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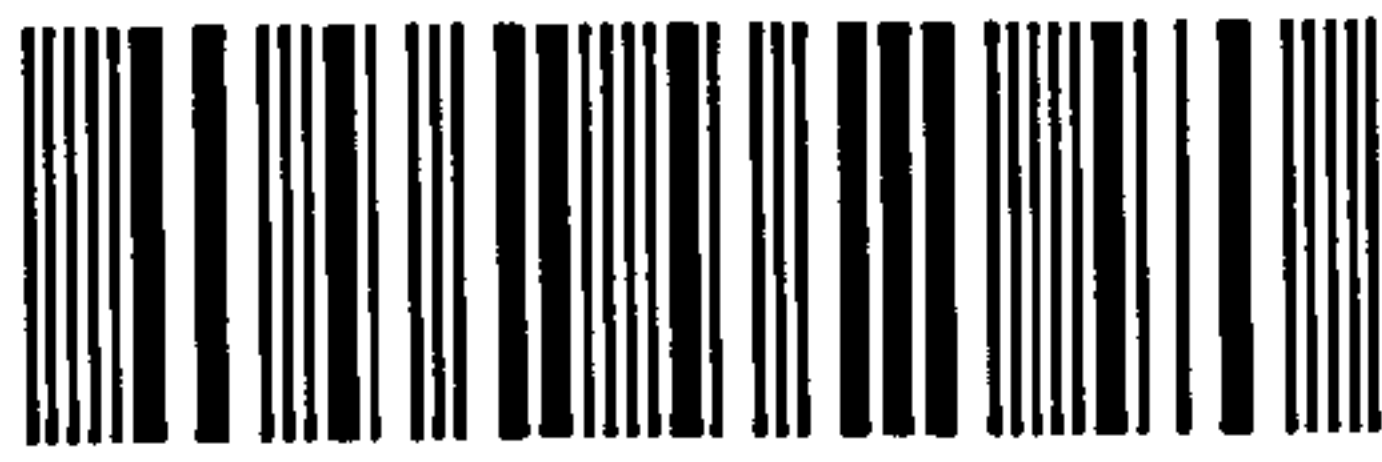
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Sub-Unit Vaccines for *Brucella*

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This work was completed at the Defence Science and
Technology Laboratory, Porton Down, Salisbury, UK.

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Abstract

Brucella species are the causative agents of brucellosis, which is regarded as the world's most prevalent zoonotic disease. Brucellosis is endemic to the Middle East, Mexico, Asia, South America and the Mediterranean and causes significant economic losses in livestock in these areas of the world, as well as being a reservoir for human brucellosis. Although there are live attenuated vaccines available for brucellosis, they have many drawbacks when used in animals, and are still infectious to humans. It is clear from this that there is a need for a new effective vaccine for brucellosis for both animal and human use.

There are increasing instances of ATP-binding cassette (ABC) systems being identified as virulence factors or potential vaccine candidates. In this study inventories of all the ABC systems encoded by five sequenced *Brucella* strains were compiled. These inventories were compared and differences have been found that could aid in the identification of virulence factors of *Brucella*. This study also explores the potential of ATP-binding cassette transporters as sub-unit vaccines against *Brucella melitensis* 16M. Eight ABC transporter proteins (PotD, PotF, Cgt, CydD, LolE, FbpA, OppA and ZnuA) have been produced using recombinant protein technologies, and their protective efficacy was evaluated in a number of studies using the murine model of *B. melitensis* infection. Of the eight vaccine candidates selected two have shown potential as novel vaccines against brucellosis. PotD and PotF are putative polyamine binding proteins of *Brucella* and show protection against experimental challenge of approximately 1×10^4 CFU of *B. melitensis* 16M when expressed by DNA vaccines or used in a PotD/PotF combination vaccine. Through this work a dual adjuvant system (ISCOMs and CpG) has been identified as a more effective adjuvant for *Brucella* vaccines than others previously used.

Declaration

I declare that this thesis is composed of my own work and has been compiled by myself to conform to the submission regulations of the Open University. During the course of this work I have received help from others when needed, these instances have been declared below:

Dr Simon Smith oversaw my use of the electron microscope used to produce the image of ISCOMs.

Dr Elie Dassa checked the accuracy of the re-annotation I performed of the ABC systems of all the *Brucella* species.

The *Brucella melitensis* 16M genomic DNA was made and kindly supplied by Dr Nicky Commander.

The original PotD and PotF protein expression constructs and first batch of protein was made by David Harland. The original FbpA protein expression construct was made by Sarah Montague.

Dr Sophie Smither and Dr Steve Lonsdale kindly provided the irradiated *Brucella* strains.

All but one of the *Brucella* challenge experiments were carried out at the Veterinary Laboratories Agency by Dr Nicky Commander and the *Brucella* immunology team using experiments designed by myself.

The *Brucella* challenge experiment carried out at Dstl was completed by Sophie Smither and Carwyn Davies, using experiments designed by myself.

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List of Abbreviations

µm	micro meters
2xYT	2x Yeast Tryptone
ABC	ATP-binding cassette
ABCISSE	ABC systems: Information on Sequence Structure and Evolution
ADP	Adenosine diphosphate
AEC	3-Amino-9-Ethylcarbazole
APCs	Antigen-presenting cells
ATP	Adenosine triphosphate
BCV	<i>Brucella</i> containing vacuole
BCV	<i>Brucella</i> chemical vaccine
Blotto	Phosphate buffered saline containing 2% (w/v) skimmed milk powder
BLS	<i>Brucella</i> Lumazine synthase
BMEI	Prefix to genes from <i>Brucella melitensis</i> 16M chromosome 1
BMEII	Prefix to genes from <i>Brucella melitensis</i> 16M chromosome 2
BOV	Prefix to genes from <i>Brucella ovis</i> ATCC 28541 chromosome 1
BOV_A	Prefix to genes from <i>Brucella ovis</i> ATCC 28541 chromosome 2
bp	Base pairs
BP	Binding protein
BR	Prefix to genes from <i>Brucella suis</i> 1330 chromosome 1
BRA	Prefix to genes from <i>Brucella suis</i> 1330 chromosome 2
BruAb1	Prefix to genes from <i>Brucella abortus</i> biovar 1 chromosome 1
BruAb2	Prefix to genes from <i>Brucella abortus</i> biovar 1 chromosome 2
BSA	Bovine serum albumin
DMEM	Dulbecco's modified eagle medium
CD	Cluster of differentiation (e.g. CD4)
CFA	Complete Freund's adjuvant
CFT	Complement Fixation Test
CFU	Colony forming units
chr 1	Chromosome 1
chr 2	Chromosome 2

Conc.	Concentration
CpG	Oligodeoxynucleotides containing CpG motifs
CT	Cholera toxoid
CTL	Cytotoxic T lymphocyte
CV	Column volume
DAB	3,3'-Diaminobenzidine
ddH ₂ O	Ultra pure distilled water
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediamine Tetra-acetic Acid
ELISA	Enzyme-linked Immunosorbant assay
ELISPOT	Enzyme-linked Immunosorbant spot
ER	Endoplasmic reticulum
FFB	FACS flow buffer
FPLC	Fast Protein Liquid Chromatography system
GDA	Glycerol Dextrose Agar
GM-CSF	Granulocyte – macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
H ₂ O	Water
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidase
i.m.	Intramuscular
i.p.	Intraperitoneal
IFA	Incomplete Freund's adjuvant
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IM	Inner membrane protein
IM-ABC	Inner membrane protein – ATP-binding cassette fusion protein
IMAC	Immobilised Metal Affinity Chromatography

IPTG	Isopropyl-Beta-d-thiogalactopyranoside
ISCOMs	Immunostimulatory complexes
kDa	Kilo Daltons
L	Litres
L-agar	Luria agar
L-broth	Luria broth
LPS	Lipopolysaccharide
M	Molar
Mb	Mega base pairs
MFP	Membrane fusion protein
mg	milligrams
MHC	Major histocompatibility complex
ml	milli litres
mM	milli Molar
MLST	Multi-locus sequence typing
MLVA	Multiple locus variable number tandem repeats analysis
MWCO	Molecular weight cut-off
N/A	Not applicable
<i>Nramp1</i>	Natural resistance-associated macrophage protein 1)
nm	nano metres
°C	Degrees centigrade
OD	Optical density
Omp	Outer membrane protein
ORFs	Open reading frames
PA	Protective Antigen
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.05% (v/v) tween-20
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
PRRs	Pattern-recognition receptors
PU	Protective units

RBPT	Rose Bengal Plate Test
rBLS	Recombinant <i>Brucella</i> Lumazine Synthase
rBLSOmp31	Recombinant <i>Brucella</i> Lumazine synthase coupled to 10 Omp31 protective epitopes
rDnaK	Recombinant DnaK
Rev.1	<i>Brucella melitensis</i> Rev.1
rIL-12	Recombinant Interleukin-12
rpm	Revolutions per minute
SDA	Serum Dextrose Agar
<i>S. pneumoniae</i>	<i>Streptococcus pneumoniae</i>
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
SRBC	Sheep Red Blood Cells
rSurA	Recombinant SurA
TAE	Tris acetate EDTA
TF	Trigger factor protein
TLR9	Toll-like receptor 9
TLRs	Toll-like receptors
TMHMM	Transmembrane Hidden Markov model
TNF- α	Tumour Necrosis Factor-alpha
VLA	Veterinary Laboratories Agency
VNTR	Variable Number Tandem Repeats
v/v	Volume in volume
w/v	Weight to volume
<i>Y. pestis</i>	<i>Yersinia pestis</i>

Chapter 1 – Introduction

1.1 *Brucella*

The first official isolation of *Brucella* was achieved by David Bruce in 1887 [34]. Earlier reports describe outbreaks of animal abortions of epidemic proportions that could have been attributed to *Brucella* infections [127]. There is evidence of *Brucella* being discovered in two thousand year old cheese [43] and there are suggestions that Florence Nightingale died from brucellosis [84,21,367]. In both animals and humans *Brucella* species are the causative agents of brucellosis, also known as Malta fever or undulant fever [67]. The bacteria are small (0.5-0.7 μm diameter, 0.6-1.5 μm length) non-motile, gram-negative, non-spore forming coccobacilli that are part of the sub-phylum $\alpha 2$ -*proteobacteria*, which also includes nitrogen-fixing bacteria of the genus *Nitrobacter*, *Rhizobium*, *Agrobacterium* and *Rickettsia* [249]. *Brucella* species are described as facultative intracellular pathogens. There are currently nine (*B. melitensis*, *B. suis*, *B. abortus*, *B. canis*, *B. ovis*, *B. neotomae*, *B. pinnipedialis*, *B. ceti* and *B. microti*) recognised species of *Brucella* and their differences are determined by their host preferences (see section 1.1.1).

Growth of *Brucella* species can be achieved on many different types of media, such as serum dextrose agar (SDA), glycerol dextrose agar (GDA), Farrell's antibiotic medium or commercially available media such as chocolate agar (Becton Dickinson). Although *Brucella* species will grow at a range of temperatures between 20 and 40 °C their optimal growth occurs at 37°C. Only two *Brucella* species (*B. abortus* and *B. ovis*) require supplementary CO₂ for optimal growth. Different biovars of *Brucella* species can be distinguished experimentally by identifying different growth properties including: CO₂ requirement, Hydrogen Sulphide production and by looking at their serological properties [204].

1.1.1 Animal brucellosis

In animals brucellosis is a non-lethal infection that predominantly causes infectious abortions in females and orchitis in males. The route of animal infection is via the mouth, eyes, nose and skin if open. Once inside the animal host, the *Brucella* are phagocytosed by macrophages and trafficked through the regional lymph nodes to placental trophoblasts in the females and the testis in males [237]. *Brucella*'s ability to preferentially utilise erythritol rather than other carbon

sources was once thought to be the reason for its quick colonisation of the female reproductive tract in ruminants as they have high levels of erythritol in this area [96]. However, *Brucella* also colonises the reproductive tracts of rodents, which only have trace amounts of erythritol in their tissues [96].

The control of animal brucellosis is paramount in reducing the incidence of human brucellosis. Many areas of the world that are brucellosis-free have strict control programs and mandatory pasteurisation of all milk products. Successful eradication of brucellosis depends upon strong regulation and diagnostic testing of live stock to prevent spread and to control the disease [245].

1.1.1.1 *Brucella melitensis*

The natural hosts of *Brucella melitensis* are goats and sheep, in which it causes infectious abortions. *B. melitensis* is considered the least host-specific of the *Brucella* species and can sometimes infect other animals including dogs and camels [369]. It is highly pathogenic to humans and is thought to be one of the world's most prevalent zoonotic diseases. *B. melitensis* was the first of the *Brucella* species to be isolated by David Bruce in 1887, isolated from a patient's splenic tissue and originally called *Micrococcus melitensis* [67]. In 1917 the name was changed to *Brucella* in honour of Bruce. According to the International Committee on Systematics of Prokaryotes – Subcommittee on the taxonomy of *Brucella* (ICSP) there are three recognised biovars of *B. melitensis*; *B. melitensis* 16M, *B. melitensis* 63/9 and *B. melitensis* Ether.

1.1.1.2 *Brucella suis*

There are five different biovars of *Brucella suis*, which are categorised according to their microbiological phenotypic characteristics. *Brucella suis* strains 1330, Thomsen, and 686 can cause infections in wild and domesticated swine, whereas *B. suis* strains 40 and 513 infect reindeer and murine species, respectively. In female swine *B. suis* causes military brucellosis of the uterus leading to infectious abortions, and spreads rapidly between animals [237]. All of the *B. suis* strains, with the exception of Thomsen, have been found to cause disease in humans, although to a lesser degree than *B. melitensis*. The first description of brucellosis in swine was by Traum in 1914, but it was not named as a separate species until 1931 by Huddleson [140]. Infection of pigs with *B. suis* results in an acute disease characterised by abortions, infertility,

orchitis, epididymitis and arthritis. *B. suis* also causes frequent abscesses in infected organs and tissues along with the onset of spondylitis [6].

1.1.1.3 *Brucella abortus*

The natural host of *Brucella abortus* is cattle. In 1897 Bernhard Bang, a Danish veterinarian isolated *Brucella abortus* as the agent that caused brucellosis in cattle, bison, water buffalo and African buffalo. Although *B. abortus* host preference is cattle, there have been reports of *B. abortus* infections in camels [5]. Infection with *B. abortus* occurs through exposure to infectious abortions during calving or when an abortion occurs. *B. abortus* is also able to infect and cause disease in humans although the disease caused is considered less severe than that of *B. melitensis* [237]. The geographical distribution of *B. abortus* is still widespread however, there are countries that have effectively eradicated bovine brucellosis (defined as the absence of any reported cases in the last five years). These countries include, Australia, Canada, Cyprus, Denmark, Finland, The Netherlands, New Zealand, Norway, Sweden and the United Kingdom [293]. It is difficult to assess the natural incubation time of *B. abortus* in infected cattle as it is not possible to determine the exact moment of infection. In female cattle the predominant symptom is abortion, whereas in bulls *Brucella* localise in the testicles causing enlargement [6].

1.1.1.4 *Brucella canis*

The natural host species for *Brucella canis* is the dog. *B. canis* was first isolated from aborted pups in pregnant beagles by Carmichael in 1966 [44]. Most canine *Brucella* infections are acquired through the consumption of infected milk, or contact with infectious vaginal secretions and male urine or semen [346,237]. Routes of entry into the body include the genitals, the nose or the eyes. Infection with *Brucella* often results in the end of the dog's breeding career. Dogs infected with *B. canis* present with a range of symptoms including infectious abortions, bacteraemia, prostatitis, epididymitis, scrotal dermatitis, lymphadenitis and splenitis [6]. Although primarily a pathogen of the dog, *B. canis* can occasionally infect humans with mild symptoms [344,197,198].

1.1.1.5 *Brucella ovis*

The sheep is the natural host of *Brucella ovis*. It predominately causes testicular complications such as genital lesions leading to low fertility in rams, whereas ewes appear relatively resistant,

although they do suffer from occasional abortions. Rams often shed *Brucella* in their semen during early infection but over time the numbers decrease [6] *B. ovis* was first isolated from sheep in New Zealand by MacFarlane *et al.* in 1950 [215], and was given its name by Buddle in 1955 [37,36]. There are no documented cases of *B. ovis* infections in humans.

1.1.1.6 *Brucella neotomae*

The natural host of *Brucella neotomae* is the wood rat. *B. neotomae* was first identified by Stoenner and Lackman in 1957 [308], and has only ever been isolated from wood rats.

1.1.1.7 *Brucella pinnipedialis* and *Brucella ceti*

Brucella pinnipedialis and *Brucella ceti* are relatively new species in the *Brucella* field. *B. pinnipedialis* was isolated by Ewalt *et al.* in 1994 from a bottlenose dolphin [97]. Its primary hosts are seals, sea lions, and walruses. *Brucella ceti* was isolated from North sea seals and cetacean populations in 1996 by Ross *et al.* [280]. There have been three reported cases of human infection from marine mammal *Brucella* species; one report was a laboratory worker with clinical signs of brucellosis [32], the second was two patients from Peru diagnosed with neurobrucellosis caused by marine mammal strains [301] and the third was from a patient in New Zealand infected with a *Brucella* strain originating from the United States of America [212].

1.1.1.8 *Brucella microti*

Brucella microti is the most recently identified *Brucella* species. Originally isolated from the common vole *Microtus arvalis* [289], it has also been identified in soil samples [288] and in wild red foxes located in lower Austria [287].

1.1.2 *Brucella* genome comparisons

Of all the *Brucella* species/strains there are currently nine sequenced strains (available at <http://patric.vbi.vt.edu/>) : *B. melitensis* 16M [78], *B. abortus* 9-941 [128], *B. suis* 1330 [258], *B. ovis* 63/290 [323], *B. canis* RM6/66, *B. abortus* 2308, *B. suis* Thomsen (biovar 2), *B. melitensis* 63/9, *B. abortus* S19. The characteristics of the *Brucella* genomes are very similar, with the exceptions of *B. suis* biovars 2, 3 and 4 whose genomes are slightly smaller they all have approximately 3.3 Mbp genomes comprising two chromosomes, one of 2.10 Mbp (Chr I) and a smaller chromosome approximately 1.2 Mbp (Chr II) in length [155]. Both chromosomes have a

G-C content of around 57% [128,258,78] and comparisons between the genomes of *Brucella* species have shown that they are very similar with an almost identical selection of genes and gene organisation [128]. There is a clear functional difference between the two chromosomes, Chr I encodes the vast majority of core metabolic process, such as transcription, translation and protein synthesis. Whereas, Chr II encodes a large amount of genes involved in membrane transport, energy metabolism and regulation [128,258,78]. The first direct genome comparison between *B. melitensis* 16M and *B. suis* 1330 has shown that the sequence identity for the majority of open reading frames (ORFs) is $\geq 99\%$ [258]. However, despite the high degrees of similarity between *B. melitensis* 16M and *B. suis* 1330 there have been differences observed. *B. suis* 1330 contains 42 unique genes located in 22 genetic islands, whereas *B. melitensis* 16M contains 32 unique genes on 11 genetic islands, these differences seem to mainly effect genes with either unknown function such as hypothetical proteins or probable surface-exposed genes like outer membrane proteins (Omps) and membrane transporter genes [258,322]. The sequence of *B. abortus* 9-941 was released later than *B. melitensis* 16M and *B. suis* 1330, and comparisons between these three species have highlighted the high sequences homologies between these three strains even further [128]. Many of the genomic differences between *B. melitensis* 16M, *B. abortus* 9-941 and *B. suis* 1330 relate to small ORFs, where functional assays such as microarrays or proteomics studies are needed to help assess their function [128].

Perhaps the more interesting comparisons in *Brucella* genomics are between *Brucella* species and other members of the $\alpha 2$ -proteobacteria such as *Agrobacterium tumefaciens*, *Sinorhizobium meliloti* and *Mesorhizobium loti*. The genomes of *A. tumefaciens* (5.67 Mbp, [357]), *S. meliloti* (6.7 Mbp, [109]) and *M. loti* (7.60 Mbp, [159]) are considerably larger than that of *Brucella*. However, despite this, comparisons between these organisms and *B. suis* have shown that 1,902 (of 3388) *B. suis* ORFs were conserved in *A. tumefaciens*, *S. meliloti* and *M. loti* genomes [258]. Further analysis by Paulsen *et al.* has shown that *B. suis* Chr I shares large regions of gene order with *M. loti* [258]. Indicating that these bacteria are very closely related.

1.1.3 Human brucellosis

Human brucellosis is a non-lethal debilitating disease known to be caused by six of the nine species of *Brucella* (*B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. pinnipedialis* and *B. ceti*). Of these six, it is *B. melitensis* that causes the most severe infection and *B. abortus*, *B. suis*, *B. canis*, *B. pinnipedialis* and *B. ceti* causing milder forms of the disease in descending order of severity. As brucellosis is endemic in animals in many parts of the world these animals act as a reservoir for human infection [251]. The route of transmission from animal to human takes place via three recognised channels: 1. the consumption of infected animal produce (e.g. unpasteurised milk and cheese products or undercooked animal meat); 2. direct contact with infected animal birth products; 3. the inhalation of aerosolised *Brucella*. Brucellosis is a common occupational hazard for shepherds, abattoir workers, veterinarians, industrial dairy workers and laboratory personnel [249].

Once inside the human body *Brucella* infect professional and non-professional macrophages and they are then disseminated throughout the body via regional lymph nodes and subsequently into the circulation. After they enter the circulation the *Brucella* can be distributed to multiple organ systems high in reticuloendothelial tissue such as the spleen, liver, and haematopoietic system [249,283]. Infection usually takes one to eight weeks to incubate, dependant on inoculum size, *Brucella* species/strain and individual host resistance. After dissemination throughout the body brucellosis usually takes the form of either an acute disease or a chronic disease.

The acute form of the disease usually presents itself within two to four weeks of infection. Brucellosis presents itself with a variety of flu-like symptoms including fever, sweats, chills, malaise and nausea coupled with anorexia, headaches, myalgias and back pain [285]. The clinical manifestations of brucellosis include a wide variety of non-specific symptoms including anaemia, enlargement and inflammation of the liver, spleen, uvea and vertebra, a decrease in blood platelets and meningitis [368,285].

Chronic brucellosis is described as symptoms that persist for more than a year after diagnosis [101]. Symptoms for chronic brucellosis include chronic fatigue, depression, weight loss and

arthritis. *Brucella* can also invade the central nervous system and cause neurobrucellosis, occurring in about 5% of cases in non-endemic areas rising to approximately 18% in endemic areas [253]. Neurobrucellosis presents itself as acute or chronic meningitis, with other clinical symptoms including encephalitis, intracerebral abscess, demyelination and radiculoneuritis [253].

If left untreated brucellosis symptoms can worsen and patients might eventually die. Treatment for brucellosis is complex, often consisting of a cocktail of two or more antibiotics over a six week period. The current recommended treatment for brucellosis is a combination of either doxycycline and rifampicin for six weeks, or doxycycline for six weeks and streptomycin for two or three weeks [253]. In uncomplicated cases of brucellosis, these treatments are usually effective in 90% of cases and relapses can often be treated with the same regimens [303,252]. Where rare complications occur, the most common being spondylitis, there is no consensus on the optimal antibiotic regimen [252]. Where neurobrucellosis occurs, regimens often take the form of triple or quadruple antibiotic cocktails for prolonged periods of time. Endocarditis is also a major complication of brucellosis and considered a medical emergency, surgery to remove the infected heart valve is usually required and antibiotic treatment is also used for patients to make a full recovery. However, endocarditis is still responsible for the bulk of mortality caused by brucellosis [252].

1.1.4 *Brucella* as a biological warfare agent

Brucella species have traditionally been cited as potential agent of bioterrorism by the US Centers for Disease Control and Prevention (CDC) [1]. The reasoning behind this is that *Brucella* species are infective by the aerosol route and can cause a chronic debilitating disease that can be difficult to diagnose, which would cause wide spread panic and chaos after a deliberate release. The United States of America (USA) started developing *B. suis* as a biological weapon in 1942, testing it in animal field trials in 1944 and 1945, before stopping the offensive *Brucella* program in 1969 [267]. The USA is not the only country to experiment with *Brucella* as a biological weapon. In the former Soviet Union *Brucella* was developed as a biological warfare agent as part of the Soviet biological weapon program [250]. Reports have stated that both dry and liquid forms of *Brucella* were developed as biological weapons and that

they were tested on the island of Vozroshdeniye during the height of the Soviet biological weapon program [250]. Although *Brucella* species have not been directly used as a biological weapon they still pose a threat to armed forces personnel deployed to endemic areas of the world. A case of diagnosed brucellosis in a soldier six weeks after returning from active duty in Iraq was later revealed to be caused by the consumption of unpasteurised goat's cheese [235].

