCINES ENCODING *B. MELITENSIS omp25* AND *ialB*

6.1: Summary

DNA vaccines based upon the B. melitensis genes omp25 or ialB were studied to confirm the protective effects observed in previous investigations and to determine the immunological effectors elicited by vaccination. The data obtained substantiated the previous finding that four inoculations of either omp25 or ialB based DNA vaccines were immunogenic and able to provide significant protection against virulent challenge.

Antigen specific induction of the essential cytokine IFN-γ was recorded from Rev.1, p-omp25 and p-ialB vaccinated mice. The anti-Brucella response in Rev.1 group mice was Th 1 dominated with production of IFN-γ mainly contributed by CD4+ effector cells. The antigen specific response in p-ialB vaccinated mice was characterised by CD4+ dominated IFN-γ production and high balanced IgG1: IgG2a titres, whereas the specific response observed from p-omp25 vaccinated mice was characterised by relatively lower antibody titres and CD8+ dominated IFN-γ production. Each vaccine provided equivalent protective activity in the murine infection model.

6.2: Introduction

In previous investigations, the p-*omp25* [1], p-*omp25* [2], and p-*ialB* plasmids were shown to be both protective and immunogenic, and therefore selected for further investigation. The first aim of the experiments discussed in this chapter was to measure and compare the principal immune effectors of the DNA

vaccines and the gold standard live vaccine Rev.1, in order to expand understanding of the immunological correlates of protection in murine brucellosis, and advance the knowledge regarding the role of Omp25 and IalB as protective antigens.

The earlier studies had established the presence of antibodies recognising *B. melitensis* 16M antigens in the serum of p-*omp25* or p-*ialB* vaccinated mice. Although high titre specific antibody responses are usually associated with smooth strain *Brucella* infection, specific antibody alone is insufficient to protect animals against infection. Moreover, cell mediated immunity is known to be essential in the control of intracellular infections such as *Brucella* (Mackaness, 1971, Cheers 1984). Although the full range of cellular immune effectors required for protection are not yet understood, it is widely accepted that activation of a Th 1 type response is essential for clearance of a *Brucella* infection. In particular the ability of a vaccine to evoke production of antigen specific IFN-γ is critically important: IFN-γ is essential for the clearance of *Brucella* (Murphy *et al,* 1996). Other Th 1 cytokines support and bolster the action of IFN-γ (Baldwin and Parent, 2002), and both CD4+ and CD8+ cells contribute to *Brucella* control (Oliveira and Splitter, 1995).

B. melitensis omp25 based DNA vaccines were selected for study. The immunogenicity of outer membrane proteins has been investigated for a wide variety of pathogens. Notable examples include the use of Omp preparations as vaccine components for protection against human infection with *Neisseria meningitidis* (Rosenstein *et al,* 2001., Urwin *et al,* 2004). The protective effect of *Neisseria* Omps appears to be largely antibody dependant. More significantly Omp induced Th 1 immune responses have been shown to confer protection against *Chlamydia trachomatis* (Eko *et al,* 2004), indicating that cell mediated responses against Omps can be effective in the control of intracellular pathogens. The conserved Omp25 antigen of *Brucella* spp. is known to be immunogenic and has already been investigated for protective efficacy in a number of studies. Significantly, passive transfer studies using *Brucella* Omp specific antibodies, and attempts to use recombinant Omp25 as a subunit vaccine have not been entirely successful (Bowden *et al,* 1998). In contrast, the

omp25 based DNA vaccines created in this study show significant efficacy against *B. melitensis* 16M challenge, suggesting that the DNA vaccine approach is particularly beneficial for this antigen. One of the principal objectives of the studies described in this chapter is to elucidate and compare important effectors of protective immunity induced by the successful DNA vaccine and the live vaccine Rev.1.

The p-*ialB* was also selected for further study. A role for IalB in *Brucella* virulence has yet to be determined. However, it has been recently documented that the *B.suis ialB* gene contains a frame shift mutation, which abrogates expression of this protein in the wild type *B. suis* (Paulsen *et al,* 2002). Thus, expression of the *ialB* may be restricted to *B. melitensis* infections and as such this antigen may be a useful species-specific marker, or a determining factor for the observed host preference of this species. However, in the context of vaccination the absence of this antigen from *B. suis* means that a p- *ialB* vaccine would not be useful for prophylaxis against this particular challenge.

A homologue of *Brucella ialB* exists in *Bartonella spp.,* where it is believed to play an important role for in establishing infection. The *iaiA* and *ialB* genes of *Bartonella* were shown to confer an invasive phenotype onto *E. coli* strains DH5α and HB101, and postulated to play a role in the invasion of erythrocytes (Mitchell and Minnick, 1995). Whether or not this important virulence factor is a protective antigen for the *Bartonella* pathogens was not assessed in these investigations. *Brucella* IalB protein shares between 30 – 35% amino acid identity with *Bartonella* spp. homologues, but whether *ialB* plays a significant role in *Brucella* virulence remains to be determined. Current studies have demonstrated a significant protective effect from the p-*ialB* DNA vaccine, and thus determination of the immune effectors triggered by this vaccine is an important step towards understanding IalB as a protective antigen.

A protective brucellosis vaccine is expected to prime Th 1 cellular immune responses, resulting in production of IFN-γ, TNF-α, IL-12 and IgG2a isotype specific antibodies. Conversely, Th 2 cellular responses represented by IL-4 and IL-5 production are not effective at combating *Brucella* infection. Successful

Brucella vaccines are associated with a strongly Th 1 biased immunity. Moreover, virulent or attenuated *Brucella* strain infections or exposure to killed *Brucella* organisms triggers Th 1 cytokine production (Zaitseva *et al,* 1995). This property of *Brucella* has been exploited in an adjuvant capacity for promoting Th 1 biased responses to unrelated pathogens (Scott *et al,* 1998). The capacity of whole *Brucella* based vaccines to stimulate Th 1 responses is crucial to their success, and conversely the absence of strong Th 1 priming from traditional sub-unit vaccines limits their effectiveness. Notably, DNA vaccines are recognised to promote strong Th 1 responses. Thus, the aim of the work described in this chapter is to characterise the immune response induced by both the control vaccine Rev.1 and the novel DNA vaccines, with particular emphasis on the ability of each to promote production of the crucial cytokines IFN-γ, IL-12 and TNF-α, or counteractive Th 2 and T_{reg} type immune responses (IL-4 and IL-10 respectively). Comparing the Rev.1 and DNA primed antigen specific immune responses is necessary to reveal any qualitative or quantitative distinctions in antigen specific response brought about by the different modes of delivery: live versus DNA vaccine. Furthermore, measurement of the immune response is critical to understanding the action of the vaccines and to enable extrapolation of results and hypotheses from the BALB/c mouse infection model to the intended targets of vaccination: small ruminants or humans.

6.3: Experimental procedures and aims

Two separate investigations assessing the *omp25* and *ialB* based vaccines were carried out. Each study compared the efficacy and immunogenicity of the selected DNA vaccine against that provided by the gold standard live vaccine Rev.1. In the first investigation, a DNA vaccine preparation based upon *omp25* was assessed. The p-*omp25* [1] and p-*omp25* [2] DNA vaccines (*omp25* cloned into the pCR3.1 (Invitrogen) and pTargeT (Promega) vectors, respectively) were previously shown to have equivalent protective activity, indicating that the different vector formulations offered no practical advantages in terms of vaccine efficacy in this model. Furthermore, significant quantitative and qualitative differences were not observed in either antigen production from plasmid transfected Cos 7 cells, or in the induction of basic anti-*Brucella* antibody

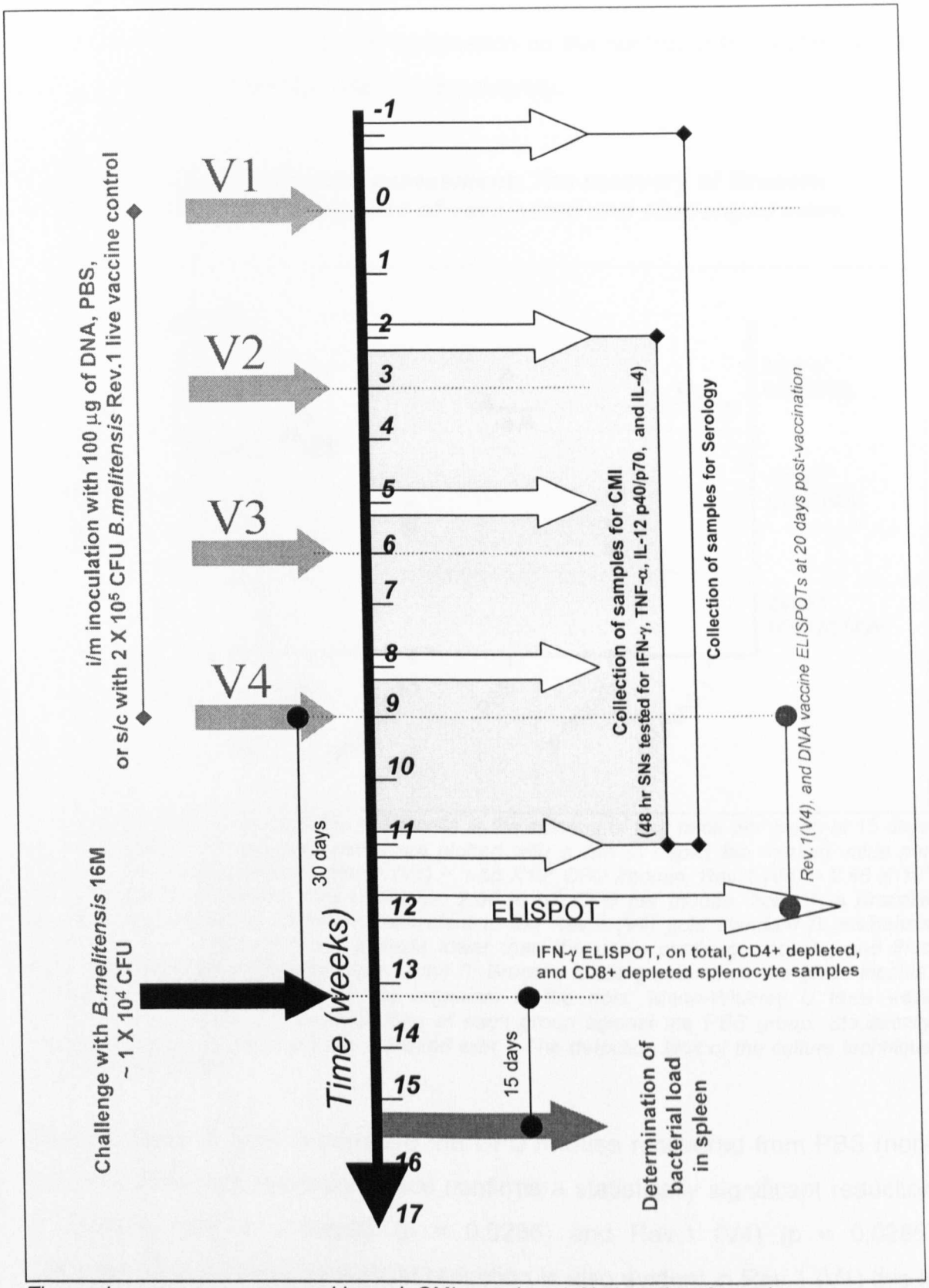
responses. Therefore, as it was not possible to identify one vaccine as being superior to the other, a mixture of both plasmids (referred to as p-*omp25*) was prepared for use in all subsequent studies. In the second study, the *ialB* DNA vaccine was assessed.

For these investigations each of the DNA vaccines: pCR3.1-*omp25* (p-*omp25* [1]), pTargeT-*omp25* (p-*omp25* [2]), *p- ialB,* and the pcDNA3.1 vector control were produced by Plasmid Factory GmbH & Co. KG. (Bielefeld, Germany) to GMP standards. Plasmids were suspended in sterile Dulbecco's Phosphate Buffered Saline (D-PBS) to a final concentration of 1 mg/ml. Preparations were certified as > 98% closed circular supercoiled (CCC) structure and determined to have an endotoxin content of less than or equal to 0.1 E.U. (endotoxin units) per μg of DNA. p-*omp25* [1] and p-*omp25* [2] were combined in equal ratios to produce the vaccine formulation p-*omp25*. Sufficient p-*omp25* was prepared as a single batch for use throughout this vaccine trial. All plasmid stocks were maintained at –80°C in 600 μl aliquots until required for use.

The BALB/c murine brucellosis model was used. Protective efficacy was assessed through challenge with 1 X 10^4 CFU per mouse as previously described. Antigen specific immune responses against killed *B. melitensis* 16M, recombinant IalB or recombinant Omp25-GST were measured in terms of antibody or specific cytokine production from *in vitro* restimulated splenocytes by ELISA and ELISPOT. Killed *B. melitensis* antigen preparations were prepared in-house. Recombinant *E. coli* were created in-house but recombinant protein purification was subcontracted to Lionex GmbH (Germany).

A flow chart summarising the investigations described in this chapter is provided in Figure 6.1.

Figure 6.1: Experimental outline for the evaluation of p-omp25 or p-ialB protective efficacy and immunogenicity in the BALB/c mice



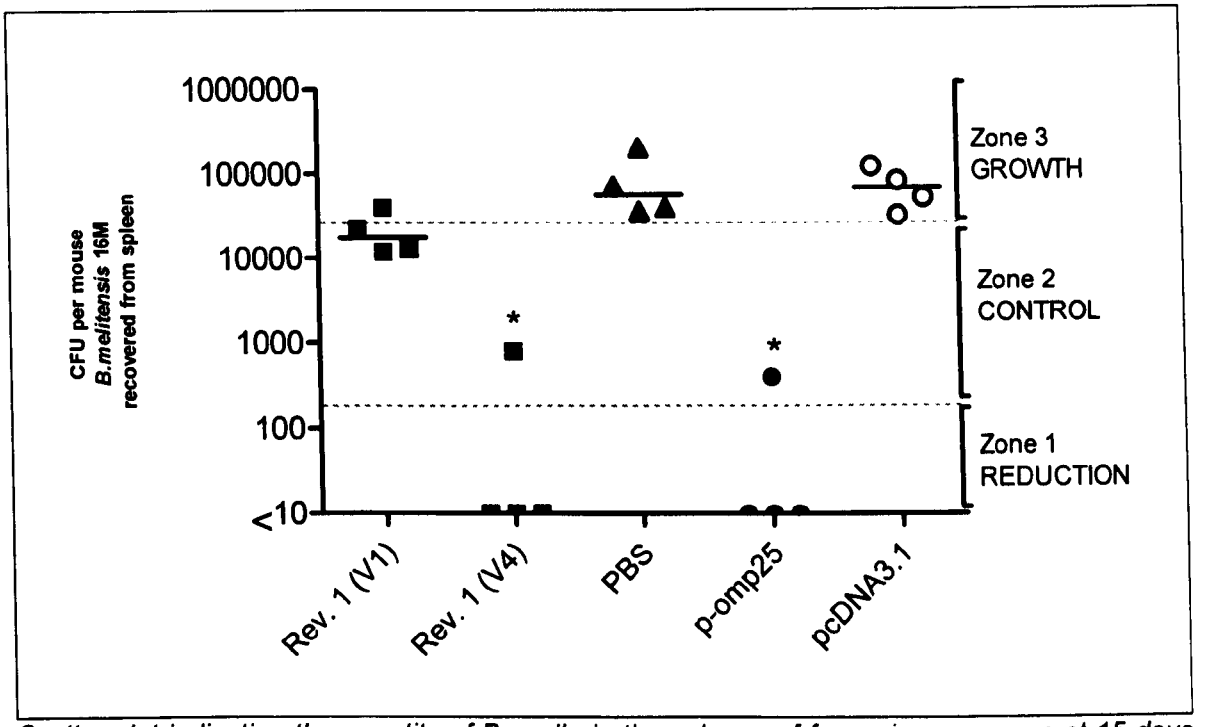
Three mice per group were used in each CMI experiment. No less than four mice per group were used for protection assay. Five mice per group were used in ELISPOT assay. Procedures and methods are described in chapter 2.

6.4: Results

6.4.1: Protective efficacy results

The effects of p-*omp25* or p-*ialB* vaccination on the control of *B. melitensis* 16M are illustrated in figures 6.2 and 6.3 respectively.

Figure 6.2: p-omp25 vaccine assessment: The recovery of Brucella melitensis 16M from the spleens of vaccinated and challenged mice.

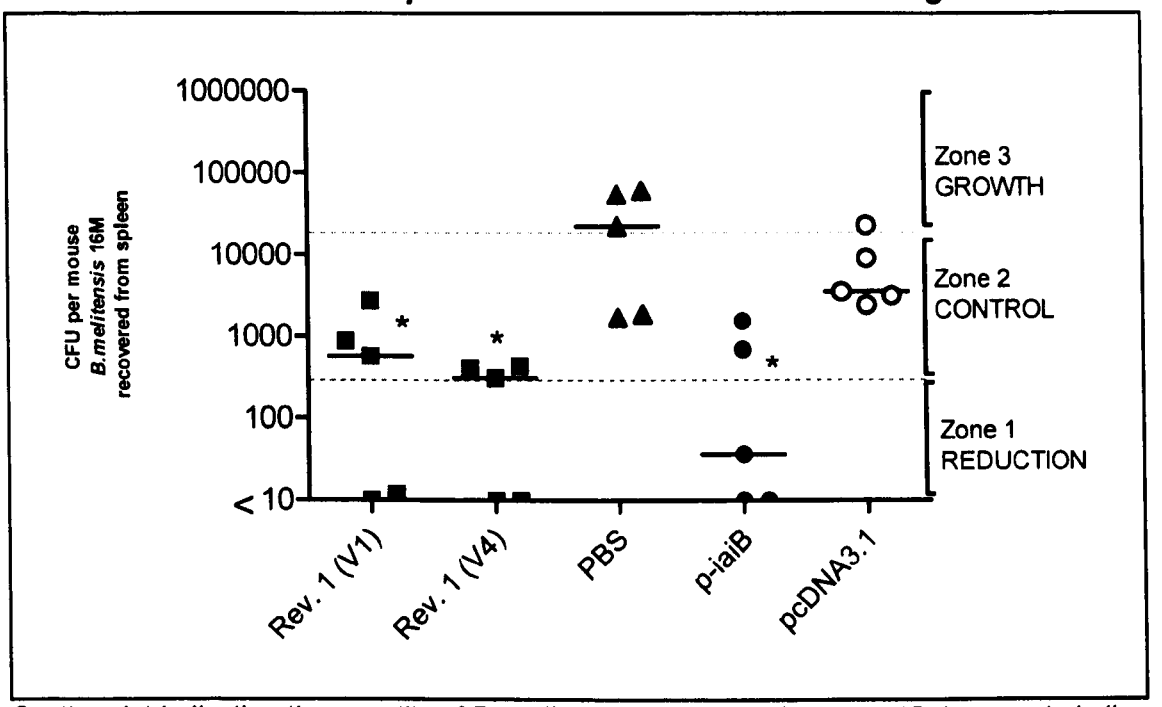


*Scatter plot indicating the quantity of Brucella in the spleens of four mice per group at 15 days post-challenge. Individual data points are plotted with a line to depict the median value per group. Actual bacterial inocula: Rev.1 (V1) ~ 1.55 X10^5 CFU /mouse, Rev.1 (V4) ~ 2.66 X 10^5 CFU/ mouse. B. melitensis 16M challenge: 2.66 X 10^4 CFU per mouse. Zone 1: a Brucella reduction is observed of protection equivalent to the Rev.1 (V4) gold standard B. melitensis vaccine. Zone 2: delimits Brucella loads lower than the given challenge inocula, and thus reflects control of Brucella replication. Zone 3: Brucella loads greater than the given inocula, representing active replication of the organism in the host. Mann-Whitney U tests were performed to compare the bacterial load of each group against the PBS group. Statistically significant reductions (p< 0.05) are indicated with *. The detection limit of the culture technique is 10 CFU per spleen.*

Mann-Whitney U Test to compare the CFU /mouse recovered from PBS (non-immune) mice and vaccinated mice confirms a statistically significant reduction in *Brucella* load in p-*omp25* (p = 0.0286) and Rev.1 (V4) (p = 0.0286) vaccinated mice. Although a slight reduction is also evident in Rev.1 (V1) this is not quite significant in this assay (p = 0.0571). There is no evidence of a protective effect in pcDNA3.1 inoculated mice (p = 1.0).

A comparison of the *Brucella* CFU per mouse recovered from each of the groups clearly shows a reduction in bacterial load for the p-*omp25* and Rev.1 (V4) vaccinated mice: In terms of protection units (Mean Log CFU per spleen of PBS group – Mean Log CFU per spleen vaccinated group) the Rev.1 (V4) and p-*omp25* vaccines provide 3.45 and 3.42 protection units respectively. In each of these groups *Brucella* were not cultured from three out of the four mice, suggesting less than ten *Brucella* per spleen. The fourth mouse had significantly fewer *Brucella* CFU in its spleen than the non-immune (PBS) control mice. The bacterial load measured from the p-*omp25* vaccinated mice was not found to be significantly different to that of the Rev.1 (V4) mice ($p = 0.8857$), suggesting an equivalent reduction of bacterial load in these two vaccine groups. Mice vaccinated with Rev.1 at V1 (90 days prior to challenge) also demonstrated a slightly lower number of *Brucella* recovered from spleens compared to PBS mice, although this reduction was not found to be statistically significant. The mean *Brucella* per spleen in this group was approximately 2.17×10^4 CFU, indicating a 0.08 log reduction from the given challenge inocula, and a 0.54 protection units compared to the *Brucella* load of the non-immune mice.

Figure 6.3 : p-ialB vaccine assessment: The recovery of Brucella melitensis 16M from the spleens of vaccinated and challenged mice.



*Scatter plot indicating the quantity of Brucella in the spleens of mice at 15 days post-challenge. Individual data points are plotted with a line to depict the median value per group. Actual bacterial inocula: Rev.1 (V1): 0.95 X 10^5 CFU per mouse, Rev.1 (V4): 1.95 X 10^5 CFU per mouse, B. melitensis 16M challenge: 2.26 X 10^4 CFU per mouse. Zone 1: a Brucella reduction is observed of protection equivalent to the Rev.1 (V4) gold standard B. melitensis vaccine. Zone 2: delimits Brucella loads lower than the given challenge inocula, and thus reflects control of Brucella replication. Zone 3: Brucella loads greater than the given inocula, representing active replication of the organism in the host. Mann-Whitney U tests were performed to compare the bacterial load of each group against the PBS group. Statistically significant reductions ($p < 0.05$) are indicated with *. The detection limit of the culture technique is 10 CFU per spleen.*

In the investigation represented in Figure 6.3, Mann-Whitney U Test comparing the CFU / mouse recovered from PBS (non-immune) mice and vaccinated mice, confirmed a statistically significant reduction in *Brucella* load in p-*ialB* and Rev.1 (V4) ($p = 0.0079$) vaccinated mice. A significant effect was also evident in Rev.1 (V1) vaccinated mice ($p = 0.0317$). Rev.1 vaccination is protective when given at time V1 or V4 in this study. All mice in these group had *Brucella* loads lower than the inocula dose: the mean reduction was around 1.5 logs. In the Rev.1 (V1) group *Brucella* were recovered from all mice, albeit in very low numbers. In the Rev.1 (V4) group viable *Brucella* was not recovered from two of the mice, indicating a bacterial load of less than ten CFU per spleen from these mice. The protective effect recorded for the p-*ialB* vaccination was similar to that demonstrated in the Rev.1 (V4) group, whereby two of the mice in each group did not harbour detectable levels of *Brucella* (≤ 10 CFU per spleen) post-

challenge. A statistically significant difference was not indicated between the Rev.1 (V4) and p-*ialB*, or Rev.1 (V1) and p-*ialB* bacterial loads (p = 0.8413 and 0.6905 respectively). In terms of protection units, p-*ialB* ≥ Rev.1 (v4) > Rev.1 (V1) (2.15, 2.12, and 1.83 respectively).