1.1.5 Diagnosis of brucellosis

Diagnosis of brucellosis is difficult and the best way to confirm an infection is to isolate the organism from the host's blood or other tissue. Cultures from bone marrow are considered the absolute diagnosis due to the high numbers of *Brucella* in the reticuloendothelial system, although this is an invasive and painful technique [249]. There are also a number of serological tests available for the diagnosis of brucellosis.

The Rose Bengal Plate Test (RBPT) is an antigen agglutination test that consists of *Brucella* cells stained with Rose Bengal and mixed with a buffer at pH 3.65. The test involves mixing the antigen with 30 µl of serum on a glass slide or enamel strip. After a brief incubation the slide is then examined for the presence of agglutination. Samples are usually confirmed positive if there is any agglutination present, although results can be graded depending on the speed of the agglutination [237,105]. However, to use the RBPT for *B. canis* diagnosis, specific *B. canis* antigens must be used due to the difference in lipopolysaccharide structure [283] and its cross reactivity with other bacterial species such as *Escherichia coli* O116 and O157, *Francisella tularensis* and *Yersinia enterocolitica* [249]. Indirect enzyme-linked immunosorbent assays (ELISAs) can be used as a diagnostic tool and routinely use purified O chain antigens. ELISAs overcome some of the problems associated with the RBPT and show a higher sensitivity and specificity [11].

Another serological test for brucellosis is the Complement Fixation Test (CFT). The CFT utilises anti-sheep red blood cell (SRBC) antibodies, whole SRBC, complement and antigen. Serum samples are mixed with *Brucella* antigen (usually whole *Brucella* cells) leading to the formation of antigen-antibody complexes. With the addition of anti-SRBC and whole SRBC, complexes form between the two. When the complement is added, and if there are no *Brucella* antibodies present the complement is utilised by the anti-SRBC and SRBC complexes causing SRBC lysis.

If there are *Brucella* antibodies present then the complement binds the anti-*Brucella* – *Brucella* antigen complexes and minimal SRBC lysis occurs. In sheep and goats the CFT is considered the most specific and sensitive method of serological diagnosis of *B. melitensis* infection [203].

In more recent years, the development of polymerase chain reaction (PCR) techniques have helped in both the typing and diagnosis of brucellosis. Positive PCR results can sometimes be obtained from tissue samples taken as little as 10 days after infection. The major genes used in the development of PCR diagnostic techniques are the 16S ribosomal RNA genes [239]. However, approaches originally used to evaluate the taxonomy and evolution of *Brucella* species could have applications as diagnostic tools. For example, multi-locus sequence typing (MLST), based on PCR analysis of DNA sequences of internal fragments of specific housekeeping genes, enables the characterisation of *Brucella* by their unique sequences [325,289]. Similarly, multiple locus variable number tandem repeats (VNTR) analysis (MLVA) takes advantage of polymorphisms in tandemly repeated DNA sequences for analytical purposes. MLVA technology for evaluation of *Brucella* species is a new technology and, in 2006, two publications described MLVA markers that may be used for identification of *Brucella* species and, in some cases, specific *Brucella* strains [350,181]. These MLVA panels have been used to type strains of *Brucella* isolated from humans in Peru [300]. MLVA technology has been compared to other PCR-based typing methods, providing evidence that it is a comparable or, in some cases, a more useful diagnostic tool [110].

1.1.6 The intracellular trafficking of *Brucella*

Brucella are characterised as facultative intracellular bacteria since they spend most of their life within host cells. They can infect either non-professional phagocytic cells like epithelial cells and fibroblasts, or professional phagocytic cells including murine peritoneal macrophages and human monocytes, neutrophils and bovine mammary gland macrophages [224]. The trafficking of *Brucella* through each type of cell differs between non-professional and professional phagocytic cells.

1.1.6.1 *Brucella* trafficking through non-professional phagocytic cells

The entry of *Brucella* into epithelial cells occurs via the use of unknown cell receptor molecules, and through phagocytosis using actin filaments and activation of GTPases (figure 1.1 A). During

early infection *Brucella* are routed to the early phagocytic compartments of the cells (figure 1.1B) and at this stage, the host cell has developed its late endosome which will develop into the lysosome and then the phagolysosome (figure 1.1 C). The majority of *Brucella* that are ingested into non-professional phagocytic cells are routed to the endoplasmic reticulum (ER) via the autophagocytic pathway. Here they join and fuse with the ER, using type 1 transmembrane glycoproteins called lysosome-associated membrane protein 1 (LAMP-1 depicted by ●, figure 1.1 D & E). *Brucella* do not travel through the late endosome or golgi apparatus throughout intracellular trafficking. Once inside the ER, *Brucella* replicate without interfering with cellular function [224]. During this trafficking *Brucella* avoid phagosome contact and are therefore not in danger of being digested in the phagolysosome.

1.1.6.2 *Brucella* trafficking through professional phagocytic cells

Brucella enters professional phagocytic cells via binding to cell receptors such as FcR, C3bR, mannose receptor and the fibronectin receptor [347] (figure 1.2). Once bound, *Brucella* enters via lipid rafts (membrane areas rich in cholesterol, glycosylphosphatidylinositol (GPI) and glycosphingolipids). Specifically, *Brucella* use cholesterol and GPI-anchored proteins as their lipid rafts [347,224]. Opsonised *Brucella* gain entry via the cell's Fc receptors [224] (figure 1.2 A) and, once *Brucella* have gained entry into the cell, development of the early *Brucella*-containing vacuole (BCV) occurs (figure 1.2 B). During the development of the BCV, the host cell starts to develop the early endosome, which will become the late endosome, then subsequently the lysosome and the phagolysosome (figure 1.2 C). The intermediate BCV forms by the acquisition of lysosomal-associated membrane proteins (LAMP) (figure 1.2 D) and fusion with the cells ER occurs within the first few hours of infection (figure 1.2 E). The *Brucella* enter the final stages of cell infection by formation of the replicative BCV which occurs without the need for LAMP-1, but with the recruitment of other ER markers including calnexin and calreticulin (depicted by ●), before replicating in the cisternae compartment of the ER [51] (figure 1.2 F & H). *Brucella* do not travel through the

Figure 1.1: *Brucella* entry and evasion of non-professional phagocytic cell killing; adapted from [224]

- A.** Entry of *Brucella* into non-professional phagocytic cells via actin filaments
- B.** *Brucella* routed to early phagocytic compartments
- C.** Development of the late endosome
- D & E.** Trafficking of *Brucella* through the autophagocytic pathway to the ER
- F.** Phagosome and lysosome fusion to form the phagolysosome resulting in bacterial breakdown

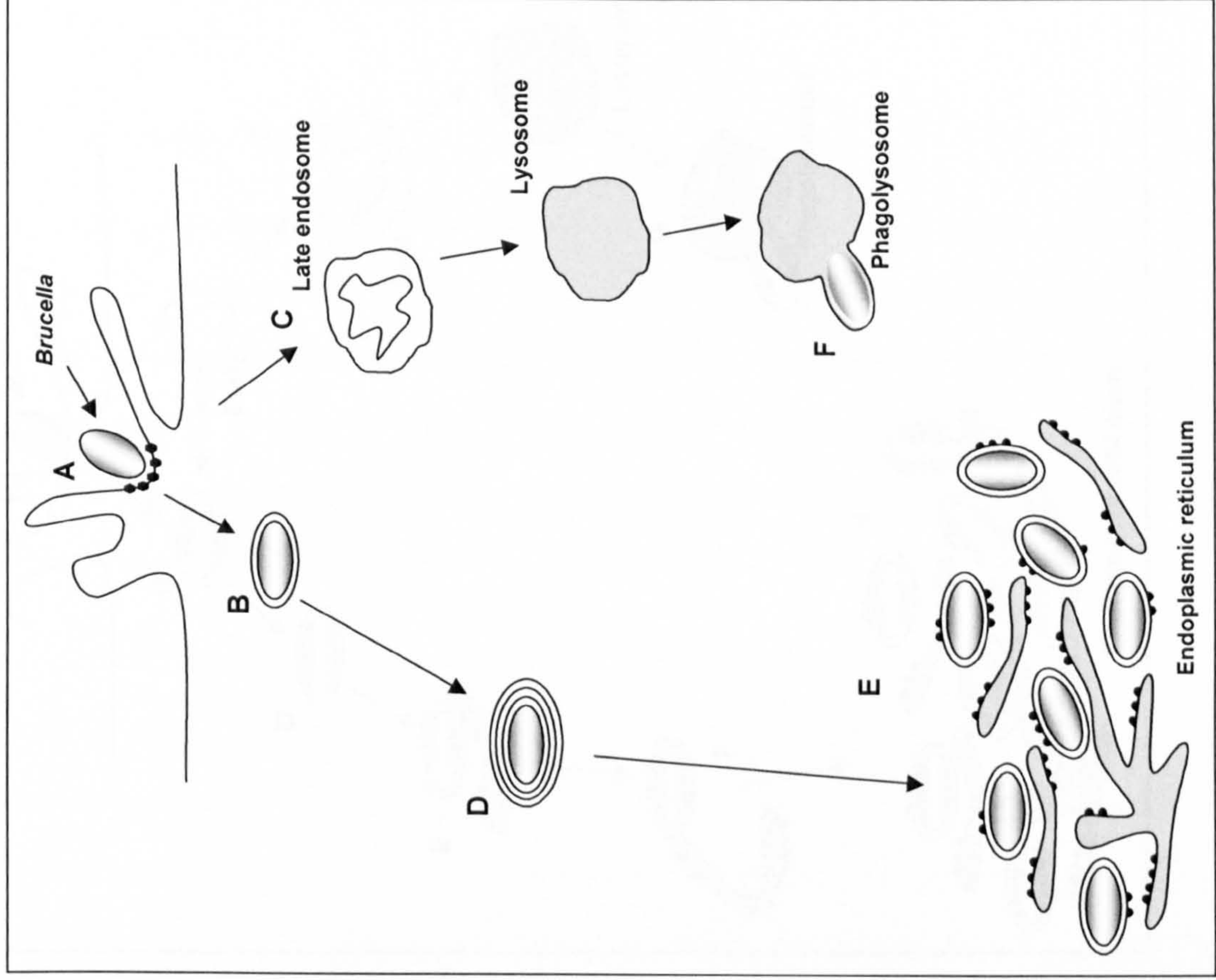
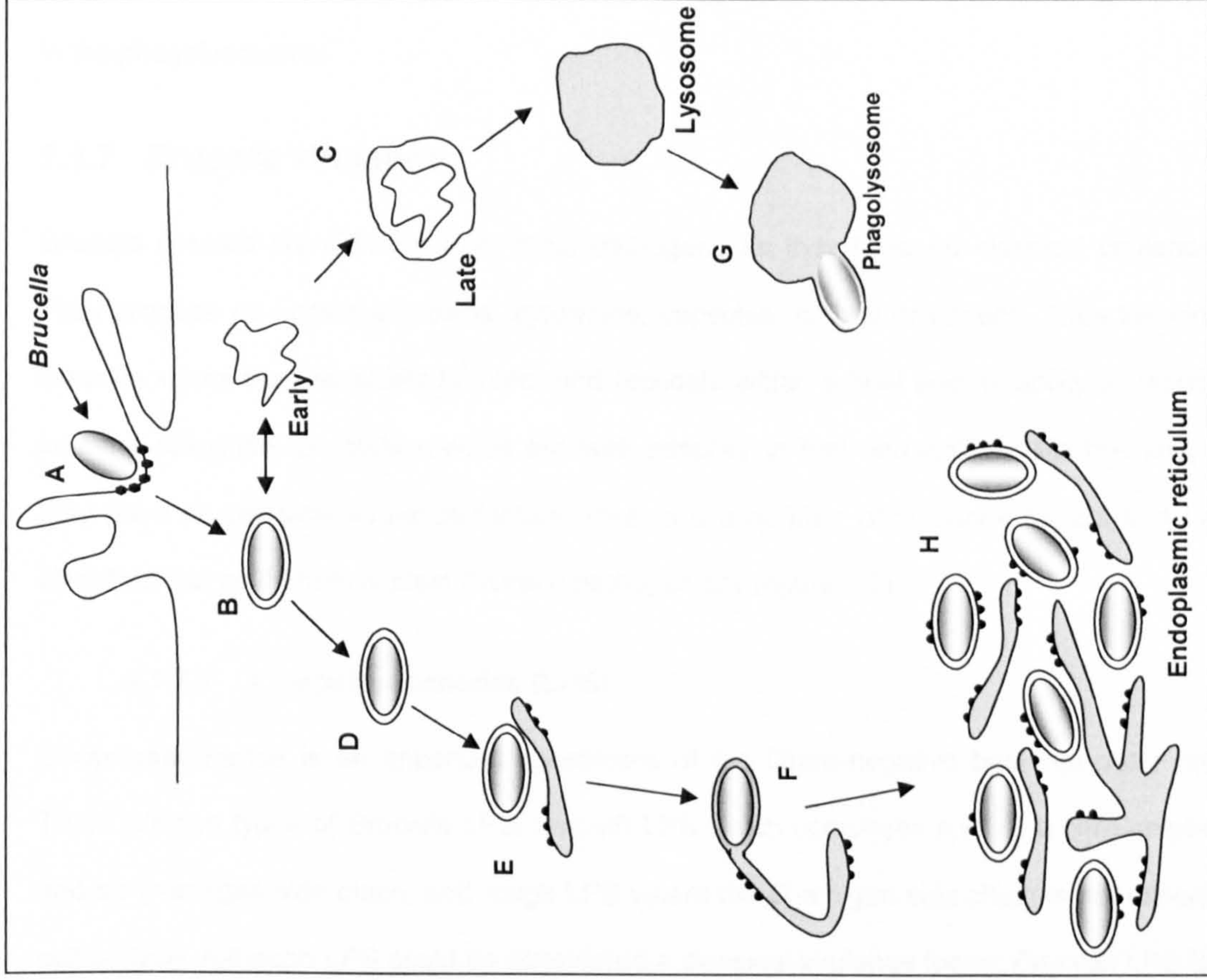


Figure 1.2:

Brucella entry and evasion of professional phagocytic cell killing; adapted from [52]

- A.** Entry of *Brucella* to professional phagocytic cells via FcR and C3bR
- B.** Development of the early *Brucella* containing vacuole (BCV)
- C.** Development of professional phagocytic cells endosomes and lysosome
- D.** Formation of the intermediate BCV
- E.** Fusion of the BCV to the ER
- F & H.** Recruitment of ER markers and replication of *Brucella*
- G.** Digestion of opsonised *Brucella* in the phagolysosome



late endosome (figure 1.2 C) or golgi apparatus during intracellular trafficking. Once inside the ER *Brucella* inhibit apoptosis and start to replicate [224]. Again, during intracellular trafficking the *Brucella* do not come into contact with the cells phagosome and so are not in danger of breakdown in the phagolysosome.

1.1.7 *Brucella* virulence

Brucella species are different from most pathogens as they have no classical virulence factors. They produce no known exotoxins, cytolytins, capsules, fimbria or flagella. Thus the virulence of *Brucella* stems from its ability to infect and replicate within a host and its ability to resist the host immune response. *Brucella* species are well adapted to their intracellular lifestyle and, although they have no classical virulence factors, there are a number of virulence factors that have been identified that could help explain *Brucella* pathogenicity (figure 1.3).

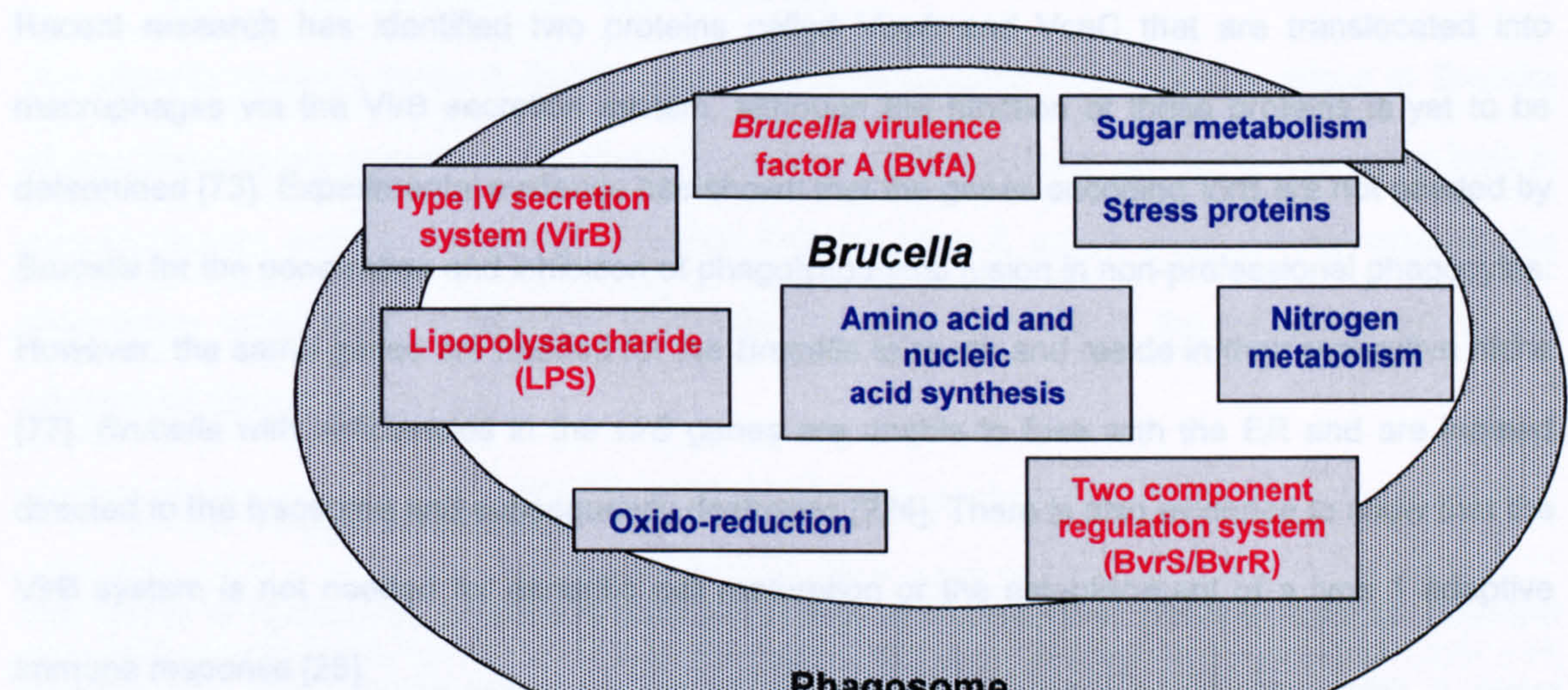
1.1.7.1 Lipopolysaccharide (LPS)

Lipopolysaccharide is an important constituent of the Gram-negative bacterial outer membrane. There are two types of *Brucella* LPS: smooth LPS which comprises lipid A, a core polysaccharide and an O-antigen side chain, and rough LPS where the O-antigen side chain is not exported to the cell surface. Although LPS could be considered a classical virulence factor, *Brucella* LPS is different from that of most other Gram-negative bacterial LPS. It is many times less toxic than the LPS of *E. coli* [119] and is highly resistant to macrophage degradation [103], offering some protection against the host immune response. Studies on *B. melitensis* LPS have characterised the reduced toxicity of *Brucella* LPS further [324]. The difference between the LPS of rough and smooth species of *Brucella* is thought to alter the way in which different species of *Brucella* enter cells [324]. Smooth *Brucella* species are thought to interact with lipid rafts on the cell surfaces, whereas rough *Brucella* species (*B. canis* and *B. ovis*) seem not to interact with lipid rafts and more frequently fuse with lysosomes [178].

1.1.7.2 Type IV secretion system VirB

Type IV secretion systems are a group of multi-protein complexes that enable bacteria to secrete molecules and proteins across the cell envelope [30]. For example, *Bordetella pertussis* secretes

the pertussis toxin through the P0 system [25] and *Legionella pneumophila* export proteins through the GpsII system [22]. The *Brucella* VirB system is an analogue of VirB in *Agrobacterium tumefaciens* [243] and is encoded by the virB operon which includes 12 proteins. The composition of the VirB system and the proteins that it secretes are still largely unknown, although progress is being made in discovering what the VirB system secretes [73].



1.1.7.3 Two component regulatory system (bvrS/bvrR)

The two-component regulatory system (bvrS/bvrR) of *Brucella* spp. [124, 155] which is needed for encystation and pathogenicity in *Brucella* spp. [156].

Red text = possible virulence factors; Blue text = important in intracellular survival factors. Adapted from [152]

has been located in the genomes of *B. melitensis*, *B. suis* and *B. abortus* and the homology between each species differs only by several amino acids [302]. It is thought that the TCRS is responsible for sensing whether the bacteria are living in an intracellular or extracellular environment, and for turning off unnecessary genes and turning on essential genes [302]. Sequencing of the *Brucella* genomes has found approximately 21 predicted TCRS [71, 258, 128] the best characterized being the BvrS/BvrR system. Mutations in the BvrS/BvrR cause problems in the production of outer membrane proteins 22 and 25 (Omp22 and Omp25) which, in turn, leads to reduced virulence in mice [302, 134]. The Omp25 protein, which is regulated by the BvrS/BvrR

the pertussis toxin through the Ptl system [65] and *Legionella pneumophila* export proteins through the Dot/Icm system [22]. The *Brucella* VirB system is an analogue of VirB in *Agrobacterium tumefaciens* [240] and is encoded by the *virB* operon which includes 12 proteins. The composition of the VirB system and the proteins that it secretes are still largely unknown, although progress is being made in discovering what the VirB system secretes [73].

Recent research has identified two proteins called VceA and VceC that are translocated into macrophages via the VirB secretion system, although the function of these proteins is yet to be determined [73]. Experimental evidence has shown that the genes encoding VirB are not needed by *Brucella* for the penetration and inhibition of phagolysosomal fusion in non-professional phagocytes. However, the same genes are needed for the *Brucella* to reach and reside in their replicative niche [77]. *Brucella* with deficiencies in the *virB* genes are unable to fuse with the ER and are instead directed to the lysosome and subsequently destroyed [224]. There is also evidence to show that the VirB system is not needed for dendritic cell maturation or the establishment of a type 1 adaptive immune response [28].

1.1.7.3 Two component regulatory system (BvrS/BvrR)

The two-component regulatory system (TCRS) in *Brucella* is a homologue of the ChvI/ChvG system of *Rhizobium meliloti* and *A. tumefaciens* [124,193] which is needed for endosymbiosis and pathogenicity in *Bryophyllum diagremontiana* and *Medicago sativa* plants, respectively [55,56]. The TCRS consists of a regulatory protein, BvrR, and a sensory protein, BvrS. This regulatory system has been located in the genomes of *B. melitensis*, *B. suis* and *B. abortus* and the homology between each species differs only by several amino acids [302]. It is thought that the TCRS is responsible for sensing whether the bacteria are residing in an intracellular or extracellular environment, and for turning off unnecessary genes and turning on essential genes [302]. Sequencing of the *Brucella* genomes has found approximately 21 predicted TCRS [78,258,128], the best characterised being the BvrS/BvrR system. Mutations in the BvrS/BvrR cause problems in the production of outer membrane proteins 22 and 25 (Omp22 and Omp25) which, in turn, leads to reduced virulence in mice [302,124]. The Omp25 protein, which is regulated by the BvrS/BvrR

system may be involved in virulence through a mechanism that inhibits TNF- α release in human macrophages [89,151]. *B. melitensis* and *B. ovis* Omp25 mutants are attenuated in mice [88] and although *B. abortus* Omp25 mutants display different phenotypes to their wild-type strains, they are still virulent in mice, whereas BvrS/BvrR mutants are attenuated in mice [87,207], indicating that BvrS/BvrR influences other genes in *Brucella* that could be involved in virulence [207].

1.1.7.4 *Brucella* virulence factor A (BvfA)

The *Brucella* virulence factor A (BvfA) is an 11 kDa periplasmic protein that is unique to *Brucella* with no homologues currently in GenBank [180]. *B. suis* BvfA mutants are highly attenuated both *in vitro* and *in vivo*, and the expression of BvfA is induced when *B. suis* is within macrophages during phagosome acidification, suggesting that BvfA could be involved in the establishment of the intracellular niche [180].

1.1.7.5 Sugar metabolism

The ability to metabolise different sugars for energy is important in bacterial survival. *Brucella* preferentially utilise an uncommon sugar called erythritol which provides them with a major advantage over competing bacteria [96]. There are only a few genes that have been shown to be important in the intracellular survival of *Brucella* that relate to sugar metabolism [168]. Erythritol metabolism is one example with the identification of two genes, *eryD* and *eryC* that are thought to be important for *Brucella* intracellular growth since mutants in *eryD* and *eryC* exhibit slower intracellular growth than their wild-type counterparts [39]. It is known that genes involved in the glycolysis pathway (glucose degradation) are absent in *Brucella* [78].

1.1.7.6 Nitrogen metabolism

As nitrogen constitutes a large part of many different components of bacteria including proteins, nucleic acids and enzymes, the ability to metabolise nitrogen is extremely important in bacterial survival. There have been three genes identified in *Brucella* that encode for nitrogen metabolism. They are *glnA*, *glnD* and *glnL* and are genes involved in NH₄⁺ utilisation [168]. A homologue of *GlnA* in *Salmonella typhimurium* has been shown to be important in its virulence [166].

1.1.7.7 Amino acid and nucleic acid synthesis

Due to the low nutrient nature of the intracellular environment, it may not be possible to gain all nutrients required for bacterial survival and growth from the environment. Therefore bacteria may have to synthesise their own amino acids and nucleic acids. Genes have been identified in *B. suis* that demonstrate its ability to make amino acids and nucleic acids (*purD*, *purE*, *purF* (purine synthesis) and *carAB*, *pyrB* and *pyrD* (pyrimidine synthesis)) [168]. Previous studies have shown that *purE* mutants of *B. melitensis* are attenuated in both mice and human monocyte-derived macrophages [66,85], highlighting that, whilst residing inside host cells, *Brucella* species may need to synthesise nucleotides. Similarly, a *B. suis* mutant of the *aroC* gene (involved in the synthesis of aromatic amino acids) was attenuated both *in vitro* and *in vivo* [104].

1.1.7.8 Oxidoreduction

Brucella species contain the *cydDCAB* operon which encodes for cytochromes that are important in respiration. Within the operon is the *cydD* gene which is important in the intracellular survival of *B. suis* [168], a finding that has been strengthened by the discovery that a *B. abortus cydB* mutant was attenuated in the mouse model of infection [93].

1.1.7.9 Stress proteins

Three genes have been identified that could be required for adaptation to the intracellular environment. These are *hfg* encoding host factor 1, *htrA* encoding a protease and *lon* also encoding a protease [168]. *B. abortus* strains containing mutations in the *hfg* gene are more sensitive than wild type bacteria to hydrogen peroxide and low pH and they also fail to replicate in host macrophages [273]. Similarly, *B. abortus lon* mutants show increased sensitivity to hydrogen peroxide and to certain antibiotics, and are attenuated *in vitro* and *in vivo* [272]. Mutants of the *htrA* gene show attenuation *in vitro* but are fully virulent *in vivo*, indicating that the gene may not be needed for full virulence in natural infections [263].

1.1.8 Animal models of brucellosis

As brucellosis is a disease associated with a range of large animal species it is difficult to identify a suitable small animal model that accurately reflects brucellosis in nature.

1.1.8.1 Mouse model of brucellosis

The most widely used and well characterised animal model of brucellosis is the mouse model. There are two strains of mice that are commonly used for studying brucellosis. These are the *Brucella*-resistant C57BL/6 strain and the more susceptible Balb/C strain [20]. Mice are used because they are bred, housed, and handled for animal studies. Furthermore, the mouse immune response, although complex, is also well characterised, aiding the understanding of the immune response to *Brucella* species.

The growth of *Brucella* species in mice varies depending on which mouse strain is used. Early studies into *Brucella* growth have evaluated its growth in four different strains; Balb/C, CBA/H, C57Bl/10 and B10Br. When mice were dosed intravenously with 5×10^5 Colony Forming Units (CFU) *B. abortus*, the splenic bacterial burden peaked at two weeks post infection, in all strains. In the Balb/C, C57Bl/10 and B10Br strains the bacterial loads decrease steadily until around seven weeks post infection. However, in CBA/H mice bacterial loads decreased at three weeks post infection before persisting in the spleen at a constant level for seven weeks post infection, indicating that in this strain *Brucella* infection presents itself as a chronic infection [135]. In all these strains clearance of *Brucella* from the liver was relatively efficient [135]. Longer experiments have shown that in Balb/C mice growth of *B. abortus* reaches a plateau phase approximately eight weeks post infection before gradually declining up until 24 weeks post infection. In C57Bl/10 mice the plateau phase is shorter with bacterial loads lower after eight week of infection. After 16 weeks *Brucella* recovery can be difficult in C57Bl/10 mice [222].

Mice infected with *Brucella* do not produce any outward clinical signs of disease, so protection is measured by comparing the number of recoverable *Brucella* CFU from naïve non-immunised mice with mice inoculated with a novel vaccine candidate or other treatment. Typically, mice immunised with a current animal vaccine are also included in the studies as positive controls. The majority of protection studies completed using the mouse model of brucellosis involve infection via the intraperitoneal (i.p.) route with approximately 1×10^4 CFU of *B. melitensis* and, although this is an effective experimental technique, it does not truly represent a natural route of infection. Recently

there have been two studies published which specifically evaluate exposure of mice to *Brucella* via the aerosol route [246,156]. Kahl-McDonagh *et al.* demonstrated that inhalational infection with *B. abortus* causes a chronic infection in the lungs and peripheral organs at lower doses than inhalational infection with *B. melitensis* [156]. Olsen *et al.* used a jet nebuliser to dose mice with *B. melitensis* or *B. abortus*, reporting consistent *Brucella* growth in the liver, lungs and spleen between the two species, although they were unable to demonstrate protection from a 1×10^7 CFU dose of *B. abortus* vaccine strain RB51 against aerosol challenge [246].

Immunocompromised mice have also been used to study *Brucella* virulence and the *Brucella* specific immune response. Mice deficient in interferon regulatory factor-1 (IRF-1^{-/-}; deficient in IL-12, functionally impaired natural killer (NK) cells and cytotoxic CD8⁺ T cells) and mice lacking recombination-activating gene 1 (*Rag1*^{-/-}; deficient in mature T and B cells) have both been studied [167,148]. IRF-1^{-/-} mice still have some control over a *Brucella* infection at low bacterial concentrations as challenge with 5×10^5 CFU of *B. abortus* are needed to cause death [167]. More recently IRF-1^{-/-} mice were reported to succumb to infection after 9 days when given 1×10^7 CFU of *B. melitensis* [269]. Studies using *Rag1*^{-/-} mice have indicated that a T- and B-cell independent immune response can control a *Brucella* infection at a high level in the murine spleen, but not the liver. However, the presence of T and B cells is required for full clearance of the infection [148].

1.1.8.2 Other animal models of brucellosis

Although the mouse model of infection is the best characterised experimental model for brucellosis, there are other animal models reported in the literature. These include infections in goats [217,92], beagles [248] and monkeys [260]. Research using these different models is pathologically more realistic and therefore important in furthering our knowledge of brucellosis. However, the mouse model is immunologically better characterised, cheaper, and easier to work with and so is more often the model of choice for *Brucella* research.

1.2 The immune response

The immune response is a complex interaction of many different chemical signals, molecular pathways and cellular networks, the function of which is to defend the body against infection from pathogenic organisms. Although there is significant cross-over between different branches of the immune response it can be broadly split into two main categories: the innate immune response and the adaptive immune response.

1.2.1 The innate immune response

The innate immune response is the body's first line of defence against pathogens, consisting of various non-specific defence mechanisms, including physical barriers, phagocytosis, natural killer cell activation and initiation of the alternate complement cascade.

1.2.1.1 Physical and chemical barriers

The first major line of defence against pathogens is the skin and epithelium. The constant regeneration of the epithelium helps remove bacteria that have adhered to it. Flushing mechanisms such as tears and saliva aid in the prevention of bacterial colonisation of the mouth and eyes. The gastrointestinal and respiratory tracts are lined with mucus that assist in trapping bacteria which prevents infection of the lungs and digestive systems [200]. The enzyme lysozyme breaks down the bacterial cell wall, which reduces bacterial growth. Lysozyme can be found in bodily secretions such as sweat, tears and saliva. The general low pH of sweat and gastrointestinal fluids also aids in the prevention of infection [23].

1.2.1.2 Complement cascade

The complement cascade ultimately leads to cell lysis via the creation of a pore in the cell membrane known as the membrane attack complex (MAC). There are three pathways of complement activation: the classical pathway, the lectin pathway and the alternative pathway. Only the alternative pathway is part of the innate immune system as it does not require any antigen specific cell signals for activation. This pathway is activated by the pathogen cell surface creating an environment favourable for complement binding and activation. Once activated, the complement

cascade leads to the generation of a C5 convertase whose function is to catalyse the first steps in the production of the MAC. The alternative complement cascade starts with the continuous low level breakdown of complement molecule C3 into C3b, a very reactive molecule with the ability to bind many different cell surface markers. When C3b binds to a bacterial cell surface then a molecule called Factor B is activated and cleaved into Bb which binds on to C3b on the microbe. C3bBb complex (C3 convertase) is an enzyme that increases the cleavage of C3 to C3b, creating a positive feedback for increasing the activation of complement [200]. C3bBb then further binds more C3b creating C3bBb3b complex or C5 convertase. C5 convertase cleaves C5 into C5a and C5b and, from this point on, the generation of the MAC is a non-enzymatic process. C5a is a strong immune modulator molecule which causes neutrophil activation, adhesion, migration and chemotaxis, and monocyte activation causing the release of cytokines such as IL-1 and IL-6, and it can cause mast cell degranulation. C5b is crucial for the formation of the MAC as it binds C6 and C7 forming C5b67, which then binds C8 and anchors itself in the bacterial membrane. C5b678 causes the insertion and polymerisation of many C9 molecules causing the formation of the MAC. The MAC causes bacterial cell lysis and eventually cell death [276].

1.2.1.3 Innate pathogen recognition and activation

The cells of the innate immune response include dendritic cells, macrophages, natural killer (NK) cells and neutrophils, all of which have important roles in the initiation of the immune response. Neutrophils are a major class of phagocytic cells that are abundant in the blood stream. Upon early infection neutrophils migrate to the site of infection where they play essential roles in the phagocytosis of the invading pathogen. Macrophages and dendritic cells have the ability to recognise general markers on pathogens called microbe-associated molecular patterns (MAMPs). These are recognised by a group of receptors called the Toll-like receptors (TLRs), which recognise a range of different MAMPs, both extracellular and intracellular. For example, TLR4 recognises lipopolysaccharide, and TLR9 recognises short methylated lengths of DNA called CpGs. Other TLRs include TLR5 (recognises flagellin), TLRs 6 and 2 (a TLR dimer that recognise diacyllipopeptides), TLRs 1 and 2 (another TLR dimer that recognise triacyllipopeptides) [276]. Activation of TLRs leads to the increased phagocytosis by macrophages and release of pro-

inflammatory cytokines such as tumour necrosis factor- α (TNF- α), interleukin-12 (IL-12), and IFN- γ . Activation via TLRs also causes the maturation of dendritic cells, leading to their migration to regional lymph nodes and the initiation of the adaptive immune response. Pro-inflammatory cytokines assist in the recruitment of further phagocytic cells such as neutrophils and NK cells [200]. NK cells develop in the bone marrow once fully developed, they circulate in the blood stream and are activated by macrophage derived cytokines. Upon activation NK cells kill infected cells via the release of cytotoxic granules and perforin. NK cells are also a major producer of IFN- γ generated during an innate immune response. Production of cytokines such as IL-12 in conjunction with tumour necrosis factor – alpha (TNF- α) by macrophages can also stimulate release of IFN- γ by NK cells. Many of the cytokines produced by the innate immune response also play important roles in shaping the adaptive immune response.

Both macrophages and dendritic cells also play major roles as antigen presenting cells (APCs). After a pathogen has been phagocytosed antigens are displayed on the surface of the cell in conjunction with major histocompatibility complexes (MHC) II. These are recognised by cells of the adaptive immune response which helps in the development of the immune response.

1.2.2 The adaptive immune response

The adaptive immune response is another branch of the immune response that takes longer to establish itself during an infection than the innate response. However, once established, it is specific to particular pathogens and it is responsible for the clearance of an invading pathogen. The adaptive immune response is also responsible for the generation of immunological memory which makes vaccination possible. There is significant cross-over in elements of the adaptive immune response, but it can be broadly split into two categories: the humoral immune response and the cellular immune response.

1.2.2.1 T cell maturation and differentiation

T cells are all derived from the same progeny of lymphoid cells produced in the bone marrow, only reaching full maturity in the thymus. During their early development T cells express both CD4 and

CD8 molecules, and it is only when T cells are simulated to recognise either MHC I or II that they become designated as CD4⁺ T cells or CD8⁺ T cells. T cells that are able to recognise MHC I molecules develop into CD8⁺ T cells and those that recognise MHC II molecules become CD4⁺ T cells. At this point both populations of T cells are referred to as naïve CD4⁺ T cells or naïve CD8⁺ T cells.

Once naïve CD4⁺ T cells are activated by MHC II complexes the differentiation into Th1 or Th2 cells begins. The CD4⁺ T cells first differentiate into an intermediate cell type known as a Th0 cell, and the production of IL-2 by Th0 cells stimulates it to proliferate further. Th0 cells have the ability to differentiate into either Th1 or Th2 cells, largely determined by the type of invading pathogen and by cytokine signalling. Intracellular pathogens generally cause CD4⁺ T cells to differentiate into Th1 cells, whereas extracellular pathogens generally give rise to Th2 cell populations. Cytokine signalling also affects the differentiation of Th0 cells into Th1 or Th2 cells; cytokines such as IL-12 (produced from macrophages), IL-23 (dendritic cells) and IL-18 (macrophages) lead to the rise of Th1 cells, whereas IL-4 (mast cells/T cells) and IL-13 (T cells) lead to the rise of Th2 cells. Th1 and Th2 cells are the co-ordinators of the immune response modulating the function of other cells via direct contact or secretion of different cytokines that direct the most appropriate response to a pathogen. The differentiation of naïve CD4⁺ T cells into either Th1 or Th2 cells determines which type of immune response will be produced, either a cellular (Th1) or humoral (Th2) immune response.

The activation of naïve CD8⁺ T cells is a simpler process as they are pre-designated to become cytotoxic T lymphocytes (CTLs). The differentiation of naïve CD8⁺ T cells into CTLs takes place via activation from mature dendritic cells although in some cases the differentiation requires additional signalling from CD4⁺ T cells. The result of activation from both sources is the production of IL-2 by the naïve CD8⁺ T cells which drives their proliferation and differentiation into CTLs. Once T cells have matured and differentiated into Th1 cells, Th2 cells or CTLs, their functions in the immune response have different effects upon different cell populations.

1.2.2.2 The cellular immune response

The main effector cells of the cellular immune response are the Th1 cells and CTL populations. The array of cytokines produced by Th1 cells defines their function within the immune response. Th1 cells produce cytokines such as IFN- γ (increasing activation of NK cells, macrophages and dendritic cells), TNF- α (increases nitric oxide production from macrophages), and granulocyte macrophage - colony-stimulating factor (GM-CSF, increases the production of macrophages and dendritic cells). The function of CTLs is to kill infected cells expressing foreign peptide presented in conjunction with MHC I via the release of cytotoxic granules and perforin similar to that of NK cells. Upon recognition of the antigen there is a clonal expansion of CTLs, which gives rise to two populations of CD8⁺ T cells, specifically CTLs or memory T cells. Activated CTLs secrete further cytokines such as IFN- γ and IL-2. IFN- γ stimulates macrophage activation and increases antigen presentation via MHC class I and II, which starts the cycle over again, acting as a positive feedback loop driving the cellular immune response [42].

1.2.2.3 The humoral immune response

The humoral immune response is generated by a subset of cells known as B cells, which are generated in the bone marrow from common lymphoid progenitor cells. B cell antigen specificity arises from the B cell receptor (BCR) a membrane bound immunoglobulin coupled with two transmembrane signalling molecules Ig α and Ig β . The BCR is capable of recognising free antigen, however, when antigen binds to the BCR it is the two signalling molecules (Ig α and Ig β) that cause the signalling cascade that activates the B cells. B cell activation, leads to clonal expansion of the activated B cell population [276]. B cells then differentiate into plasma cells that produce pathogen-specific antibodies of the same specificity as the BCR on their cell surface. Antibodies themselves function in a number of different ways including neutralisation of toxins by binding active sites, binding directly to pathogens (opsonisation) thereby increasing phagocytosis of the pathogens, and activation of complement via the classical pathway [200]. Cytokines such as IL-4 and IL-5 produced by CD4⁺ Th2 cells also act upon B cells aiding in their maturation and differentiation.

1.2.2.4 Cross-talk and negative regulation of the immune system

There is significant cross-talk between both the humoral and cellular immune responses. For example, secretion of IFN- γ by CTLs causes plasma cells to switch antibody isotype from IgG1 to IgG2, which are better at opsonising bacteria and so causing increased phagocytosis by macrophages. Similarly, there are negative regulators of the immune response. For example, the secretion of IL-10 by Th2 cells acts to stop the production of cytokines by Th1 cells and macrophages and increase MHC class II expression thus depressing the cellular immune response [276]. In some cases IL-10 can be produced by some Th1 cells during a self-limiting cellular immune response.

1.2.2.5 Immunological memory

One of the key functions of the adaptive immune response is the generation of immunological memory. Immunological memory is the capacity of the immune system to rapidly recognise and react to a second exposure of a previously encountered pathogen with an aggressive immune response quicker than the first encounter. The mechanisms behind immunological memory are complex, but it is known that immunological memory is maintained by a small population of specialised memory cells. The generation of antigen specific memory cells occurs following the first exposure of an antigen to the immune system, during the clonal expansion of B and T cell populations. Upon activation from presented antigens the clonal expansion of T or B cells leads to a small population of either cell type becoming memory T or B cells.

The memory T cell population can be further split into memory CD4⁺ T cells or CD8⁺ T cells. Most of these cells are dormant until a second exposure to a pathogen. However, there is a small population of memory cells that are dividing at any one time. It is thought that this division is induced by cytokines. For example, IL-7 is responsible for maintenance of CD4⁺ memory T cells and IL-15 contributes to maintenance of CD8⁺ memory T cells [282]. Memory CD8⁺ T cells also express the anti-apoptotic marker called Bcl-2 which promotes cell survival and is thought to be one reason for the long-term survival of this cell type. CD4⁺ and CD8⁺ T cells can become one of two types of memory cell, either effector memory cells or central memory cells. Effector memory cells

quickly migrate to inflamed peripheral tissues where they rapidly mature into effector T cells and secrete large quantities of cytokine more quickly than naïve T cells, and also display receptors for inflammatory cytokines for quick re-stimulation. In comparison, central memory cells have little or no effector function and reside in the peripheral lymphoid tissues (such as lymph nodes and the spleen) where, upon activation, they readily proliferate and differentiate into effector cells [282].

The immunological memory of B cells starts during clonal expansion and differentiation of activated antigen-specific B cells into either plasma cells or memory B cells. Upon activation memory B cells division is much quicker than naïve B cells, and production of antibody is also quicker. Not only is the antibody produced by memory B cells released quicker, it is also of a different isotype than that produced by naïve B cells. On first exposure to a pathogen the initial antibody to be produced is IgM (which has low affinity binding). It is only later during infection that IgG (which has high affinity binding) is produced. Upon activation memory B cells produce IgG, a better antibody for bacterial opsonisation and anti-toxin responses.

Immunological memory is what makes vaccination possible. The process of vaccination exposes the body to the first encounter with material from a pathogen. Upon a second natural exposure to the same pathogen it is the immune system's memory cells that create the quicker immune response required to clear the infection before it establishes itself.

1.2.3 The immune response to *Brucella*

Defining an immune response to a pathogen can aid in the development of effective vaccines. The complete immune response to *Brucella* is not yet fully understood. Most of the research into the immune response generated to *Brucella* has been evaluated in the mouse model of infection. The *Brucella*-specific immune response can be broadly split into two categories: the innate immune response and the adaptive immune response.

1.2.3.1 The innate immune response to *Brucella*

1.2.3.1.1 The roles of antigen presenting cells (APCs) during brucellosis

APCs make up an important aspect of the immune response. APCs consist of macrophages and dendritic cells, and are one of the earliest lines of defence of the innate immune response. Early studies have shown that macrophages from infected animals such as guinea pigs [216,265] and cattle [102] have an increased rate of killing when compared to macrophages from uninfected mice, showing that following *Brucella* infection macrophages do become activated. Stimulation of human monocytes with heat killed *B. abortus* leads to the secretion of the proinflammatory cytokines TNF- α (increases macrophage activation and local inflammatory response), IL-1 β (increases macrophage activation and induces T cell activation) and IL-6 (T and B cell growth and differentiation) [139], all important cytokines for inducing and shaping the immune response. Interestingly, during *in vitro* *B. suis* infection studies the production of TNF- α is inhibited by Outer membrane protein 25 (Omp25) [151], which is highly conserved between *Brucella* species [58] leading to the theory that all *Brucella* can inhibit TNF- α production. Inhibiting the production of a major cytokine like TNF- α means the immune response is lacking in a key cytokine. The inhibition of TNF- α could lead to decreased macrophage activation and a decrease in the inflammatory response which, in turn, leads to less cell recruitment and so a decrease in phagocytosis, a key component of the immune response against brucellosis. Dendritic cells have also been shown to produce cytokines upon infection with *Brucella* species. Infected human DCs not only increase the expression of certain cell surface receptors and MHC class I and II, but also produce a range of pro-inflammatory cytokines such as TNF- α , IL-6, IL-10 and IL-12. [375].

In the mouse the innate immune response differs from the human response in that there is no inhibition of TNF- α production. *In vitro* studies using murine macrophages infected with *Brucella* have shown that a similar array of cytokines is produced such as IL-1, IL-6 and TNF- α [83], with TNF- α being the first cytokine produced and production peaking at six to seven hours post infection. In the mouse TNF- α is needed for full *Brucella* clearance as mice deficient in the TNF- α receptor genes have exacerbated *Brucella* infections [371,372].

1.2.3.1.2 The importance of IFN- γ during murine brucellosis

IFN- γ is crucial for survival during a murine *Brucella* infection since, in the absence of IFN- γ , Balb/C and C57BL/6 mice die of brucellosis [229]. However, in Balb/C IFN- γ deficient mice the bacterial burdens at 1 week post-infection are equivalent to that in wild-type mice. Balb/C IFN- γ deficient mice die at 10.5 weeks post-infection, whereas wild-type mice start clearing *Brucella* infections around this time [229]. IFN- γ patterns differ in C57BL/6 mice as they produce IFN- γ throughout the infection and, in its absence, have increased bacterial loads until they succumb to infection after 6 weeks post infection [229]. Balb/C mice stop producing IFN- γ during the first week of infection and this cessation of IFN- γ production continues until the end of the infection. Yet Balb/C mice are able to control and clear the *Brucella* infection. Murphy *et al.* have shown that during this IFN- γ deficient period, removing TNF- α and CD8⁺ T cells results in an increase in *Brucella* CFU recovered [228], suggesting that it is TNF- α and CD8⁺ T cells that are providing the control during IFN- γ deficient periods [228]. Baldwin *et al.* have reported that macrophages from both resistant (natural resistance macrophage protein 1 (*Nramp1*) positive) and susceptible (*Nramp* negative) mice can control a *Brucella* infection *ex vivo*, showing that control of *Brucella* infection is not related to inherent differences in the macrophage ability of each strain [20]. Furthermore, it has been demonstrated *ex-vivo* that the decrease in IFN- γ production could be attributed to a decrease in expression of the IL-12 receptor, IL-12R β 2 [284]. If susceptible Balb/C mice are treated with recombinant IL-12, the production of IFN- γ increases, leading to a 1000-fold reduction of *Brucella* CFU during primary infection and increased survival during secondary infection [284].

1.2.3.1.3 The role of natural killer cells during brucellosis

On first contact with *Brucella*, APCs phagocytose the *Brucella*, causing cell activation and release of cytokines such as IL-12 and IL-2. IL-12 activates NK cells, causing them to initiate killing of infected cells and production of their own cytokines such as IFN- γ , which then causes macrophage stimulation, increasing proliferation and MHC class I and II expression [276]. It has been shown that NK cells are not of paramount importance in the early stages of a *Brucella* infection, as removal of NK cells *in vivo* does not alter the ability of mice to clear a *Brucella* infection [99]. Although this does not mean that NK cells don't play an important role in the immune response against *Brucella*, it

does demonstrate that in mice there is still a sufficient immune response for *Brucella* clearance without NK cells [99].

1.2.3.2 The adaptive immune response to *Brucella*

The different arms of the adaptive immune response appear to have different roles in the clearance of a *Brucella* infection.

1.2.3.2.1 The humoral immune response to *Brucella*

Due to the intracellular nature of *Brucella*, antibodies are not as effective at clearing infection as they may be for an extracellular pathogen because antibodies access the intracellular environment. Important antibodies that aid in the clearance of a *Brucella* infection are IgG2a isotype antibodies in mice and the IgG3 isotype antibodies in humans [118]. IgG2a and IgG3 are effective for opsonisation of bacteria, thus increasing phagocytosis by macrophages [118]. The production of IFN- γ causes class switching of IgG production from IgG1 isotypes to IgG2a in mice and IgG3 isotypes in humans [276]. Antibodies generated against the O-antigen of *Brucella* LPS are also thought to be important in stopping the spread of *Brucella* throughout the body [365]. Additionally, older studies show that passive transfer of sera or monoclonal antibody recognising the LPS or OPS can protect against *B. abortus* infections, confirming that antibody may be important in protecting against *Brucella* infections in mice [222,355].