It should be noted that these data show a number of PBS and pcDNA3.1 group mice with *Brucella* levels falling within the range defined as the control zone (zone 2), where fewer *Brucella* than the challenge dose were recovered from the spleen. For example, mice in the pcDNA3.1 group have *Brucella* splenic loads within the Control Zone (Zone 2). However, comparison against the PBS (unvaccinated control) mice indicates that this apparent reduction is not statistically significant (p = 0.8413).

The results of the two studies, summarised in figures 6.2 and 6.3 confirmed that p-*omp25* and p-*ialB* are protective against *B. melitensis* 16M challenge in the mouse, and indicate that the protective effect is not significantly different to that achieved by Rev.1 vaccination.

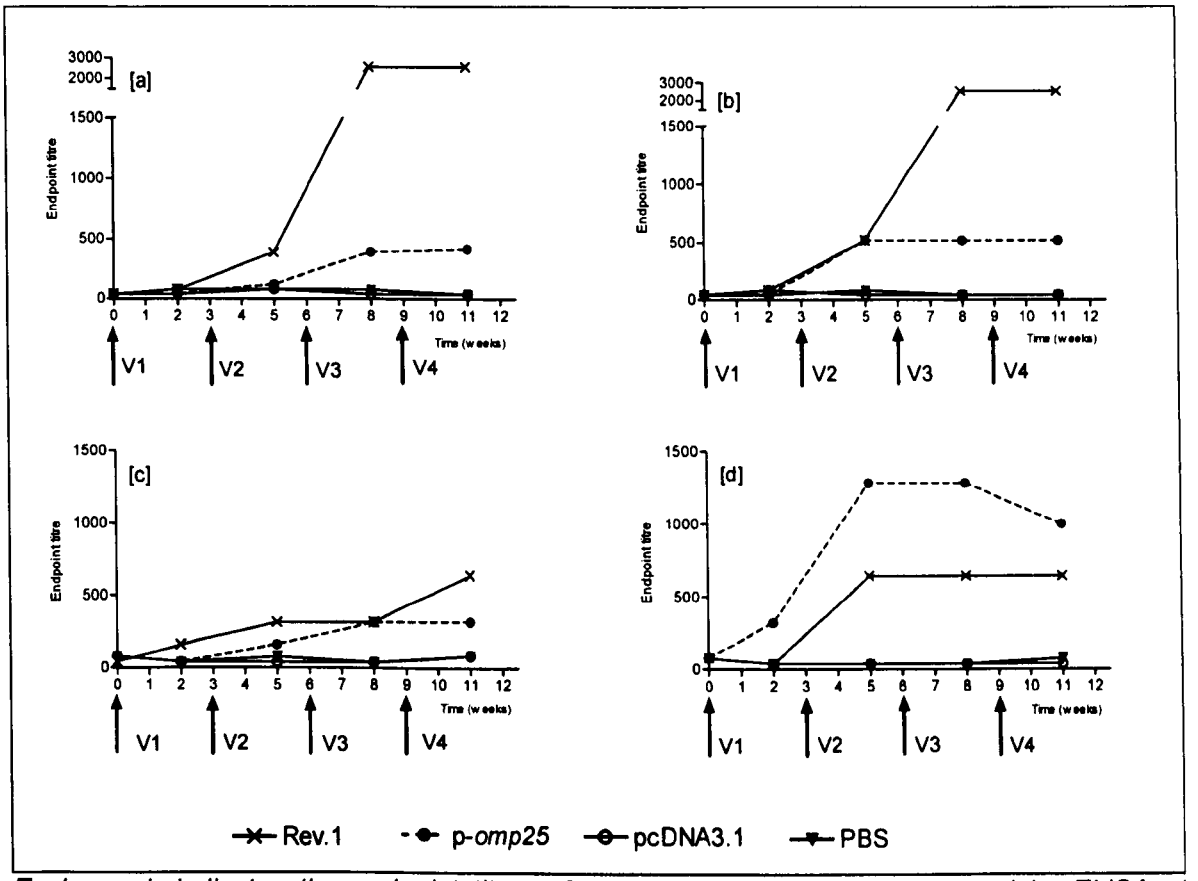
6.4.2: The specific immune response to vaccination

The ability of each of the vaccines to induce specific antibodies and cell mediated immune responses was measured.

Humoral immune responses

For p-*omp25* vaccine assessments, p-*omp25* , pcDNA3.1, PBS and Rev.1 group antisera (pooled by group) were assayed by simple indirect ELISA against *B. melitensis* 16M antigen, recombinant Omp25-GST and GST antigens. Each group sample was assayed in serial dilution from 1/40 (antisera/ diluent) through to 1/5120. Figure 6.4 shows the antibody responses, in terms of specific IgG1 and IgG2a titres, as measured in the Omp25-GST and 16M ELISAs during the experiment.

Figure 6.4: Specific antibody production in response to vaccination observed in the p-omp25 investigations

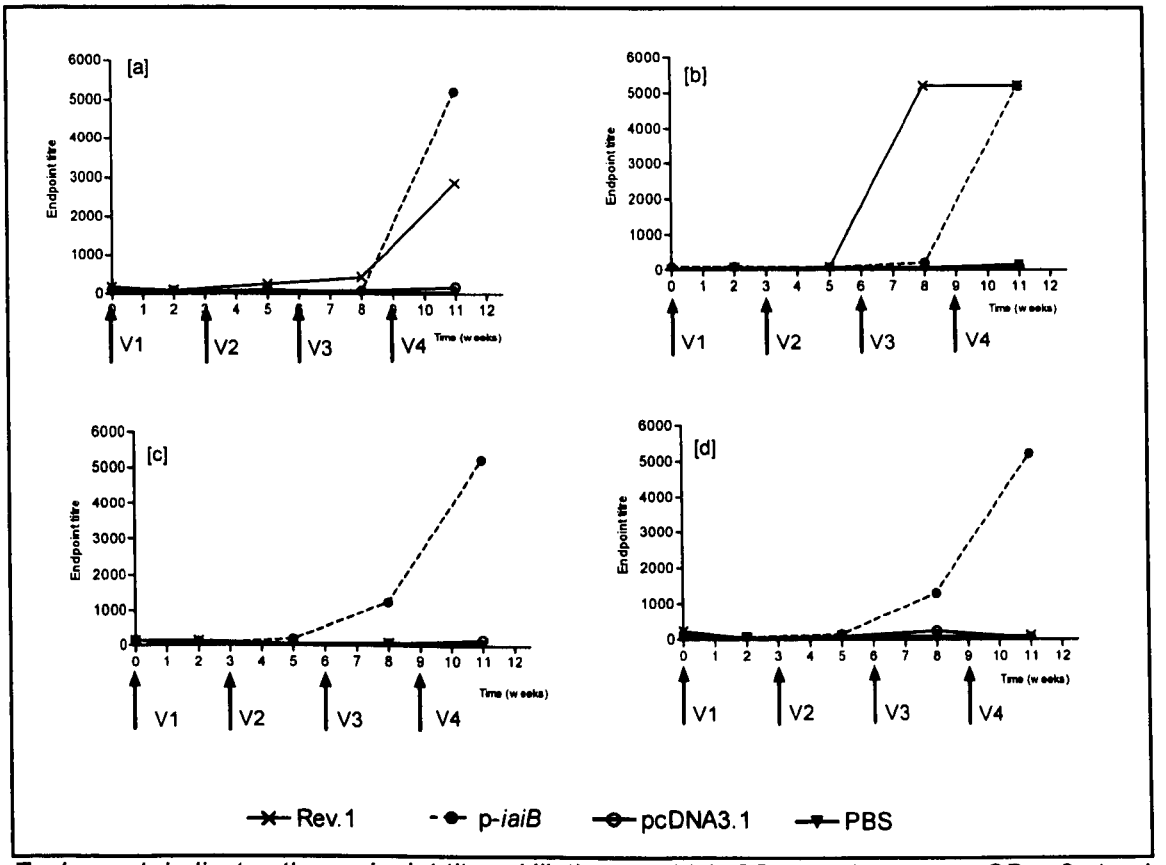


Each graph indicates the endpoint titres of pooled serum samples measured by ELISA at intervals post-vaccination. [a] IgG1 antibody detected by 16M ELISA, [b] IgG2a detected by 16M ELISA, [c] IgG1 detected by recombinant Omp25-GST ELISA and [d] IgG2a detected by recombinant Omp25-GST ELISA. The times at which p-omp25, PBS or pcDNA3.1 inoculations were given are indicated as V1, V2, V3 and V4. Rev.1 inoculation was given at V1 only.

Figure 6.4 illustrates that anti-*Brucella* and anti-Omp25 antibodies were confirmed from both Rev.1 vaccinated and p-*omp25* vaccinated mice. Specific antibody was demonstrable from the vaccinated mice from week two of the study. Individual serum samples were also assayed at a dilution of 1/100 at week eleven of the study: none of the samples were positive in the GST ELISA, but all mice in the Rev.1 and p-*omp25* groups were detected as positive in the Omp25-GST and 16M ELISAs (data not shown). Specific responses were not detected from either PBS or pcDNA3.1 inoculated mice at any time during this study.

In the p-*ialB* investigations, serological responses were measured in ELISA against 16M or recombinant IalB antigen. The data is summarised in figure 6.5.

Figure 6.5: Specific antibody production in response to vaccination observed in the p-ialB investigation



Each graph indicates the endpoint titres (dilution at which OD sample ≤ mean OD + 2 standard deviations, negative control) of pooled serum samples measured by ELISA at intervals post-vaccination. [a] IgG1 antibody detected by 16M ELISA, [b] IgG2a detected by 16M ELISA, [c] IgG1 detected by recombinant IalB ELISA and [d] IgG2a detected by recombinant IalB ELISA. The times at which p-ialB PBS or pcDNA3.1 inoculations were given are indicated as V1, V2, V3 and V4. Rev.1 inoculation was given at V1 only.

Figure 6.5 indicates the development of anti-IalB and Anti-*Brucella* antibodies following vaccination. *Brucella* specific IgG1 and IgG2a antibodies are detected from both Rev.1 and p-*ialB* vaccinated mice. Individual antisera were also assayed at week eleven of this study: all five mice in the p-*ialB* vaccine group were positive in both the 16M and IalB ELISA (data not shown). IalB specific antibodies were not observed in either the Rev.1, PBS or pcDNA3.1 control mice at any sampling time.

The development of antibodies in Rev.1 vaccinated mice

In the studies summarised in Figure 6.4 and 6.5, Rev. 1 vaccination resulted in high titre IgG1 and IgG2a isotype antibodies detected in the 16M ELISA. 16M specific antibodies were first detectable at week five during the p-*omp25*

investigation (Figure 6.4 [a] and [b]) and week eight during the p-*ialB* investigation (Figure 6.5 [a] and [b]). In both studies, high titres of both IgG1 and IgG2a were recorded at the final sampling time (eleven weeks post-vaccination).

A Rev.1 response to Omp25 antigen was detectable early post-vaccination: low titre specific IgG1 was detected in the sera of the Rev.1 vaccinated mice at two weeks post-vaccination. By five weeks post-vaccination relatively high titre Omp25 specific antibodies were evident in these mice, suggesting that the Omp25 antigen is immunologically significant in Rev.1 vaccination. In contrast, a significant reaction specific to the IalB antigen was not detected from the Rev.1 group mice at any stage during the p-*ialB* investigations, suggesting that Rev.1 vaccination does result in immunological recognition of this particular antigen.

The development of antibodies in p-*omp25* vaccinated mice

The maximal antibody titres detected from the p-*omp25* vaccinated mice in the 16M antigen ELISA are 1/520 for IgG2a and 1/360 for IgG1. When using the recombinant Omp25-GST assay the titre of IgG1 specific antibody is approximately 1/420, which is comparable to that achieved in the 16M ELISA. However, the IgG2a titre measured by this assay is significantly greater than that measured by 16M ELISA (1/1280). Furthermore, IgG2a responses were detectable in the Omp25-GST assay at week two of the investigation after a single immunisation with *p-omp25,* and were maximal by week five, following a single booster immunisation. IgG1 responses were first detected at week five, and reached their maximum by week eight, following a third DNA inoculation.

The development of antibodies in p-*ialB* vaccinated mice

High titre (≥ 1/5120) IgG1 and IgG2a isotype antibodies were detected in antisera from mice receiving p-*ialB* inoculations (Figure 6.5). Antibodies were detected by both the 16M and the recombinant IalB ELISA. These responses became detectable at week eight for the IalB ELISA and week eleven for the 16M ELISA. Responses were maximal at week eleven of the study.

Cell mediated immune responses

Splenocytes were harvested from three individual mice per group and cultured in the presence of *Brucella* specific antigens: 16M antigen, Brucellergene™, recombinant IalB or recombinant Omp25-GST (depending upon the particular investigation), mitogen control (Concanavalin A) or in media (DMEM alone). Splenocytes were harvested and assayed at three week intervals throughout the study period. After 48 hours, SN was removed from these cultures and tested for the presence of cytokines. The concentration of IL-10, IL-4, IFN-γ , IL-12: p70 and p40 and TNF-α in SNs was determined using Biosource Quantikine ELISAs.

IFN-γ production measured in splenocyte supernatants

IFN-γ production in response to mitogen stimulation

Concanavalin A stimulation of mouse splenocytes resulted in the generous production of IFN-γ (≥ 500 pg/ml) from all groups of mice except for those in the Rev.1 group during the p-*omp25* investigation at week 11 of the study. Thus indicating that except for these samples all cells were viable and capable of proliferation and cytokine production. Negligible IFN-γ (or indeed any cytokine) was detected from Rev.1 immunised mice at week 11 of the p-*omp25* investigation, in response to any of the stimulating antigens. The time delay incurred through processing the splenocytes at containment level III most probably contributed to a decline in viability of this particular set of splenocyte cultures: consequently these data were not representative of the IFN-γ production from Rev.1 vaccinated mice at this time. Concurrent samples from the PBS, pcDNA3.1 and p-*omp25* groups in this investigation were not adversely affected: the expected response to mitogen stimulus was recorded in these samples. An appropriate mitogen response was observed at week eleven of the p-*ialB* investigation from the Rev.1 vaccinated mice, confirming the ability of mice to produce IFN-γ at this stage post-vaccination.

Significant differences in ConA induction of IFN-γ between mice that were inoculated with p-*omp25*, p-*ialB*, pcDNA3.1 or PBS were not noted. The mean concentration of IFN-γ in all of these samples was between 350 and 850 pg/ml,

indicating that the ability to respond to mitogen stimulus was not influenced by the different vaccine inoculations. However, mice vaccinated with Rev.1 showed overall lower IFN-γ concentrations (in response to ConA stimulation) at both five and eight weeks post-vaccination in both investigations (mean IFN-γ concentrations between 50 and 450 pg/ml) suggesting a reduced capacity to produce IFN-γ following Rev.1 vaccination. However, this effect was not found to be statistically significant (Mann-Whitney U test, $p \geq 0.05$).

IFN-γ production in response to Brucella specific antigen stimulation

Brucella specific antigen stimulation resulted in different IFN-γ responses from each group of mice depending upon vaccine and time of sampling. These results are summarised in table 6.1.

Table 6.1: IFN-γ production in response to specific antigen stimulation

	Time and [# DNA inoculations]			
	Week 2 [1]	Week 5 [2]	Week 8 [3]	Week 11 [4]
	Brucellergene™ stimulation			
Rev.1	86.7 ± 92.9	315.8 ± 156.6 *	323.6 ±177.23 *	390.3 ± 239.3 *
p-omp25	ND	18.6 ± 2.3 *	1.66 ± 0.99	92.9 ± 130.3
p- ialB	≤ 1.0	2.9 ± 2.8	2.2 ± 1.9	≤ 1.0
	Omp25-GST stimulation			
Rev.1	ND	5.9 ± 6.9	11.2 ± 6.9 *	F
p-omp25	ND	15.1 ± 9.1 *	136.5 ± 164.47	97.1 ± 127.5
	IalB stimulation			
Rev.1	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0
p-ialB	≤ 1.0	≤ 1.0	2.9 ± 2.8	11.0 ± 13.5

Table showing mean ± standard deviation IFN-γ concentration (pg/ml) in the SN of stimulated splenocyte cultures after 43 ± 6 hours.
All DNA vaccine related data derived from assay of three mice per group at each sampling time. Rev.1/Brucellergene stimulation data derived from six mice group (except for week 11 data, which used only three mice per group).
ND: Not determined.
F: failed assay.
** indicates samples with IFN-γ concentrations significantly different to those measured in the corresponding antigen naïve (PBS or pcDNA3.1 vaccinated) samples (Mann Whitney U test). Detection limits the assay = 1.0 pg/ml.*

Brucella specific IFN-γ production in Rev.1 vaccinated mice

Brucellergene™ stimulation resulted in consistently high mean quantities of IFN-γ production (between 80 – 1000 pg/ml) from Rev.1 vaccinated mice at weeks five, eight and eleven of the experiments. Omp25-GST stimulation was able to promote limited IFN-γ production from the Rev.1 vaccinated mice. In contrast, IalB stimulation of Rev.1 mice did not result in detectable production of IFN-γ. A summary of IFN-γ responses to antigen stimulation is given in table 6.1, and Omp25-GST specific IFN-γ responses are summarised in figure 6.6

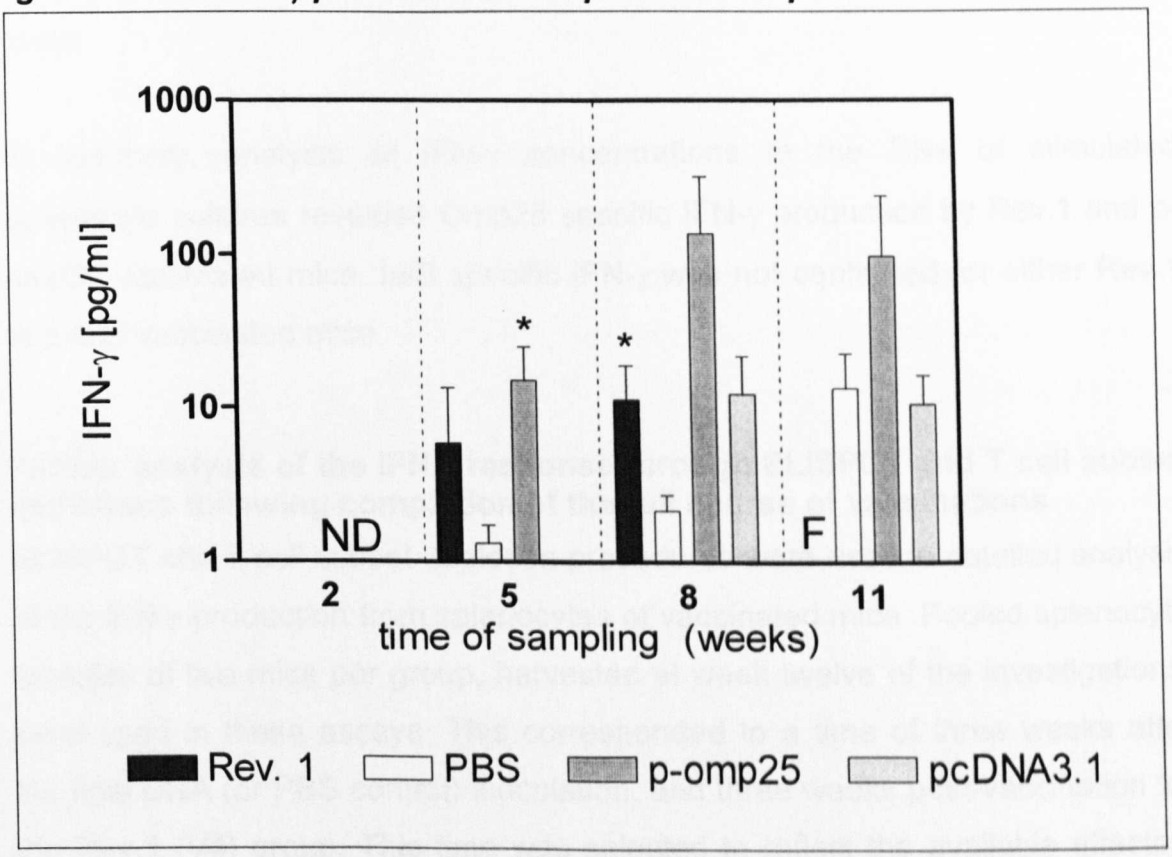
Brucella specific IFN-γ production in DNA vaccinated mice

Brucellergene™ stimulation did not reveal consistent or statistically significant IFN-γ production from p-*ialB* or p-*omp25* vaccinated mice. Furthermore, significant IFN-γ was not observed from IalB stimulated p-*ialB* group mice. Limited IFN-γ production was recorded from p-*ialB* inoculated mice in response

to IalB stimulation at week eight and week eleven of the study (summarised in Table 6.1), but these responses were not determined to be statistically significant. It is possible that the quantity of antigen specific IFN-γ observed (≤ 30 pg/ml) is of biological significance and contributes to the protective response, but a larger sample size or more sensitive method for IFN-γ detection would be required to validate these observations. These data suggest that despite the detection of high titres of IgG2a antibody, significant IFN-γ production is not evoked by the p-*ialB* vaccinations. These results are summarised in Table 6.1.

In contrast, Omp25 specific induction of IFN-γ was observed in p-*omp25* group mice (data summarised in Figure 6.6).

Figure 6.6: Net IFN-γ production in response to Omp25-GST stimulation



*Bars represent the mean NET IFN-γ concentration [pg/ml], and error bars the standard deviation. * indicates samples for which the concentration of IFN-γ is significantly greater than in the corresponding PBS samples. ND: not determined. F: failed assay.*

Mann-Whitney U Test of each data set presented figure 6 f was carried out to determine if the IFN-γ concentration of a sample from vaccinated mice was significantly different to that from PBS vaccinated mice. Production of IFN-γ in

response to Omp25-GST antigen stimulation is clearly evident in both p-*omp25* and Rev.1 vaccinated mice. However, because of high non specific responses these responses were not always considered statistically significant in comparison to the responses of antigen naïve mice (PBS or pcDNA3.1 groups).

At week 5, significant IFN-γ production was detected in the p-*omp25* inoculated mice ($p = 0.0022$) samples that had been stimulated with Omp25-GST. At week eight only Rev.1 samples showed any statistically significant IFN-γ production ($p = 0.0022$), and at week 11 none of the responses were determined to be statistically significant (Note that the Rev.1 assay failed). Note that although responses are observed at both week eight and week eleven from the p-*omp25* inoculated mice they are not determined to be significant in the Mann-Whitney U analysis because of variability in the response between individuals in the group.

In summary, analysis of IFN-γ concentrations in the SNs of stimulated splenocyte cultures revealed Omp25 specific IFN-γ production by Rev.1 and p-*omp25* vaccinated mice. IalB specific IFN-γ was not confirmed for either Rev.1 or p-*ialB* vaccinated mice.

Further analysis of the IFN-γ response through ELISPOT and T cell subset depletions following completion of the full course of vaccinations

ELISPOT and T cell subset depletion procedures were used in detailed analysis of the IFN-γ production from splenocytes of vaccinated mice. Pooled splenocyte samples of five mice per group, harvested at week twelve of the investigations, were used in these assays. This corresponded to a time of three weeks after the final DNA (or PBS control) inoculation, and three weeks post-vaccination for the Rev.1 (V4) group. This time was selected to reflect the available effectors present after the full course of vaccinations and shortly before challenge in these investigations. The summarised results of these studies are shown in tables 6.2 and 6.3.