1.2.3.2.2 The roles of T cells in the immune response to *Brucella*

The role of T cells in *Brucella* infection has mainly been assessed in the mouse model of infection. A range of studies have shown roles for the CD8⁺ T cell and the CD4⁺ T cell in the control of *Brucella* infection [242,15]. Specifically, primary roles for CD8⁺ T cells have been demonstrated when infecting β 2-microglobulin depleted C57BL/10 mice (which have no cell surface MHC class I and therefore no CD8⁺ T cell response) with *Brucella*. Mice harbour an increased *Brucella* load during the infection, indicating that CD8⁺ T cells are needed to reduce *Brucella* numbers [242]. Adoptive transfer of CD4⁺ or CD8⁺ T cells from infected Balb/C mice to naïve mice provides them with similar levels of protective efficacy against *B. abortus* 2308 [15]. This suggests a role for both CD4⁺ and CD8⁺ in *Brucella* infections.

In human infection specific subsets of T cells have been shown to be important. For example, Bertotto *et al.* have demonstrated that $\gamma\delta$ T cells increase dramatically among circulating blood lymphocytes during the acute phase of infection, although the mechanisms by which they help in the clearance of infection are as yet unknown [26]. There is also a subset of $\gamma\delta$ T cells known as V γ 9V δ 2 T cells which increase in numbers in the peripheral blood of patients with brucellosis [241]. These V γ 9V δ 2 T cells are a population of cells only found in primate species and so their importance in *Brucella* clearance has to be studied in brucellosis patients. It has been demonstrated that V γ 9V δ 2 T cells can decrease the growth of *Brucella* using either contact-dependant cytotoxicity such as lytic granules or by increased activation via signalling by soluble factors release by infected monocytes [241,247].

1.2.3.2.3 The roles of cytokines in the immune response to *Brucella*

Of the range of cytokines induced by *Brucella* species there are some which appear to have a more important role during infection than others. Specifically, IFN- γ is critical for *Brucella* clearance in mice as resistant mice strains with disruptions in the IFN- γ gene die between six to ten weeks post-infection [229]. Additionally, both IL-12 and TNF- α are thought to play important roles in resistance to *Brucella* infections [371,372], and studies have shown that reducing IL-12 or TNF- α using specific antibodies four hours before challenge with virulent *B. abortus* results in an exacerbated infection [372]. Data has also indicated that IFN- γ , TNF- α and IL-6 are present in at higher levels in serum from patients with acute brucellosis than in healthy volunteers [79]. In comparison, there is no difference between levels of IL-1 β , TGF- β 1, IL-2, IL-4 and IL-8 [8].

1.3 Vaccination against brucellosis

The adaptive immune response can take days to establish which, in some cases, can mean that an infectious agent has already had time to produce an established infection within the body, causing the generated immune response to be less effective. Vaccination uses the immune system's ability to produce specific memory cells against pathogens to speed up the immune response to infection. On a second exposure to an antigen, both memory T cells and memory B cells recognise the

antigen and start multiplying immediately. This decreases the response time of the immune system limiting the time in which an infectious agent can establish itself.

1.3.1 The history of human vaccination against brucellosis

Although the current live attenuated vaccine strains of *Brucella* used in animals are not suitable for human use there are examples of human vaccination with live attenuated strains of *Brucella*, in the former Soviet Union (USSR) and China. In the former USSR there was a high prevalence of brucellosis in sheep and goats. This reservoir of brucellosis was the cause of 85 – 95 % of the cases of brucellosis reported in humans, because of this since 1953 – 54 the problem has been kept under control by the routine vaccination of the so called “at risk” populations [340]. Many of the live attenuated *Brucella* vaccines have the ability to induce a protective immune response against brucellosis, *B. melitensis* Rev.1 and *B. abortus* strain 19 are two examples. However, both of these strains are still virulent in humans and so a new strain was developed in the former Soviet Union which was derived from *B. abortus* strain 19, called *B. abortus* 19-BA [340]. Strain 19-BA was administered as a dose of 1×10^9 cells via skin scarification and protection was effective for upto one year (although maximal protection was only for five-six months) [63,281]. The vaccine was usually well-tolerated in healthy adults but the number of general reactions to the vaccine increased in people who had previously had brucellosis and repeated vaccination had a tendency to cause hypersensitivity [281]. Epidemiological studies did show that the vaccine effectively reduced the incidence of acute human brucellosis by five to eleven fold [340]. Despite the well tolerated nature of strain 19-BA independent studies have shown that it can still cause acute brucellosis in some people [304]. In China another strain of *B. abortus* was used for human vaccination called 104M. This strain was administered as a dose of $7-10 \times 10^{10}$ cells also via skin scarification. Strain 104M is reported to be more virulent than 19-BA and can give rise to serious illness if given by the subcutaneous route [63]. Nether the BA-19 or 104M vaccines would meet the requirements for efficacy and safety needed to be licensed in the western world [62].

There are also two examples of non-living *Brucella* vaccines that have been used in humans in France and the former USSR. In France a phenol-insoluble residue of lipid extract was prepared

from *B. abortus* S19 cells [63,194]. This vaccine was used to dose occupationally exposed groups of workers, who were administered two doses of 1 mg at two week intervals via the subcutaneous route [125]. The vaccine was very well-tolerated and there were no signs of hypersensitivity from multiple doses and reports stated the vaccination lasted upto two years [63]. Even though the vaccine was used for over two decades, evidence of clinical efficacy of the vaccine is not available and the vaccine is no longer used [63]. The other non-living *Brucella* vaccine used in the former USSR was derived from their live attenuated *Brucella* vaccine 19-BA. It was called *Brucella* chemical vaccine (BCV) and is made from a cell wall preparation of 19-BA extracted using 0.1 M acetic acid [63]. The optimal dose of BCV was 1 mg via the intramuscular route, which only induced mild local reactions [341]. The protection afforded by BCV was found to be comparable to the live 19-BA vaccine and it caused less adverse reactions and no multiple dosing hypersensitivity indicating that this vaccine could warrant further study [311,63].

1.3.2 Live attenuated vaccines against *Brucella*

Currently the most efficacious animal vaccines available for brucellosis are the live attenuated vaccines. These are live *Brucella* species that have known or unknown mutations resulting in attenuation of virulence. Live attenuated strains of *Brucella* species have been available for many years and are considered the most likely type of vaccine to elicit the type of immune response needed to clear or prevent infection. However, many of these live attenuated strains have issues regarding their use. For example, many may still cause infection or have the potential to revert back to full virulence. However, the main problem with live attenuated vaccines is confusion of diagnosis since there is no way of determining a vaccinated animal from a naturally infected animal.

1.3.2.1 *Brucella melitensis* Rev.1

B. melitensis Rev.1 was created by Elberg and Faunce and is derived from a streptomycin-dependant mutant that, upon subculture, lost its dependence but retained its resistance to streptomycin [90]. It was originally developed for the immunisation of sheep and goats but is now also used for cattle, delivered subcutaneously with a dose of approximately 10^9 CFU [236]. Rev.1 induces serum-specific antibodies that persist in the host and help in clearance of infection. The

stability of Rev.1 has been proven by passage in several animal species [236]. However, there are some drawbacks to Rev.1. Although it is attenuated, depending upon the time of vaccination and dose given, it can still cause infectious animal abortions. It also induces positive serology which interferes with diagnostic testing so that it is not possible to differentiate between vaccinated animals and field-infected animals [291].

1.3.2.2 *Brucella abortus* strain 19

Created by Buck in 1930, *B. abortus* strain 19 was derived from a virulent isolate from a Jersey cow. After being left in the laboratory at room temperature for approximately one year, the isolate was found to be attenuated [35]. Strain 19's attenuation is thought to be due to its inability to grow on erythritol, and therefore it is slow to colonise the reproductive tracts of its ruminant hosts [291]. The efficiency of strain 19 is dependant on a number of variables, including the age of animal, dose given and the vaccination route used. The optimal dose for strain 19 has not been determined, although the minimal dose is thought to be between 5×10^8 and 10^9 CFU [236]. Although it is rare, strain 19 can still cause infectious abortions if given during pregnancy and isolated bacteria from these occurrences have been able to grow in the presence of erythritol, demonstrating that the mutation is reversible [291,96]. Strain 19 also produces positive serology so vaccinated animals are indistinguishable from field infected animals [291].

1.3.2.3 *Brucella suis* strain 2

B. suis strain 2 was produced by Xie Xin in 1986, derived from an old laboratory strain of *B. suis* biovar 1 that had become naturally attenuated after serial transfer on culture media for several years [359]. Strain 2 has been used in China since 1971 for the vaccination of sheep, pigs, goats and cattle. The vaccine is given orally and is not known to cause abortions in animals and persistence in tissues has not been reported either [236].

1.3.2.4 *Brucella abortus* RB51

B. abortus RB51 was created by Schurig *et al* in 1991. It is derived from a rifampin-resistant mutant of *B. abortus* 2308 that was repeatedly passaged on trypticase soy agar with varying concentrations of rifampin or penicillin [290]. Being a rough mutant of *B. abortus*, the RB51 O-chain component of

the LPS is located intracellularly. This roughness was found to be very stable after multiple *in vivo* and *in vitro* passages of the strain [290]. RB51 is attenuated in many animals including mice, guinea pigs, goats and cattle [291]. RB51 is a licensed vaccine and it has been found that it has an insertion sequence 711 (IS711) in the gene which encodes for glycosyltransferase (called *wboA* gene), an enzyme essential in the biosynthesis of O-chain of LPS [336].

1.3.2.5 Novel live attenuated *Brucella* vaccines

Modern molecular manipulation techniques have enabled easier engineering of live attenuated strains of *Brucella* species for evaluation. There is a vast array of literature describing novel attenuated *Brucella* species [76]. For example, Kahl-McDonagh and Ficht have defined one *B. melitensis* deletion mutant named $\Delta asp24$ which persists for longer *in vivo* and provides superior protection against *B. melitensis* challenge in mice [158]. This mutant has also been tested for protective efficacy in a goat model, providing protection against infection abortions [157]. A *B. abortus* attenuated mutant recently created by Paulley *et al.* has mutations in the *bhuA* (*Brucella* heme utilisation) gene and exhibits reduced pathogenicity in both *in vitro* and *in vivo* experiments suggesting that heme utilisation is important for *Brucella* virulence [257]. The main problem with developing live attenuated vaccines for human use is the possibility of reversion back to full virulence and persistence of some strains. Two approaches have been used to try and overcome this problem. The first involves the creation of mutants in existing attenuated strains, creating highly attenuated live vaccines which are less likely to revert back to a virulent form [16]. The second is the microencapsulation of highly attenuated mutants for slow release and enhanced immunogenicity [17].

1.3.3 Non-living *Brucella* vaccines

1.3.3.1 *Brucella abortus* 45/20

B. abortus 45 was originally isolated from a cow in 1922. A rough mutant was obtained after 20 passages through guinea pigs and was so named *B. abortus* 45/20. *B. abortus* 45/20 was able to protect guinea pigs and cattle from *Brucella* infections [213,214]. However, when used as a live vaccine it had the ability to revert back to its smooth virulent form, thus losing its attenuation and

interfering with diagnostic testing. As the live form had a tendency to revert to wild-type [225], 45/20 was used as a bacterin (a suspension of killed bacteria) and incorporated with adjuvant, usually based on water/oil emulsions [291]. Protection from the killed 45/20 was variable and studies often gave mixed results when compared to strain 19. Killed 45/20 did not cause abortions or provide positive serology results. However, because of batch-to-batch variation, unpredictable efficacy, and severe local reaction at the injection site, vaccination with 45/20 was discontinued [291].

1.3.3.2 Recombinant sub-unit protein vaccines

Recombinant sub-unit vaccines are attractive options for vaccination due to their ease of production and safety record. They are also proven to be licensable vaccines as shown by the development of the sub-unit hepatitis B vaccine [306]. There has been substantial research published describing the development of recombinant sub-unit vaccines to protect against brucellosis (table 1.1). As all the experimental techniques, *Brucella* species and strains used for the development of novel sub-unit vaccines are different it is difficult to compare all the research from the literature directly. Some of the more recent work to develop sub-unit vaccines against *Brucella* has been completed by Pasquevich *et al.* who have identified two novel protective recombinant outer membrane proteins, Omp16 and Omp19, from *B. abortus* [255]. Omp16 and Omp19 were tested in two different forms, lipidated and unlipidated, both in combination with incomplete Freund's adjuvant (IFA), Alum, or Cholera toxoid (CT). Vaccination with two 30 µg doses of the lipidated forms of Omp16 and Omp19 in combination with IFA conferred significant protection against *B. abortus*. Interestingly, immunisation with the unlipidated forms of Omp16 and Omp19 induced greater protection against *B. abortus* infection and similar levels of protection to *B. abortus* strain 19. Immunisation with unlipidated Omp16 and Omp19 adjuvanted with Alum or CT also conferred protection against *B. abortus* challenge [255].

Increasing the efficacy of already identified vaccine candidates is also important. For example, the vaccine candidate L7/L12 is a well characterised novel protective antigen against *Brucella* and it has already been shown to be protective in the mouse model of brucellosis when administered as a DNA vaccine or recombinant protein [199,176,243]. Two more recent studies have shown that

Table 1.1: *Brucella* antigens assessed as sub-unit vaccines against *Brucella* species
Protection Units = Mean Log₁₀ CFU negative control mice – mean Log₁₀ CFU vaccinated mice

Antigen	Function	<i>Brucella</i> species/strain	Adjuvant	Best Protection Units elicited	Reference
22.9KDa protein	Unknown	<i>B. abortus</i> 2308	Incomplete Freund's adjuvant	1.92 PU	[53]
32.2KDa protein	Unknown	<i>B. abortus</i> 2308	Incomplete Freund's adjuvant	0.83 PU	
Recombinant <i>Brucella</i> Lumazine Synthase (rBLS)	Lumazine Synthase	<i>B. abortus</i> 544	Aluminium Hydroxide gel	1.40 PU	
rBLS	Lumazine Synthase	<i>B. abortus</i> 544	Monophosphoryl lipid A	1.31 PU	[334]
rBLS	Lumazine Synthase	<i>B. abortus</i> 544	Incomplete Freund's adjuvant	1.26 PU	
rSurA	Periplasmic peptidyl cis-trans isomerase	<i>B. abortus</i> 2308	Complete Freund's adjuvant and incomplete Freund's adjuvant	0.95 PU	
rDnaK	Molecular chaperone of hsp70 family	<i>B. abortus</i> 2308	Complete Freund's adjuvant and incomplete Freund's adjuvant	1.45 PU	[75]
rSurA + rDnaK	-	<i>B. abortus</i> 2308	Complete Freund's adjuvant and incomplete Freund's adjuvant	1.66 PU	
Omp16	Unknown	<i>B. abortus</i> 544	Incomplete Freund's adjuvant	1.97 PU	
Omp19	Unknown	<i>B. abortus</i> 544	Incomplete Freund's adjuvant	1.85 PU	[255]
Omp16 + Omp19	Unknown	<i>B. abortus</i> 544	Incomplete Freund's adjuvant	1.89 PU	
BLS + 27 amino acids from Omp31	-	<i>B. ovis</i>	Incomplete Freund's adjuvant	2.69 PU	[47]
L7/L12-maltose binding protein fusion	Ribosomal protein	<i>B. abortus</i>	Immunepius adjuvant system	1.21 PU	[243]
P39	Periplasmic binding protein	<i>B. abortus</i> 544	CpG	2.48 PU	[10]
Bacterioferin (BFR)	Iron-binding protein	<i>B. abortus</i> 544	CpG	Non-protective	
Omp31	Haemin-binding protein	<i>B. melitensis</i> H38S	Incomplete Freund's adjuvant	1.16 PU	[46]
Omp31	Haemin-binding protein	<i>B. ovis</i> PA76250	Incomplete Freund's adjuvant	2.00 PU	
GroEL & GroEL-GroES fusion	Heat shock proteins	<i>B. abortus</i> 2308	Incomplete Freund's adjuvant	Non-protective	
GroES & GroEL-GroES-HtrA fusion	Heat shock proteins	<i>B. abortus</i> 2308	Incomplete Freund's adjuvant	Non-protective	[19]
HtrA	Heat shock protein	<i>B. abortus</i> 2308	Incomplete Freund's adjuvant and Ribi	Non-protective	

liposomised and escherisome-mediated delivery of L7/L12 can help to increase the efficacy of this protective antigen [205,206].

Another sub-unit vaccine candidate to be evaluated builds upon two already identified sub-units, *Brucella* lumazine synthase (BLS) [334] and Omp31 [46]. Created by Cassataro *et al.*, this protein is a combination of BLS with the 27 amino acid exposed loop of Omp31 attached to its N-terminus. When mice are immunised with BLS, Omp31 or a combination of the two proteins and subsequently challenged with *B. ovis*, protection is observed. However, when mice are immunised with the hybrid protein the level of protection seen against *B. ovis* increases to better than that of *B. melitensis* Rev.1. Immunisation with this hybrid protein also confers protection to challenge with *B. melitensis* but to a lesser degree than against *B. ovis* [47]. The main problem associated with recombinant sub-unit vaccines is that they are generally less immunogenic than live attenuated or killed whole cell preparations [262]. To overcome this problem protein vaccines are often administered with an adjuvant.

1.3.4 Adjuvants

Adjuvants are used for various purposes such as increasing the immunogenicity of a recombinant protein, to reduce the number of vaccinations needed for protective immunity, to act as a delivery system to increase uptake of antigen by the mucosa, or to cause slow release of the antigen leading to a longer lasting immune response. Although important features of any vaccine formulation there are only a handful of licensed adjuvants available for human use. They are aluminium compounds, a micro-fluidised oil/water emulsion called MF59 and some virosomes [162]. Due to the limited amount of adjuvants available for human use the search is on for the next big breakthrough in adjuvant technology.

1.3.4.1 Aluminium salt adjuvants

After being discovered in the 1920s aluminium salts remain the only adjuvant licensed for human use in the United States by the Food and Drug Administration (FDA). First described in the open literature by Glenny *et al.* [117], aluminium salts have been widely adopted as the most common

adjuvants used in humans. This is due to the fact that aluminium salts are well tolerated and there is a large array of data to support their safe use over the years [189]. The most common of the aluminium salt adjuvants are aluminium phosphate and aluminium hydroxide (also known as Alhydrogel). The mechanisms of action for aluminium salt-based adjuvants are three fold: (i) they create a depot effect at the site of immunisation leading to slow release of the antigen [189], (ii) they stimulate immune cells via activation of complement, and (iii) they increase the activation of macrophages [162]. Licensed vaccines that currently use aluminium salt technologies as an adjuvant include the adsorbed Diphtheria and Tetanus toxoids and the recently licensed recombinant Hepatitis B vaccine [4]. The limiting factor of aluminium salt adjuvants is their induction of an antibody response and bias toward the generation of a Th2 type immune response.

1.3.4.2 Freund's adjuvants

One of the most potent adjuvants developed to date is Complete Freund's Adjuvant (CFA) [106]. Developed in the mid 1930s by Freund, it is composed of an water-oil emulsion containing killed mycobacteria. Although it is a potent stimulator of the immune system, CFA is very reactogenic and so deemed unsuitable for human use [342]. Incomplete Freund's adjuvant (IFA) is a less potent version of CFA which does not contain the mycobacteria. IFA is more suitable for human use and, although in extreme cases it can still cause granulomas in humans, it has been used in some influenza vaccines in the United Kingdom [342].

1.3.4.3 Immunostimulatory complexes

The immunostimulatory complexes (ISCOMs) (figure 1.4), were first discovered by Morein *et al.* in 1984 [223]. Originally designed as a presentation method for proteins from enveloped viruses, the ISCOMs function has developed and they have become a well characterised adjuvant technology. ISCOMs are approximately 40 nm cage-like structures that form naturally when saponins derived from the *Quillaja saponaria* tree in South America, cholesterol and phospholipids are mixed under specific conditions [259]. The use of ISCOMs in humans and animals can be limited due to the toxicity of the saponins used. To remove the impurities a partially purified fraction of the saponins such as Quil A was produced. However, Quil A was also found to be unsuitable for human use as it has associated toxicity [24], and so it is now used in the veterinary field. The development of further



ISCOM structures

Figure 1.4: Electron micrograph of immunostimulating complexes (ISCs (Abisco-100 Isconova, Uppsala, Sweden)) at a concentration of 2.4 mg/ml. The image shows the 40 nm cage like structures that naturally form when saponins, cholesterol and phospholipids are mixed.

purified fraction of the saponins has led the way in the development of ISCOMs for human use, the most common of which is ISCOPREP®, a highly purified fraction of saponin [259]. The immune response generated by ISCOMs is generally broad, stimulating both antibody and cellular immune responses. Mice immunised with ISCOMs show increased recruitment of neutrophils, mast cells, macrophages and dendritic cells, all of which also show signs of increased activation via the secretion of immune modulators such as IL-1, IL-6, IL-12 and IFN- γ [298,7]. There are a number of currently licensed ISCOM-based vaccines on the market in the veterinary medicine field, including Canvac CCI (made by Pfizer, a vaccine for *Bordetella bronchiseptica* in dogs) and Equilis® Prequenza (an equine vaccine against influenza) [3].

1.3.4.4 Oligodeoxynucleotides (CpG)

Oligodeoxynucleotides containing CpG motifs (CpG) are short lengths of unmethylated CpG dinucleotides that are recognised by the innate immune system [171]. Unmethylated CpG dinucleotides are relatively common in the genomes of most bacteria but are suppressed and methylated in vertebrate genomes [172]. The innate immune system contains a range of pattern-recognition receptors (PRRs) that bind a range of molecules that are expressed across a variety of pathogens [173]. The best known PRRs are the TLR protein family. The stimulation of TLR9 (which recognises CpG) leads to the secretion of cytokine such as IL-6 and IL-10 from B cells and IFN- γ and TNF- α from dendritic cells. Although there are no licensed CpG in use currently there have been a number of ongoing clinical trials to confirm the safety of CpG technology [2,61,174].

1.3.5 DNA vaccination

DNA vaccination is a relatively new technology that was first described in the 1990s. In 1992, Tang *et al.* first described the basic principle of eliciting an immune response from DNA directly administered to the skin of mice [317]. The construction of DNA vaccines involves the insertion of a protective antigen gene into a plasmid vector with a eukaryotic expression system. The purified plasmid is then directly inoculated into the animal or human and the hosts own cells synthesises the antigen, using RNA transcription and translational mechanisms in ribosomes. The antigen is then presented to the immune system, producing an immune response that aids in the clearance of the

infection [274]. DNA vaccines are able to elicit good cellular immune responses which are generally considered the appropriate immune response for intracellular pathogens such as *Brucella* [123]. The first experiments completed in small animals [317,108,275] and in non-human primates [196,192] spawned large amounts of optimism about this new technology. It was the transfer of this technology from small animals to humans that caused problems since early DNA vaccine trials in humans gave poor levels of immunity [201] and cast a shadow over the early enthusiasm of DNA vaccination for human use. However, in recent years there has been a resurgence in DNA vaccine technology. With the ever increasing improvements in molecular techniques and DNA vaccine construction, DNA vaccines are becoming increasingly effective at eliciting strong immune responses. Recently, four DNA vaccines have been licensed in the veterinary medicine fields against a range of diseases including West Nile virus [71] in horses and infectious haematopoietic necrosis virus [114] in salmon. There is also now a strong body of work that has shown DNA vaccine to have some efficacy in humans when specific vaccination regimes are used [195].