Table 6.2: A comparison of the frequency and phenotype of IFN-γ secreting cells from mice receiving p-omp25 or control vaccine Rev.1. Δ IFN-γ secreting cells per million in response to stimulation with specific antigens

Vaccine	Net responsive cells		
	Total cell population	**CD4+ depleted cell population**	**CD8+ depleted cell population**
Stimulation with recombinant Omp25-GST [10 μg/ ml]			
Rev1 @ 3weeks	**15.5 ± 1.44**	4.0 ± 1	18.5 ± 2.5
pcDNA3.1 [X4]	**0**	0	0
p-*omp25* [X4]	**73.3 ± 20.3**	41.0 ± 5.2	10.8 ± 3.5
Stimulation with Brucellergene™ [40 μg /ml]			
Rev.1 @ 3 weeks	**41.36 ± 13.66**	10.0 ± 15.04	28.0 ± 10.76
pcDNA3.1 [X4]	**0**	0	0
p-*omp25* [X4]	**0**	0	0

The number of IFN-γ secreting antigen specific cells detected in splenocyte preparations from vaccinated mice. Data is displayed as ΔSFC / million, where Δ SFC is derived by subtraction of the background response of corresponding unstimulated cells. Data is displayed as mean Δ SFC /million ± Standard deviation. Two way ANOVA analysis confirms statistically significant differences between the vaccine groups and the T cell subset responses ($p < 0.0001$)
SFC: Spot Forming Cells.

Results presented in Table 6.2 illustrate that Brucellergene™ stimulation resulted in the greatest frequency of antigen specific IFN-γ secreting cells from Rev.1 vaccinated mice. T cell subset depletion indicated that the CD4+ cell subset was largely responsible for the production of IFN-γ in response to this antigen, since depletion of CD4+ cells from the reaction resulted in a 76% reduction in the number of Brucellergene™ specific IFN-γ secreting cells detected. In contrast depletion of CD8+ cells resulted in 33% reduction in the number of Brucellergene™ reactive cells detected. A response was also detected in Rev.1 group splenocytes following stimulation with the recombinant Omp25-GST antigen. Within this Omp25 specific population depletion of the CD4+ subset contributed the greatest reduction in IFN-γ secreting cells, resulting in a 74% decrease in the mean number of responsive cells. CD8+ depletion of splenocytes from these mice did not result in detectable reduction in the number of Omp25 responsive cells.

The ELISPOT data reveals that p-*omp25* vaccination produced greater total numbers of IFN-γ secreting cells in response to stimulation with Omp25-GST than Rev.1 vaccinated mice (73.3 Spot Forming Cells (SFC) versus 15.5 SFC, respectively). Measurement of depleted cell populations in the p-*omp25* samples indicated a significant decrease (~85%) in the number of responsive cells in the CD8+ depleted population. CD4+ subset depletion from this splenocyte population resulted in a 44% decrease in the number of responsive cells. This data suggested that that both CD4+ and CD8+ antigen specific IFN-γ secreting cells were induced by inoculation of p-*omp25* but there was a bias toward the CD8+ population. This result was in direct contrast to that observed for Rev.1 vaccinated mouse samples stimulated with the Omp25 antigen where CD4+ cells appeared to be the main population of responder cells.

Brucellergene™ or Omp25-GST specific IFN-γ producing T cells were not detected from vector or PBS control groups (data not shown).

In a second study (data presented in table 6.3) the CD4+ dominated response to Brucellergene™ antigen was confirmed in the Rev.1 vaccinated mice, with CD4+ depletion causing an 81% reduction in detectable responder cells, and CD8+ depletion resulting in a less pronounced effect (58% reduction). Interestingly, IFN-γ secreting cells were not detected following recombinant lalB stimulation of splenocytes harvested from Rev.1 vaccinated mice at this time.

Table 6.3: A comparison of the frequency and phenotype of IFN-γ secreting cells from mice receiving p-ialB or control vaccine Rev.1. Δ IFN-γ secreting cells per million in response to stimulation with specific antigens

Vaccine	Net responsive cells		
	Total cell population	**CD4+ depleted cell population**	**CD8+ depleted cell population**
Stimulation with recombinant IalB [20 μg/ml]			
Rev.1 @ 2 weeks	**0**	0	0
pcDNA3.1 [X4]	**0**	0	0
p-*ialB* [X4]	**75.02 ± 18.37**	11.0 ± 4.85	36.25 ± 2.92
Stimulation with Brucellergene™ [40 μg /ml]			
Rev.1 @ 2 weeks	**42.50 ± 20.06**	8.1 ± 4.03	18.0 ± 6.02
pcDNA3.1 [X4]	**0**	0	0
p-*ialB* [X4]	**0**	0	0

The number of IFN-γ secreting antigen specific cells detected in splenocyte preparations from vaccinated mice. Data is displayed as ΔSFC / million, where ΔSFC is derived by subtraction of the background response of corresponding unstimulated cells. Data is displayed as mean ΔSFC /million ± Standard deviation. Two way ANOVA revealed a statistically significant difference between the SFC detected in different vaccine groups and for different subsets ($p < 0.05$).

From the p-*ialB* vaccinated samples a high number of IalB specific cells were detected. T cell depletion studies revealed that elimination of the CD4+ subset resulted in the most noticeable reduction in IFN-γ secreting cells. CD4+ depletion effected an 85% reduction in the number of responsive cells whereas CD8+ depletion only achieved a 51% reduction, suggesting that p-*ialB* vaccination results in priming of both CD4+ and CD8+ antigen specific cells, but CD4+ cells are the dominant phenotype.

In summary the ELISPOT results presented in Tables 6.2 and 6.3, indicate a CD4+ dominated IFN-γ response from Rev.1 and p-*ialB* vaccinated mice, and a CD8+ dominated response from p-*omp25* .

IL-4, IL-12, IL-10 and TNF-α production

In addition to IFN-γ, the antigen specific generation of two other key Th.1 cytokines (IL-12 and TNF-α) was determined from the SN samples of each group at each sampling time. Th 2 cytokine production (IL-4) and

immunoregulatory cytokine production (IL-10) was also assessed. All cytokines were measured using Biosource Quantikine ELISAs to assess the concentration of cytokine in 48 hour splenocyte cultures. These data are summarised in table 6.4.

Table 6.4: TNF-α, IL-12p 40 and p70, IL-4 and IL-10 production in response to Brucella specific antigen stimulation

	Time			
	Week 2	Week 5	Week 8	Week 11
Net TNF-α production (Mean ± standard deviation) pg/ml				
Brucellergene™ stimulation				
Rev.1	22.7 ± 27.7	48.8 ± 69.4	104.2 ± 95.5	195.9 ± 12.9*
p-omp25	1.6 ± 1.0	31.2 ± 6.1	19.4 ± 14.8	34.4 ± 10.8
p- ialB	99.9 ± 153.3	64.0 ± 92.1	64.5 ± 89.6	26.83 ± 29.3
Omp25-GST stimulation				
Rev.1	ND	7.0 ± 1.0	70.0 ± 71.7	F
p-omp25	6.5 ± 5.6	33.2 ± 23.1	13.7 ± 16.6	32.0 ± 13.1
IalB stimulation				
Rev.1	≤1.0 pg/ml recorded in each sample			
p-ialB	10.3 ± 14.1	5.2 ± 9.37	≤ 1.0	2.8 ± 4.1
Net IL-12 p40 and [IL-12 p70] production (mean only) pg/ml				
Brucellergene™ stimulation				
Rev.1	34.4 [≤1.0]	35.0 [5.1]	62.0 [6.9]	53.8 [12.3]
p-omp25	33.8 [29.9]	44.9 [9.1]	151.7 [≤1.0]	68.5 [≤1.0]
p-ialB	≤1.0 pg/ml recorded in each sample			
Omp25-GST stimulation				
Rev.1	ND	62.9 [5.4]	42.5 [12.0]	F
p-omp25	111.0 [≤1.0]	51.5 [≤1.0]]	500 [7.5]	189.1 [17.0]
IalB stimulation				
Rev.1	≤1.0 pg/ml recorded in each sample			
p-ialB				
Net IL-4 production (mean only) pg/ml				
Brucellergene™ stimulation / Omp25-GST stimulation / IalB stimulation				
Rev.1	≤10.0 pg/ml recorded in each sample			
p-omp25				
p- ialB				
Net IL-10 production (mean only) pg/ml				
Brucellergene™ stimulation / Omp25-GST stimulation / IalB stimulation				
Rev.1	≤10.0 pg/ml recorded in each sample			
p-omp25				
p-ialB				

*ND: not determined, F: failed assay, * significant quantity of IFN-γ compared to corresponding antigen naïve control (PBS or pcDNA3.1). Each data point was obtained from three mice per group. Samples were assayed in duplicate.*

TNF-α production from vaccinated mice

As with IFN-γ stimulation, TNF-α was found in all ConA stimulated samples, except those from the failed Rev.1 assay at week 11 of the p-*omp25* study. The average concentration of net TNF-α in the ConA stimulated samples was around 95.48 ± 70.66 pg/ml (Mean ± standard deviation). Significant differences in the TNF-α concentration of different vaccine groups was not observed ($p \geq 0.05$). The average concentration of TNF-α in the SNs from unstimulated cells was 15.81 ± 7.69 pg/ml. Significant differences in the TNF-α concentration of resting cultures (unstimulated) from each vaccine group was not observed ($p \geq 0.05$).

The net concentration (Concentration of antigen stimulated TNF-α minus resting concentration) of TNF-α produced in response to Brucellergene™, recombinant Omp25-GST or IalB stimulation was measured. TNF-α production was detected. However, variation of TNF-α concentrations within groups (n = 3) meant that overall the responses of the vaccinated mice were not significantly different (Mann-Whitney U test, $p \geq 0.05$) to those observed from non-immune PBS inoculated mice stimulated with the same antigen.

IL-12 production from vaccinated mice

IL-12 p40 and p70 were measured in the SNs of cultured splenocytes by Biosource ELISAs. For these assays group samples were pooled, such that each test sample consisted of the pooled SN from a single group (n = 3) of mice stimulated with a designated antigen at a designated time.

A low level of IL-12 p40 was detected intermittently in SNs of p-*omp25* and Rev.1 vaccinated mice following stimulation with Omp25-GST or Brucellergene™. The average IL-12 p40 concentration in the SNs of unstimulated pooled splenocyte cultures from these mice was 53.46 ± 33.37 (Mean ± standard deviation) pg/ml, but an antigen specific effect on IL-12 p40 production was not observed. A similar pattern of IL-12 p70 production was observed, although IL-12 p70 was not detected in unstimulated SNs and concentrations observed in stimulated SN never exceeded 30 pg/ml. Notably,

neither IL-12 p40 nor p70 were detected in the SNs from Rev.1 vaccinated mice in response to stimulation with recombinant IalB. Furthermore, IL-12 p40 and p70 were not determined following stimulation of p-*ialB* group splenocytes with either Brucellergene™ or recombinant IalB.

IL-4 production from vaccinated mice

IL-4 was recorded intermittently, at concentrations of ≤10 pg/ml in the SNs from unstimulated, IalB, Omp25 and Brucellergene™ stimulated samples. A significant vaccine or stimulation specific effect on IL-4 concentration was not detected ($p > 0.05$). IL-4 was consistently detected in the ConA stimulated SNs.

IL-10 production from vaccinated mice

SNs were tested for the presence of IL-10. Each sample contained between 1 and 10 pg/ml of IL-10. Neither antigen nor vaccine specific effects on IL-10 concentration were detected.

6.5: Discussion

The main aims of the investigations described in this chapter were to assess a vaccine formulation based upon a mixture of the two *omp25* constructs for protective efficacy and also to measure the antigen specific immune responses elicited by both p-*omp25* and p-*ialB* vaccines.

The protective efficacy of the combined pCR3.1-*omp25* and pTargeT-*omp25* vaccine: p-*omp25* was confirmed. Mice receiving the p-*omp25* vaccine, given as four intramuscular inoculations of 100 μg per dose at three week intervals, were able to control *Brucella* replication in the spleen to a similar extent to those mice vaccinated with the 'gold standard' live strain vaccine, *B. melitensis* Rev.1 (V4). Similarly, the p-*ialB* vaccine resulted in an equivalent protective effect to the Rev.1 (V4) vaccine. Both the p-*omp25* and p-*ialB* vaccines effected a mean 2 – 3 log reduction of splenic *Brucella* compared to the PBS unprotected control (3.42 or 2.15 protection units or ≥ 99% reduction of actual numbers). This represents a considerable improvement over the protective efficacy so far

described for other *Brucella* specific DNA vaccines. In addition this represents the first report of protection mediated by DNA vaccines against brucellosis caused by *B. melitensis*. A DNA vaccine encoding the *B. abortus* GroEL was found to be immunogenic but not protective against *B. abortus* 544 challenge (LeClerq *et al,* 2002). The immunodominant cytosolic P39 protein (Al-Mariri *et al,* 2001) resulted in only a modest reduction of *B. abortus* 544 at eight weeks post-challenge (0.73 Log reduction in splenic *Brucella* compared to control mice), and a statistically insignificant reduction (0.15 Log) at an earlier sampling time (4 weeks post-challenge). A more successful result was recorded from a DNA vaccine based upon *Brucella* Lumazine synthetase, which afforded a 1.25-1.65 log control of splenic *Brucella* following *B. abortus* 544 challenge (Velikovsky *et al,* 2002). Similarly, a DNA vaccine encoding *Brucella* Superoxide dismutase (SOD) resulted in a 1.15 log protective effect through intramuscular inoculation which was moderately improved (1.52) by delivery of the vaccine directly to the spleen (Munoz-Montesino *et al,* 2004). Thus far *B. melitensis* specific DNA vaccines with demonstrable protective efficacy have not been reported.

Importantly, the immune mediators of the protective effect were examined and the induction of critical Th 1 biased effector responses were shown for the novel p-*omp25* and *p-ialB* vaccines. The presence of relatively high titre antibodies of the IgG2a isotype in the sera of DNA and Rev.1 vaccinated mice, suggested the involvement of IFN-γ rather than IL-4 in the specific response to vaccination (Snapper *et al,* 1988). Antigen specific IFN-γ secretion was directly measured following specific antigen stimulation of splenocytes. Other Th 1 cytokines were also measured in the SNs of stimulated splenocyte cultures: TNF-α and IL-12 p40 and p70 were produced in response to stimulation with *Brucella* antigens although a consistent statistically significant antigen specific stimulation effect could not be seen. Overall the cytokine analysis revealed a Th 1 dominated immune response specific antigen stimulation in the DNA and Rev.1 vaccinated mice. This response is expected to contribute to the protection of mice following *B. melitensis* 16M challenge.

The established method for estimating optimal protective efficacy of live vaccines in the BALB/c infection model involves challenge at 30 days post-vaccination (Bosseray *et al*, 1991). This suggests that the most appropriate time to measure corresponding immunological effectors of protection is around 30 days post-vaccination. Specific secretion of cytokines was measured from the Rev.1 vaccinated mice at weeks 2, 3, 5, 8, and 11 post-vaccination during the current experiments. Thus, the response measured between weeks two and five of the study corresponded to the time when the mouse was optimally protected against challenge. The data obtained in these studies indicated that period of optimal protection from Rev.1 correlated well with the detection of *Brucella* specific IFN-γ and the first appearance of specific IgG2a. Thus suggesting that these are significant effectors of the protective response. The ability of the Rev.1 vaccinated mice to recognise the Omp25 and lalB antigens was also measured.

The expected humoral immune response to Rev.1 was observed. IgG1 and IgG2a specific antibodies were detected using the 16M ELISA. The Omp25-GST ELISA confirmed the specific immunological recognition of the Omp25 antigen in Rev.1 vaccinated mice (Figure 6 e [c] and [d]), and the kinetics of the response measured in the Omp25-GST ELISA was similar to those measured in the 16M ELISA. The maximum titres achieved in the 16M ELISA were considerably higher than in the Omp25-GST ELISA, reflecting the fact the dominant serological response from Rev.1 vaccinated mice is directed against the S-LPS present in 16M antigen. Additionally, possible structural differences between the recombinant Omp25-GST and native Omp25 in the 16M antigen that result from the absence of Omp25- LPS association in the recombinant preparation and possible differences in protein folding may impact upon availability of conformational epitopes. Therefore, the detection of responses from Rev.1 vaccinated mice in the Omp25-GST ELISA was significant because it indicated that the *E. coli* derived recombinant Omp25-GST preparation was not prohibitive to the binding of anti-Omp25 antibodies raised against the native protein delivered by the live Rev.1. Notably, the IgG2a Omp25-GST specific antibodies were detected earlier than IgG1 Omp25-GST specific antibodies in the Rev.1 vaccinated mice. This IgG2a dominance persisted for the duration of

the experiment. Thus, indicating that although both IgG1 and IgG2a antibodies are produced on exposure to Rev.1, the response directed against Omp25 antigen is IgG2a dominated. Thereby suggesting native Omp25 antigen promotes a Th 1 type immune response in the context of protective vaccination.

In contrast, IalB specific antibody responses were not detected in Rev.1 vaccinated mice. Transcription of *ialB* from the *B. melitensis* strain 16M was confirmed through RT-PCR studies described in Chapter 3, and the expression of protein from cultured 16M has been confirmed through the proteome studies of Wagner *et al,* (2002). Moreover, the same group directly compared the cultured proteomes of *B. melitensis* 16M and Rev.1 demonstrating significant differences in the expression of certain proteins, but differential expression of IalB was not recorded (Eschenbrenner *et al,* 2002). Together this data suggests that IalB expression is a consistent and persistent phenomenon from cultured *B. melitensis* strains, and therefore the antigen is expected to be accessible for interaction with the immune system following Rev.1 vaccination. Notably, these expression studies are restricted to measuring IalB expression under artificial culture conditions and do not necessarily reflect the changing environment and stimuli encountered *in vivo*. It is therefore possible that the lack of IalB specific antibodies in the vaccinated mice reflects a lack of expression of this antigen from Rev.1 *in vivo*.

Specific antibody responses were recorded from the mice that received inoculation with the novel DNA vaccines. Relatively high titre IgG1 and IgG2a specific responses were detected from the p-*omp25* inoculated mice in both the 16M and Omp25-GST ELISAs. The confirmation of specific antibody from the p-*omp25* group in the 16M ELISA proved that antibodies induced by this vaccine were able to bind native Omp25. Furthermore, demonstration of p-*omp25* 16M reactive antibodies supported a possible *in vivo* interaction between p-*omp25* generated antibodies and viable *B. melitensis* 16M following challenge. The titre of anti-Omp25 in the antisera from p-*omp25* vaccinated mice exceeded that recorded for the Rev.1 vaccinated mice, indicating an enhanced response to this antigen elicited by the monospecific DNA construct compared to the recommended live vaccine strain Rev.1. In addition, Omp25 specific serum

antibodies were apparent after a single inoculation, suggesting that immunity might be achieved from less than four inoculations of p-*omp25*.

High titre IalB specific antibodies were measured following vaccination with p-*ialB*. In the 16M ELISA (expected to contain native *Brucella* IalB protein) the response was not detected until the final sampling time (week eleven) of the study. Due to differential sensitivities of the two ELISAs, a response was determined earlier in the recombinant IalB ELISA, with seropositive results apparent from week eight of the study. These data suggest that at least three inoculations were required to elicit detectable specific antibody, and that four inoculations resulted in production of high titre antibody responses. Again, it is significant that the reactivity is demonstrable in the 16M ELISA as this denotes recognition of native IalB protein by antibodies raised through p-*ialB* vaccination, and such reactivity would be essential for these antibodies to have an impact on live *Brucella* challenge *in vivo*.

In the 16M ELISA the IgG1 titre from p-*ialB* exceeded that measured from the Rev.1 vaccinated animals. Since the reactivity of Rev.1 group antisera in the 16M ELISA is polyclonal but antisera from DNA vaccinated mice is monospecific, this result suggests that high affinity and avidity response antibodies are induced by p-*ialB* vaccine. In contrast to the IgG2a biased response to the Omp25 antigen from p-*omp25* vaccinates, the balance of IgG1 and IgG2a in p-*ialB* antisera does not suggest a strong Th 1 bias.

In summary, both DNA vaccines (p-*omp25* and p- *ialB*) promote development of specific antibodies. IgG2a dominant Omp25 specific responses are achieved by both Rev.1 and p-*omp25* vaccination, whereas IalB specific antibodies are only observed in p-*ialB* vaccinated mice: Rev. 1 vaccinated mice do not produce IalB specific antibodies.

Historically antibodies have not been considered important for protection against intracellular pathogens (for review see Casadevall, A., 2003). This view is increasingly being challenged, and there is evidence for antibody mediated protection against obligate intracellular pathogens such as *Ehrlichia chaffensis*

(Winslow *et al*, 2000), and *Listeria monocytogenes* (Edelson 1999 and 2001). The role of antibodies in protection against brucellosis is ambiguous. Protection has been described in mice following passive transfer of *Brucella* specific antibodies (Montaraz *et al,* 1986), but in a natural infection situation their impact is difficult to determine. The detection of *Brucella* specific antibodies is more often associated with diagnosis of active infection, rather than considered as an indicator of immunity. Indeed, Duran-Ferrer *et al,* (2004) noted a reduction in magnitude and duration of antibody response in pregnant 'immune' Rev.1 vaccinated sheep following challenge with virulent *Brucella* in comparison to previously naïve sheep. Nevertheless, it is well known that opsonisation of bacteria by specific antibody enhances the efficiency of phagocytic uptake, and this has been documented for both virulent and attenuated *Brucella* strains in cultures of both activated and quiescent phagocytic cells. Moreover, opsonisation appears to decrease survival time and replication of internalised *Brucella* (Harmon *et al,* 1988., Caron *et al,* 1994., Gross *et al,* 1998), suggesting that antibodies can affect the outcome of infection.

Protection has been observed in passive transfer studies using monoclonal antibodies (Mabs) against *Brucella* LPS and a number of outer membrane proteins (Montaraz *et al,* 1986., Cloeckaert *et al,* 1991, Bowden *et al,* 1995). However, to date the protection mediated by antibodies specific for proteins has been difficult to demonstrate against smooth strain *Brucella* challenge and significantly, protection mediated through Omp25 specific antibodies has been repeatedly dismissed. For example, Montaraz (Montaraz *et al,* 1986) was unable to protect BALB/c mice against *B. abortus* challenge through passive transfer of IgM raised against 'porins' from *Brucella* outer membrane fractions. Furthermore, IgG2a Mab specific to the Omp25 protein (A59/05F01/CO9) was unable to provide significant protection against challenge with the naturally rough *B. ovis* (Bowden *et al,* 2000) suggesting that even in the absence of interference from LPS and peptidoglycan on the *Brucella* surface antibodies against this protein as are not sufficient for protection. The role of IalB specific antibodies in brucellosis immunity has not previously been assessed. Significantly, over 70% of *Brucella* convalescent antisera were reactive to this protein, suggesting widespread humoral recognition of this protein. It is possible

that Omp25 and IalB specific antibodies contribute to protection through increasing the efficiency of the phagocytosis and subsequent destruction of the internalised *Brucella*. Alternatively, interaction of the specific antibody and *Brucella* antigen may promote protection through neutralisation of an essential virulence function. The data obtained in this study confirms that specific antibodies capable of recognising and binding with native IalB and Omp25 are generated by the DNA vaccines, but their relative contribution to the observed protection remains undefined.

Irrespective of any direct role in protection, the demonstration of specific IgG2a antibody is significant since immunoglobulin class switching to generate IgG2a requires the involvement of the critical cytokine effector IFN-γ. The actual measurement of IFN-γ and other cytokines was achieved through *in vitro* restimulation of the splenocytes of vaccinated mice and measurement of cytokine concentration in the 48 hour stimulated splenocyte cultures. For the Rev.1 vaccination this data reflects the changes in IFN-γ response occurring with time post-vaccination. The Rev.1 vaccinated mice were shown to produce IFN-γ upon stimulation with the Omp25-GST or Brucellergene™ antigens, but not upon stimulation with the IalB antigen. Apart from a modest and statistically insignificant reduction in the quantity of IFN-γ elicited following ConA stimulation at weeks five and eight, there was no direct evidence for a disruption of IFN-γ production from Rev.1 vaccinated (infected) mice, which would correlate with the observed IFN-γ hiatus observed in BALB/c mice exposed to virulent *Brucella* infection (Murphy *et al,* 2001a).

Rev.1 vaccination resulted in a Th1 cytokine profile in which IFN-γ was the principal effector released upon encounter with *Brucella* specific antigens. Importantly, IFN-γ release was also observed when Rev.1 mice were stimulated with recombinant Omp25-GST indicating that native Omp25 primes T cells following Rev.1 vaccination. In contrast, IFN-γ was not observed in the SNs of IalB stimulated splenocytes from Rev.1 mice, suggesting that the IalB antigen is not involved in Rev.1 mediated immunity.