1.3.5.1 DNA vaccines for brucellosis

Several studies indicate that DNA vaccines are able to provide protective efficacy against brucellosis (table 1.2). For example, Yang *et al.* tested a bank of potentially immunogenic antigens taken from the *B. melitensis* 16M genome as DNA vaccines in the Balb/c mouse model of protection against *B. melitensis* [364]. From their panel of antigens, the two best performing were a chaperone protein called trigger factor/immunophilin protein (TF) and bp26 (a periplasmic diagnostic antigen). When animals were dosed with both bp26 and TF in combination a 20-fold reduction in *B. melitensis* numbers was observed when compared to control mice [364]. Similarly, Commander *et al.* have identified two protective antigens which produced 2-log reductions in *Brucella* spleen loads compared to control immunised mice [59]. These proteins are invasion protein B (InvB) and Omp25. InvB shows good protection as a DNA vaccine against *B. melitensis* challenge but there is a frame-shift mutation in the *B. suis* homologue meaning that this vaccine might not work against *B. suis*. Omp25 is also a good vaccine candidate and is well conserved between all species of *Brucella*. L7/L12, a ribosomal antigen, has been used to provide a 1-log reduction in *Brucella* spleen loads when used as a DNA vaccine [176]. Luo *et al.* have improved upon the protective efficacy of L7/L12

Table 1.2: *Brucella* antigens assessed as DNA vaccine against *Brucella* species
 Protection Units = Mean Log₁₀ CFU negative control mice – mean Log₁₀ CFU vaccinated mice

Antigen(s)	Function	Delivery vector	<i>Brucella</i> species/strain	Best Protection Units elicited	Reference
L7/L12	Ribosomal protein	pCDNA3.1	<i>B. abortus</i> 544	1.59 PU	[199,176]
L7/L12	Ribosomal protein	pCDNA3	<i>B. abortus</i> 2308	1.26 PU	[176]
L7/L12-Omp16	-	pCDNA3.1	<i>B. abortus</i> 544	2.05 PU	[199]
Superoxide dismutase (SOD)	Cu-Zn superoxide dismutase	pCDNA3	<i>B. abortus</i> 2308	1.52 PU	[227]
SOD-IL-2 fusion	Cu-Zn superoxide dismutase, IL-2 cytokine	pSecTag	<i>B. abortus</i> 2308	1.50 PU	[120]
Omp31	Unknown	pCI	<i>B. melitensis</i> H38S	1.30 PU	[50]
Omp31	Unknown	pCI	<i>B. ovis</i>	2.24 PU	[50]
BLS	Luminase synthase	pCDNA1	<i>B. abortus</i> 544	1.65 PU	[333]
<i>bp26</i>	Diagnostic antigen	pCDNA3.1	<i>B. melitensis</i> 16M	1.16 PU	[364]
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase enzyme	pCMV-link	<i>B. abortus</i> 2308	Non-protective	[279]
Omp25	Unknown	pCDNA3.1	<i>B. melitensis</i> 16M	2.54 PU	[59]
IaIB	Possible involved in invasion of macrophages	pCDNA3.1	<i>B. melitensis</i> 16M	2.70 PU	[59]
P39	Putative periplasmic binding protein	pCI	<i>B. abortus</i> 544	0.73 PU	[9]
BCDP31-SOD-L7/L12	-	pET22b	<i>B. abortus</i> 2308	3.58 PU	[370]
GroEL	Heat shock protein	pCMV-link	<i>B. abortus</i> 2308	Non-protective	[182]

by immunising mice with an L7/L12-Omp16 DNA vaccine. Mice then challenged with *B. abortus* had a 2-fold reduction in *B. abortus* CFU recovered [199]. One of the most effective DNA vaccines developed was a tripartite DNA vaccine encoding BSCP31 (an immunodominant protein in *Brucella*), superoxide dismutase and L7/L12. When mice were immunised with three doses of 50 µg DNA vaccine it induced a cytokine profile showing a Th1 bias, with increased INF-γ and TNF-α production, and an increase in CD8⁺ T cells. When immunised mice were challenged intravenously with 5x10⁶ CFU of *B. abortus* 2308 mice displayed a 3.58 PU reduction in spleen loads

1.3.5.2 DNA vaccine prime-boost strategies

The principle of DNA prime-boost strategies is to first prime the immune system to an antigen using a DNA vaccine vector and then to boost the immune response generated by immunisation with the antigen in a different form, for example, as a protein [358]. This is known as a DNA prime protein boost strategy. This heterologous prime-boost strategy can lead to a stronger immune response than when only homologous antigen delivery is used. Prime-boost strategies have helped to enhance the effectiveness of DNA vaccines in human clinical trials and, in early 2008, two phase I clinical trials proved that prime-boost strategies using antigens from Human Immunodeficiency Virus (HIV) were successful at inducing strong immune responses in humans [129,345]. There has been only one known example of a prime-boost strategy being evaluated for *Brucella* species. Cassataro *et al.* used a DNA vaccine encoding Omp31 to prime the immune system and recombinant Omp31 protein to boost [49]. Results from this study indicated that the prime boost strategy induced higher levels of IFN-γ, an important cytokine for protection against *Brucella*, but also higher levels of IL-10, a negative regulator of the immune system that can dampen down the Th1 immune response. There was also no difference between cytotoxic T cell responses in any of the immunisation groups. Protection studies against *B. ovis* and *B. melitensis* both revealed that mice immunised under the prime-boost vaccination regimen did not have a significantly lower bacterial burden in their spleens when compared to mice immunised with the individual components.

1.4 ATP – binding cassettes

ATP-binding cassettes (ABC) proteins are one of the largest protein super families and they are found in all prokaryotes and eukaryotes. Although ABC proteins are extremely versatile they all contain one defining feature, the ability to hydrolyse ATP to ADP, providing the energy needed for active transport. The import and export of molecules across cell membranes is essential for bacterial survival, and ABC transporters are responsible for much of this movement. In fact, 5% of the *E. coli* genome encodes for ABC proteins [191].

1.4.1 Structure of ABC systems

ABC proteins are well conserved proteins. The primary sequences and configuration of the sub-unit proteins in ABC systems are especially well maintained throughout the family of ABC systems [112]. Typically, ABC transporters consist of a highly conserved ATPase domain (the ATP-binding cassette, also known as the nucleotide binding domain), the role of which is to bind and hydrolyse ATP to ADP, which provides the energy needed for transport [326]. The ABC protein contains two conserved motifs called Walker A (G-X-X-G-X-G-K-S/T, where X represents any amino acid residue) and Walker B (ϕ - ϕ - ϕ - ϕ -D, where ϕ designates a hydrophobic residue) [328]. Together the Walker A and Walker B motifs form the structure for ATP-binding [327]. These two motifs can also be found in any ATP-binding proteins, including those not associated with transport. ABC transporters also contain a signature sequence (LSGGQ/KQR) that is very well conserved between all ABC transport proteins. This sequence is also known as the linker peptide or C motif [112]. Although the configuration of ABC systems varies (figure 1.5 and 1.6), the majority of ABC systems comprise two hydrophilic ABC domains associated with two hydrophobic membrane-spanning domains (inner membrane (IM) proteins). Import systems are only found in prokaryotic organisms and contain both ABC domains and IM domains, along with extra-cytoplasmic binding proteins (BP) designed to bind the specific allocrite of that ABC system. In Gram-negative bacteria the BP are located in the periplasm whereas, in Gram-positive bacteria, they are anchored to the outer membrane of the cell via N-terminal lipid groups [261]. In comparison, ABC systems involved in export functions usually contain only IM and ABC domains fused together either via the N-terminus (IM-ABC) or the C-terminus (ABC-IM), which homodimerise to create a functional system [68].

Figure 1.5: Schematic of importing ABC transporter system

Basic components and layout of importing ABC transporter systems in Gram-negative and Gram-positive organisms

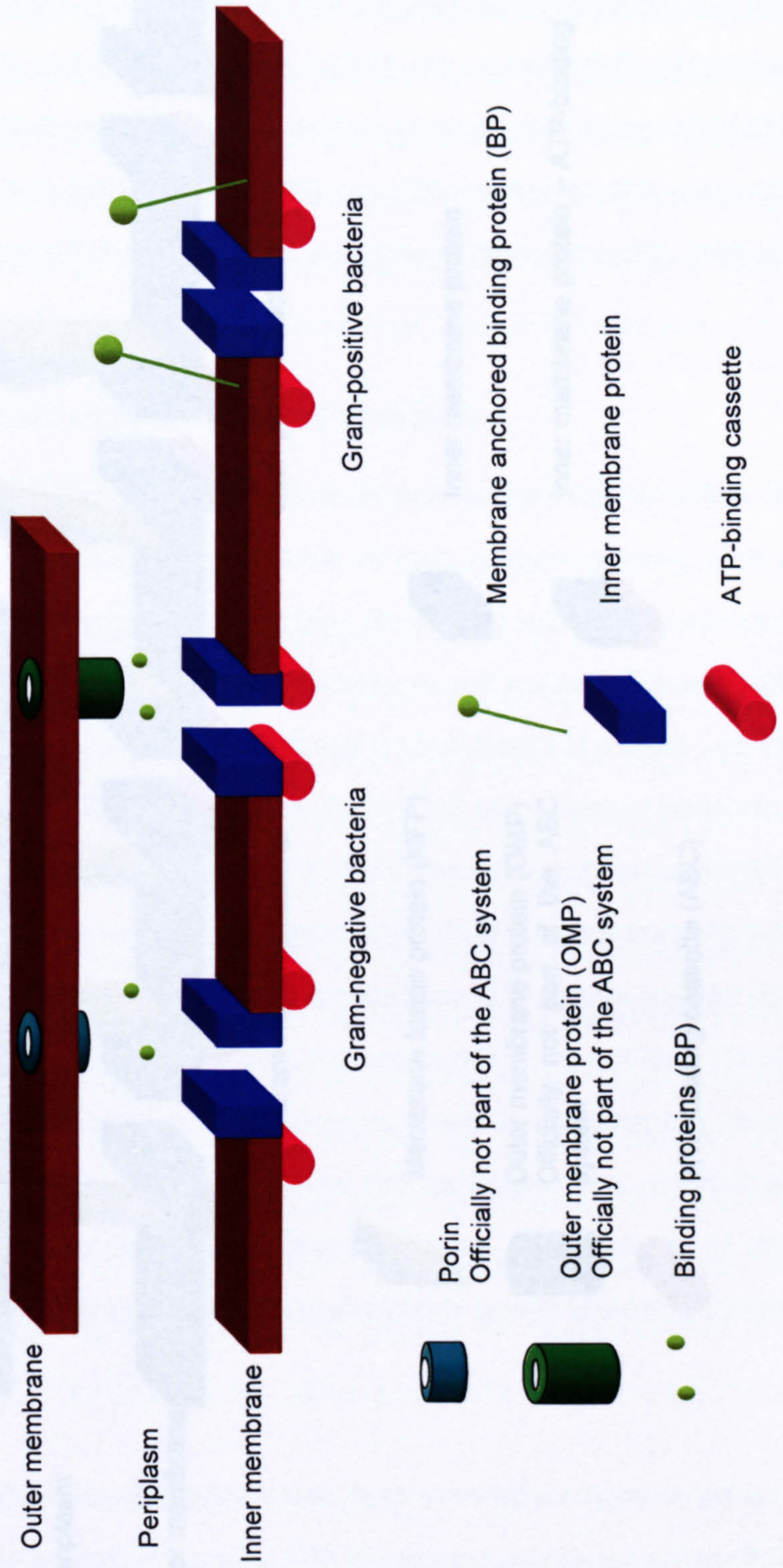
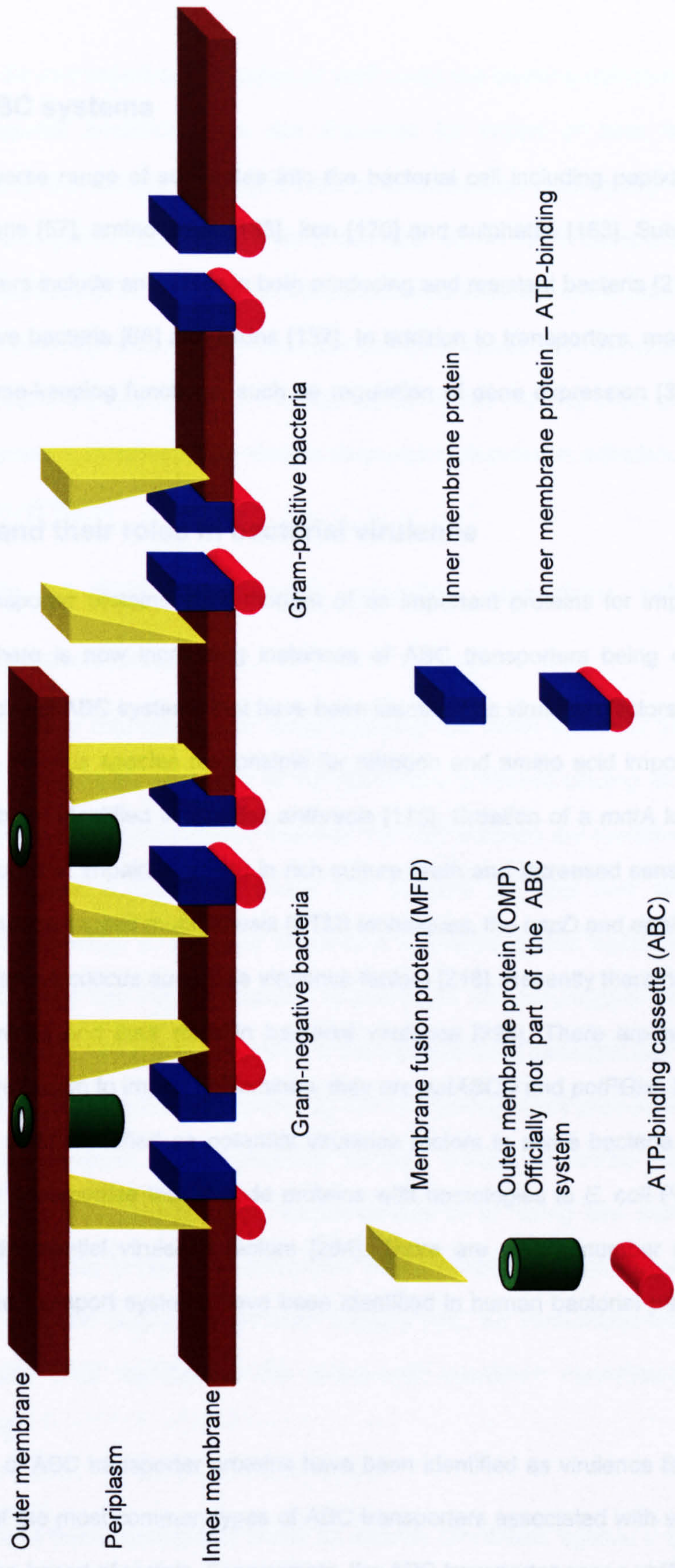


Figure 1.6: Schematic of exporting ABC transporter system

Basic components and layout of exporting ABC transporter systems in Gram negative and Gram positive organisms

organisms



1.4.2 The roles of ABC systems

ABC systems import a diverse range of substrates into the bacterial cell including peptides [82], polyamines [142], metal ions [57], amino acids [138], iron [170] and sulphates [163]. Substances exported by ABC transporters include antibiotics in both producing and resistant bacteria [219,330], fatty acids in Gram-negative bacteria [68] and toxins [137]. In addition to transporters, many ABC proteins have roles in house-keeping functions, such as regulation of gene expression [332] and DNA repair [68,121].

1.4.3 ABC systems and their roles in bacterial virulence

For a long time ABC transporter proteins were thought of as important proteins for import and export processes only. There is now increasing instances of ABC transporters being cited as virulence factors [112]. Bacterial ABC systems that have been identified as virulence factors include putative ABC importers in *Yersinia* species responsible for nitrogen and amino acid import [160]. The *mntA* gene has also been identified in *Bacillus anthracis* [115]. Creation of a *mntA* knockout mutant of *B. anthracis* resulted in impaired growth in rich culture broth and increased sensitivity to oxidative stress. Using signature-tagged mutagenesis (STM) techniques, the *oppD* and *oppF* ABC genes were identified in *Staphylococcus aureus* as virulence factors [218]. Recently there has been a lot of interest in polyamines and their roles in bacterial virulence [296]. There are two ABC transporter systems that are known to import polyamines, they are *potABCD* and *potFGHI*. Proteins from these systems have been identified as potential virulence factors in some bacteria. Genes identified in *Streptococcus pneumoniae* that encode proteins with homologies to *E. coli* PotD and PotA have been identified potential virulence factors [264]. There are also a number of other occasions where polyamine transport systems have been identified in human bacterial pathogens [202,362,296].

Although different families of ABC transporter proteins have been identified as virulence factors in pathogenic bacteria, one of the most common types of ABC transporters associated with virulence are those responsible for the import of metals. For example, the ABC transporter genes *ybtPQ* in *Y.*

pestis genes are required for iron import and mutation of *ybtP* make the bacteria avirulent in mice [100]. *Streptococcus pyogenes* *mtsABC* genes are important for import of both iron and manganese, and mutations in the *mtsABC* genes gives rise to an attenuated phenotype [149]. The Zinc importer *znuA* in *Haemophilus dureyi* is another example of an ABC transporter gene associated with virulence. Mutants in the *znuA* gene show decreased virulence in the rabbit model [188]. There is also some evidence that ABC transporters can be involved with cell attachment, which is also classed as a virulence characteristic. It has been shown that mutants in the glutamine transporter gene *glnQ* in group B Streptococcus show a decrease in fibronectin adherence along with reduced invasion *in vivo* [315].

1.4.4 Immunogenicity of ABC transporter proteins and their roles as potential vaccine candidates

Proteins that are most commonly associated with immunogenicity are those visible to the host's immune system. For ABC transporters, this would be the membrane-anchored binding proteins in Gram-positive bacteria. However, in Gram-negative bacteria, the ABC systems are contained within the periplasmic space. However, it has been shown that proteins that are held within the periplasmic space or even embedded in the inner membrane in Gram-negative bacteria can be immunogenic [112]. The concept that ABC transporter proteins can be immunogenic when not visible to the immune system seems unlikely. However, if the bacteria have damaged cell walls or are phagocytosed and presented to the immune system by APCs it is likely these proteins would be displayed. Examples of immunogenic ABC transporter proteins include an amino acid ABC transporter protein of *Brucella ovis* identified by Teixeira-Gomes *et al.* using two dimensional electrophoresis and immunoblotting [319]. ABC transporter proteins have also been identified in *Enterococcus faecium* as potential targets for antibody therapy [40]. An immunodominant ABC transporter protein has also been identified in the problematic bacterium methicillin-resistant *Staphylococcus aureus* [41].

ABC transporter proteins have been successfully tested as vaccine candidates against a variety of different pathogens. An example is the LolC protein in *Burkholderia pseudomallei* [130] which is

part of the putative LolCDE ABC system in *B. pseudomallei*, whose likely function it is to sort lipoproteins between the inner and outer membranes. When administered along with ISCOMs and CpG adjuvant, LolC offers good protection against lethal *B. pseudomallei* challenge [130]. Similarly, PiuA and PiaA are ABC transporter proteins associated with iron uptake in *Streptococcus pneumoniae*. PiuA and PiaA have been shown to have protective properties against *S. pneumoniae* in the mouse model of protection [33]. In addition, ABC transporter proteins have also shown potential as novel vaccines for *Y. pestis* infection, the causative agent of plague. OppA is an ABC transporter binding protein putatively involved in the uptake of oligopeptides that has been shown to induce a protective immune response against experimental challenge with *Y. pestis* [316].

1.4.5 ABC transporters in *Brucella*

A number of studies involving *Brucella* ABC proteins have been published in the open literature (table 1.3). Most of the research focuses on ABC protein-deficient mutants and their phenotypes and virulence. Some *Brucella* ABC transporter proteins can be considered virulence factors which, when mutated, result in the creation of an attenuated mutant. The *znuA* gene is putatively involved in zinc import in *B. abortus*, and is important in intracellular survival and virulence in mice [164]. Deletions of the *znuA* gene in *B. abortus* creates an attenuated mutant that is capable of protecting mice against wild-type *B. abortus* challenge [363]. Similarly, Kim *et al.* created 4400 mutants of *B. abortus* to identify genes involved in internalisation and intracellular growth, reporting the identification of 25 genes, two of which were ABC transporter genes *znuA* and *cydC* [165]. Additionally, other genes of the *cydDCAB* operon (putatively involved in cytochrome *bd* biogenesis) are considered important in *B. abortus*, as a *cydB* mutant is defective in intracellular survival and lacks virulence in the mouse model of infection [93]. The *cydD* gene has been identified as essential for intracellular replication by *B. suis* [168]. Together, this suggests that cytochrome *bd* biogenesis is required for replication in the intracellular environment. Furthermore, iron import also may be important for bacterial survival since genes identified in *B. abortus* show that the ABC transporter protein FbpA is required for intracellular growth [94].

Table 1.3: Brucella ABC system genes researched in the literature

Gene	Function	Brucella species used	Model used	Phenotype	Reference
<i>rbsC</i>	Ribose ABC transporter protein	<i>Brucella melitensis</i>	Macrophages, HeLa cells and Mice	Reduced growth in all models used	[185]
BMEI10923	ABC transporter protein	Unknown	Mice	Unknown	[76]
<i>ZnuA</i>	Zinc uptake	<i>Brucella abortus</i>	Macrophages, HeLa cells and Mice	Reduced growth in Zinc chelated media and no replication in HeLa cells and macrophages. Attenuated in mice	[363,164]
<i>nikA</i>	Nickel uptake	<i>Brucella suis</i>	J774A.1 cells	No difference from wild-type <i>B. suis</i> . Activated after internalisation in macrophages	[153,169]
<i>fbpA</i>	Iron uptake	<i>Brucella abortus</i>	Macrophages	Activated after 24 hrs after macrophage infection	[94]
<i>dppA</i>	Dipeptide uptake	<i>Brucella melitensis</i>	Macrophages, HeLa cells and Mice	Reduced growth in all models used	[186]
<i>artI</i>	Arginine transport	<i>Brucella suis</i>	J774A.1 cells	Activated after internalisation in macrophages	[169]
<i>exsA</i>	ABC transporter protein	<i>Brucella abortus</i>	Mice	Attenuated in mice	[278]
<i>dbpA</i>	Ribose uptake	<i>Brucella melitensis</i>	Macrophages and HeLa cells	Unknown	[76]
<i>malK</i>	Maltose uptake	<i>Brucella melitensis</i>	Macrophages and HeLa cells	Unknown	[76]
<i>araG (gguA)</i>	L-arabinose transport	<i>Brucella suis</i>	Macrophages	Not needed for survival inside macrophages	[13]
<i>ugpA</i>	SN-glycerol transport	<i>Brucella melitensis</i> & <i>Brucella abortus</i>	Macrophages, HeLa cells and Mice	Reduced growth in all models used	[186,94]
<i>cydC</i>	Cytochrome biogenesis	<i>Brucella abortus</i>	HeLa cells	Deficient in intracellular growth	[165]
<i>cydD</i>	Cytochrome biogenesis	<i>Brucella suis</i>	Macrophage like THP-1 cells	Reduced intramacrophagic growth at 48 hours	[168]
BMEI1258	Unknown	<i>Brucella suis</i>	J774A.1 cells	Activated after internalisation in macrophages	[169]
BMEI10336	Unknown	<i>Brucella melitensis</i>	Macrophages and HeLa cells	Unknown	[76]
<i>cgt</i>	Cyclic β -1,2 glucan transporter	<i>Brucella abortus</i>	HeLa cells and mice	Reduced virulence in mice and defective intracellular growth in HeLa cells	[277]

1.5 Project aims

There is currently no licensed human vaccine against brucellosis. Although there are live attenuated vaccines available for animal use, many of these can cause infection in humans, making them unsuitable for licensing for human use. The development of a sub-unit vaccine would be the safer alternative to a live attenuated vaccine. Although significant research into sub-unit vaccines has been initiated, to date no sub-unit vaccine has been effective enough for further development in humans and animals. Recently, ABC transporter proteins have been identified in bacteria as potential virulence factors [112] and there is evidence in the literature that ABC transporter proteins could make effective novel vaccine candidates [130,316,295,33]. Therefore, ABC transporter proteins could be tested as novel vaccine candidates against *Brucella* species. The aim of this project is to select, produce and evaluate ABC transporter proteins as potential sub-unit vaccines against *Brucella melitensis*.

Chapter 2 - Materials and Methods

2.1 Bioinformatic techniques

2.1.1 Identification of ABC systems in *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella canis* & *Brucella ovis*

The prediction of ABC systems in sequenced bacterial genomes is based on the homology of experimentally identified or predicted ABC proteins from other bacterial systems. The Artemis viewer (available from www.sanger.ac.uk) was used to visualise the annotated *Brucella* genomes [78,258,128]. Using the annotated genomes, ABC proteins were searched for using an array of related words, specifically “ATP-binding cassettes”, “binding protein”, or “outer membrane protein”. For completeness all proteins that were labelled as hypothetical or conserved hypothetical proteins were also checked. Hits were compiled and then genes upstream and downstream were also checked to ensure that all genes from one system were identified. After the genome searches were completed protein sequences were aligned using the basic local alignment search tool (BlastP) against other ABC proteins using the ABC systems: Information on Sequence Structure and Evolution (ABCISSE) database [68]. The ABCISSE database comprises 24000 proteins from 9500 annotated systems over 795 different organisms. Where searches on ABCISSE were unclear, proteins were aligned using BlastP searches against the Genbank database to clarify function. Proteins searched against ABCISSE that scored a threshold e-value of 10^{-6} were assigned to an ABC family and sub-family where applicable. This method was completed for all sequenced strains of *Brucella*, then ABC system lists were compiled and compared.