The measurement of antigen specific IFN-γ is significant since IFN-γ is considered crucial to anti-*Brucella* immunity (Murphy *et al*, 2001a/b). Cytokine production following Omp25 stimulation was qualitatively similar in p-*omp25* and Rev.1 vaccinated mice. A rapid induction of IFN-γ response was observed, with considerable IFN-γ evident following the second p-*omp25* inoculation. At week eight and week eleven (after the third and fourth p-*omp25* vaccinations respectively) the mean concentration of Omp25 specific IFN-γ was elevated in the SNs of p-*omp25* inoculated mice, compared to those from other mice. However, this was not considered statistically significant because of the high degree of variability within the groups. Although desirable, statistical significance in the assays cannot be taken as an absolute measure of success or failure because of low sample numbers. Nonetheless, the data confirms antigen specific induction of IFN-γ in p-*omp25* inoculated mice, which is evident in SN from week five of the study after one booster inoculation.

TNF-α and IL-12 were also measured intermittently in the SN from *Brucella* antigen stimulated splenocytes. However, the production of these cytokines in vaccinated mice was never significantly different to that observed in the antigen naïve controls. TNF-α is a primary mediator of inflammatory responses and important in the regulation of immune responses. It is expressed by a number of activated cell types, including macrophages, neutrophils and NK cells of the innate immune response as well as antigen specific T and B cells. Thus, a large proportion of the TNF-α observed in these studies from any group of mice is a result of innate response to bacterial antigen by the splenocyte populations, and consequently innate TNF-α production may obscure measurement of antigen specific effects.

Despite the absence of a convincing vaccine mediated augmentation of TNF-α production, the demonstration of production of this cytokine from p-*omp25* is noteworthy because of the reported inhibitory effects of native Omp25 on TNF-α production by cultured human macrophages (Jubier-Maurin *et al*, 2001). The inhibition of this key mediator is known to be significant in the control of *Brucella*, particularly early on in the infection (Zhan *et al*, 1996., Murphy *et al*,

2001) and thus Omp25 has an important immunomodulatory role that possible contributes to the virulence or survival of the *Brucella*. The reported inhibition of TNF-α by Omp25 has yet to be confirmed *in vivo* or indeed demonstrated from cell cultures other than human THP-1 macrophages, but the possibility of such effects naturally invites questions regarding the safety of an Omp25 based vaccine. Judging from the fact that the p-*omp25* vaccinated mice are capable of TNF-α generation and that these mice are protected against challenge it would appear that such immunomodulation does not feature in this particular model. Nevertheless, because this is often cited as a host specific effect there remains a concern TNF-α inhibition could occur in humans inoculated with this vaccine. The investigation of this potential problem in humans is outside the scope of this thesis but would need to be addressed before the application of this vaccine in humans could be considered.

IalB specific IFN-γ, TNF-α or IL-12 were not recorded in the splenocyte SNs from either Rev.1 or p-*ialB* vaccinated mice. In the absence of demonstrable IalB specific Th 1 cytokines, IL-10 and IL-4 production was assessed in order to check for a possible mechanism of IL-12 inhibition, and the involvement of regulatory T cells in the immune response to p-*ialB* vaccination or IalB stimulation. IL-10 is known to inhibit the synthesis of Th 1 type cytokines, and reduce activation of monocytes, macrophages and dendritic cells (For review see de Waal Malefyt and Moore, 1998). However, no evidence of elevated IL-10 concentrations was detected in any of the groups. In addition, *Brucella* antigen specific induction of IL-4 was not observed in any of the samples thus ruling out the generation of a strongly Th 2 biased immune response.

Although the actual *in vivo* proportion of *Brucella* reactive cells was not measured, *ex vivo* incubation with Brucellergene™, IalB, or Omp25-GST, and T cell fractionation and ELISPOT methods were used to evaluate the number of antigen specific primed and activated IFN-γ secreting cells in the splenocyte population of the mice post-vaccination. The ELISPOT data therefore provided a snapshot of the effector cell population present in the vaccinated mice prior to challenge.

The phenotype of antigen specific IFN-γ secreting cells was determined by simple *in vitro* T cell depletion procedures. Both CD4+ and CD8+ T cell have been shown to contribute to the control of *Brucella* growth in the BALB/c mouse in adoptive transfer studies (Araya *et al*, 1989). Additional studies involving *in vivo* depletion strategies have indicated that the involvement of CD8+ cells is crucial (Pavlov *et al*, 1982., Mielke, 1991., Oliveira and Splitter 1995). However, recently it has been suggested that whilst CD8+ T cells are important in the control of live attenuated *Brucella* infections, they have a less significant role in the control of virulent infections. Baldwin and Gorenka (2004) postulated that CD8+ cells are effective at controlling primary infection with virulent *B. abortus* 2308 in C57BL/6 mice at three and six weeks post-infection but not at ten weeks. The absence of a role for CD8+ cells in the later stages of infection with virulent strains is postulated to relate to an as yet undefined virulence mechanism which manipulates antigen presentation in the infected host in order to avoid MHC class I peptide loading and thereby avoid induction and the cytotoxic action of CD8+ T cells. Ultimately, this strategy would promote establishment and persistence of the virulent *Brucella* infection in the mouse, because once this virulence factor was operational infected cells would not display antigen for recognition by CD8+ effector cells. Therefore, any protective effect from CD8+ T cells is limited to the early stages of infection prior to the activation of the virulence factors that reduce MHC class I peptide loading and hide the virulent *Brucella* from activated cytotoxic T cells. This hypothesis ultimately indicates that CD4+ T cells are crucial for the control of chronic brucellosis.

For the Rev.1 (V4) mice the ELISPOT data showed that CD4+ cells were largely responsible for the production of Brucellergene™ specific IFN-γ. When these splenocytes were stimulated with the Omp25-GST antigen rather than Brucellergene™ a qualitatively similar CD4+ dominated response was observed. Thus indicating that an Omp25 specific CD4+ effector cells population is generated following exposure of the host to native Omp25 antigen delivered by live *Brucella*. The relative contribution of the Omp25 specific response to the overall protective effect of Rev.1 vaccination cannot be directly determined in this experiment, but is clear that a significant number of effector

cells are produced soon after exposure, suggesting that the immune response against this antigen is important. In contrast, IalB specific IFN-γ effector cells were not detected from Rev.1 vaccinated mice. This finding correlates with the humoral immune response and SN cytokine data, and supports the hypothesis that IalB is not a significant antigen in Rev.1 mediated immunity.

Omp25 specific effector cells were also confirmed in p-*omp25* vaccinated mice. A significant number of Omp25-GST responsive cells were observed in the total splenocyte population from these mice. In fact, a greater frequency of Spot Forming Cells (SFC) was measured from these samples than observed in the concurrent Brucellergene™ stimulation of the Rev.1 (V4) derived splenocytes. Since the Brucellergene™ response of the Rev.1 (V4) vaccinated mice indicates the magnitude of IFN-γ priming achieved through a successful protective vaccine in response to multipartite stimulus, the high number of Omp25-GST specific SFC suggests observed following p-*omp25* vaccination suggests highly effective priming of biologically significant numbers of T cells. Interestingly, the T cell subset depletion studies indicated that CD8+ T cells were mainly responsible for the production of IFN-γ following p-*omp25* vaccination. Depletion of the CD8+ fraction of the splenocyte population resulted in an 85% reduction in the number of detectable responsive cells, whereas CD4+ depletion only caused a 44% reduction. This result contrasted with that observed for the Rev.1 vaccine where CD4+ cells were responsible for the majority of the Omp25 specific IFN-γ production. Whether this difference is significant in terms of the longevity of protective immunity or for the extrapolation of findings to the target species is speculative.

Despite the absence of confirmed specific IFN-γ in the SN of cultured stimulated splenocytes, IalB specific effector cells were observed in p-*ialB* vaccinates using the ELISPOT assay. Notably the total number of IalB specific SFC detected in the ELISPOT for p-*ialB* vaccinated mice was not significantly different to the number of Omp25 specific SFC demonstrated following p-*omp25* vaccination despite differences in the quantity of this effector recorded in the SN of each set of stimulated cells. This finding suggests that both the p-*ialB* and p-

omp25 vaccines result in quantitatively similar numbers of primed T cells, but there is a difference in the capacity of the primed T cells to respond to stimulus with cognate antigen. That is, IalB specific T cells secrete less IFN-γ upon ligation with cognate antigen than Omp25 specific T cells. Interestingly, the IalB specific IFN-γ secreting cells were shown to be mainly CD4+ cells. Depletion of CD4+ cells resulted in an 85% reduction in the IFN-γ secreting SFC per million detected in ELISPOT assay. CD8+ cells were also primed by this vaccine, as depletion of this subset resulted in a 51% decrease in detected effector cells.

The priming of different T cell subsets is usually related to the mechanisms of antigen presentation. DNA vaccines promote CD8+ T cell priming when antigen is presented by APCs in the context of MHC class I. Direct transfection of APCs results in endogenous production and processing of the encoded antigen to produce strong CD8+ effector cells responses, whereas transfected myocytes are thought to promote CD8+ T cell priming mainly through cross-presentation mechanisms. The generation of CD4+ effector cells following DNA vaccination is generally thought to occur following expression of the encoded gene in bystander cells, and subsequent uptake and MHC class II restricted processing of the exogenous antigen by APCs. However, autophagy occurring in dendritic cells as part of normal cell homeostasis has also been shown to deliver endogenous antigens into the phagolysomal complex and thus access the MHC class II processing pathway (For review see Howarth and Elliot, 2004).

The differential priming of antigen specific CD4+ and CD8+ subsets by Rev.1, p-*ialB* and p-*omp25* vaccines may be related to differences in the antigen presentation pathways targeted by the different vaccines. Rush *et al* (2002) have shown that cell effective priming of T cell subsets through DNA vaccination is dependent upon both the quantity of presented antigen and the cellular location of the expressed antigen, with cell associated antigen accessing to MHC class I processing routes delivering stronger CD8+ priming. Omp25 specific CD4+ and CD8+ priming were observed from both p-*omp25* and Rev.1 vaccines. However, the relative frequencies of CD4+ and CD8+ cells suggests that class II presentation of Omp25 derived peptides is dominant

following Rev.1 vaccination, whereas class I presentation is dominant following DNA vaccination. From this observation it can be concluded that DNA derived Omp25 is probably cell associated and effectively targeted to MHC class I peptide loading through either direct or cross-presentation routes. In contrast, the strong antibody response and CD4+ priming bias of p-*ialB* reflects dominance of the MHC class II presentation of the antigen. During the production of the recombinant IalB protein it was observed that the N terminal signal sequence of the *ialB* gene was active in *E. coli* resulting in the export of the nascent protein to the cell membrane and cleavage of the GST fusion tag from the mature protein (Mahavir Singh, personal communication). Furthermore, although it was not conclusively demonstrated, the IFA results obtained in chapter 3 suggested that the secretion signal of the *Brucella ialB* gene was also functional in eukaryotic cells. The signal is expected to result in the export of the protein to the cell surface and potential secretion into the extracellular environment. If such processing occurs *in vivo* following expression of the p-*ialB* plasmid by transfected host cells, or following expression of the *ialB* gene directly from *Brucella* during infection, the secreted protein will be readily accessible for exogenous antigen processing via MHC class II restricted mechanisms leading to CD4+ priming, thus accounting for the CD4+ bias in the IFN-γ producing cells. Secretion of the antigen also makes it accessible for interaction with B cells and antibody binding, and thus explains the high antibody titres observed following p-*ialB* vaccination.

Despite a difference in T cell priming this study does not reveal any discernable difference between the ability of p- *ialB*, p-*omp25* or Rev.1 vaccinated mice to protect mice against *B. melitensis* 16M challenge.

In conclusion, current results have confirmed that protective vaccination with Rev.1 results in the generation of a CD4+ dominated effector response and negligible numbers of CD8+ effector cells. Similarly, the protective p-*ialB* vaccine promotes a CD4+ dominated response, with a lower but significant population of CD8+ effector cells. In contrast, p-*omp25* vaccination results in a CD8+ dominated response with a relatively low number of CD4+ cells. The impact of these differences in T cell priming upon protective efficacy are not

immediately apparent from this investigation as each vaccine promoted an equivalent protective effect following challenge at 30 days post-vaccination. Baldwin and Gorenka indicated that CD8+ T cells were still effective for the control of *B. abortus* 2308 in the C57BL/6 mouse up to six weeks post-infection, and therefore extrapolation of this finding to the current investigations suggests that CD8+ cells would contribute to the control of infection observed at 15 days post-challenge but may not be sufficient to control any residual *Brucella* remaining after six weeks. In this case the CD4+ effector cells become essential for the complete elimination of infection, and thus vaccines such as Rev.1 or p-*ialB* which induce high frequency CD4+ priming are likely to be most effective for long term control of infection. In contrast vaccines promoting CD8+ priming have to induce suitably potent effectors to eradicate infection prior to the subversion of antigen processing by virulent organisms. The absence of viable *Brucella* recovery from 75% of the p-*omp25* vaccinated mice suggests effective deployment of anti-*Brucella* effectors, but sterile immunity in 100% of vaccinates is not achieved. Consequently, although the CD8+ priming by the p-*omp25* vaccine is significant, the demonstration of an appreciable Omp25 specific CD4+ population is encouraging as this is likely to be essential for the clearance of residual infection.

This investigation of p-*omp25* represents the first report of protective immunity against smooth strain *Brucella* challenge effected by Omp25 specific immune responses. Other researchers have reported that the delivery of viable recombinant *E. coli* expressing *B. melitensis* Omp25 on the surface was unable to confer protection against *B. melitensis* 16M challenge in the BALB/ mouse model despite the generation of significant titres of specific IgG2a and a doubling of antigen specific IFN-γ compared to unmodified *E. coli* controls (Bowden *et al,* 1998). It is not realistic to directly compare the IFN-γ response described by Bowden *et al,* with that generated through p-*omp25* immunisation as the influence of the *E. coli* specific response in the former study cannot accurately be delimited and may obscure the antigen specific response. *E. coli* LPS will undoubtedly account for a large proportion of the IFN-γ induction observed in this study. However, a comparison of the capacity of the *E. coli*

Omp25 and p-*omp25* vaccines to promote specific responses may provide some insight into the differential protective efficacies. For instance, although the *E. coli* Omp25 vaccine induces far greater quantities of IFN-γ (in the ng/ml range rather than pg/ml), there is a greater relative enhancement of antigen specific IFN-γ (a mean ten-fold increase) between the p-*omp25* vaccine and it's relevant vector control (*E. coli* pUC19 for the Bowden study and pcDNA3.1 vector control in this study). It is therefore possible, that Omp25 specific priming may be more potent from the DNA vaccine than the live *E. coli,* and this improved IFN-γ response may be directly related to the enhanced protective efficacy.

It is not possible to determine the impact of potential differences in the IFN-γ secreting cell phenotypes between the DNA and *E. coli* Omp25 vaccines as this was not measured in the *E. coli* study. However, as the parent strain *E. coli* is not normally considered to be an intracellular pathogen, delivery of the Omp25 antigen for MHC class I presentation and generation of CD8+ T cells is anticipated to be less significant than seen with DNA vaccines. Thus, the potent induction of Omp25 specific CD8+ cells may also contribute to the improved efficacy of the p-*omp25* vaccine.

The specific immune response to *Brucella* IalB antigen has not previously been investigated, and this study represents the first report indicating this to be an important protective antigen in brucellosis. The p-*ialB* vaccination resulted in an equivalent protective effect to that observed from the Rev.1 or p-*omp25* vaccines. Immune responses to p-*ialB* vaccination were characterised by strong antibody responses and a CD4+ dominated IFN-γ secreting effector population. The *Brucella* IalB antigen is postulated to be involved in a specific receptor defined interaction with host cells, which facilitates internalisation. This function is well characterised for the homologous *Bartonella ialB* product (Mitchell and Minnick 1995). In *Brucella* IalB is likely to be most important in cells such as placental trophoblasts, as specific receptor mediated internalisation is a redundant mechanism in professional phagocytes. However, IalB receptor interaction may facilitate entry of the bacteria into phagocytes without the

activation of bactericidal responses associated with Fc, complement or fibronectin (Fn) mediated internalisation. Alternatively, the *ialB* gene product may play a role in the subversion of pathways trafficking internalised bacteria to the replicative niche. A major immunological output of p-*ialB* vaccination is antibody. It is therefore hypothesised that the generation of high affinity anti-IalB IgG may act to neutralise the function of the IalB protein. Moreover, the secretion or surface location of the IalB protein allows antibodies to be effective in a neutralising, complement fixing, or opsonising capacity. A thorough investigation of the role of the *ialB* locus in *Brucella* pathogenesis is required. The production of *B. melitensis* ΔialB mutants and comparison of *in vitro* and *in vivo* growth would be facilitate understanding of the role of this antigen in pathogenesis and protective immunity. Importantly, IalB specific immune responses were shown to be protective, despite their notable absence in Rev.1 mediated immunity.

In conclusion, the data generated in this investigation confirms that mice were protected through vaccination with the p-*omp25* or p-*ialB* DNA vaccines and that the four-dose vaccination schedule resulted in the generation of antigen specific IFN-γ and serum antibodies of IgG1 and IgG2a isotype. The value of Omp25 as a protective antigen is supported by the observation of significant Omp25 antibody and IFN-γ production in Rev.1 vaccinated mice. In contrast IalB specific antibody and IFN-γ were not detected from Rev.1 vaccinated mice suggesting that this antigen is not contributory to protective immunity in Rev.1 vaccinated mice. The demonstration of p-*omp25* and p-*ialB* protective efficacy and characterisation of antigen specific responses in the BALB/c mouse is a significant first step towards the development of a non-living vaccine that could be applied to target species: small ruminants or man.

Chapter 7: AN INVESTIGATION OF CATIONIC LIPOSOME DELIVERY SYSTEMS TO IMPROVE DNA VACCINE EFFICIENCY

7.1: Summary

The DNA vaccines p-omp25 and p-ialB had been shown to have equivalent protective efficacy against B. melitensis 16M challenge when delivered as four 100 μg doses at three week intervals (p-omp25 [X4] and p-ialB [X4]). In order to reduce the amount of DNA and the number of inoculations needed for the protective effect, modifications to the vaccination strategy were assessed. A single dose i/m inoculation of either naked DNA vaccine (p-omp25 [X1] or p-ialB [X1]), or liposome adsorbed DNA (L-p-omp25 [X1] or L-p-ialB) was unable to provide the same level of protection as the p-omp25 [X4] or p-ialB [X4] vaccination.

Differences were recorded in the specific immune responses elicited by the different vaccine preparations. The specific antibody responses were greatest following the multiple dose vaccinations with naked DNA (p-omp25 [X4] or p-ialB [X4]. Single dose naked DNA (p-omp25 [X1] or p-ialB [X1]) vaccinations produced only low titres or an absence of detectable antibodies, but delivery of equivalent quantities of DNA surface adsorbed to cationic liposomes (L-p-omp25 or L-p-ialB) resulted in improved titres. IFN-γ secreting CD4+ or CD8+ effector cells were not demonstrable in either p-ialB [X1] or L-p-ialB [X1] vaccinated mice. However, an anamnestic IFN-γ effector cells was detected from L-p-ialB [X1] vaccinated mice post-challenge, suggesting that modest enhancement of T cell priming had occurred. Although significant, this improvement was insufficient for protective immunity from a single dose of vaccine, suggesting that antibody alone is insufficient and that CD4+ effectors are required for protection. In contrast, fewer total IFN-γ secreting cells were observed for the L-p-omp25 [X1] vaccinated mice than in the p-omp25 [X1] mice, and a reduction in the capacity for CD8+ T cell priming was observed in L-p-omp25 [X1] vaccinates. The superior efficacy of the p-omp25 [X4] versus the

p-omp25 [X1] strategy was also correlated with a modest increase in CD8+ effector cells. Together the data suggest that both cell subsets contribute to protection but that the CD8+ cells are essential for clearance under the conditions assessed in this experiment.

In conclusion, four doses of p-omp25 or p-ialB naked DNA vaccines are protective. A single dose of these naked DNA vaccines is not sufficiently immunogenic to be protective. Liposome delivery of these vaccines is effective for promoting more rapid induction of antibody responses. However, a single dose of liposome DNA vaccine does not promote appropriate cellular responses for protection against Brucella challenge.

7.2: Introduction

In the previous chapters two novel DNA vaccines based upon the *B. melitensis* 16M genes *ialB* and *omp25* have been shown to be protective in the BALB/c mouse model of brucellosis. Through these studies it was concluded that p-*omp25* protection was effected through a Th 1 biased cell mediated response to vaccination, characterised by the generation of antigen specific IFN-γ secreting CD8+ and CD4+ cells, with a simultaneous IgG2a dominated specific antibody response. Furthermore, facets of this response (IFN-γ and IgG2a) were demonstrable after two inoculations of the p-*omp25* plasmid, suggesting that it may be possible to achieve protection from a reduced number of inoculations of the vaccine. The p-*ialB* vaccine was also shown to have significant protective efficacy in the mouse model, and evoked a high titre specific antibody responses and CD4+ dominated effector responses.

Before these vaccines can be tested for efficacy in a small ruminant host, further work is required to improve the efficiency of the vaccination protocol. The present protective protocol requires four inoculations of 100 μg of plasmid, given at three week intervals. The extrapolation of this vaccination protocol to the intended host is not necessarily simple and the quantities and frequency of DNA inoculations required for effective vaccination of livestock need to be

determined. Since increased quantities of DNA are perceived as necessary to elicit immune reactions from larger animals in comparison to the mouse models, this currently makes large scale DNA vaccination of livestock economically untenable. For this reason methods to improve the efficiency of vaccination, in terms of reducing the amount of DNA required to promote an effective immune response are required before vaccine trials.

Secondly, in addition to a drastic reduction in the amount of DNA required, the impracticality of booster vaccinations needs to be addressed. The need for repeated booster vaccinations invokes a need for multiple farm visits by a veterinarian for administration of the vaccine, or precise and regular direct application of the licensed product by the stockowner. In the case of human vaccination the current vaccination schedule would require patients to make multiple follow-up visits to health care professionals. In both a veterinary and medical setting this requirement not only increases the cost of vaccination but also increases the opportunity and propensity for non-compliance with the protective protocols. Therefore, in order to make DNA vaccination a useful alternative to current strategies an efficacious vaccine consisting of a single immunisation of a minimal amount of DNA is required. And thus in order to progress with the development of p-*ialB* and p-*omp25* for use in target species demonstrable protective efficacy and defined immunological correlates of protection in the BALB/c mouse model following a single inoculation of DNA needs to be demonstrated. The principal aim of work undertaken in this study was to assess the protective efficacy and immune response achieved following a single inoculation of the selected DNA vaccines (p-*omp25* and p-*ialB*).

Unfortunately, naked DNA vaccination is an inefficient procedure. Indeed, to the best of the authors knowledge, there are no convincing examples of a protective effect demonstrable from subjects receiving only a single intramuscular immunisation with a naked DNA vaccine. Methods for improving the efficiency of DNA vaccination in livestock and non-human primates have been widely investigated (van Drunen Littel-van den Hurk *et al*, 2004., O'Hagan *et al*, 2001., Singh and O'Hagan, 2003). Most of these concentrate on improving the expression capacity of the plasmid or improving the delivery of the plasmid to

the host cells. The capacity of a DNA vaccine to induce an immune response is naturally dependent upon its capacity to express encoded antigen in-vivo. Numerous studies have shown that engineering of vaccines in order to increase the level of antigen expression through the inclusion of molecular adjuvants, non-specific intron elements and Kozak signal sequences, along with promoter and codon optimisation can all be useful in improving the level of expression of encoded antigen from plasmids (For review see Garmory *et al*, 2003). However, the expression of antigen is only possible from appropriately transfected host cells and therefore the effectiveness of the vaccination is ultimately restricted by the efficiency with which the plasmid is delivered to the nucleus of the target host cell.

From biodistribution studies aimed toward assessing the fate of parenterally administered naked DNA it is estimated that as much as 99% of inoculated plasmid is destroyed almost immediately by extracellular nucleases. Therefore, the vast majority of DNA inoculated into the host is not appropriately internalised by host cells and therefore cannot contribute to the induction of an immune response in terms of generation and presentation of specific antigen. This 'waste' DNA is not entirely redundant as it is thought to contribute to the overall effect of DNA vaccination through CpG specific stimulation of innate receptors such as TLRs, and thus functions as an inherent adjuvant in the vaccine preparation (Klinman *et al*, 2004). Nevertheless, parenteral delivery of naked DNA is a very inefficient use of the plasmid preparations. One consequence of this is that the success of this naked DNA vaccine approach in both murine models and target species often appears to be somewhat dose dependent. For any DNA vaccine there appears to be a critical threshold dose of DNA below which the number of transfected cells is too low to result in measurable immune reaction. Increasing the quantity of DNA above this threshold does not always increase the effectiveness of the vaccine but decreasing the quantities of DNA generally results in decline or indeed abrogation of the response.

Numerous mechanical and formulation strategies have been investigated for ability to increase *in vivo* transfection efficiencies. Among the widely reported

mechanical strategies for improving the delivery of DNA vaccine plasmids to host cells are ballistic devices referred to "Gene guns", and the increasingly popular in-vivo electroporation strategies. In addition to the mechanical strategies, the physical formulation of a vaccine can impact upon immunogenicity and efficacy. The formulation of vaccines with classical adjuvants such as alum, and oil and water emulsions are the most notable examples of this. Both mechanical and formulation strategies have been investigated for improving DNA vaccination in livestock species (For review see van Drunen Little-van de Hurk *et al,* 2004).

Studies of delivery strategies used with DNA vaccination are summarised in table 7.1.

Table 7.1: A summary of some of the more widely investigated DNA vaccine delivery strategies

Reference	*Detail*
I: Classical adjuvants	
Fischer et al, (2003)	The formulation of a Rabies DNA vaccine with Aluminium phosphate results in a rapid high titre serological response.
Van Rooij et al, (2002)	Neutralising antibody production and reduced shedding of Porcine Pseudorabies Virus (PPRV) in Pigs, following administration of DNA vaccine formulated with DDA adjuvant.
II: Biological adjuvants	
Sobol et al, (2003)	Local mucosal immune responses achieved through DNA vaccination with a plasmid encoding Influenza virus Haemagglutinin antigen (HA) co-delivered with Cholera Toxin (CT).
Wong et al, (2002)	Improved efficacy of a Foot and Mouth Disease Virus DNA vaccine in Pigs through co-administration of IL-2.
Somasundaram et al, (1999)	Improved efficacy of a PRV DNA vaccine, using porcine GM-CSF as an adjuvant.
Min et al, (2001)	Assessment of the adjuvant potential of cytokines on a DNA vaccine for avian coccidiosis, caused by *Eimeria acervulina*.

Continued....

Table 7.1 continued…

III: Mechanical delivery strategies	
Braun et al, (1999)	Gene gun delivery of a DNA vaccine encoding the Bovine Herpes Virus gD antigen shown to be protective in cattle
Lodmell et al, (2002)	Gene gun delivery of Rabies DNA vaccines results in more rapid induction of significant neutralising antibody titres in non-human primates.
De Rose et al, (2002)	A review discussing various delivery routes for the administration of DNA vaccines to sheep.
Babiuk et al, (2002), Tollefsen et al, (2003)	Intramuscular inoculation of plasmid DNA followed by in-vivo electroporation improves the efficiency of plasmid uptake and subsequent immune responses in ruminants.
Babiuk et al, (2003)	Topical application and electroporation of a DNA vaccine improves transgene expression in porcine skin.

Spanning the division between delivery strategy and classical adjuvant are liposome and microparticle based approaches. Liposomes and microparticles have proven efficacious for the mucosal delivery of drugs and proteinaceous effectors, and more recently the adjuvant properties of these delivery systems have been exploited in vaccine development. DNA or proteins encapsulated within appropriate size liposomes or microparticulate formulations are readily taken up by host cells thereby resulting in increased transfection efficiency for DNA vaccines. Moreover, lipid formulation of DNA is frequently used to improve transfection efficiencies *in vitro*. Appropriate particle design can also provide a level of protection for the DNA plasmids against degradation by nucleases. Studies comparing particle mediated DNA vaccination with naked DNA vaccination show that lower quantities of DNA are required to elicit equivalent immune responses when using the particle approach. Furthermore, particle based formulation permits the investigation of alternate delivery routes, such as oral inoculation or direct administration to mucosal surfaces, which otherwise would not be applicable for naked DNA immunisation. The applicability of these systems for mucosal delivery is particularly appealing in the context of brucellosis vaccine development, as promoting local mucosal protective responses is a potential strategy for preventing the establishment of infection. Thus particle based delivery strategies offer a number of advantages over the direct intramuscular inoculation of naked DNA vaccine.

The use of microparticles based upon the variants of the polymer PLG for the delivery of vaccines has been extensively reviewed (O'Hagan *et al,* 2004., Singh *et al,* 2004). Similarly, the use of phospholipid vesicles, or liposomes, for the improved delivery of vaccines has been widely investigated (Felnerova *et al,* 2004). Liposomal delivery of protein antigens has an adjuvant effect but unlike many classical adjuvants does not induce local or systemic toxicity (Allison and Gregordiadis, 1974., Alving, 1991., Gregoriadis, 1990), which is a distinct advantage in a veterinary or clinical setting. Variations of liposome formulas incorporating immunostimulatory compounds such as MPL, or Quil A (ISCOMS), or viral membrane proteins (virosomes) have been investigated for antigen delivery, and have shown usefulness in improvement of immune responses in a number of model systems (Singh and O' Hagan, 2002). Neutrally charged non-ionic surfactant based liposomes, or niosomes, have also been shown promote increased potency response to model antigens in small animal models (Brewer *et al,* 1998), and cationic cholesterol based liposomes have proven to be an effective in augmenting the immune response to an influenza vaccine (Guy *et al,* 2001).

In the context of DNA vaccination, delivery of the plasmid directly to the APC is the primary advantage of particle-based formulations. Lipid based transfection agents have been proven highly effective at promoting DNA uptake by cells in culture (Felgner, 1991), and *in vivo* (Gregoriadis, 1996). Gregoriadis *et al,* (1997) demonstrated significant improvement of antibody and cytokine responses from mice receiving DNA vaccines entrapped in cationic liposomes compared to mice receiving equivalent quantities of naked DNA. In addition, cationic liposome preparations have proven effective adjuvants for the mucosal delivery of HIV-1 DNA vaccines in mice (Ishii *et al,* 1997). In large animal targets, liposome based DNA vaccines have proven effective for induction of serological responses in ponies against rabies virus (Fischer *et al,* 2003a), and improving the protective efficacy of a DNA vaccine for canine distemper virus (CDV) in dogs (Fischer *et al,* 2003b).

The immunopotentiation effect of liposomes is believed to derive from both the increased uptake of DNA into professional antigen presenting cells, and

subsequent trafficking to the local draining lymph node for efficient T cell priming. In support of this claim, liposome complexed DNA does not appear to be taken up by muscle cells at the site of inoculation in significant quantities, but is detectable at the draining lymph node presumably following phagocytosis by APC (Velinova *et al,* 1996). Following phagocytosis it is presumed that the liposome fuses with the phagosomal membrane and releases the DNA into the cytoplasm leading eventually to the expression of the encoding antigen, and processing for immune recognition (Legendre, 1995).

Thus, the use of a liposome based delivery strategy with the p-*omp25* and p-*ialB* vaccines may be able to improve the immunogenicity of these vaccines. Liposomes can protect the DNA from nuclease attack, whilst enhanced uptake leads to more efficient induction of immune systemic immune responses in the lymph node. Such an approach is hypothesised to improve the efficiency of the DNA vaccination process such that a reduction of the quantity of DNA or the number of inoculations required to generate the protective response may be possible. The achievement of protective efficacy from a single inoculation of vaccine would go some way toward promoting DNA vaccination as a practical alternative to current live vaccines for brucellosis.

In summary, the investigations described in this chapter were devised to investigate the immunogenicity and protective efficacy of a single shot of the naked DNA vaccines p-*omp25* and *p-ialB* . Since intramuscular naked DNA vaccination is known to be a relatively ineffective method, an alternative delivery strategy involving the passive adsorption of DNA to cationic liposomes was also investigated as a means to improve vaccine efficacy.

7.3: Experimental procedures and aims

The protective efficacy and immunogenicity of naked DNA and liposome formulated DNA vaccination was assessed. As this investigation required the comparison of protective efficacy and immunogenicity of a considerable number of groups of mice, it was not possible to conduct all experiments concurrently. Thus, the data presented in this chapter is the result of three separate in-vivo investigations. The BALB/c mouse brucellosis model was used in all studies, and appropriate control groups of unvaccinated mice, pcDNA3.1 inoculated mice and Rev.1 vaccinated mice were included in each experiment. These studies can be summarised as follows: -

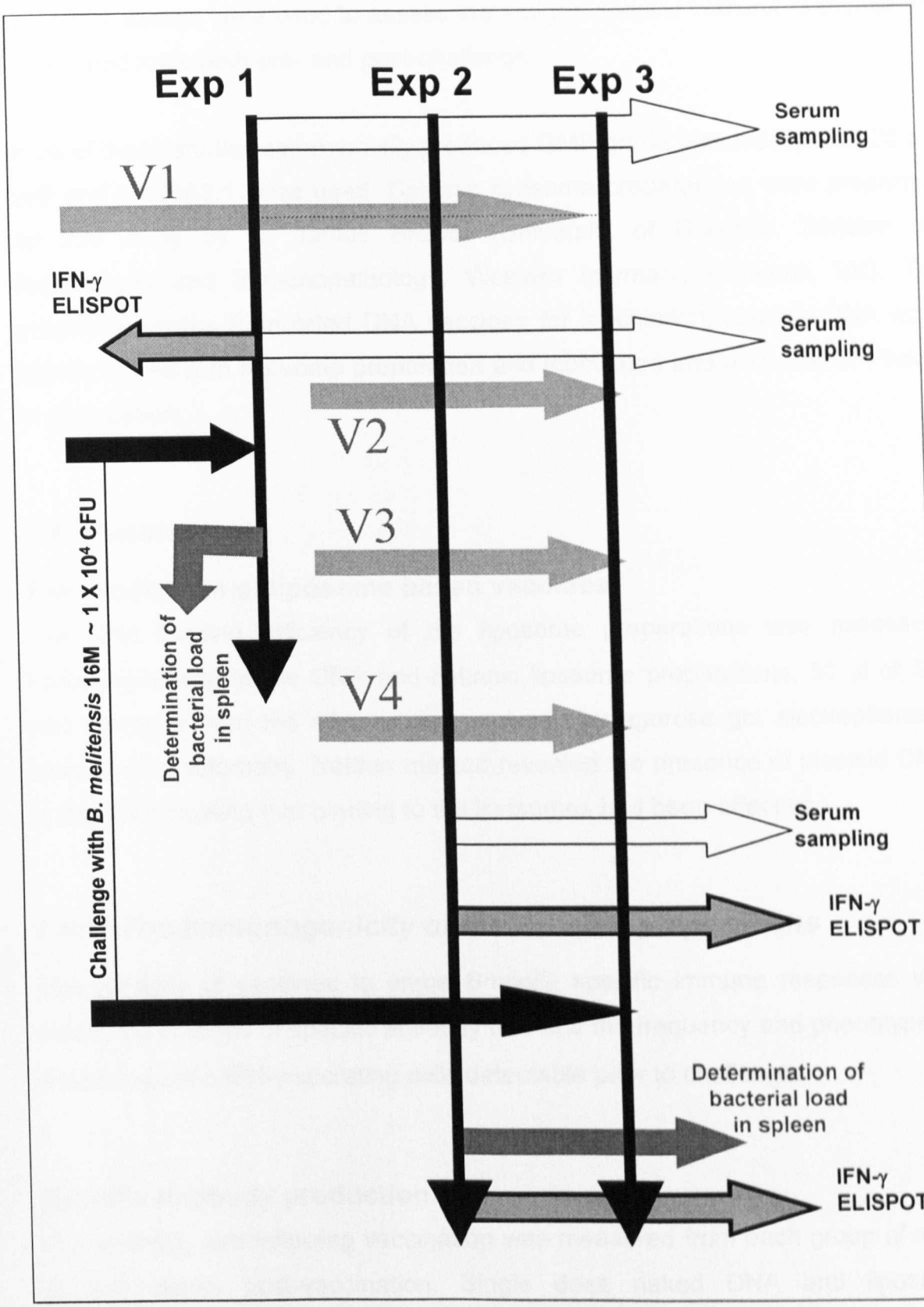
1. p-*omp25* [X1] vs p-*ialB* [X1]: A comparison of the protective efficacy of single dose naked DNA vaccination with either p-*omp25* or *p-ialB* .

2. L-p-*omp25* [X1] vs p-*omp25* [X4]: A comparison of the protective efficacy of a single dose of liposome adsorbed p-*omp25* (L-p-*omp25* [X1] [X1]) with multiple dose naked p-*omp25* (p-*omp25* [X4]) vaccination.

3. L-p-*ialB* vs p-*ialB* [X4]: A comparison of the protective efficacy of a single dose of liposome adsorbed p-*ialB* (L-p-*ialB* [X1]) with multiple dose naked p-*ialB* [X4] vaccination.

The general experimental schedule is outlined in figure 7.1.

Figure 7.1: Schematic representation of experiments to evaluate different doses and vaccine formulations



All details for materials and methods for each protocol within these experiments are described in Chapter 2.

For immunogenicity assessments antibody production was measured by ELISA (16M antigen, recombinant Omp25-GST and recombinant IalB), and IFN-γ ELISPOT assays were used to assess the antigen specific immune response of vaccinated mice both pre- and post-challenge.

In all of these studies commercially produced GMP grade plasmids, p-*omp25* p-*ialB* and pcDNA3.1 were used. Cationic liposome preparations were prepared for this study by Dr James Brewer (University of Glasgow, Division of Immunology and Immunopathology, Western Infirmary, Glasgow, UK). To prepare liposome formulated DNA vaccines for inoculation, plasmid DNA was directly mixed with liposome preparation and inoculated into mice within 1 hour of preparation.

7.4: Results

The production of liposome based vaccines

The DNA binding efficiency of the liposome preparations was assessed. Following mixing of the DNA and cationic liposome preparations, 50 μl of SN was removed from the mixture and analysed by agarose gel electrophoresis and spectrophotometry. Neither method revealed the presence of plasmid DNA in the SN indicating that binding to the liposomes had been effective.

7.4.1: The immunogenicity of the vaccine preparations

The capacity of vaccines to prime *Brucella* specific immune responses was measured in terms of specific antibody titre and the frequency and phenotype of antigen specific IFN-γ secreting cells detectable prior to challenge.

Specific antibody production

The antibody titre following vaccination was measured from each group of mice at two weeks post-vaccination. Single dose naked DNA and liposome vaccination titres were compared with data obtained from previous assessment of multiple dose naked DNA vaccinations. The data is summarised in table 7.1

Table 7.2: A comparison of antibody responses following a single immunisation with either naked DNA or liposome adsorbed DNA.

	Titre of specific IgG1 and IgG2a antibodies in ELISA					
ELISA	*Omp25-GST*		*IalB*		*16M*	
Isotype	*IgG1*	*IgG2a*	*IgG1*	*IgG2a*	*IgG1*	*IgG2a*
p-*omp25* [x1]	-	**1/320**	ND	ND	-	-
p-*omp25* [X4]	**1/640**	**1/1280**	ND	ND	**1/420**	**1/520**
L-p-omp25 [X1]	**1/320**	**1/1000**	ND	ND	**1/640**	**1/1280**
p-*ialB* [X1]	ND	ND	-	-	-	-
p-*ialB* [X4]	ND	ND	**1/5210**	**1/5210**	**1/5210**	**1/5210**
L-p-*ialB* [X1]	ND	ND	**1/270**	(1/30)	**1/500**	**1/120**
pcDNA3.1 [X1]	-	-	-	-	-	-
pcDNA3.1 [X4]	-	-	-	-	-	-
L-pcDNA3.1	-	-	-	-	-	-
Liposome only	-	-	-	-	-	-
Rev.1 @ 2 weeks post-vaccination	-	-	-	-	-	-
Rev.1 @ 11 weeks post-vaccination	**1/640**	**1/640**	-	-	**1/2560**	**1/2560**

ND: Not tested. - : Negative sample (OD below assay cut off)

Data in Table 7.2 shows that a single intramuscular inoculation of naked p-*ialB* plasmid was not sufficient to generate a detectable level of specific antibodies in either the IalB recombinant antigen ELISA or the 16M antigen ELISA Whereas, multiple inoculations of naked p-*ialB* elicited strong and equal magnitude antibody titres of both IgG1 and IgG2a isotype. However, a single inoculation with 100 μg of p-*ialB* surface adsorbed to cationic liposomes (L-p-*ialB*) was able to generate detectable specific IgG1 and low titre IgG2a. The titre from L-p-*ialB* group mice was low, and IgG1 isotype antibodies were the dominant class of antibody whereas high titre balanced IgG1 and IgG2a responses were detected following p-*ialB* [X4]. Overall, the use of liposome delivery for the p-*ialB* plasmid resulted in detectable antibody responses after a single

inoculation, which was not demonstrable from mice inoculated with an equivalent quantity of naked DNA.

IgG1 and IgG2a Omp25 specific antibodies were detected following multiple inoculations with p-*omp25* vaccine (p-*omp25* [X4]). IgG2a titres were in excess of IgG1 titres. Notably, Omp25 specific IgG2a was recorded after a single inoculation (p-*omp25* [X1]). Specific antibody was also detected in the serum of mice receiving liposome formulated L-p-*omp25*. A higher titre IgG2a response was observed from L-p-*omp25* [X1]vaccinated mice than naked p-*omp25* [X1] vaccinated mice. Furthermore, a detectable IgG1 response is achieved after a single inoculation with L-p-*omp25* [X1]but not p-*omp25* [X1]. The antibody response measured from L-p-*omp25* [X1]p-*omp25* [X1] and p-*omp25* [X4] was dominantly IgG2a isotype.

IFN-γ production from vaccinated mice prior to challenge

For mice receiving the full course of four inoculations with plasmid DNA the ELISPOT assays have previously been described in Chapter 6. These results are reiterated here for direct comparison against the results obtained for mice inoculated with a single dose of naked DNA or liposome adsorbed DNA.

The results of the ELISPOT investigations to determine the frequency and phenotype of primed antigen specific IFN-γ effector cells are summarised in table 7.3.

Table 7.3: A comparison of the frequency and phenotype of IFN-γ secreting cells from mice receiving various doses and formulations of DNA vaccines. Δ IFN-γ secreting cells per million in response to stimulation with specific antigens

Vaccine	Net responsive cells		
	Total cell population	**CD4+ depleted cell population**	**CD8+ depleted cell population**
Stimulation with recombinant Omp25-GST [10 μg/ ml]			
Liposome [X1]	**12.0 ± 2.21**	0	0
pcDNA3.1 [X1]	**0**	0	0
pcDNA3.1 [X4]	**0**	0	0
L-pcDNA3.1 [X1]	**0**	0	0
p-*omp25* [X1]	**50.8 ± 24.9***	27.8 ± 2.3 {↓45%}	11.3 ± 2.3{↓78%}
p-*omp25* [X4]	**73.3 ± 20.3***	41.0 ± 5.2 {↓44%}	10.8 ± 3.5 {↓85%}
L-p-omp25 [X1]	**25.0 ± 0.77***	2.8 ± 0.08 {↓89%}	13.0 ± 0.9 {↓48%}
Stimulation with recombinant IalB [20 μg/ml]			
Liposome [X1]	**10.25 ± 1.75**	0	2.25 ± 1.77
pcDNA3.1 [X1]	***17.25 ± 2.00***	32.0 ± 17.58	11.25 ± 5.57
pcDNA3.1 [X4]	**0**	0	0
L-pcDNA3.1 [X1]	**28.75 ± 19.12**	7.05 ± 4.05	0
p-*ialB* [X1]	**12.75 ± 1.75**	0	0
p-*ialB* [X4]	**75.02 ± 18.37***	11.0 ± 4.85 {↓85%}	36.25 ± 2.92 {↓52%}
L-p-*ialB* [X1]	**23.25 ± 6.06**	0	0

The number of IFN-γ secreting antigen specific cells detected in splenocyte preparations from vaccinated mice. Data is displayed as Δ SFC / million, where Δ SFC is derived by subtraction of the background response of corresponding unstimulated cells. Data is displayed as mean Δ SFC /million ± Standard deviation.

** indicates SFC values significantly different to the vector and liposome control groups (ANOVA and Bonferroni's multiple comparison test, p >0.05).*

{↓ percentage decrease or inhibition of responder cell frequency from total cells}

The effector response of Rev.1 vaccinated mice was also measured in these investigations (see Chapter 6 for full discussion). Results showed that IFN-γ is mainly produced by CD4+ cells following vaccination with Rev.1.

The mean frequency of antigen specific IFN-γ secreting cells in the splenocyte populations of p-*omp25* [X1] inoculated mice was modestly reduced in comparison to that observed under identical stimulation conditions for the p-*omp25* [X1] vaccine: p-*omp25* [4] mean 50 SFC per million compared with p-

omp25 [X4] mean 73 SFC per million. A direct statistical comparison of the number of SFC per well for the four replicate wells of each group sample showed no significant difference between the data sets (Mann-Whitney U test, $p > 0.05$), suggesting an equivalent effect for the two vaccine strategies. IFN-γ production from both p-*omp25* [X1] and p-*omp25* [X4] mice was dominated by CD8+ T cells. Thus indicating that for this particular construct booster vaccinations do not significantly increase the frequency of antigen specific effector cells or alter the bias in CD4+: CD8+ priming.

For the L-p-*omp25* [X1] vaccinated mice, IFN-γ effector cells were detected at a lower frequency than the p-*omp25* [X1] vaccinated mice but this difference was not determined to be statistically significant (Mann-Whitney U test, $p > 0.05$). However, a statistically significant difference in the SFC from p-*omp25* [X4] and L-p-*omp25* [X1] vaccinated mice was observed (Mann-Whitney U Test, $p = 0.0286$). Interestingly, T cell subset depletion assays revealed that the majority of IFN-γ secreting cells in the splenocyte population from L-p-*omp25* [X1] samples were CD4+ cells. This is in direct contrast to the data from the samples obtained from mice inoculated with naked p-*omp25* where both single and four dose vaccination protocols elicited cells largely of the CD8+ phenotype.

For p-*ialB* vaccination the benefit of booster inoculations was more apparent. The p-*ialB* [X1] was unable to elicit statistically significant numbers of IalB specific SFC when compared to the SFC observed from pcDNA3.1 [X1] inoculation (Mann-Whitney U test, $p> 0.05$). However, p-*ialB* [X4] group produced approximately 75 SFC per million, and Mann-Whitney U test revealed that this response was significantly different to that observed in both the pcDNA3.1 and p-*ialB* [X1] groups ($p = 0.0286$).

The dominant phenotype of responder cells in p-*ialB* [X4] samples was shown to be CD4+ cells. L-p-*ialB* vaccination resulted in a low and statistically insignificant total number of SFC, when compared to the response observed from the control L-pcDNA3.1 group ($p> 0.05$), and a lower mean total SFC than observed for p-*ialB* [X1]. Both CD4+ and CD8+ subset depletion eliminated the detectable response from the p-*ialB* [X1] or L-p-*ialB* samples.

7.4.2: The protective efficacy of the various vaccination protocols

Due to the considerable numbers of mice involved in this investigation, it was not possible to conduct a single efficacy trial to compare all the selected vaccination strategies. Therefore, the protective efficacy of single dose naked DNA vaccination, multiple dose naked DNA vaccination, or single dose liposome formulated DNA vaccination was assessed in a number of separate experiments, described on page 210.