2.1.2 Identification of membrane-spanning domains

Genes of interest had to be checked for membrane-spanning domains since, if present, they could cause problems with protein purification techniques in downstream processing. Sequences of the genes were obtained using the annotated genomes downloaded from Genbank. An internet-based program called, TMHMM v 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) was used to identify membrane-spanning domains [175]. Once membrane-spanning domains had been identified primers were designed to exclude these sections of the protein. If there were too many membrane-spanning domains to avoid, primers were designed to amplify the entire gene.

2.2 Microbiology Techniques

2.2.1 Materials

Chemical reagents and materials were mainly sourced from Sigma-Aldrich Ltd (Poole, UK). All restriction enzymes were acquired from Roche Diagnostics Ltd (Lewes, UK). Distilled water (dH₂O) and phosphate-buffered saline (PBS) were obtained from Gibco (Paisley, UK). Bacto agar, yeast extract and tryptone were purchased from Oxoid Ltd (Basingstoke, UK). *Brucella melitensis* 16M DNA was kindly provided by Dr. N. Commander (Veterinary Laboratories Agency VLA, Weybridge, Surrey).

2.2.2 Media Preparation

Bacteria were cultured in Luria broth (L-broth), Luria agar (L-agar) or 2x Yeast Tryptone (2xYT) broth. L-broth consists of 1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract and 0.5% (w/v) sodium chloride in dH₂O. 2x YT media constituents are 1.6% (w/v) Bacto tryptone, 1% (w/v) Bacto yeast extract, 0.5% (w/v) sodium chloride and 0.5% (v/v) glucose in dH₂O. L-agar was made by the addition of 2% (w/v) bacto agar to L-broth. All media was sterilised before use by autoclaving and subsequently stored at room temperature. Antibiotics were used when necessary. Stock ampicillin was made at a concentration of 50 mg/ml, and was used to supplement L-broth or L-agar to a final concentration of 100 µg/ml. Chloramphenicol was made at a stock concentration of 34 mg/ml and added to media to a final concentration of 34 µg/ml. All stock antibiotics were filter sterilised through a 0.22 µm filter before use in any media.

2.2.3 Growth of *Escherichia coli*

Escherichia coli strains were routinely cultured for cloning, expression and purification purposes. *E. coli* were grown in L-broth at 37°C, shaking at 180 rpm. Alternatively, *E. coli* was grown on L-agar plates incubated at 37°C.

2.2.4 Maintenance of *E. coli* strains

E. coli strains were maintained in 15% glycerol. Strains were grown overnight and 850 µl of culture was mixed with 150 µl of sterile glycerol. Stocks were then transferred to -70°C for long term storage.

2.2.5 Culture of *Brucella melitensis* 16M and inoculum preparation

B. melitensis 16M and Rev.1 were cultured on serum dextrose agar (SDA) or serum dextrose (SD) broth. Cultures on plates were incubated at 37°C, 10% CO₂. Challenge inoculum preparations were prepared from *Brucella* cultured on SDA for three days, harvested into PBS. Inoculum CFU was calculated using nephelometry readings (where a transmission value of 42-46% equates to a 1x10¹⁰ CFU) and concentrations were adjusted accordingly. Administration of *Brucella* to mice was completed within 1 hour of inoculum preparation and exact CFU doses were enumerated following serial dilution of inoculum on SDA plates.

2.3 Molecular Biology Techniques

2.3.1 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was used to amplify the genes of interest for use in the cloning of recombinant proteins. Oligonucleotide primers were designed to amplify the genes (Table 2.1). Genes were amplified using varying PCR conditions and cycling temperatures (Tables 2.2 and 2.3). Basic PCR components were 10 µl 10x PCR buffer, 0.5 µl dNTP mix, 0.4 µl Taq polymerase, 15.6 µl nuclease-free water, 0.5 µl forward primer and 0.5 µl reverse primer. Basic PCR temperatures were 96°C (10 minutes), (96°C (30 seconds), 50°C (30 seconds), 72°C (1 minute) for 30 cycles), 72°C (10 minutes). All temperature cycling was performed in either a Thermo Cycler 9600 or 2400 (Applied Biosystems, Warrington, UK). All oligonucleotide primers were synthesised by MWG Biotech (Milton Keynes, UK). Nuclease-free water (Promega UK, Southampton UK) was used as a negative control in all PCR reactions. Once PCR reactions were complete, all reactions were cooled to 4°C.

Table 2.1: Oligonucleotide sequences used to amplify target genes in the *Brucella melitensis* genome for cloning purposes.

Primer	Oligonucleotide sequence and built in restriction site (5' to 3')	Description
BMZnuA/F1	C <u>GAATTC</u> GC TTG TTT CTT GCT TCT GCC TTT CTG EcoR1	ZnuA forward primer, full
BMZnuA/R1	CG <u>GCGGCCGC</u> CG TTA TTT CGA CAG GCA GTC TTT CAG Not 1	ZnuA reverse primer, full
BMCgt/F1	C <u>GAATTC</u> GC GTG CGC CGT ACG GGC CGG TAT ATT GAT ATG ACC EcoR1	Cgt forward primer, truncate
BMCgt/R1	CG <u>GCGGCCGC</u> CG TCA CGA AGG TCG TCT TGC CGC AGC CGG AAT GCC Not 1	Cgt reverse primer, truncate
BMOppA/F1	G <u>CTGCAG</u> GC ATG GCA CAA ACA GTG CTC AAC CGG GGT AAC Pst 1	OppA forward primer, full
BMOppA/R1	G <u>CTCGAG</u> GC TTA GTT CTT GAC GGA TAG CCA GCG CGT GCC Xho 1	OppA reverse primer, full
BMCydD/F1	GC <u>GAATTC</u> G ATG GGT TTC CAT CTG CTC GGC ACG CTG CAA EcoR1	CydD forward primer, truncate
BMCydD/R1	GC <u>CTCGAG</u> G TCA TTC CGC AGC CCT CCG TTG GAT GAC AGC Xho 1	CydD reverse primer, truncate
BMLoIE/F1	C <u>GAATTC</u> GC GTG AAG GCG CCG AAG TCC EcoR1	LoIE forward primer, truncate
BMLoIE/R1	G <u>CTCGAG</u> CG TTA GTT GCG CTC CAC CAC Xho 1	LoIE reverse primer, truncate

Blue code denotes a start codon

Red code denotes a stop codon

Underlined text denotes a restriction enzymes site, enzyme is noted under the underlined text.

Table 2.2: Optimised PCR conditions for amplifying genes for cloning

Gene	Components & volume (µl)									
	PCR Buffer	MgCl ₂	dNTP	Forward Primer	Reverse Primer	Template	Enzyme (<i>Taq</i> or <i>Pfu</i> polymerase)	dH ₂ O	DMSO	Total volume
ZnuA	10	6	1.8	1	1	3	0.5 <i>Taq</i>	24.2	2.5	50
Cgt	10	6	1.8	1	1	3	0.5 <i>Taq</i>	24.2	2.5	50
LolE	10	N/A	1.8	1	1	1	0.5 <i>Pfu</i>	34.7	N/A	50
CydD	10	6	1.8	1	1	3	0.5 <i>Taq</i>	24.2	2.5	50

Table 2.3: Optimal PCR temperature cycles for PCR reactions

	Optimal PCR temperature conditions					
	Amplification Cycle					
	Den. °C¹ (Time minutes)	Den. °C² (Time minutes)	Ann. °C³ (Time minutes)	Ext. °C⁴ (Time minutes)	Ext. °C⁵ (Time minutes)	Cyc. °C⁶
ZnuA	96 (5)	96 (0.5)	59 (0.5)	72 (1)	72 (10)	30
Cgt	96 (5)	96 (0.5)	59 (0.5)	72 (1)	72 (10)	30
LolE	96 (3)	96 (0.5)	60 (0.75)	72 (2)	72 (5)	30
CydD	96 (5)	96 (0.5)	59 (0.5)	72 (1)	72 (10)	30
OppA	96 (5)	96 (0.5)	59 (0.5)	72 (1)	72 (10)	30

1 = Denaturing step

2 = Amplification denaturing step

3 = Amplification annealing step

4 = Amplification extension step

5 = Final extension step

6 = Number of amplification cycles

2.3.1.1 *Pfu* polymerase PCR

Pfu polymerase was also used during some PCR methods to produce DNA fragments for cloning. This is a proof-reading polymerase designed to reduce sequence errors during amplification. However, *Pfu* leaves blunt ends on PCR products. In order to clone into the pCR®T7/NT-TOPO® vector, the PCR product required the addition of 'sticky ends' before ligation.

2.3.1.2 Addition of sticky ends to blunt end PCR products

PCR product was purified using a Qiagen QIAquick PCR purification kit using manufacturer's instructions. Purified PCR (5 µl) product was mixed with 1.5 µl Taq polymerase and 1.5 µl dNTP. This mix was then heated to 72°C for 10 minutes, and then chilled to 4°C.

2.3.2 Agarose gel electrophoresis

DNA was visualised using agarose gel electrophoresis. Gels were prepared using 0.7 or 1% (w/v) UltraPure agarose (Amersham Biosciences, Chalfont-St Giles, UK) dissolved in 1x TAE buffer (40mM Tris-acetate, 1mM EDTA). Ethidium bromide was added to a final concentration of 0.5 µg/ml. DNA was prepared by mixing samples in a ratio of 1:6 with 6x DNA loading buffer (40% (w/v) sucrose and 0.4% Bromothemol blue (w/v) in dH₂O). Up to 20 µl of DNA was loaded in to a single well of a gel, along with suitable molecular weight ladders (Roche Diagnostics Ltd Lewes, UK). Gels were run in Sub-Cell GT gel tanks (Bio-Rad laboratories Ltd., Hemel Hempstead, UK) at a voltage of 70-100 V for 60-90 minutes. The gels were visualised and photographed under ultraviolet light using a GelDoc 1000 Molecular Analyst system (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

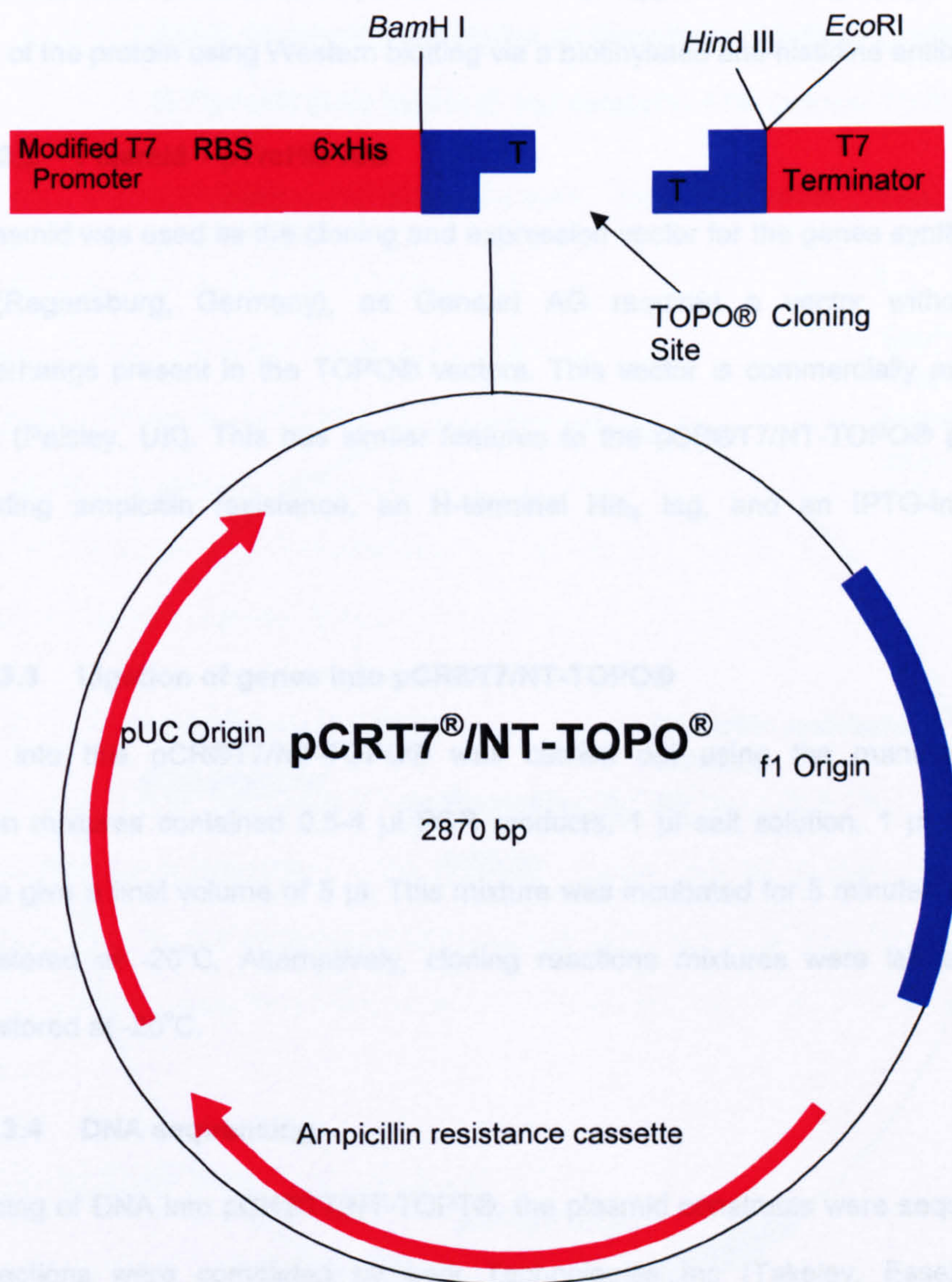
2.3.3 Cloning Techniques

2.3.3.1 Plasmid – pCR®T7/NT-TOPO®

The pCR®T7/NT-TOPO® (Invitrogen Ltd, Paisley, UK) plasmid (Figure 2.1) was used for the initial cloning of genes in this study. This vector was chosen because it has several features that are useful for cloning, protein expression and purification. This plasmid has a single deoxythymidine

Figure 2.1: pCR[®]T7/NT-TOPO[®] plasmid map

Schematic diagram showing important features of this plasmid, such as the T7 promoter, His₆ Tag and the ampicillin resistance gene (adapted from Invitrogen manual pCR[®]T7 TOPO[®] TA Expression Kits Version I 21st October 2002).



overhang which can be used for the effective ligation of PCR products amplified using *Taq* polymerase as *Taq* polymerase leaves a single deoxyadenosine overhang on PCR products. It also has an ampicillin resistance gene, which allows for the effective selection of transformed *E. coli* colonies on selective media. The modified T7 promoter in the plasmid is an inducible promoter which allows expression of the protein to be switched on by the addition of isopropyl-Beta-d-thiogalactopyranoside (IPTG). Also encoded within the plasmid is an N-terminal 6x histidine tag (His 6). This enables the expressed proteins to be purified via affinity-tagged chromatography and also allows the detection of the protein using Western blotting via a biotinylated anti-histidine antibody.

2.3.3.2 Plasmid – pTrcHis A/B

The pTrcHis A/B plasmid was used as the cloning and expression vector for the genes synthesised by Genart AG (Regensburg, Germany), as Genart AG required a vector without the deoxythymidine overhangs present in the TOPO® vectors. This vector is commercially available from Invitrogen Ltd (Paisley, UK). This has similar features to the pCR®T7/NT-TOPO® plasmid (Figure 2.2), including ampicillin resistance, an N-terminal His₆ tag, and an IPTG-inducible promoter.

2.3.3.3 Ligation of genes into pCR®T7/NT-TOPO®

Cloning of genes into this pCR®T7/NT-TOPO® was carried out using the manufacturer's instructions. Ligation mixtures contained 0.5-4 µl PCR products, 1 µl salt solution, 1 µl TOPO® vector, and dH₂O to give a final volume of 5 µl. This mixture was incubated for 5 minutes at room temperature then stored at -20°C. Alternatively, cloning reactions mixtures were left at 16°C overnight and then stored at -20°C.

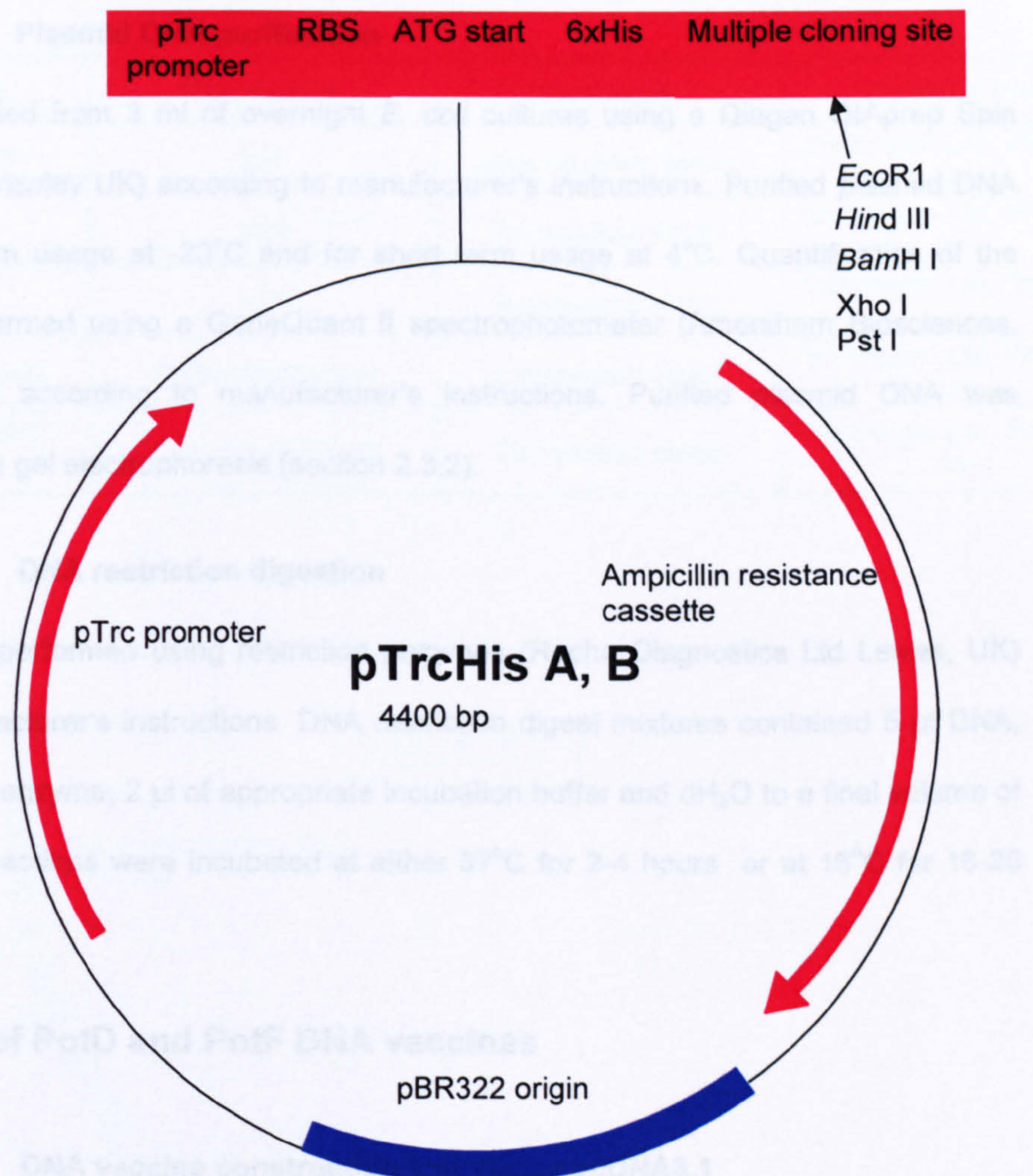
2.3.3.4 DNA sequencing

After PCR and cloning of DNA into pCR®T7/NT-TOPT®, the plasmid constructs were sequenced. All sequencing reactions were completed by Lark Technologies Inc (Takeley, Essex, UK). Sequencing results were visualised and analysed in SeqMan™ II (DNASar Inc, Madison WI, USA) for errors that may have occurred during amplification. Insert-specific sequencing primers were designed using Clone Manager professional suite version 8 (Scientific & Educational Software,

Carv, NC, USA). The universal primer T7 forward primer (5' - TAATACGACTGACTATAGGG - 3') and the reverse primer pRSET reverse primer (5' - TAGTTATTGCTCAGGGGTGG - 3') were also used for sequencing.

Figure 2.2: pTrcHis A/B plasmid map

Schematic diagram showing important features of this plasmid, including the pTrc promoter, His₆ Tag and the ampicillin resistance gene (adapted from Invitrogen manual pTrcHis A, B and C vectors for expression of recombinant proteins containing N-Terminal 6xhis tags in *E. coli* version G 17th October 2005).



2.3.4. Production of PotD and PotF DNA vaccines

DNA vaccines encoding the PotD and PotF genes were constructed by GenSart (Rappitzburg, Germany). PotD and PotF genes were inserted into the DNA vaccine vector pDNA3.1 (figure 2.2). The pDNA3.1 vector was chosen for use as the DNA vaccine vector as it has had extensive use in

Cary, NC, USA). The universal primers T7 forward primer (5' – TAATACGACTCACTATAGGG – 3') and the reverse primer pRSET reverse primer (5' – TAGTTATTGCTCAGCGGTGG – 3') were also used for sequencing.

2.3.3.5 Transformation of plasmid constructs into competent maintenance or expression cells

Plasmid DNA constructs were transformed into different *E. coli* strains for maintenance or expression. Table 2.4 outlines the *E. coli* strains used in this study. All transformations were carried out according to the *E. coli* strain manufacturer's instructions (Invitrogen).

2.3.3.6 Plasmid DNA purification

Plasmid DNA was purified from 3 ml of overnight *E. coli* cultures using a Qiagen QIAprep Spin Miniprep Kit (Qiagen, Crawley UK) according to manufacturer's instructions. Purified plasmid DNA was stored for long term usage at -20°C and for short term usage at 4°C. Quantification of the plasmid DNA was performed using a GeneQuant II spectrophotometer (Amersham Biosciences, Chalfont-St Giles, UK) according to manufacturer's instructions. Purified plasmid DNA was visualised using agarose gel electrophoresis (section 2.3.2).

2.3.3.7 DNA restriction digestion

Digestion of DNA was performed using restriction enzymes (Roche Diagnostics Ltd Lewes, UK) according to the manufacturer's instructions. DNA restriction digest mixtures contained 5 µl DNA, 10 U of each restriction enzyme, 2 µl of appropriate incubation buffer and dH₂O to a final volume of 20 µl. DNA digestion reactions were incubated at either 37°C for 2–4 hours or at 16°C for 16–20 hours, as appropriate.