The assessments of protective efficacy of liposome formulations of L-p-omp25 [X1]and L-*p-ialB* (Experiments 2 and 3) were actually conducted concurrently with the studies described in chapter 6. A direct comparison of the protective efficacy of a single dose of the naked DNA vaccinations (p-*ialB* and p-*omp25*) was conducted in a separate study (Experiment 1). The data for protective efficacy is summarised in table 7.4.

Table 7.4: The protective effect of vaccination with naked DNA or liposome formulated DNA.

Vaccine group	*Brucella* CFU per spleen	*Brucella* per spleen as a % of challenge dose	Protection units
Experiment 1: A comparison of single dose naked DNA vaccine efficacy			
Rev.1 (V4)	2.95 ± 0.35*	67.8	2.04*
PBS	5.00 ± 0.05	114.8	0
pcDNA3.1 [X1]	4.56 ± 0.31	104.8	0.43
p-*omp25* [X1]	4.45 ± 0.44	102.3	0.55*
p-*ialB* [X1]	4.19 ± 0.42*	96.4	0.80*
Experiment 2: p-*omp25* [X4] compared with L-p-omp25 [X1]			
Rev.1 (V1)	4.28 ± 0.21	96.8	0.55
Rev.1 (V4)	1.47 ± 0.82*	33.2	3.35*
PBS	4.83 ± 0.29	109.2	0
PcDNA3.1 [X4]	4.80 ± 0.21	108.5	0.02
p-*omp25* [X4]	1.40 ± 0.69*	31.7	3.42*
L-p-omp25 [X1]	4.01 ± 0.14	90.8	0.81
L-pcDNA3.1	4.86 ± 0.44	109.8	-0.03
Experiment 3: p-*ialB* [X4] compared with L-p-iaiB			
Rev.1 (V1)	2.2 ± 1.0*	51.2	1.83*
Rev.1 (V4)	1.94 ± 0.77*	44.8	2.12*
PBS [X4]	4.10 ± 0.68	93.6	0
PcDNA3.1 [X4]	3.75 ± 0.36	86.1	0.32
p-*ialB* [X4]	1.91 ± 0.93*	44.0	2.15*
L-*p-ialB* [X1]	3.07 ± 1.08	70.5	1.00
L-pcDNA3.1	3.37 ± 0.58	77.5	0.70

Brucella CFU per spleen: Log Brucella CFU per spleen ± standard deviation.
Protection units = Log Brucella CFU per spleen of unvaccinated mice – Log Brucella CFU per spleen of vaccinated mice. % Challenge dose: (Log CFU Brucella per spleen / Log CFU challenge dose) X 100.
** indicates statistically significant reduction of Brucella CFU per spleen compared to PBS controls (Mann Whitney U test, $p < 0.05$).*

The data obtained in these investigations confirms a protective effect for Rev.1 (V4) vaccination in each experiment, and a protective effect from the four dose vaccination protocols for p-*omp25* or *p-ialB* ($p < 0.05$). Statistically significant reduction of bacterial load is also observed for p-*ialB* [X1] vaccination ($p < 0.05$), in experiment 1. However, in terms of protection units this effect is slight and does not compare favourably with the protective effect of the multiple dose vaccines or the Rev.1 controls. Furthermore, the reduction is not significantly

different ($p > 0.05$) when compared to that observed in the pcDNA3.1 vaccinated mice.

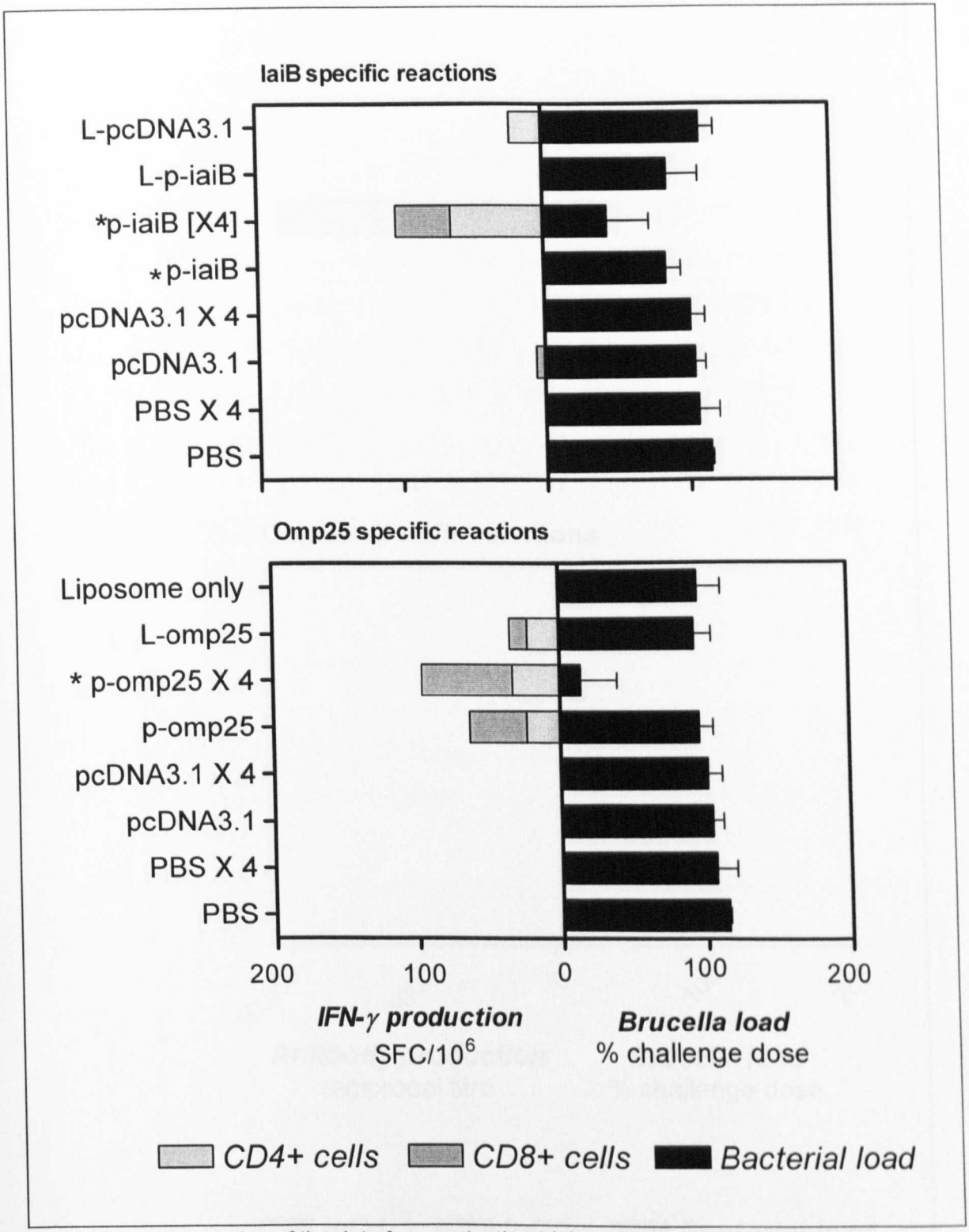
In experiment 2, L-p-omp25 [X1]vaccination provided a 0.88 protection unit effect. Although a reduction in splenic *Brucella* was apparent this was not found to be statistically significant ($p > 0.05$) in comparison to the unprotected controls.

L-p-*ialB* vaccination provided 1.0 unit of protection in experiment 3, although this effect was not significant compared to the PBS, L-p-*ialB* [X1] or vector (pcDNA3.1) controls.

The relationship between protective efficacy and vaccine primed antigen specific immune responses

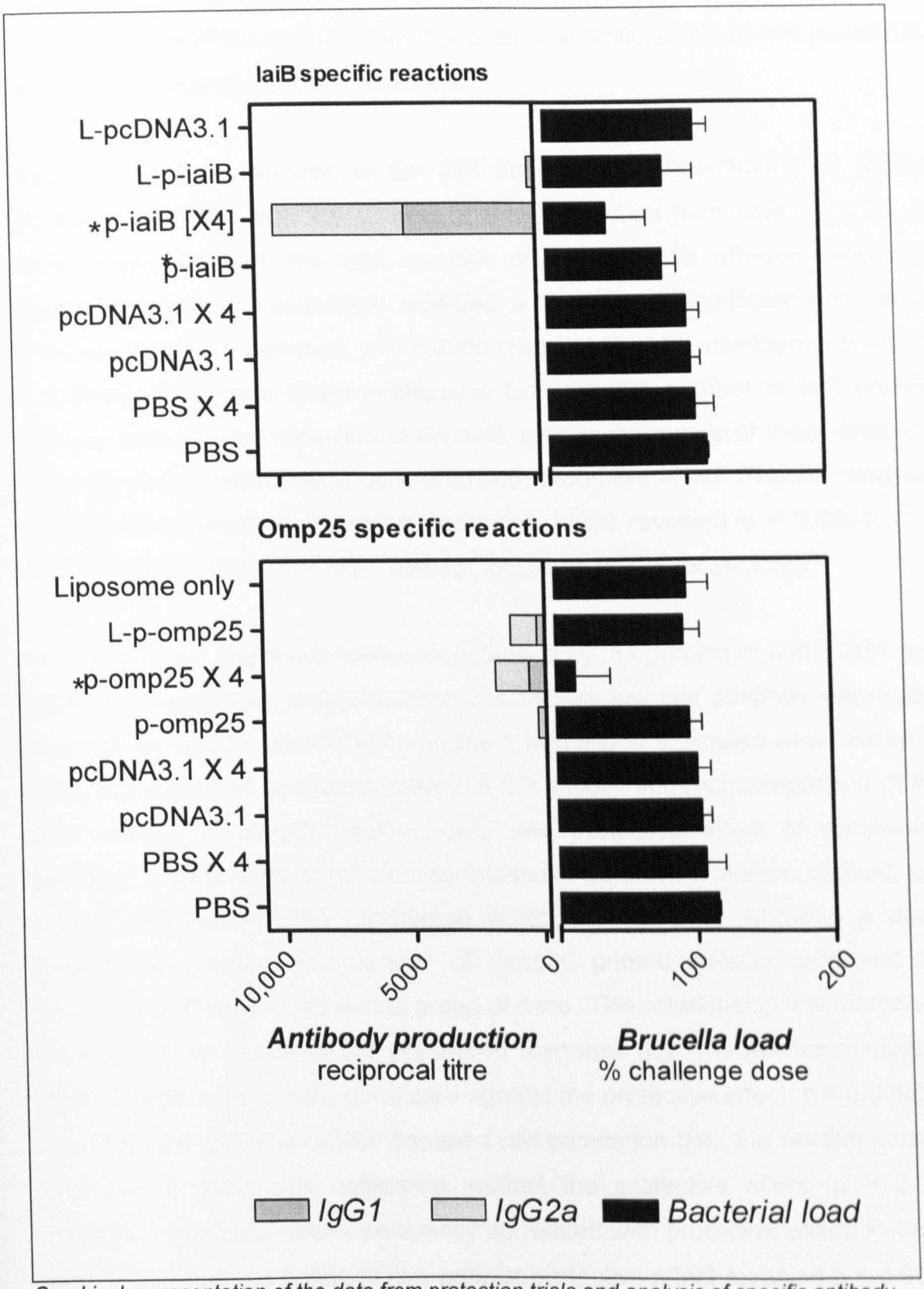
To permit comparison of protective efficacy between experiments the *Brucella* load post-challenge for each vaccine group was presented as the percentage of the challenge dose. The mean log *Brucella* per spleen was calculated from the combined data from all three experiments. These values for protective effect were directly compared against the vaccine specific immune effectors measured during the investigations. These comparisons of protection against humoral immune responses and protection against IFN-γ secreting cells are presented in figures 7.2 and 7.3.

Figure 7.2: The relationship between the protective efficacy of vaccination and the generation of antigen specific IFN-γ secreting effector cells



Graphical representation of the data from protection trials and analysis of IFN-γ producing antigen specific cells detected in mice two weeks prior to challenge
Bacterial load represented as a percentage of the challenge dose in each study. Groups marked with were shown to have a statistically significant protective effect.*
CD4+ cells = [Total SFC – CD4+ depleted SFC], CD8+ cells = [Total SFC – CD8+ depleted SFC].

Figure 7.3: The relationship between the protective efficacy of vaccination and the generation of specific antibodies



Graphical representation of the data from protection trials and analysis of specific antibody production detected in mice two weeks prior to challenge. Bacterial load represented as a percentage of the challenge dose in each study. Groups marked with were shown to have a statistically significant protective effect.IgG1 and IgG2a titres measured.*

Figures 7.2 and 7.3 show the immune responses alongside the observed protective effect of vaccination. The data shows that antigen specific immune effectors, either IFN-γ secreting effector cells or specific antibody, are present in the protected animals prior to challenge.

Immune responses specific to the IalB antigen are characterised by strong antibody responses and the priming of IFN-γ secretion from CD4+ T cells. A direct comparison of the total number of IalB specific effector cells and protective effect of vaccination revealed a statistically significant correlation (Pearson correlation method, $p = 0.0066$ (two-tailed), 95% confidence limits, $r^2 = 0.7344$), showing a direct relationship between the number of IalB primed effector cells and the reduction of *Brucella* load in that group of mice. Antibody responses were also directly correlated with protective effect: Pearson analysis to compare antibody titre against protective effect revealed $p = 0.0024$, $r^2 = 0.8076$, and $p = 0.0035$, $r^2 = 0.7836$, for IgG1 and IgG2a respectively.

Omp25 specific responses were characterised by the priming of both CD8+ and CD4+ T_{em} responses, and production of relatively low titre antibody responses. Each of the *omp25* based DNA vaccine formulations assessed were shown to have some level of protective activity in this model. A direct comparison of the total number of Omp25 specific cells and protective effect of vaccination revealed a statistically significant correlation (Pearson correlation method, $p = 0.0087$ (two-tailed), 95% confidence limits, $r^2 = 0.7095$), showing a direct relationship between the number of Omp25 primed effector cells and the reduction of *Brucella* load in that group of mice. This relationship was preserved when the CD4+ depleted cell population response (i.e.: the relative number of CD8+ T cells) was directly compared against the protective effect: $p = 0.0099$, $r^2 = 0.6793$, but not when CD8+ depleted cell population (i.e.: the relative number of CD4+ T cells) was compared against the protective effect ($p > 0.05$). Antibody responses were also directly correlated with protective effect: Pearson analysis to compare antibody titre against protective effect revealed $p = 0.0015$, $r^2 = 0.8345$, and $p = 0.0139$, $r^2 = 0.6631$, for IgG1 and IgG2a respectively.

7.4.3: IFN-γ production from vaccinated mice post-challenge

IFN-γ ELISPOT was conducted on splenocyte samples harvested during each of the protective efficacy bacterial enumeration studies. The aim of this investigation was to determine whether prior exposure to the candidate antigens resulted in measurably different responses to *Brucella* challenge. The results are summarised in table 7.4.

Table 7.4: A comparison of the frequency of IFN-γ secreting cells from vaccinated mice that had been challenged with ~1 X10^4 CFU B. melitensis 16M. Δ IFN-γ secreting cells per million in response to stimulation with specific antigens

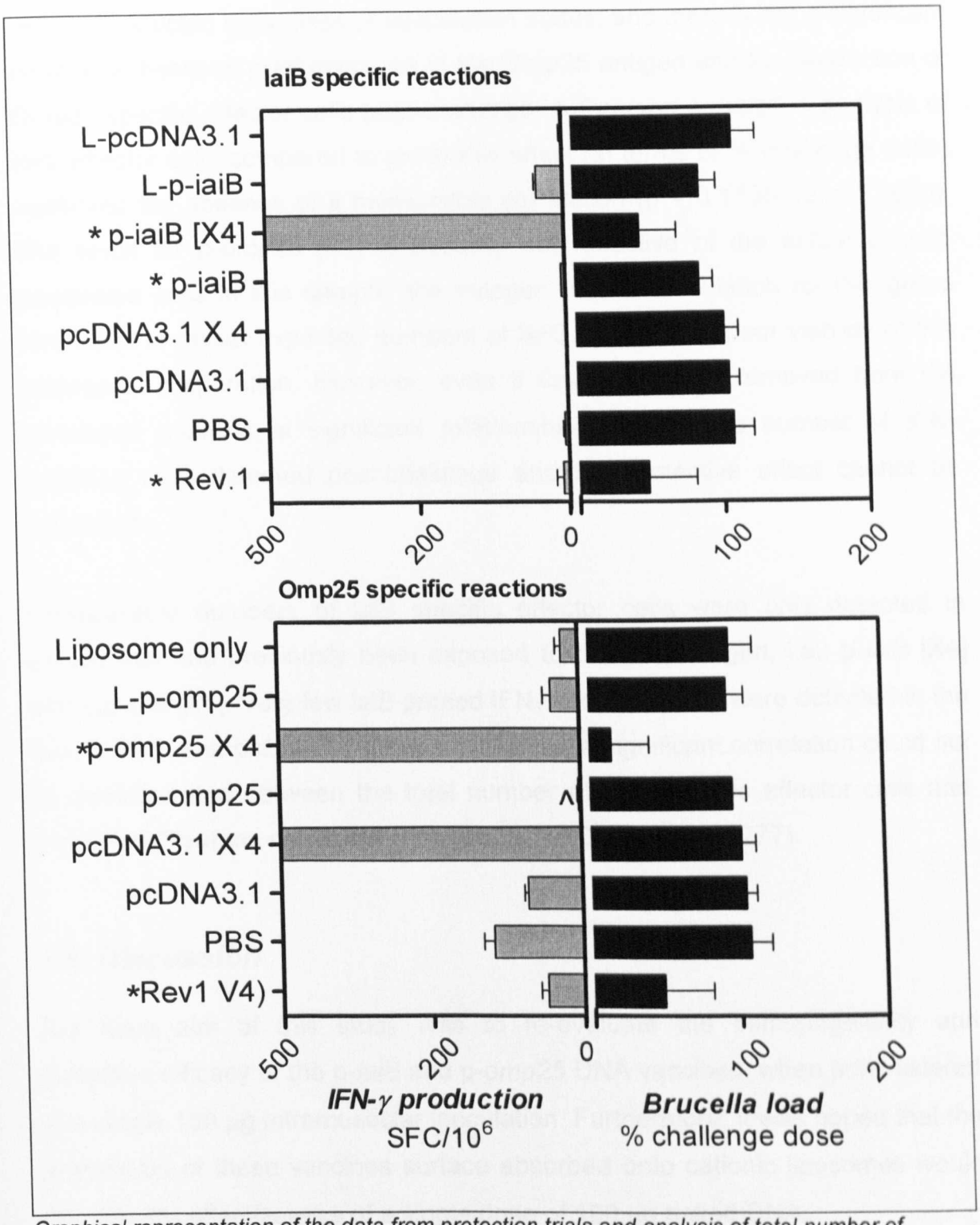
Vaccine group	Omp25 stimulation	IalB stimulation
Rev.1	60.00 ± 10.68	9.50 ± 11.50
PBS	146.75 ± 18.23	5.25 ± 2.73
Liposome [X1]	27.25 ± 8.99	1.00 ± 3.00
pcDNA3.1 [X1]	89.00 ± 6.69	0
pcDNA3.1 [X4]	>500	0
L-pcDNA3.1 [X1]	>500	0
p-omp25 [X1]	3.75 ± 1.77*	ND
p-omp25 [X4]	>500	ND
L-p-omp25 [X1]	46.25 ± 12.87	ND
p-iaiB [X1]	ND	6.00 ± 0.62
p-iaiB [X4]	ND	>500
L-p-iaiB [X1]	ND	43.50 ± 3.97

The number of IFN-γ secreting antigen specific cells detected in splenocyte preparations from vaccinated mice. Data is displayed as Δ SFC / million, where Δ SFC is derived by subtraction of the background response of corresponding unstimulated cells. Data is displayed as mean Δ SFC /million ± Standard deviation.

*ND: Not determined. > 500 indicates saturated colour development in the wells. * sub-optimal assay: <200 SFC recorded following ConA stimulation for this group.*

Table 7.4 shows the results of IFN-γ ELISPOT assays performed post-challenge. Splenocytes used in this assay were harvested and stimulated concurrently to the assays for enumeration of bacterial load. These data were directly compared against the protection data. This comparison in depicted in Figure 7.4.

Figure 7.4: The relationship between the protective efficacy of vaccination and the number of antigen specific effector cells detected post-challenge



Graphical representation of the data from protection trials and analysis of total number of specific IFNγ producing cells detected in mice at 15 ±1 days post- challenge. Bars represent mean values and error bars on protection data reflect standard deviation.
Groups marked with were shown to have a statistically significant protective effect. ^ indicates assay with sub-optimal positive control (ConA) responses (< 50 SFC per well) in IFNγ ELISpot. Detection limits of assay are 500 SFC per well.*

Figure 7.4 shows the mean total IFNγ secreting cells for each group compared to the bacterial load recovered from challenged mice. Omp25-GST reactive cells are detected regardless of vaccination status, and there is not a significant correlation between prior exposure to the Omp25 antigen and the production of Omp25 specific effector cells post-challenge. A Pearson correlation analysis of total effector cells compared to protective effect (in terms of % challenge dose) confirmed the absence of a measurable correlation ($p = 0.1136$, $r2 = 0.3620$). The result for p-*omp25* [X1] is possibly not reflective of the actual Omp25 responsive cells in this sample: the mitogen control stimulation for this group also failed to reveal expected numbers of SFC, suggesting poor viability of this splenocyte preparation. However, even if these data are removed from the correlation analysis, a significant relationship between the number of IFNγ secreting cells detected post-challenge and the protective effect cannot be measured.

Considerable numbers of IalB specific effector cells were only detected in groups that had previously been exposed to the IalB antigen, i.e.: p-*ialB* [X4] and L-*p-ialB* [X1]. Very few IalB primed IFNγ secreting cells were detected in the Rev.1, PBS, and pcDNA3.1 groups. However, a significant correlation could not be demonstrated between the total number of IalB specific effector cells and protection (Pearson correlation analysis, $p = 0.0785$, $r^2 = 0.4277$).

7.5: Discussion

The main aim of this study was to re-evaluate the immunogenicity and protective efficacy of the p-*ialB* and p-*omp25* DNA vaccines, when administered as a single 100 μg intramuscular inoculation. Furthermore, it was hoped that the formulation of these vaccines surface absorbed onto cationic liposomes would improve the effectiveness of a single dose of 100 μg naked DNA.

For both the p-*omp25* and p-*ialB* vaccines a multiple booster protocol consisting of four 100 μg doses of plasmid at three week intervals was the most successful approach in terms of protective efficacy. A slight reduction in bacterial load was

observed for the p-*omp25* vaccine in a single dose protocol when the plasmid was delivered as either naked DNA (p-*omp25* [X1]) or liposome adsorbed (L-p-*omp25*), although these were not considered statistically signficant. A slight protective effect (deemed statistically significant) was observed for the p-*ialB* [X1] vaccination, but protection was not indicated for the L-p-*ialB* vaccination. Overall, the data showed that single doses of vaccine given either as naked DNA or as liposome adsorbed DNA was unable to provide equivalent protective efficacy to that observed following multiple inoculations. ELISPOT and ELISA techniques were used to analyse the capacity of the different vaccination strategies to prime IFN-γ secreting T cells and antibody production respectively, in the hope of explaining the qualitative differences in protection.

p-*omp25* specific immunity

Single versus multiple dose naked DNA vaccination

Antibody responses were demonstrable following a single 100 μg dose of naked p-*omp25* vaccine. Further booster inoculations served to increase the titre of the specific response. Antibody responses were persistently dominated by high titre IgG2a, suggesting a strong Th 1 bias to the immune response. However, analysis of cellular effectors indicated that multiple booster vaccinations with naked p-*omp25* (p-*omp25* [X4]) did not result in significantly more antigen specific effector cells compared to the response elicited following a single vaccination (p-*omp25* [X1]) (Mann Whitney U test $p > 0.05$). Thus suggesting that the increased protective effect of the multiple dose strategy is not achieved through generation of a greater magnitude of cellular response. Furthermore, both the multiple and single dose formulations of vaccine resulted in predominantly CD8+ IFN-γ responses. Depletion of the CD8+ subset resulted in a 77% and 85% reduction in the detected effector response for p-*omp25* [X1] and p-*omp25* [X4] respectively, suggesting a modest but statistically insignificant increase in the priming of CD8+ cells from the protective four dose p-*omp25* [X4] protocol. Similarly, depletion of CD4+ cells resulted in a 44% decrease in the total number of cells for the p-*omp25* [X4] vaccination, and a

45% decrease for the p-*omp25* [X1], implying no difference in the ability of the two vaccines to prime CD4+ T cells. Since statistically significant differences in immune responses are not seen between p-*omp25* [X1] and p-*omp25* [X4] it is not possible to determine which subset of cells has the greatest impact upon the protective effect.

Liposome versus naked DNA vaccination

Liposome adsorption of the p-*omp25* vaccine notably increased the antibody titre observed after a single inoculation, suggesting a considerable immunopotentiating effect from the liposome delivery. Notably, liposome delivery did not alter the bias of the IgG2a dominated response to this antigen. Similarly enhanced specific antibody responses following liposomal delivery of a luciferase encoding plasmid were recorded by Norman *et al*, (2000). In contrast, the frequency of Omp25 specific IFN-γ secreting cells was not improved by liposome delivery of the vaccines, but differences in responsive T cell subsets were recorded. Non specific IFN-γ secreting cells were detected by ELISPOT from mice that had received inoculation with uncomplexed (without DNA) liposomes, suggesting an enhancement of non-specific IFN-γ production presumably triggered by activation of macrophages upon phagocytosis of the liposome particles. In this way the liposomes are anticipated to act as adjuvants as well as delivery vehicles for the DNA. However, this non-specific effect was not reflected in an increase of IFN-γ secreting cells for the L-p-*omp25* [X1] vaccinated mice compared to the equivalent quantity of DNA delivered as naked DNA (p-*omp25* [X1]): comparable frequencies of total antigen specific effector cells were detected from p-*omp25* [X1] or L-p-*omp25* [X1] groups.

Although similar frequencies of total effector cells were recorded in both groups, there were significant differences in the T cell subsets responding to p-*omp25* [X1] and L-p-*omp25* [X1] vaccination. Whereas CD8+ cells were the dominant type recorded from the splenocytes of p-*omp25* [X1] vaccinated mice, CD4+ T cells were the dominant cell subset recorded from L-p-*omp25* [X1] vaccinated mice. From these data it is not possible to determine whether this difference in

phenotype of primed effector cells had any significant impact upon the development and maintenance of protective immunity, as both of these single dose preparations induced equivalent non-protective effects (weak reduction in bacterial load) despite the involvement of distinct T cell subsets.

Other researchers have shown that entrapment of DNA vaccines within cationic liposomes can augment both Th 1 and Th 2 type cytokine production in comparison to that achieved through naked DNA vaccination (Gregoriadis *et al*, 1997). It is possible that the enhanced antibody responses observed following L-p-*omp25* [X1] vaccination were a result of increased priming of Th 2 type immune responses rather than the protective Th1 cellular responses. IL-4 producing CD4+ cells were not measured in this investigation, and thus this hypothesis cannot be validated. However, measurable levels of antigen specific IL-4 could not be demonstrated in the *omp25* vaccine studies in any of the groups (data not shown), suggesting that any induction of Th 2 cytokine production is small. Furthermore, the Omp25 and 16M specific antibody response measured from L-p-*omp25* [X1] vaccinated mice is IgG2a dominated, supporting the absence of IL-4 in the specific response.

The CD4+ and CD8+ effector cells generated by naked p-*omp25* vaccination are likely to be the result of myocyte transfection and antigen production, followed by antigen uptake by a professional APC and processing via conventional MHC class II pathways and cross presentation to access MHC class I pathways. Intramuscular delivery of naked DNA is not considered an efficient way to delivery DNA directly to APCs (O'Hagan *et al*, 2004), and much evidence suggests that cross-presentation is the dominant route of CD8+ priming in this situation (Ulmer *et al*, 1996., Corr *et al*, 1999). The relative abundance of the CD8+ T cell subset demonstrated in this study suggests that the naked DNA derived Omp25 antigen readily accesses the MHC class I processing pathways, either because of effective direct transfection of professional APCs, or because of intrinsic properties of the protein which target it to the cytosol of APCs.

DNA vaccines were surface adsorbed onto cationic liposomes to promote efficient uptake of DNA into cells. Direct uptake of liposomes by professional APCs was also expected to enhance immune induction in the draining lymph nodes, and increased potential for MHC class I restricted presentation of endogenously produced DNA encoded antigens and direct priming of CD8+ T cells. However, the L-p-*omp25* [X1]vaccine was not able to produce the total numbers of effector cells achieved by either the protective p-*omp25* [X4] vaccination or the uneffective p-*omp25* [X1] vaccination. Depletion studies indicated that CD4+ T cells are the dominant responders to the L-p-*omp25* [X1] vaccine. Moreover, the actual frequency of antigen specific CD4+ T cells elicited by p-*omp25* [X1], p-*omp25* [X4], or L-p-omp25 [X1]was not significantly different ($p > 0.05$), suggesting that the principal difference between the vaccines was that liposome formulation was less effective than naked DNA for presenting antigen in association with MHC class I for priming CD8+ T cells. A clear reason for the apparent reduction in CD8+ cell priming for L-p-*omp25* [X1]compared with p-*omp25* [X1] is not apparent.

Post-challenge ELISPOT investigations revealed that all mice contained Omp25 specific effector cells post-exposure to live *Brucella*. In most cases the frequency of cells was above the analytical capacity of the assay, and resulted in saturated readings (> 500 SFC / 10^6), suggesting that native Omp25 is a prominent and significant immunogen in live *B. melitensis* 16M infections. A significant difference in IFN-γ generation between groups that had been primed by vaccination compared to that from pre-challenge antigen naïve mice was not observed. An investigation of effector cells at an earlier interval post-challenge may have revealed an anamnestic response from the Omp25 responsive mice. However, at 15 days post-challenge it appears that the strong primary Omp25 specific response observed in naïve mice may mask this effect.

Although an explanation for specific abrogation of CD8+ priming by liposome vaccination is not apparent, these data clearly indicate that the CD8+ effector cells are important contributors to the protective effect of the *omp25* based vaccines. A clear relationship between the number of CD8+ effector cells (Total

SFC – CD8+ depleted SFC) and protective effect is evident. In contrast, a clear correlation between CD4+ effector cells and protection is not observed. Overall, these results suggest that CD8+ T cells are the main effectors of the protective response of p-*omp25* vaccination. Furthermore, these data indicate that the liposome 'single inoculation' delivery strategy is not useful as a mechanism through which to achieve increased uptake and immunogenicity, and protective efficacy of the p-*omp25* vaccine.

p-*ialB* specific immunity

Single versus multiple dose naked DNA vaccination

Studies with the p-*ialB* vaccine showed that multiple inoculations of the naked DNA vaccine (p-*ialB* [X4]) was the most immunogenic and efficacious strategy. In this respect this data concurs with that from the p-*omp25* studies. However there were several differences in the data from the p-*ialB* studies compared to the p-*omp25* studies suggesting a different mode of action for these two equivalently protective vaccines.

Antibody production was the most striking output of the immune response following p-*ialB* [X4] vaccination: high titre equally balanced IgG1 and IgG2a responses were detected. A single dose of naked DNA (p-*ialB* [X1]) was unable to induce detectable levels of antibody. Similarly, significant IFN-γ secreting cells were only observed from the p-*ialB* [x4] protective vaccination regime. IFN-γ secretion was mainly attributed to CD4+ cells, with the bias toward MHC class II primed immune responses resulting from secretion of the endogenously manufactured protein and hence supplying antigen for exogenous uptake. Antigen secretion also increases the availability of the IalB protein for interaction with B cells, thus promoting strong antibody responses. In general, it was shown that that the protective effect of p-*ialB* [X4] vaccination correlated to the induction of high titre specific antibody and a CD4+ dominated cellular response.

Unlike the single dose p-*omp25* [X1] DNA vaccine the p-*ialB* [X1] vaccine did not induce measurable antigen specific IFN-γ production (detected as IFNg secreting effector cells). Since the quantity of DNA delivered in these experiments was, as near as practicable, equivalent, the discrepancy in immunogenicity suggests that the IalB antigen may be inherently less immunogenic than the Omp25 antigen. Indirect evidence suggesting that IalB is a comparatively weak immunogen can be found through measurement of the IalB specific response of Rev.1 and 16M infected mice. It is notable that IalB specific effector cells or antibodies are not detected from mice that have been vaccinated with Rev.1 or from IalB naïve mice that have been challenged with *B. melitensis* 16M. This suggests that the native antigen is either poorly expressed or poorly immunogenic, and indicates that IalB specific immune responses are not essential for the development of Rev.1 mediated protection.

Differences in plasmid structure between p-*omp25* and p-*ialB* may account for slight differences in immunogenicity. It has previously been demonstrated that the presence of an intron element (such as included in the pTargeT vector) can promote an increase in the expression of the encoded gene in-vivo (Chapman *et al,* 1991., Hartikka, *et al,* 1996). Enhanced expression from pTargeT-*omp25* may account for the more rapid induction of Omp25 specific responses that are detected after a single inoculation. This modest improvement in immunogenicity is only observed as a more rapid induction of specific response and does not the impact on the overall magnitude of the specific response. Interestingly, when pTargeT-*omp25* (with intron element) and pCR3.1-*omp25* (without intron element) were directly compared (in chapter 5) differences in the magnitude or rapidity of the Omp25 specific response of mice treated with the different vaccines was not observed.

Nevertheless, this perceived poor immunogenicity is overcome by multiple inoculations of p-*ialB* since IalB specific CD4+ and antibodies generated by p-*ialB* [X4] vaccination are highly effective for the control of *B. melitensis* in the murine host.

Liposome versus naked DNA vaccination

IalB specific antibody was detected following a single dose of L-p-*ialB* compared to three doses of naked *p-ialB* , indicating that liposome formulation of the p-*ialB* vaccine prompted more rapid induction of immune responses compared to naked DNA vaccination. This effect is presumably due to a more effective uptake of L-p-*ialB* by APCs, resulting in a faster delivery of antigen to the LNs and other sites of immune induction. Unfortunately, despite this improvement in antibody production, antigen specific effector cells were not apparent in the L-p-*ialB* vaccinated mice prior to challenge. It was interesting therefore that the post-challenge ELISPOT data from the p-*ialB* investigations indicated that only p-*ialB* [X4] and L-p-*ialB* vaccinated mice had measurable IalB specific IFN-γ production post-exposure to virulent *B. melitensis* 16M, suggesting an anamnestic response from these animals. In the post-challenge studies the number of antigen specific effector cells observed from L-p-*ialB* inoculated mice was considerably increased to that observed in p-*ialB* [X1] inoculated mice thereby providing further evidence of enhanced immunogenicity of the liposome formulation.

Conclusion

Overall, it was shown that the liposome strategy was effective for generating antigen specific immune responses from a reduced dose of DNA. However, the necessary improvements in cellular immune responses, (and in particular the antcipated increased generation of CD8+ T cells), were not observed. Thus, whilst this strategy was effective in one sense (antibody enhancement), it does not appear to be wholly appropriate for use against intracellular pathogens such as *Brucella* which are controlled by cellular responses and in particular by IFNγ production. Furthermore, this investigation has concluded that a single dose of these naked DNA vaccines is unable to provide effective control of *Brucella*. For p-*omp25* [X1] the reduction in protective effect (compared to p-*omp25* [X4]) was related to a relatively reduced level of CD8+ T cell priming. For the L-p-*omp25* [X1] vaccination the lack of protection was directly linked to the lack of production of CD8+ T cells, which therefore appear to be the crucial effectors of

p-*omp25* specific immunity. These data suggest that a CD8+ effector response is effective in the control of *B. melitensis* infection via recognition of the Omp25 antigen, despite the fact that Omp25 specific CD8+ effectors were not observed from Rev.1 vaccinated mice.

In contrast, protection afforded by the p-*ialB* based vaccines is achieved through the action of high titre antibodies and CD4+ IFN-γ secreting cells. For the p-*ialB* [X1] vaccination the immune stimulus was simply not sufficient for the generation of a significant antigen specific response. IalB specific antibody production was augmented by liposome delivery of the p-*ialB* vaccine but T cell effector generation was not detectably enhanced. These data suggest that although antibodies may be significant in protection they are insufficient to control *B. melitensis* challenge: IalB specific CD4+ IFN-γ secreting cells are needed for protective efficacy. Nevertheless, despite the absence of detectable IalB specific IFN-γ secreting cells prior to challenge, L-p-*ialB* vaccination resulted in an anamnestic response post-challenge. Thus, liposome delivery of this vaccine modestly improved the immunogenicity of a single 100 μg dose of DNA. This result is promising suggesting that, with further modifications, L-p-*ialB* may become an effective vaccine.

In conclusion, attempts to improve the efficiency of the single dose vaccination by targeting DNA delivery directly to APCs were partially successful. For both vaccines (*omp25* and *ialB* based) liposome adsorbed formulations of the vaccines were able to promote a stronger antibody responses than equivalent quantities of naked DNA, indicating that liposome delivery could enhance humoral immunity. Unfortunately, significant enhancement of cellular immune responses was not measured. Since cell mediated immunity against intracellular pathogens is essential (Mackaness 1971), and IFN-γ in particular is essential for the control of *Brucella* (Murphy *et al*, 2001a) future investigations into these vaccines may be best directed toward alternative strategies with proven potential for the enhancement of cell-mediated immunity.

Chapter 8: CONCLUSIONS AND FUTURE PERSPECTIVES

Summary of project findings

Following the selection of five candidate genes from the *B. melitensis* 16M genome and the construction and evaluation of eukaryotic expression plasmids encoding these antigens, two protective DNA vaccines were identified. These vaccines, p-*ialB* and p-*omp25* , encoded the putative invasion protein B (IalB) and 25 kDa outer membrane protein (Omp25) respectively. Although, the Omp25 antigen has previously been studied in terms of *Brucella* virulence, serodiagnosis and as a protective antigen (for review see Viscaino *et al,* 2002), this study represents the first report of a significant protective effect achieved against *B. melitensis* 16M challenge using the Omp25 antigen in a subunit vaccine approach. The *ialB* candidate was selected based upon its' putative function as an invasion protein which was assigned due to shared identity with the invasion protein B (*ialB*) of *Bartonella bacilliformis*. This candidate has not previously been investigated with regard to *Brucella* virulence or pathogenesis. This study is the first to identify the *Brucella* invasion protein B (IalB) as a novel protective antigen in brucellosis.

Naked DNA vaccination with either p-*omp25* or p-*ialB* , (with a total of four inoculations per mouse, at 100 μg DNA per inoculation, given at three week intervals) provided equivalent protection to the live vaccine *B. melitensis* Rev.1 under the model conditions used in these investigations. Immunogenicity assessment revealed the production of antigen specific IFN-γ and specific antibodies following vaccination. Attempts to reduce the number of inoculations required for successful vaccination were partially successful, with cationic liposome adsorbed formulations of DNA able to promote rapid antibody responses, suggesting a faster induction of candidate specific immune responses. Unfortunately, protective cell mediated responses were not enhanced by liposome formulation of the vaccine and consequently there was not significant improvement in protective efficacy compared to single dose

naked DNA. This work indicated that alternative approaches may be necessary to improve the efficiency or practicality of these vaccines before progression to assessment in target species can be considered.

Future perspectives

During the lifetime of this project, the *B. melitensis* 16M was sequenced. Early access to the incomplete genome data (with kind permission of Dr Vito DelVecchio, IMBM, University of Scranton, PA. USA), facilitated selection of four of the five candidate antigens investigated in this study. Prior to genome availability candidate selection was a more restricted process, reliant upon prior knowledge of the candidate and it's suggested role in *Brucella* virulence and pathogenicity. Whilst this approach is valuable, the availability of the genome sequence has significantly increased the potential choice of antigens for study. Genome sequences for the most significant pathogenic species of *Brucella*: *B. melitensis*, *B. suis* and *B. abortus,* are now available and sequencing of the naturally rough strain *B. ovis* is reported to be underway (Halling *et al,* 2004). With an ever expanding list of sequenced pathogen genomes and rapidly evolving progress in genomic comparisons and characterisation, the pool of data that can be mined for antigen selection is constantly increasing. For this study *in silico* selection was based upon putative assigned functions and identity with known immunogenic and protective antigens in other pathogens. Four novel candidates: BMEI1584 (*ialB*), BMEII0105 (*frpB*), BMEII0150 (*fliC*), and BMEII0681 (*virJ / acvB*) were chosen using this approach. The BMEI1249 (*omp25*) candidate was selected as a known immunogenic protein important for the maintenance of the *Brucella* outer membrane structure and function. Targeting immune responses to these antigens was hypothesised to interfere with important aspects of *Brucella* virulence. This panel of candidates was chosen to highlight different aspects of pathogenesis and virulence. The putative invasion protein B (IalB) was selected as an example of a *Brucella* antigen hypothesised to be involved in the establishment of infection or establishment of a replicative niche. Similarly, BMEII0150 (*fliC)* and BMEII0681 (*virJ*) were selected as examples of putative structural components of type III

and putative effectors of type IV secretion systems, respectively. Finally, candidate BMEII0105, *frpB* was selected to represent antigens involved in the acquisition or transport of essential nutrients.

This initial breadth of antigen selection, and subsequent data describing their protective efficacy indicated that the surface expressed candidates Omp25 and FrpB, and the hypothetically exported protein IalB were the most useful antigens for subunit vaccine development. Importantly, these antigens were also shown to be the most consistently expressed candidate genes during targeted transcription studies. The success of these vaccines being linked to the availability of the native antigen during *Brucella* infection for interaction with the immune system. Future work to identify similar candidates will be greatly facilitated by high throughput whole genome technologies such as microarrays and proteomics, which will increase the rate at which antigens expressed under certain infection conditions can be identified and hence selected for investigation. For each of the types of candidate targeted in this study, further representatives exist in the *Brucella* genomes for exploitation in future vaccine development projects. Based upon the results described in this thesis, focussing on candidates which are constitutively expressed, surface located or secreted, appears to be a sensible approach for selection of antigens useful as subunit vaccines.

The protective efficacy of the p-*omp25* and p-*ialB* vaccines was shown to be equivalent to that provided by the live vaccine strain Rev.1 in these investigations. Notably, this is the first study to evaluate DNA vaccine efficacy against *B. melitensis* challenge, and the first to directly compare efficacy against that achieved by the live strain Rev.1. These are promising results for *Brucella* DNA vaccines, and in terms of the ability to control splenic *Brucella* infections the results show an improvement from other *Brucella* specific DNA vaccines reported in literature to date. However, it is important to note that standardised methods were not adopted for the assessment of each different vaccine reported in literature and thus direct comparison is not entirely applicable. Most notably, the interval between vaccination and challenge and between challenge and bacterial enumeration varies between studies. A standard model for

assessing vaccine immunogenicity and residual virulence of live strains has been described by Bosseray *et al,* (1990), although only limited adherence to standardised practices is reflected in the literature. Numerous adaptations of the model have been employed in order to emphasise certain aspects of *Brucella* control and *Brucella* : host interaction.

In previous work at VLA (Mansour 2003) the kinetics of *Brucella* elimination in vaccinated and challenged mice was measured. This experiment was anticipated to allow a more thorough assessment of vaccine effects than can be inferred from a snapshot assessment of bacterial load at a single defined time post-challenge. However, the results indicated the expected pattern for *Brucella* replication and control in protected (*B. abortus* S19) and naïve (PBS) BALB/c mice and confirmed that protection was most accurately determined through measurement of *Brucella* load at 15 days post-challenge. Nevertheless, early assessment of protective efficacy revealed a delay in the onset of detectable infection in pCDNA-62 vaccinated mice, that would not have been observed in the single assessment of efficacy at 15 days post-challenge. Now that protective efficacy for p-*omp25* and p-*iaiB* in this model has been established at 15 days post-infection, it would be useful to determine the kinetics of *Brucella* elimination post-challenge, and more importantly to determine the limits and duration of immunity through challenge with different quantities and different strains of *Brucella* and at longer intervals post-vaccination. Furthermore, the current protection assays in murine models concentrate on the detection of bacterial load in the spleen. It would be advisable to also consider the effects of vaccination on dissemination of the bacteria to the other organs of the body, in particular to pathologically significant sites such as reproductive tissues. Whilst these studies are best performed in target species, preliminary data from small animal models could indicate whether the IalB protein is involved in invasion of a particular cell or tissue type.

A significant facet of this study was the measurement of immune responses induced in vaccinated mice. The investigation of the p-*ialB* and p-*omp25* vaccines revealed the generation of CD4+ and CD8+ IFNγ secreting cells, and

production of antigen specific immunoglobulins. Comparison of responses induced by the p-*omp25* and p-*ialB* vaccines and the live vaccine Rev.1 were useful for determining common immunological correlates of protection. In all vaccinated mice the absence of IFNγ production was associated with an absence of protective efficacy. Although, as for other pathogens, the presence of IFNγ is not always sufficient for control of *Brucella*. To gain a more thorough picture of protective immunity other Th 1 cytokines were measured, although an antigen specific effect was not observed in these investigations. Technologies for cytokine measurement and quantification are continually advancing, and it is probable that the use of more sensitive assays, assays for additional cytokines, or alternative and multiplexed techniques for cytokine detection (e.g.: microarray, xMAP™ Luminex® technology, and FACS) could be of value in determining a more comprehensive picture of the cytokine profile of immune mice or target species. A number of recent publications have indicated the upregulation of IL-6 and IL-8 during natural brucellosis (Refik *et al* 2005), or the relevance of IL-18 and IL-23 in the control of intramacrophagic *Brucella* (Paranavitana *et al* 2005., and Fernandez-Lago *et al* 2005). It would be pertinent in future experiments to assess a wider range of immunological responses so as to mirror the complexity of the immune response in the living host.

In this study IFNγ production was assumed to be the principal effector of the protective immune response. The production of IFNγ was studied in terms of the principal responding T cell subsets (CD4+ and CD8+ only) from the splenocyte population. From Rev.1 vaccinated mice stimulation with Omp25 and Brucellergene™ resulted in IFNγ production from mainly CD4+ cells (CD4+ effector cells), whereas it was noted that DNA vaccines primed a greater number of antigen specific CD8+ effector cells. For p-*omp25* [X1] and [X4] vaccination a slight (not statistically significant) increase in total effector cells was associated with increasing numbers of inoculations and significant improvement in protective efficacy. For p-ialB [X1] vaccination, IFN-γ secreting cells were not detected, but four inoculations with this vaccine (p-*ialB* [X4]) generated significant numbers of effector cells. In terms of responsive cell type,

Omp25 specific responses from p-*omp25* vaccinated mice were dominated by CD8+ cells, whereas Omp25 specific responses from Rev.1 vaccinated mice were dominated by CD4+ cells. IalB specific IFNγ production was dominated by CD4+ cells. Furthermore, despite demonstrable protective efficacy from the p-*ialB* vaccine, an IalB specific response was not demonstrable from Rev.1 vaccinated mice, suggesting that this antigen was not involved in the protective immune response attributed to the live vaccine. Now that production of antigen specific effector cells has been established, It would be of value to study of the contribution of the different Omp25 and IalB specific T cell subsets in control of brucellosis, through the use of $CD4^{-/-}$ and $CD8^{-/-}$ mice, and through adoptive transfer studies. Such experiments would help to determine whether the reduction of CD8+ involvement in L-p-*omp25* compared to p-*omp25* (X4) vaccinated mice was significant, or whether the reduced efficacy is due to an overall quantitative reduction in IFNγ levels, or additional parameters that have not been monitored in this study. Furthermore, further characterisation of the phenotype of responsive cells would be useful in defining immunological correlates of protection for extrapolating data to target species. A role for CD4+ and CD8+ T cells in control of *Brucella* has previously been established (Oliveira *et al* 1995) and is supported by this study. However, looking at these cells in isolation may not be sufficient to elucidate their role in protection; other possible contributors should be assessed. A role for natural killer cells in control of brucellosis has been disputed (Golding *et al* 2001). Proliferation of γδ T cells early in infection has been reported in humans (Dornand *et al* 2004, Bertotto *et al* 1993), and the relatively high frequency of γδ T cells in ruminants may be significant in the pathology of brucellosis in these animals. Recently, $CD4^-/CD8^-$ cells have been shown to contribute to the control of important intracellular pathogens such as *Mycobacterium tuberculosis* and *Francisella tularensis* (Cowley *et al* 2005). Therefore, in addition to evaluating the role of CD4+ and CD8+ cells, the involvement of γδ, NKT1.1 and $CD4^-/CD8^-$ cells in p-*omp25* and p-*ialB* vaccine induced responses should also be investigated for a fuller picture of cellular immunity.