2.3.4 Production of PotD and PotF DNA vaccines

2.3.4.1 DNA vaccine construction and vector pcDNA3.1

DNA vaccines encoding the PotD and PotF genes were constructed by Geneart (Regensburg, Germany). PotD and PotF genes were inserted into the DNA vaccine vector pcDNA3.1 (figure 2.3). The pcDNA3.1 vector was chosen for use as the DNA vaccine vector as it has had extensive use in

Table 2.4: *E. coli* strains used in this study

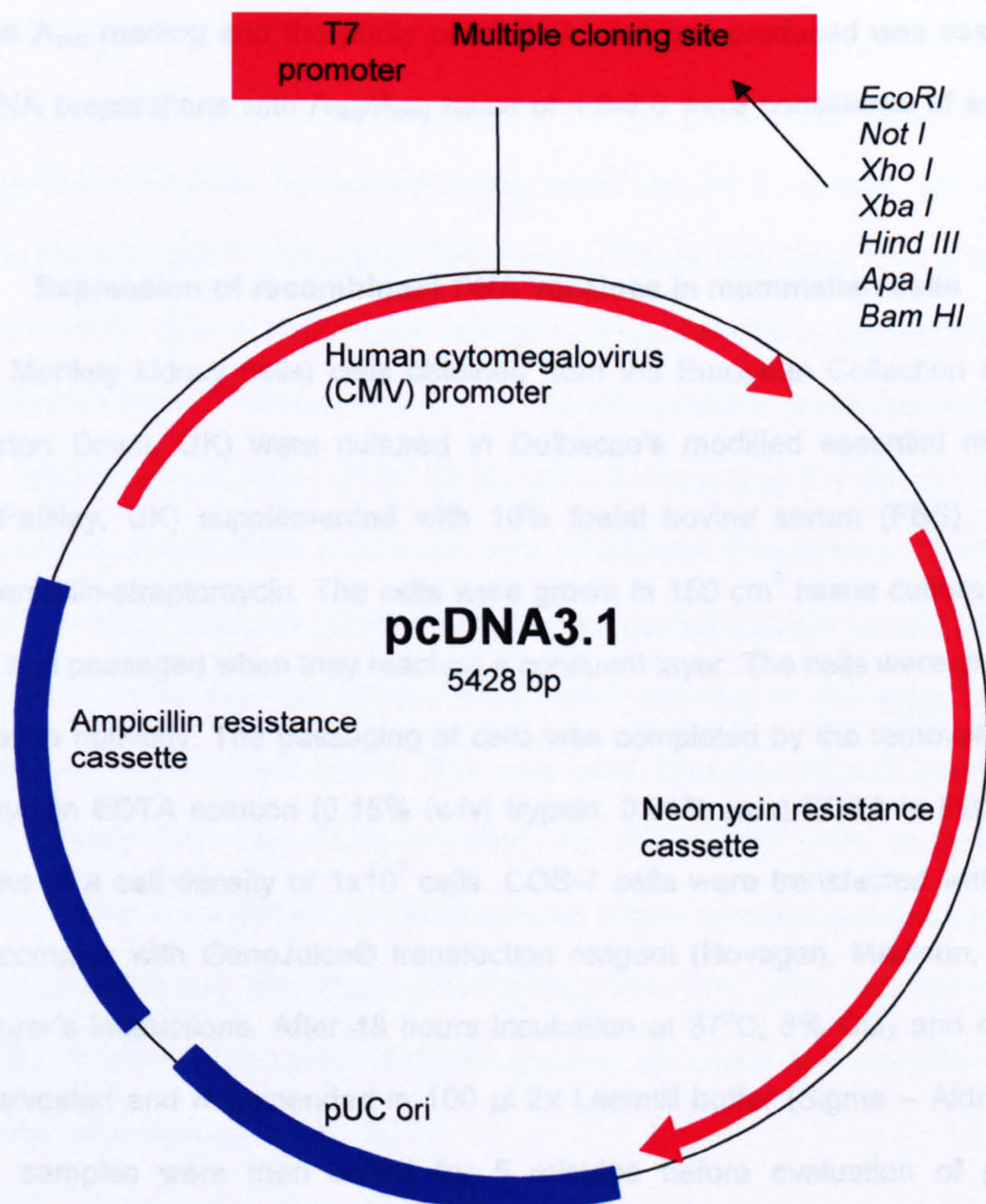
N/A = Not applicable

<i>E. coli</i> strain	Resistance	Comments	Available from
<i>E. coli</i> TOP10F'	N/A	Maintenance strain	Invitrogen Ltd, Paisley UK
<i>E. coli</i> BL21	N/A	Expression strain	Invitrogen Ltd, Paisley UK
<i>E. coli</i> BL21*	N/A	Expression strain	Invitrogen Ltd, Paisley UK
<i>E. coli</i> BL21 pLysS	Chloramphenicol	Expression strain	Invitrogen Ltd, Paisley UK
<i>E. coli</i> BL21* (DE3) pLysS	Chloramphenicol	Expression strain	Invitrogen Ltd, Paisley UK

2.3.4.2 Transformation and storage of DNA vaccines into *E. coli*

Figure 2.3: pcDNA3.1 plasmid Map

Schematic diagram showing important features of this plasmid, including the human cytomegalovirus promoter needed for expression in mammalian cells, ampicillin resistance gene for growth and selection in bacterial cells, the neomycin resistance cassette for expression of the protein in mammalian cell lines and the pUC origin for high copy number replication in *E. coli* (adapted from Invitrogen manual pcDNA3.1 (±) Version I 1997-2001).



the *Brucella* field [59,199,364,10].

2.3.4.2 Transformation and storage of DNA vaccines into *E. coli*

DNA vaccine constructs were transformed into *E. coli* TOP10 host cells for long term storage and large scale DNA vaccine production. Glycerol stocks of *E. coli* TOP10 cells containing DNA vaccines were made and stored at -80°C .

2.3.4.3 Large scale production of DNA vaccines

Large scale production of DNA vaccines was achieved using Qiagen endotoxin free giga prep kits (Qiagen, Crawley UK), used according manufacturers instructions. Preparations of DNA vaccines were ensured endotoxin free by the use of virgin plasticware and the pre-soaking of any glassware in 0.5 M NaOH for at least 4 hours before use in the assay. The concentration of the DNA vaccines was calculated using an A_{260} reading and the purity of all DNA vaccines produced was assessed using A_{260}/A_{280} ratio. DNA preparations with A_{260}/A_{280} ratios of 1.8-2.0 were considered of suitable purity for further use.

2.3.4.4 Expression of recombinant DNA vaccines in mammalian cells

COS-7 (African Green Monkey kidney cells) cells obtained from the European Collection of Cell Cultures (ECACC, Porton Down, UK) were cultured in Dulbecco's modified essential medium (DMEM, Gibco BRL, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine and 1 mM penicillin-streptomycin. The cells were grown in 150 cm³ tissue culture flasks in a monolayer culture, and passaged when they reached a confluent layer. The cells were grown at 37°C, 5% CO₂ and relative humidity. The passaging of cells was completed by the removal of the cell monolayer using trypsin EDTA solution (0.15% (w/v) trypsin, 0.01% (w/v) EDTA in PBS) and seeding into fresh flasks at a cell density of 1×10^7 cells. COS-7 cells were transfected with DNA vaccine constructs in complex with GeneJuice® transfection reagent (Novagen, Madison, USA), according to manufacturer's instructions. After 48 hours incubation at 37°C, 5% CO₂ and relative humidity, cells were harvested and resuspended in 100 µl 2x Laemilli buffer (Sigma – Aldrich Co Ltd, Poole, UK). The samples were then boiled for 5 minutes before evaluation of protein expression by Western blotting (section 2.4.1.2). Western blots were probed with mouse anti-sera

raised against the protein of interest, PotD or PotF, generated from the animal trial performed during this work (section 2.5.3).

2.4 Protein techniques

2.4.1 General protein techniques

2.4.1.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Preparation of samples for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) differed depending on the type of sample. *E. coli* cells were centrifuged at 13000 rpm for 2 minutes before being mixed with SDS reducing buffer (4 ml dH₂O, 1 ml 0.5M Tris-HCl pH 6.8, 0.8 ml glycerol, 1.6 ml 10% (w/v) SDS, 0.4 ml 2-β-metcaptoethanol, 0.05% (w/v) Bromophenol blue) in a ratio of 3:2, then lysed in a boiling water bath for 5 minutes. Purified protein was mixed in a ratio of 3:2 with SDS reducing buffer, and then added to a boiling water bath for 5 minutes. SDS-PAGE was performed using the PhastSystem™ (Amersham Biosciences Chalfont-St Giles, UK) according to the manufacturer's instructions. Briefly, 1 µl of each sample was loaded onto an application comb and applied to a PhastSystem™ gel. Proteins were run on 10-15% gradient polyacrylamide gels, and suitable molecular weight makers were added to the gels to determine the size of relative bands. Low molecular weight makers were purchased from Amersham Biosciences (Chalfont-St Giles, UK) and the markers were prepared in the same way as purified protein for SDS-PAGE. When SDS-PAGE gels were to be Western blotted, biotinylated markers or full-range rainbow markers (Amersham Biosciences Chalfont-St Giles, UK) were used. PhastGel Blue R (Amersham Biosciences, Chalfont-St Giles, UK) was used to stain the SDS-PAGE gels according to manufacturer's instructions. Gels were stained for 20-60 minutes before immersing in destain (25% (v/v) methanol, 5% (v/v) acetic acid and 70% dH₂O) until the blue colour had gone, and destain was changed where necessary. Gels were then fixed using 5% glycerol, 5% acetic acid and 90% dH₂O. Gels were dried in a drying oven overnight at 37°C.

2.4.1.2 Western blotting

Electrophoresis of proteins was performed using SDS-PAGE gels as previously described. Immobilon™-P nitrocellulose membrane (Millipore, Bedford, MA, US) was activated by soaking in methanol for 1 minute, then washed in dH₂O for 1 minute and soaked in transfer buffer (25 mM Tris, 150 mM glycine, 10% (v/v) methanol, pH 8.3) for 1 minute. Six pieces of filter paper were also soaked in transfer buffer. Once electrophoresis was complete the gel, nitrocellulose and filter paper was assembled on the PhastSystem™. The transfer of proteins from the gel to the nitrocellulose membrane was performed using the PhastSystem™ according to manufacturer's guidelines. To develop Western blots the nitrocellulose membrane was blocked (to reduce non-specific binding of primary and secondary antibodies to the nitrocellulose membrane) using 5% (w/v) skimmed milk powder dissolved in PBS (Blotto) for 16-20 hours at 4°C. Primary antibody (mouse anti-histidine IgG, Amersham™ Biosciences, Chalfont-St Giles, UK) was added at a dilution of 1:1000 and membranes were then incubated at room temperature for 1 hour. Membranes were rinsed using Blotto and then washed three times using PBS containing 0.05% (v/v) tween-20 (PBST). Secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase (HRP), (Bio-Rad laboratories Ltd., Hemel Hempstead, UK)), along with avidin-HRP (to allow marker detection, Bio-Rad laboratories Ltd, Hemel Hempstead, UK) was added at a dilution of 1:1000. The membrane was incubated at room temperature for 1 hour. The membrane was rinsed and washed again using PBST and developed by the addition of 3,3'-Diaminobenzidine (DAB peroxidase substrate, Sigma-Aldrich Co. Ltd., Poole, UK) according to manufacturer's instructions. Reactions were stopped by rinsing the membrane for 30 seconds in dH₂O. Membranes were then left to dry at room temperature overnight. Alternatively, Western blots were developed using Enhanced Chemiluminescence (ECL) detection reagents (Amersham™ Biosciences, Chalfont-St Giles, UK). In this case, nitrocellulose membranes were placed on a plastic cover and 1 ml of detection reagent (ECL detection reagent 1 and 2 in a 1:1 mix) was added to the membrane. Membranes were then incubated for 1 minute at room temperature. The membrane was then sandwiched between two plastic covers and the detection reagent was squeezed off. Membranes were then exposed to Amersham ECL hyperfilm (Amersham™ Biosciences, Chalfont-St Giles, UK) for 30 seconds – 2 minutes in a darkroom under red light conditions. ECL hyperfilm was then developed by submersion

in ECL developer solution (Sigma-Aldrich, Poole, UK) for 1 minute, then washed in H₂O for 1 minute before being exposed to ECL fixer solution (Sigma-Aldrich, Poole, UK) for 1 minute, and a final wash in H₂O for 2 minutes. Developed and fixed hyperfilms were then left to air dry for at least 4 hours.

2.4.2 Expression and Purification techniques

2.4.2.1 Protein expression

To evaluate protein expression glycerol stocks of expression strains of *E. coli* (section 2.2.4) were streaked onto L-agar plates supplemented with the appropriate antibiotics and incubated at 37°C for 16-20 hours. One colony from this plate was used to inoculate a 100 ml L-broth culture containing appropriate antibiotics. This was then incubated for 16-20 hours at 37°C. *E. coli* cultures were started by inoculating 400 ml L-broth culture containing appropriate antibiotics and 4 ml of 10% (v/v) sterile glucose with 10 ml of overnight culture. Cultures were grown to 0.4-0.6 OD₆₀₀, (mid-log phase) and 1 ml of culture was removed as a pre-induction sample. Cultures were then induced by the addition of IPTG to a final concentration of 1 mM, then cultures were left for 3-4 hours before a 1 ml post-induction sample was taken. Cultures were centrifuged at 10000 rpm, the supernatant was removed and the pellets were stored at -20°C for future use. Pre- and post-induction samples were evaluated by SDS-PAGE (section 2.4.1.1) and Western blotting (section 2.4.1.2). Gels and blots were examined for protein bands of the correct size (as predicted using EditSeq DNASTar Inc, Madison WI, USA). If appropriate sized band were present, then protein solubility was addressed (section 2.4.2.2). Table 2.5 shows the optimal expression conditions for all of the protein vaccine candidates.

2.4.2.2 Solubility of proteins

To assess the solubility of the expressed proteins, protein pellets generated in section 2.4.2.1 were resuspended in 4 ml of PBS containing 300 µg/ml DNAase I and one complete EDTA-free protease inhibitor tablet (Roche Diagnostics Ltd Lewes, UK). Suspensions were sonicated 4 times (SoniPrep 150, Sanyo MSE, Leicester UK) at a amplitude of 10 microns for 30 seconds with 30 second rests between. The suspension was then centrifuged at 15,000 rpm for 15 minutes at 4°C.

Table 2.5: Optimal expression conditions for individual vaccine candidate production

Protein	<i>E. coli</i> expression strain	Media	Growth temperature	Length of expression period	IPTG concentration	Solubility
PotD	BL21*	2x YT	37°C	3-4 hours	0.1 mM	Soluble
PotF	BL21 DE3 pLysS	L-broth	37°C	3-4 hours	1 mM	Soluble
CydD	BL21 DE3 pLysS	L-broth	37°C	3-4 hours	1 mM	Insoluble
Cgt	BL21 DE3 pLysS	L-broth	20°C	16-20 hours	0.1 mM	Soluble
LoIE	BL21*	L-broth	37°C	3-4 hours	0.1 mM	Insoluble
FbpA	BL21* DE3 pLysS	L-broth	26°C	16-20 hours	1 mM	Soluble
ZnuA	BL21*	L-broth	37°C	3-4 hours	1 mM	Soluble
OppA	BL21*	L-broth	20°C	16-20 hours	1 mM	Soluble

The supernatant and pellet were separated and the pellet was resuspended in 4 ml PBS. Both the supernatant and pellet were processed for SDS-PAGE (section 2.4.2.1) and Western blotting (section 2.4.2.2). SDS-PAGE gels and Western blots were then examined for appropriate sized bands, and proteins that appeared in the supernatant samples were deemed soluble and proteins that appeared in the pellet samples were deemed insoluble.

2.4.2.3 Large scale expression

Large scale protein expression was carried out as outlined in section 2.4.2.1 using optimal growth conditions for expression of each protein. Culture volumes of 2 L and 4 L, supplemented with appropriate antibiotics where necessary, were used for large scale expression. Cultures were centrifuged for 20 minutes at 10000 rpm, and supernatants were discarded and pellets were stored at -20°C until needed.

2.4.2.4 Urea extraction of insoluble proteins

Where expressed proteins were found to be insoluble, urea extraction was performed. Pellets were resuspended in 30 ml of PBS containing 300 µg/ml DNAase I and one complete EDTA-free protease inhibitor (Roche Diagnostics Ltd Lewes, UK). Suspensions were sonicated and centrifuged (section 2.4.2.2), and the supernatant was discarded and the pellets were resuspended in 15 ml extraction buffer (8 M urea, 40 mM tris, 750 mM sodium chloride, 2 mM reduced glutathione, 0.2 mM oxidised glutathione pH 7.5). This re-suspension was left to incubate at room temperature for 1-2 hours or, if longer extraction time was needed, re-suspensions were stored at 4°C for 16-20 hours. Samples were then filtered through a 0.45 µm filter followed by a 0.22 µm filter before purification.

2.4.2.5 Preparation of soluble proteins

Pellets of soluble protein were resuspended, sonicated and centrifuged as in section 2.4.2.2. Pellets were resuspended in 15 ml PBS containing 300 µg/ml DNAase I and one complete EDTA-free protease inhibitor (Roche Diagnostics Ltd Lewes, UK). Suspensions were filtered through a 0.45 µm filter followed by a 0.22 µm filter before being purified.

2.4.2.6 Purification equipment

All purification steps were completed using an AKTA Fast Protein Liquid Chromatography system (FPLC) (Amersham Biosciences, Chalfont-St Giles, UK) under the control of Unicorn software version 4.0 (Amersham Biosciences, Chalfont-St Giles, UK). All buffers used on the FPLC were made using ultra pure distilled water (ddH₂O) and filter sterilised through a 0.22 µm filter prior to use. Purification of proteins was achieved using Immobilised Metal Affinity Chromatography (IMAC). Pre-packed 1 ml HisTrap™ columns were obtained from Amersham Biosciences (Chalfont-St Giles, UK) and used according to manufacturer's instructions.

2.4.2.7 Purification of insoluble proteins

Expressed insoluble proteins were urea extracted as described in section 2.4.2.4. Filtered denatured proteins were applied to the column using extraction buffer (section 2.4.2.4). Once loaded onto the column the concentration of extraction buffer was lowered by mixing the extraction buffer with start buffer (40 mM Tris, 750 mM Sodium chloride pH 7.5) gradually over a 30 column volume (CV) (30 ml) period. This causes the His₆ tag protein bound to the column to refold into a natural conformation. After refolding, the column was washed using 100% start buffer. All unbound protein and wash step fractions were collected. Elution of His₆-tagged bound protein was achieved using elution buffer (40 mM tris, 750 mM sodium chloride, 500 mM imidazole pH 7.5). Elution was performed using stepped increases in concentrations of imidazole starting at 10% (50 mM), 20% (100 mM), 50% (250 mM) and 100% (500 mM), by diluting elution buffer with start buffer. During the elution stage 1 ml fractions were collected, then evaluated by SDS-PAGE (section 2.4.1.1) to determine the protein fraction location and purity. Insoluble proteins which would not bind or refold on the column were purified in their denatured state using the soluble purification method (section 2.4.2.8) with the addition of 8 M urea to all buffers used. Proteins were then refolded by buffer exchange (section 2.4.2.9).

2.4.2.8 Purification of soluble proteins

Expressed soluble proteins were prepared for purification as described in section 2.4.2.5. Filtered proteins were loaded onto the column in start buffer (as in section 2.4.2.7), the column was washed using 100% start buffer, and unbound protein and wash-step elutions were collected. Proteins were

eluted from the column (as in section 2.4.2.7) and fractions were then evaluated by SDS-PAGE (2.4.1.1) to determine protein fraction location and purity.

2.4.2.9 Buffer exchange

Buffer exchange was achieved using dialysis. Different methods were used for denatured proteins and for folded or refolded proteins. All correctly folded or refolded proteins were buffer exchanged from elution buffer into PBS. Proteins were loaded into a Slide-A-Lyzer 10000 MWCO dialysis cassette (Pierce Biotechnology Inc. Rockford, Illinois, USA) using a needle and syringe and placed in 4 L PBS at 4° C for 12-20 hours. Proteins that were purified in a denatured state needed to be refolded using dialysis. Samples were eluted from the column in 8 M urea and refolding was achieved by slowly buffer exchanging into lower concentrations of urea buffers, then into PBS. The Proteins were loaded into a Slide-A-Lyzer 10000 MWCO dialysis cassette (Pierce Biotechnology Inc. Rockford, Illinois, USA) and the cassettes were suspended in 4 L of 4 M urea containing 40 mM Tris, 750 mM sodium chloride, 1 mM reduced glutathione, 0.1 mM oxidised glutathione at pH 7.5 and placed at 4°c for 2-4 hours. The dialysis cassettes were then transferred to 2 M urea buffer containing 40 mM tris, 750 mM sodium chloride, 0.5 mM reduced glutathione, 0.05 mM oxidised glutathione at pH 7.5 and placed at 4°C for 2-4 hours. Proteins at this stage could be left overnight if needed. In the final step, cassettes were transferred to PBS and left at 4°C for 12-16 hours.

2.4.2.10 Determination of protein concentration

The Bicinchoninic Acid (BCA) protein assay (Pierce Biotechnology Inc. Rockford, Illinois, USA) was used according to manufacturer's instructions to measure protein concentration. A known concentrated stock of bovine serum albumin (BSA) was diluted in PBS to produce a standard curve for the assay.

2.4.2.11 Storage of proteins

After purification, buffer exchange and concentration determination, 1 ml aliquots of all proteins were prepared and stored at -80°C. Repeated freezing and thawing of proteins was avoided to prevent damage to the proteins.

2.5 Animal Studies

2.5.1 Animals studies and suppliers

Vaccine efficacy trials were carried out at the VLA, Weybridge or Dstl, Porton Down using 6-11 week old female Balb/C mice obtained from Harlan (Oxon, UK) or Charles River Laboratories Inc (Kent, UK). All animal work carried out in this study was completed in accordance with the Scientific Procedure Act 1986 under project and personal licences authorised by the Home Office.

2.5.2 Protein and adjuvant preparation

Inoculation formulations were prepared by diluting 10 µg of recombinant protein, 12.5 µg AbISO®-100 immunostimulatory complex (ISCOMs, Isconova, Uppsala, Sweden) and 6.25 µg CpG Oligodeoxynucleotides 10103 (CpG, Coley pharmaceuticals group, Massachusetts USA), CpG 10103 sequence: 5'-TCGTCGTTTTTCGGTCGTTTT-3') in 100 µl of sterile PBS. Alternatively, inoculum was prepared by mixing 10 µg of protein with 20 % (v/v) Alhydrogel (Sigma, Poole, UK) in 100 µl PBS, or 10 µg of protein was mixed with 50 % (v/v) incomplete Freund's adjuvant (IFA, Sigma, Poole, UK), which was then homogenised until a white emulsion was formed. Where DNA vaccination was administered 100 µg DNA vaccine was dissolved in 100 µl of dH₂O.

2.5.3 Mouse inoculation schedule and serum collection

Mice were inoculated with 100 µl of the vaccine formulations (section 2.5.2), administering 50 µl intramuscularly into each hind leg muscle. Mice were inoculated with formulation three times at three week intervals where protein was used. Blood samples were taken at day 58 in all experiments via collection from tail veins. After collection blood samples were stored at 4°C for at least 1 hour but no more than 20 hours to allow blood to clot. Blood was centrifuged at 13000 rpm for 15 minutes, and the serum was collected and stored at -20°C until needed. When DNA vaccinations were being administered mice were given four doses of 100 µg DNA at three week intervals.

2.5.4 Animal challenge and splenic colonisation assays

Infection of mice with *B. melitensis* was measured by splenic colonisation. Mice inoculated with candidate vaccine formulas were left for 30 days after the final inoculation until challenge. Mice were then challenged with approximately 1×10^4 colony forming units (CFU) of *B. melitensis* 16M via the intraperitoneal (ip) route. Mice were then left 15-18 days before mouse spleens were harvested and homogenised into 0.1 ml PBS and serially diluted. Each serial dilution was plated out in triplicate and plates were incubated for 7 days at 37°C. *Brucella* colonies were enumerated to provide the CFU per spleen. Mice administered PBS or adjuvant only were used as negative controls. Mice immunised i.p. with a single dose of 2×10^5 CFU of Rev.1, the current standard *Brucella melitensis* animal vaccine, were included as positive controls.

2.6 Immunological techniques

2.6.1 Enzyme Linked Immunosorbant Assay (ELISA)

2.6.1.1 Plate coating

ELISAs were carried out using 96-well immulon 2HB plates (Corning, NY, USA). Plates were coated using two different coating solutions. Columns 1-3 were coated with anti-mouse fab-specific antibody (Sigma, Poole, UK) diluted to a final concentration of 5 µg/ml in PBS, with 100 µl per well. Columns 4-12 were coated with the antigen of interest diluted to a concentration of 5 µg/ml, with 100 µl per well. Coated plates were stored at 4°C for at least 18 hours before use but kept no longer than 5 days at 4°C.

2.6.1.2 ELISA sample and standard preparation

All dilutions were made using 2% (w/v) skimmed milk powder + 0.05% (v/v) tween 20 (Sigma, Poole UK) diluted in PBS (blotto). Antibodies used as standards were purified commercially available antibodies, isotype-specific where necessary (Sigma, Poole, UK). A starting concentration of 0.2 µg was used for the standard curve. Serum samples taken on day 58 of the experiment were diluted 1:10,000 in blotto before use in the ELISA assay.

2.6.1.3 ELISA method

All washing steps were carried out using PBST using a Skatron 96-well automated plate washer (Molecular devices, CA, USA). All incubation steps were carried out at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 1 hour unless otherwise stated. Coated plates were washed and 100 μl of coating solution was added to each well before incubation. Next, plates were washed and standard or serum samples were added; 100 μl of standard (section 2.6.1.2) was added to wells A1-A3, 100 μl samples were added to wells A4-A11. Diluted pre-inoculation sera (section 2.6.1.2) was added to wells of column 12 as a negative control for the assay. Blocking buffer was added to all other wells on the plate. Standards and samples were serially diluted down the plate, standards to row G and samples to row H, and plates were then incubated. After incubation plates were washed and 100 μl of secondary antibody ((goat anti-mouse conjugated to HRP, isotype-specific where necessary), AbDserotec Oxford, UK) was added to each well, and plates were then incubated. After incubation the plates were washed and 100 μl of substrate buffer (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) tablets (ABTS) (Sigma, Poole UK) dissolved in a phosphate-citrate buffer (11.76 g/l citric acid, 12.49 g/l sodium hydrogen phosphate pH 4.37) and 20 μl hydrogen peroxide (Sigma, Poole UK) per 50ml of buffer, prepared not more than 30 minutes before use, was added to each well. Plates were incubated at 37°C for 10-30 minutes to develop.