Notably the study described in this thesis measured the generation of the effector cell population. With further surface antigen characterisation these cells could be categorised as effector memory T cells (T_{em}), although at present it is only possible to class them as effector cells. Whilst the effector cells (potentially T_{em}) are representative of the immediate response following *in vivo* encounter with specific antigen (viable *Brucella*), it has been noted that generation of antigen specific central memory T cells (T_{cm}) is more closely associated with DNA vaccine mediated protection for some chronic disease conditions such as HIV (Seaman *et al* 2004). The presence of T_{cm} was not established was not established in this study, although a cultured ELISPOT assay could indicate whether or not delayed effector responses indicative of activation of T_{cm} were generated following vaccination. Assessment of T_{cm} and T_{em} levels induced by the *Brucella* vaccines, as well as adoptive transfer studies with antigen specific T cells, would also provide further information on the mediators of the protective responses induced by the p-*ialB* and p-*omp25* vaccines.

It is important to remember that demonstration of efficacy and immunogenicity for different DNA vaccines relies not only on appropriate selection of antigens but also on the construction of functional plasmid constructs and an appropriate delivery strategy. Notably, delivery of the p-*ialB* and p-*omp25* vaccines surface adsorbed to cationic liposomes promoted a more rapid evolution of detectable specific antibodies, and for the L-p-*omp25* vaccine promoted a qualitatively distinct IFNγ response compared to delivery via naked DNA. L-p-*omp25* [X1] vaccination resulted in a lower frequency of total responsive cells and a CD4+ dominated response, which was not protective. The attempts to enhance efficacy of the p-*ialB* vaccine through surface adsorption to cationic liposomes was partially successful with L-p-*ialB* [X1] vaccination resulting in measurable levels of IalB specific antibody and a notable anamnestic response. However, these effects were not sufficient to promote significant protection. Thus whilst the antibody promoting effect of liposome delivery could potentially be exploited for the generation of monospecific and monoclonal reagents, this is not an appropriate method for vaccine enhancement in this study.

Although liposomal delivery was not successful in this study, alterations to the liposome formulation may alter this outcome. One possible approach would be to encapsulate the DNA within the liposome rather than adsorbed to the surface. This has been a successful approach for a number of DNA vaccines Another approach would be to modify the composition of the liposomes. Recent work by Murillo and colleagues (2004) has indicated a difference in the protective effect of *Brucella* vaccines based upon protein antigen preparations which was attributable to difference in the design of the microparticulate delivery system used: PLG based microspheres are better than PLGA. Thus confirming that the carrier has a profound influence on the outcome of vaccination. Encapsulation or conjugation with protein antigens, cytokines, mannose, or even *Brucella* LPS are other possibilities. Furthermore, combining the p-*omp25* and p-*ialB* vaccines for delivery as a multivalent vaccine may also improve efficacy. Additional protective antigens could also be incorporated into these vaccines.

Alternatively, there are a multitude of other approaches for vaccine delivery, e.g.: *in vivo* electroporation (Tollesfsen *et al* 2003), PLG based microparticle generation (O'Hagan, 2004), delivery via live attenuated bacterial vehicle (Al-Mariri *et al* 2002., Garmory *et al* 2003b), or construction, e.g.: inclusion of specific targeting sequences (Kim *et al* 2005), intron elements, further CpG motifs, and codon optimised antigen sequences that could be investigated in future for improving the p-*ialB* and p-*omp25* vaccine efficiency.

Notably, rational vaccine development in the field of brucellosis has recently been weighted toward investigation of attenuated mutant strains and has focussed on identification of genes which contribute to the establishment of intracellular infection and maintenance of the chronic infection state. Targeted mutagenesis of genes to create auxotrophic mutants, egg: aromatic Δ*aroA* mutants (Foulongne *et al,* 2001), stress response (Δ*dnaK*) mutants (Kohler *et al,* 2002), LPS defective mutants (Δ*wboA*) (Winter *et al,* 1996) have resulted in attenuated strains which were considered promising vaccine candidates. Whole genome mutagenesis studies have invariably identified genes associated with

IV secretion systems, LPS biogenesis, and the maintenance of metabolism and acquisition of essential nutrients (Kohler *et al,* 2002) as essential in the pathogenesis of brucellosis. Such studies have resulted in identification of a number of promising candidates for attenuated vaccine development. For a number of reasons mutagenesis studies have so far failed to identify candidates associated directly with the initial invasion of the host cell, with the notable exception of the *Brucella* LPS. For this reason the approach taken in this thesis has been valuable for identifying important *Brucella* antigens (egg: IalB) that have been missed in mutagenesis screens to date, and as such represents a complementary approach for the identification of potentially protective antigens and candidate genes for targeted deletion. During the lifetime of this project, Edmonds *et al,* (2002) have developed and characterised *omp25* deletion mutant strains of *Brucella*, and reported attenuation and protective efficacy in both the murine model and target species. To date there are no reports describing the effects of *ialB* deletion on *Brucella* virulence, or the attenuation and protective efficacy of such strains. In light of the success of the p-*ialB* vaccine, it is important to develop defined *ialB* mutant strains in order to facilitate characterisation of this newly identified protective antigen, and elucidate the role of the antigen in *Brucella* infections. Furthermore, although only weakly effective as a subunit vaccine, the *frpB* candidate may yet prove to be of value when this gene is deleted from virulent *Brucella*. A Δ*bhuA* mutant strain of *B. abortus* 2308, in which the *frpB* gene (alternatively named as the *bhuA* gene: for *Brucella* heme uptake A), is deleted is reportedly unable to utilise heme as an iron source in the presence of chelating agents. Further studies are reported to be underway to characterise the role of *frpB* or *bhuA* in *Brucella* virulence (Roop *et al,* 2004).

Importantly, the use of live *Brucella* strains as vaccines relies of the balance between attenuation and persistence: the vaccine strains must persist within the host for a period of time in order to promote appropriate immune reactions. With this caveat comes concerns over possible reversion to virulence and potential for shedding of the strain into the environment, and each of the currently licensed live vaccines is beset by problems associated with residual virulence.

Furthermore, despite considerable research effort and widespread application to livestock, there are not any live strain *Brucella* vaccines considered safe for use in humans (For review see Hoover *et al*, 2004). Therefore, the development of an efficacious non-living vaccine may offer an alternative strategy for prophylaxis of human populations, as well as improving the practicalities of livestock vaccination. Underlying the current preference for live attenuated vaccine development is the paucity of information identifying protective antigens and the unchallenged superiority of live strains as promoters of protective responses against intracellular pathogens. These caveats are not immutable. The approach taken in this thesis highlights the value of the genome sequences for identifying novel protective antigens, and can readily be extended to assess further putative antigens. Furthermore, considerable advances are being made in the fields of immunology, vaccine delivery and adjuvant design which can be applied to improve the efficacy of subunit vaccines.

In conclusion, approaches used in this investigation have facilitated identification of a novel protective antigen (IalB) in brucellosis, and indicated that DNA vaccination possibly improves protective efficacy of the Omp25 antigen compared with the protein delivery based approaches attempted by previous researchers. The study has utilised genomic information to rapidly and simply establish whether hypothetical proteins in the *Brucella* genome are significant as protective antigens. This approach can readily be applied to other putative antigens and, unlike work to create defined mutant strains, vaccine constructs can be created safely and relatively rapidly at containment level 2. Thus minimising the risk of infection to researcher and vaccine user. Whilst the p-*ialB* and p-*omp25* vaccines offer comparable protective efficacy to the live vaccine Rev.1 in these studies, further investigations are necessary to confirm and validate these effects. Work to improve vaccine delivery efficiency was not successful in terms of promoting a rapid or significant protective effect but the results of the investigations did suggest a possible use for the cationic liposome formulation of DNA in promoting high titre antibody responses that could be exploited for reagent development.

Live vaccines remain the most popular type of *Brucella* vaccine in the world today, although it is notable that attempts to reduce the virulence of existing vaccines have resulted in poorer protective efficacy, and the most effective vaccines retain a level of virulence that makes them unacceptable for use in humans. Therefore, as a strategy to generate safer *Brucella* vaccines the subunit or non-living vaccine approach is significant. There is still plenty of work to be done to unravel the complexities of *Brucella* virulence, pathogenesis and immunity, but as this knowledge expands the possibility of developing an effective non-living vaccine also increases. The p-*ialB* and p-*omp25* vaccines constructed and evaluated in this thesis have shown promising control of *Brucella* in the murine infection model and further study is now necessary to further elucidate protective immunity, establish the duration of this immunity, and assess efficacy in target species. A number of avenues have been highlighted for further investigation.

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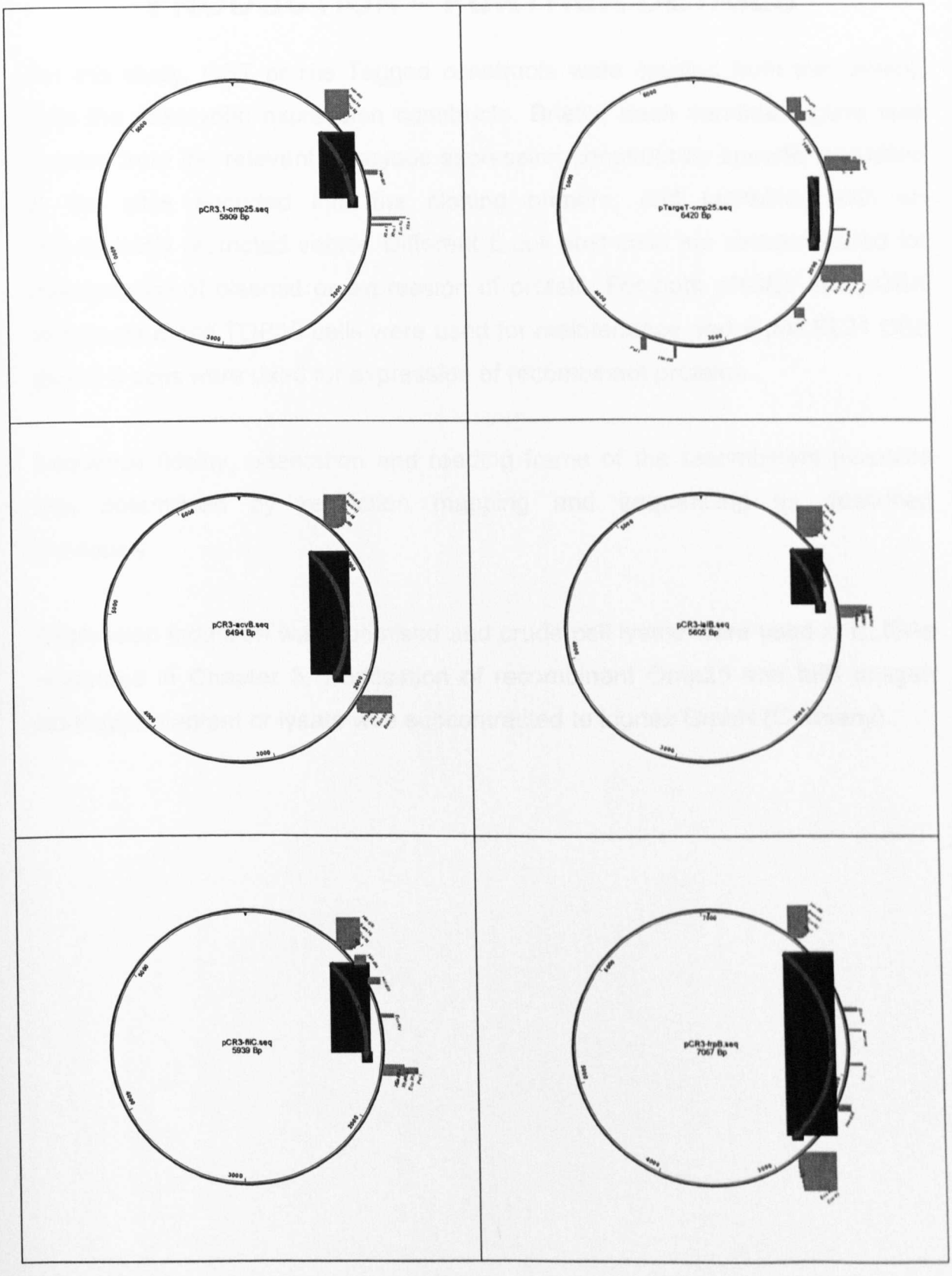
APPENDIX 1: PLASMID MAPS



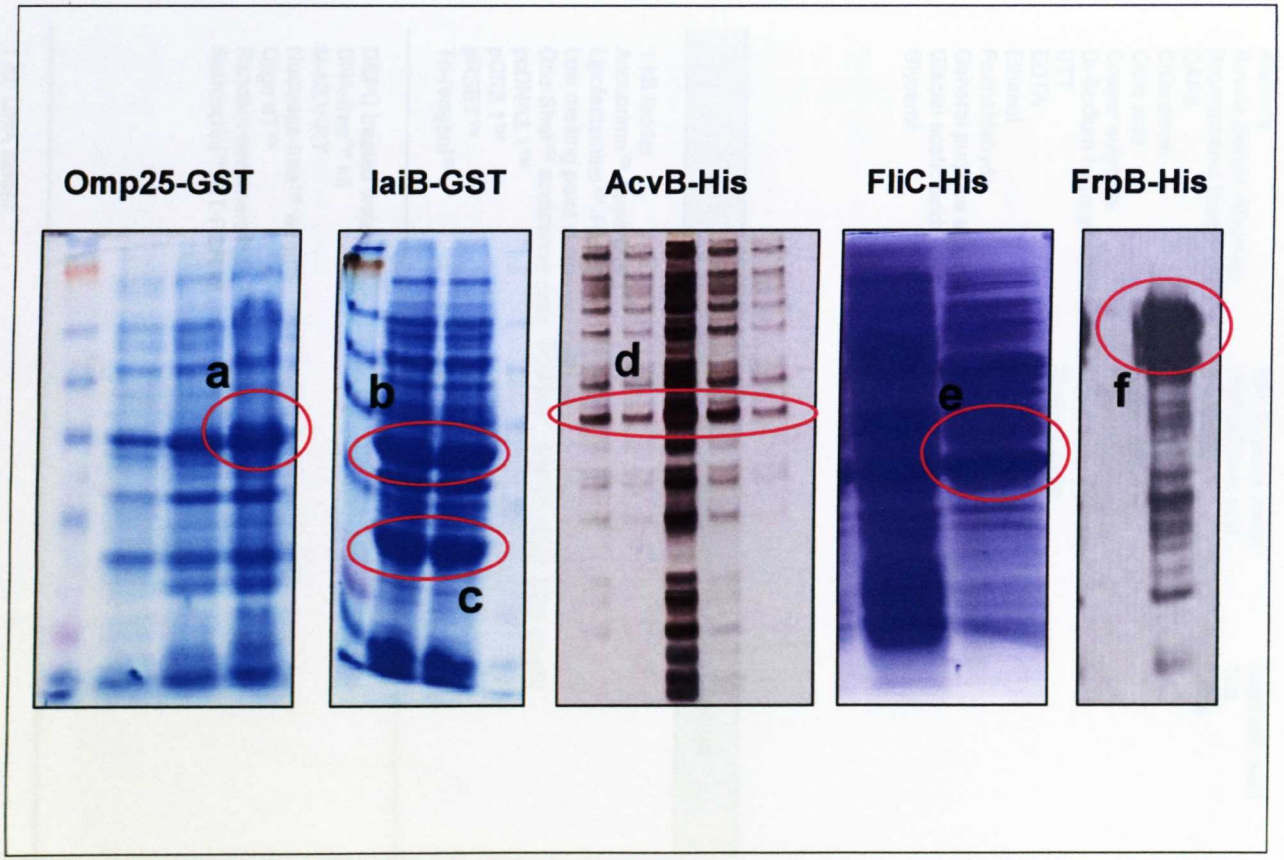
APPENDIX 2: RECOMBINANT PROTEIN PRODUCTION – FURTHER DETAILS

For this study, GST or His Tagged constructs were created from sub-cloning from the eukaryotic expression constructs. Briefly, each candidate gene was excised from the relevant eukayotic expression construct by specific restriction at the sites encoded into the cloning primers, and combined with an appropriately restricted vector. Different *E.coli* host cells are recommended for maintenance of plasmid or expression of protein. For both pRSET and pGEX constructs *E.coli* TOP10 cells were used for maintenance and *E.coli* BL21 DE3 (pLys) S cells were used for expression of recombinant proteins .

Sequence fidelity, orientation and reading frame of the recombinant plasmids was determined by restriction mapping and sequencing as described previously.

Expression induction was optimised and crude cell lysate were used in ELISAs described in Chapter 3. Purification of recombinant Omp25 and IalB antigen from supernantant or lysate was subcontracted to Lionex GmbH (Germany).

Figure A2.1: Recombinant protein crude lysates used in this study



a: Omp25-GST fusion protein in E.coli BL21 cell pellet (~55 kDa), b: IaiB-GST fusion protein in E.coli BL21 SN-2 fraction (43 kDa), c: IaiB protein (without associated fusion tag) in E.coli BL21 SN-2 fraction (~16 kDa), d: AcvB-His fusion protein in E.coli BL21 SN-2 fraction (~50 kDa), e: FrpB-His fusion protein in E.coli BL21 SN-2 fraction (~75 kDa). Gels are coomassie or silver stained.

APPENDIX 3:SUPPLIERS AND REAGENTS USED IN THIS THESIS

Product	Manufacturers
General purpose laboratory and HPLC grade chemicals	
Acetone Bovine Serum Albumen Bromophenol Blue CAPS Chloroform Citric acid Copper sulphate Di-Sodium hydrogen phosphate DTT EDTA Ethanol Formaldehyde General purpose agarose Glacial acetic acid Glycerol HPLC grade water Hydrochloric acid Imidazole Isopropanol Methanol Nickel chloride Parafomaldehyde Potassium chloride Sodium azide Sodium carbonate Sodium chloride Sodium di-hydrogen phosphate Sodium hydrogen carbonate Sterile D-PBS Sucrose Suphuric acid Tris Urea	Sigma Aldrich UK (World Headquarters: 3050 Spruce St. St Louis MO 63103 USA) **BDH / VWR international:** Merck House Poole Dorset BH15 1TD England Fisher Scientific UK Ltd Bishop Meadow Road, Loughborough, Leicestershire L011 5RG
Molecular biology reagents	
1 kB ladder Accuprime™ SuperMix 1 Lipofectamine™-Plus™ Low melting point (Nusieve) agarose One-Shot™ competent cells, TOP10F', TOP10, BL21 DE3 (pLyS) pcDNA3.1™ pCR3.1™ pRSET™ Tri-reagent™	***Invitrogen™ Life Technologies*** Invitrogen BV PO Box 2312 9704 CH Groningen The Netherlands
DEPC treated water DNA-free™ kit M-MLV-RT Nuclease-free™ water Oligo dT™ Random decamers™ Retroscript™ RT-PCR kit	Ambion Inc / Ambion (Europe) Ltd 2130 Woodward St Austin Texas 78744-1832 USA Spitfire Close Ermine Business Park Huntingdon Cambridgeshire PE29 6XY UK
1 Kb DNA ladder dNTPs JM109 competant cells pTargeT ™ Restriction endonucleases T4 ligase	Promega Corporation 2800 Woods Hollow Road Madison WI 53711-5399 USA
Limulus Amoebacyte Lysate (L.A.L) E-Toxate kit	Sigma Aldrich UK

Pyrochrome endotoxin assay	Cape Cod Inc. (ACC) 124 Bernard E. Saint Jean Drive East Falmouth, MA 02536-4445, USA
Gel extraction kit Plasmid purification kits (Mini-prep – Giga prep)	Qiagen Ltd Boundary Court Gatwick Road Crawley West Sussex RH 10 9 AX UK
Custom services	
Oligo production Plasmid and PCR product sequencing	Oswel Sequencing Services Oswel University of Southampton Boldrewood, Southampton UK.
Plasmid purification	PlasmidFactory GmbH & Co. KG Meisenstr. 96 D-33 607 Bielefeld Germany
Recombinant protein purification	Lionex GMBH 12 Mascheroder Weg Braunschweig Germany
Culture media	
DMEM Non-essential amino acids Myoclone™ Plus Foetal Bovine serum	Sigma Aldrich UK
50mM 2-Mercaptoethanol Antibiotic antimycotic solution (X100) Hanks Balanced Salt Solution (HBSS) SOC	Invitrogen™ Life Technologies
Albimi broth LB-Agar LB-broth MEM SDA + 10% horse serum TSA TSA + Penicillin	VLA Biotechnology Dept Veterinary Laboratories Agency, Weybridge Woodham Lane New Haw Addlestone Surrey KT 15 3 NB, UK
Penbrithin (Ampicillin)	SmithKline Beecham Pharamceuticals Welwyn Garden City, Hertfordshire, AL7 1EY, UK.
Protein Science Reagents	
BCA kit Dialysis cassettes (3kDa and 10 kDa CO)	Pierce Endogen Perbio Century House High St. Tattenhall Cheshire CH3 9RJ UK

Excel Gel system Protein silver staining kit Rainbow markers	Amersham BioSciences UK. Pollards Wood Nightingales Lane Chalfont St Giles Bucks, HP8 4SP, UK
0.45μM Nitrocellulose membrane Coomassie Blue Safe stain Protean II gel tanks and equipment	Bio-Rad laboratories Inc. Bio-Rad house Maylands Avenue Hemel Hempstead Herts, HP2 7TD, UK
DTT Novex Gels SDS-PAGE sample buffer (X2) See-Blue 2 markers	*Invitrogen™ Life Technologies*
Immunological reagents	
Streptavidin-Horse raddish perxoidase Streptavidin-alkaline phosphatase	Amersham Biosciences UK
Goat Anti-Mouse IgG1 Horse raddish peroxidase (STAR81P) Goat Anti-mouse IgG2a Horse raddish peroxidase (STAR82P)	Serotec Ltd 22 Bankside Station Approach Kidlington Oxford OX5 1JE UK
Anti-mouse Ig FITC Anti-Rabbit Ig FITC Anti-Sheep IgG FITC	Nordic Immunologicals Tilburg The Netherlands
Anti-sheep/goat IgG - horse raddish peroxidase Anti-sheep IgG alkaline phosphatase Anti-mouse Ig Fab' binding -alkaline phosphatase Anti-Rabbit IgG horse raddish peroxidase BCIP-NBT FastTabs™ 1-Chloro-1-napthol (1C1N) tablets	Sigma UK
AN18 Anti-mouse IFN-γ RC Biotinylated anti-mouse IFN-γ	Mabtech AB Stockholm Sweden
Anti-mouse CD4 (L3T4) conjugated beads Anti-mouse CD8+ (Ly-2) conjugated beads Midi-Macs Magnets Midi-Macs LS columns	Miltenyi Biotec Ltd. Almac House Church Lane, Bisley Surrey GU24 9DR
96 well PVDF filter plates	Millipore (U.K.) Limited Units 3&5 The Courtyards Hatters Lane Watford, WD18 8YH
FACSFlow FACS sheath fluid 100μM mesh cell sieves 80μM mesh cell sieves	BD Biosciences Erembodegem-Dorp 86 B-9320 Erembodegem, Belgium