2.6.1.4 Plate reading and analysis

Developed ELISA plates were read using an automated plate reader (Thermo Life sciences Multiskan Ascent plate reader, Basingstoke, UK). Plates were read at a wavelength of 414 nm and data was saved as a Microsoft® Office Excel file for further analysis. Data was analysed using ELISA for Windows version 2.0 software freely available from Centre for disease Control (CDC) website (<http://www.cdc.gov/ncidod/dbmd/bimb/ELISA.htm>). ELISA for Windows uses a four-parameter logistic fit curve to calculate unknown sample concentration of antibody in $\mu\text{g/ml}$.

2.6.2 ELISPOTS assays

Enzyme linked immunosorbent spot (ELISPOT) reagents were purchased from purchased from Mabtech (Nacka Strand, Sweden), Millipore™ (Watford, Hertfordshire, UK) or Sigma-Aldrich (Poole, UK). Assays were carried out in a class II biosafety cabinet unless stated otherwise.

2.6.2.1 ELISPOT plate coating

Sterile multiscreen_{HTS} - IP filter plates (Millipore, Watford, Hertfordshire, UK) were coated with anti-cytokine antibody diluted in carbonate/bicarbonate buffer (Sigma, Poole, UK) to a concentration of 15 µg/ml, and 100 µl was added to each well. Plates were then stored at 4°C for at least 18 hours but no more than 4 days before use.

2.6.2.2 Antigen and positive control preparation

Antigens were diluted in DMEM culture media (DMEM containing 10% fetal bovine serum (FBS, Sigma, Poole, UK) and 1% penicillin/streptomycin/L-glutamine solution (Gibco, Paisley, UK)) to a final on plate concentration of 10 µg/ml. A negative control consisting of cells incubated with no stimulatory antigen, and a positive control containing of 2 µg/ml concanavalin A (conA – Sigma, Poole UK) were also prepared.

2.6.2.3 Harvesting of ex-vivo splenocytes

Spleens were removed from inoculated or naïve mice and homogenised through a 40 µM nylon sieve into 5 ml of DMEM. Red blood cells were lysed by the addition of 10 ml of red cell lysis buffer (Sigma, Poole, UK) before being incubated at room temperature for 1 minute, and splenocytes were then centrifuged at 1200 rpm for 10 minutes. *Ex-vivo* splenocytes were then resuspended in 15 ml of FACS Flow Buffer (FFB) and centrifuged at 1200 rpm for 15 minutes. Splenocytes were then resuspended in between 1-2 ml of DMEM, before enumeration using a haemocytometer or Cellometer™ Auto T4 automated cell counter (Peqlab Biotechnologie GmbH, Farham, UK). Sufficient cells were removed for non-deplete ELISPOT assays before depletion steps could be completed. Cell concentrations were altered to 5×10^6 cells/ml before use in ELISPOT assays.

2.6.2.4 CD4⁺ and CD8⁺ cell depletion

All reagents for cell depletion assays were purchased from Miltenyi Biotec Ltd (Surrey, UK) unless otherwise stated. *Ex-vivo* splenocytes were harvested as in section 2.6.2.3. Harvested splenocytes were split into two separate cell populations and their volumes altered to 1 ml. One cell population received 100 µl anti-mouse CD8a (Ly-2) microbeads, while the other cell population received 100 µl anti-mouse CD4 (L3T4) microbeads, and both cell populations were incubated for 30 minutes on

ice. During this period MACS™ LS columns were loaded into a QuadroMACS™ separation unit. Columns were equilibrated by washing with 3 ml of FFB, after which microbead-labelled cell populations were added to each column and the flow-through was collected. Columns were then washed three times with 3 ml of FFB and the flow through collected. Collected cells were centrifuged at 1200 rpm for 15 minutes before being resuspended in 1-2 ml of DMEM. Cell concentrations were then determined using a Cellometer™ Auto T4 automated cell counter (Peqlab Biotechnologie GmbH, Fareham, UK). Cells concentrations were altered to 5×10^6 cells/ml before use in ELISPOT assays.

2.6.2.5 ELISPOT method

Plates were washed once with DMEM culture media and then blocked with 200 µl of DMEM culture media. Plates were incubated at room temperature for 2 hours. During this period mouse spleens were prepared as described in sections 2.6.2.3 and 2.6.2.4. Once cells preparations were completed, 100 µl of *ex-vivo* splenocytes were plated onto 96 well multiscreen HTS IP elispot plates (Millipore™, Watford, Hertfordshire, UK) along with 100 µl of stimulatory antigen. Plates were then incubated for between 16-20 hours in relative humidity at 37°C, 5% CO₂. Next, the cell and antigen mix was aspirated from the wells and plates were washed 3x with PBST and then 2x with dH₂O. Biotinylated detection antibody was diluted to 1-2 µg/ml in 10% BSA in PBS and 100 µl was added to each well. The plates were then incubated at room temperature for 2 hours. Plates were washed 3x with PBST and the enzyme conjugate (Streptavidin-HRP) was added (100 µl/well) at a dilution of 1:100. Plates were incubated at room temperature for 1 hour. BCIP/NBT developing substrate was prepared by dissolving 1 BCIP/NBT tablet (Sigma-Aldrich, Poole, UK) in 10 ml of pre-warmed dH₂O and once dissolved the solution was filtered through a 0.45 µm syringe filter. Plates were washed 4x with PBST and 2x with PBS and 100 µl BCIP/NBT substrate was added to each well. The plates were then left at room temperature to develop for 5-20 minutes. The reaction was stopped by removing the substrate solution and adding 100 µl dH₂O per well. Plates were left to air dry for 16 - 20 hours before reading using an Elispot Reader System ELR04 (Advanced Imaging Devices, Strasberg, Germany).

2.6.2.6 ELISPOT data analysis

Subtractions of cells with no stimulatory antigen were deducted from cells with stimulatory antigen and analysis of the data was performed using Graphpad Prism 4 (Graphpad software Inc, CA, USA).

2.6.3 ISCOMs & CpG assays

2.6.3.1 Cellular uptake of CpG

2.6.3.1.1 Preparation of cell populations

J774A.1 (mouse macrophages) cells obtained from the European Collection of Cell Cultures (ECACC, HPA Porton Down, UK) were cultured using DMEM culture media in 150 cm³ cell culture flasks (Corning Ltd, UK) at 37°C, 5% CO₂ and relative humidity. The cell culture flasks were seeded with 1x10⁷ cells and incubated until a confluent layer of cells had formed (approximately 2 days). The cells were then scraped from the flask and 1x10⁷ cells were seeded into a new flask to continue the cell line growth.

2.6.3.1.2 Confocal microscopy

J774A.1 cells were scraped from a flask and counted using a Cellometer™ Auto T4 automated cell counter, the cell density was adjusted to 3.5x10⁵ cells/ml using DMEM. One ml of cells was plated out onto 22 mm² glass-bottomed Wilco dishes (Intracel, Royston Hertfordshire. UK), and cells were then incubated for 16-20 hours at 37°C, 5% CO₂ and relative humidity. The supernatant from the cells was removed and 1 ml (5 µg/ml) of 5' carboxyfluorescein (FAM) labelled phosphothioate backbone CpG 10103 (FAM-CpG), synthesised by ATDBio (Southampton University, UK), ± 10 µg ISCOMs was added to the cells. The cells were then incubated for up to 20 hours. Cells were removed from the incubator at 1, 2, 4, 6, 8, and 16 hours, at which point 8 random confocal images were taken of the cells using an Olympus IX70 confocal laser-scanning microscope. An argon laser was used to excite the FAM-CpG at 488 nm and fluorescence was detected at 530 nm. Image processing was completed using Fluoview version 5.0 software (Olympus Corporation).

2.6.3.2 Stability of ISCOMs and CpG

2.6.3.2.1 Preparation of low pH buffers

Low pH citric acid buffers were used to incubate the ISCOMs & CpG. A 0.2 M solution of sodium phosphate (28.4 g/L) and a 0.1 M solution of citric acid (19.2 g/L), were mixed to get the pH 4 buffer used. The pH 4 solution was created by mixing 19.3 ml of 0.2 M sodium phosphate and 30.7 ml of 0.1 M citric acid.

2.6.3.2.2 Preparation of ISCOMs & CpG in low pH buffers

Concentrated ISCOMs \pm CpG were diluted to stock concentrations of 250 $\mu\text{g/ml}$ and 125 $\mu\text{g/ml}$ (respectively) in either pH 4 or neutral (PBS) buffers. For stimulation use in ELISPOT assays, these stocks were further diluted to 40 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ in DMEM, then 100 μl was used per well, equating to a 4 μg ISCOMs and 2 μg CpG stimulatory concentration (section 2.6.2).

2.6.4 Statistical analysis

Statistically analysis for all data was carried out using Graphpad Prism 4 (Graphpad software Inc, CA, USA).

Chapter 3 - ATP- binding cassette systems of Brucella

3.1 Introduction

ABC systems play a major role in the import and export of substances across the cell membrane, along with roles in house-keeping functions, such as regulation of gene expression [332] and DNA repair [68,121]. ABC proteins comprise one of the largest protein superfamilies in prokarya, eukarya and archaea [132]. ABC systems can play important roles in bacterial lifestyle, virulence and survival [112]. DNA-DNA hybridisations between the species had previously revealed 90% similarity between the species, leading to the suggestion that all *Brucella* species should be classified as *B. melitensis* [337,338]. However, it is widely acknowledged that the differences in host specificity and pathogenicity are related to *Brucella* genetics, although there is currently little experimental evidence to support this, a few studies have found differences between the *Brucella* species genomes that may support this hypothesis [54,128,269].

The recent sequencing of *B. melitensis* 16M [78], *B. abortus* 9-941 [128], *B. suis* 1330 [258], *B. ovis* 63/290 (NCBI: NC_009504/5 Unpublished) and *B. canis* RM6/66 (NCBI: NC_010103/4 Unpublished) has enabled the genomic comparison of the different *Brucella* species. The aim of this work was to create and compare inventories of the predicted functional ABC systems in *Brucella* from which to identify putative vaccine candidates to down select for evaluation.

3.2 Creating *Brucella* ABC system inventories

The prediction of ABC systems in sequenced bacterial genomes is based on the similarity of experimentally identified or predicted ABC proteins from heterologous bacterial systems. *Brucella* genomes were visualised using the Artemis viewer (available from www.sanger.ac.uk). ABC proteins were located within the genomes by searching for related words, specifically "ATP-binding cassette", "binding protein" or "outer membrane protein". For completeness all genes labeled hypothetical and conserved hypothetical protein were also checked. Once all searches were completed the protein sequences of each gene were aligned using the basic local alignment search tool (BlastP) [12] against other ABC proteins using the ABC systems: Information on Sequence Structure and Evolution (ABCISSE) database [68,70]. When all hits were compiled, genes located

upstream and downstream from ABC proteins were checked to ensure that all proteins from each system were located. This method was completed for all sequenced strains of wild-type *Brucella* (*B. melitensis* 16M, *B. abortus* 9-941, *B. suis* 1330, *B. ovis* 63/290 and *B. canis* RM6/66), and complete ABC system inventories were compiled and compared.

The ABC system inventories also include any system that contains mutated genes. However, these systems were not counted toward the total ABC system numbers because functionality of a system with mutated genes present cannot be addressed using bioinformatic techniques. Within the genomes of all of the *Brucella* species evaluated, single ABC system components were located that did not seem to belong to complete ABC systems. These were included in ABC system inventories and termed lone components, although these were not included in total ABC system counts as they did not comprise a complete system. Analysis of these data was achieved by grouping the ABC systems into ABC system importers, exporters, cellular processes and functionally unknown systems. The total ABC system numbers were compared, along with comparisons of the ABC systems family and function. The full inventories and alignments of the *Brucella* ABC systems, along with ABC family acronym descriptions can be seen in appendix A.

3.3 *Brucella* ABC systems – general observations

The *Brucella* strains investigated in this study all have approximately 3.3 Mb genomes comprising two chromosomes of approximately 2.1 Mb and 1.2 Mb. Despite being smaller in size it is chromosome two that encodes the most ABC system genes in all the studied *Brucella* strains. The total number of predicted functional ABC systems encoded by each of the five *Brucella* strains is similar but does show some variability (table 3.1), with *B. melitensis* 16M encoding the most systems (79 systems), followed by *B. canis* RM6/66 (74 systems), *B. suis* 1330 (72 systems), *B. abortus* 9-941 (64 systems) and, finally, *B. ovis* 63/290 (59 systems). Interestingly, of these, the four strains with the largest number of ABC systems (*B. melitensis* 16M, *B. abortus* 9-941, *B. suis* 1330, and *B. canis* RM6/66) are all of species able to cause disease in humans (whereas there are no reported cases of *B. ovis* infections in humans). These four strains also have lower numbers of predicted mutated genes within their ABC systems when compared to *B. ovis* 63/290.

Table 3.1: Total open reading frames (ORFs) for all sequenced *Brucella* strains with number of ABC system encoding genes.

Numbers in brackets denote the number of ABC system genes expressed as a percentage the total number of ORFs. Mutated genes refers to any gene with a frame shift mutation or premature stop codon.

	<i>Brucella melitensis</i>	<i>Brucella suis</i>	<i>Brucella canis</i>	<i>Brucella abortus</i>	<i>Brucella ovis</i>
Chromosome I					
Total Open Reading Frames (ORFs)	2059	2185	2102	2158	2098
ABC system ORF (%)	118 (5.7)	113 (5.2)	105 (5.0)	110 (5.1)	104 (5.0)
Total functional ABC systems	38	34	34	29	30
Pseudogenes	3	10	3	11	10
Chromosome II					
Total ORFs	1138	1203	1149	1139	1105
ABC system ORF (%)	187 (16.4)	179 (14.9)	175 (15.2)	178 (15.6)	162 (14.7)
Total functional ABC systems	41	38	40	35	29
Pseudogenes	7	7	8	14	19
Total					
Total ORFs	3197	3296	3251	3389	3203
ABC system ORF (%)	305 (9.5)	292 (8.9)	280 (8.6)	288 (8.5)	266 (8.3)
Total functional ABC systems	79	72	74	64	59
Pseudogenes	10	17	11	26	29

3.4 Numbers of ABC systems in intracellular vs. environmental bacteria

Brucella genomes encode a high percentage of ABC system genes, with an average of 8.8% of their 3.3 Mb genomes dedicated to ABC systems. This high proportion of ABC systems in *Brucella* species is also reinforced when comparing the proportion of ABC systems in other intracellular pathogenic bacteria. For example, *Burkholderia pseudomallei* and *Burkholderia mallei* have 105 (5.8%) and 77 (4.9%) predicated ABC systems, respectively, and their genomes are 7.2 Mb and 5.8 Mb in size [238,136,131]. The percentage of ABC system genes encoded in the genomes of the intracellular pathogens *Francisella tularensis* (1.8 Mb) and *Mycobacterium tuberculosis* (4 Mb) are only 1.2% and 2.5%, respectively, showing that there is no great need for intracellular bacteria to encode large numbers of ABC system genes [18,179,31]. Studying an even larger range of intracellular bacteria highlights that, in terms of numbers of ABC systems, *Brucella* species are less like their intracellular counterparts (figure 3.1). The high proportion of ABC system encoded on the *Brucella* genome could be related to their ancestry with other environmental bacteria such as *Nitrobacter* and *Agrobacterium*. These bacterial species are also members of the α -proteobacteria and have also been shown to encode high proportions of ABC systems compared to their genome sizes [132] (figure 3.2). This high proportion of ABC systems encoded by these *Brucella* strains could increase their survival rates when in diverse conditions.

3.5 ABC system functions

The predicted functionality of the ABC systems within the *Brucella* genomes is dominated by ABC systems involved in the import of nutrients (table 3.2) and, although this is not uncommon among bacteria, it is probable that *Brucella* species utilise ABC transporters to provide most of the nutrients they require [132,78]. In support of the findings of Paulsen *et al.* [258], the larger 2.1 Mb chromosome encodes a large proportion of the ABC systems involved in molecular export and cellular process whereas the ABC systems located on the smaller 1.2 Mb chromosome are largely biased toward nutrient acquisition, leading to the theory that this second chromosome is important in the acquisition and processing of nutrients in *Brucella*.

In this study, the ABC systems of these *Brucella* strains have been classified into classes, families

Figure 3.1: Intracellular bacteria genome size vs. number of ABC systems. Adapted from [132].

Brucella species do not fit into the positive correlation seen between genome size and number of ABC systems created when studying intracellular bacteria.

Intracellular Bacteria	Genome size (Mb)	N° ABC systems
<i>Brucella melitensis</i> 16M	3.3	79
<i>Brucella suis</i> 1330	3.3	72
<i>Brucella abortus</i> biovar 1	3.3	64
<i>Brucella canis</i> ATCC 23365	3.3	74
<i>Brucella ovis</i> ATCC 28541	3.3	59
<i>Buchnera aphidicola</i> Sg	0.65	4
<i>Buchnera</i> sp APS	0.66	4
<i>Chlamydia muridarum</i> Nigg	1.1	8
<i>Chlamydia pneumoniae</i> AR39	1.2	15
<i>Chlamydia pneumoniae</i> CWL029	1.2	14
<i>Chlamydia pneumoniae</i> J138	1.2	14
<i>Mycobacterium leprae</i> TN	3.2	25
<i>Mycobacterium tuberculosis</i> CDC1551	4.4	35
<i>Mycoplasma genitalium</i> G-37	0.56	10
<i>Mycoplasma pneumoniae</i> M129	0.81	13
<i>Mycoplasma pulmonis</i> UAB CTIP	0.96	18
<i>Rickettsia conorii</i> Malish 7	1.3	17
<i>Rickettsia prowazekii</i> Madrid E	1.1	14
<i>Treponema pallidum</i> Nichols	1.4	18
<i>Francisella tularensis</i>	1.9	20
<i>Burkholderia pseudomallei</i>	7.2	105
<i>Burkholderia mallei</i>	5.8	77

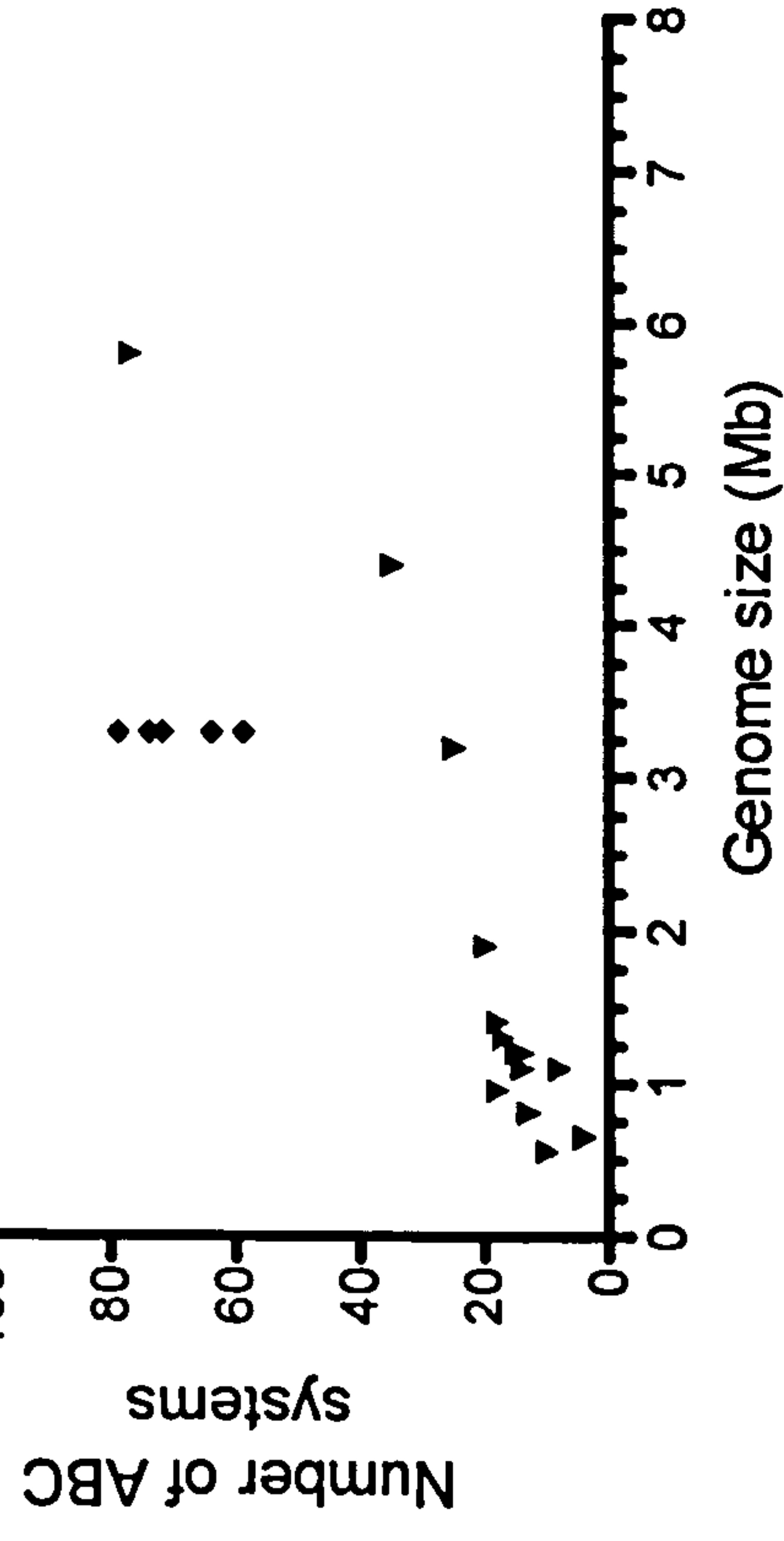


Figure 3.2: Environmental bacteria genome size vs. number of ABC systems. Adapted from [132].

Brucella species seem to fit better into the trend created by environmental bacteria when studying numbers of ABC systems.

This could be related to the ancestry of the *Brucella* species.

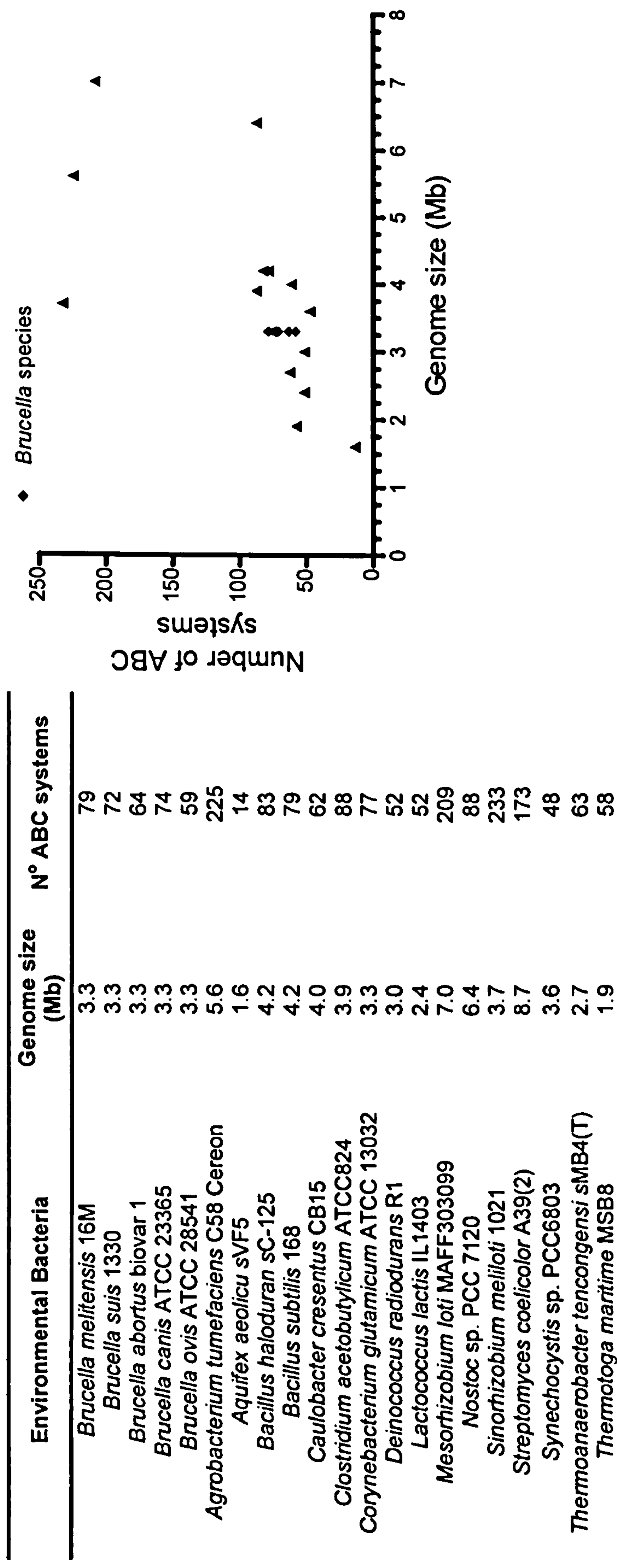


Table 3.2: *Brucella* ABC systems split into functional genres and split by chromosomal location

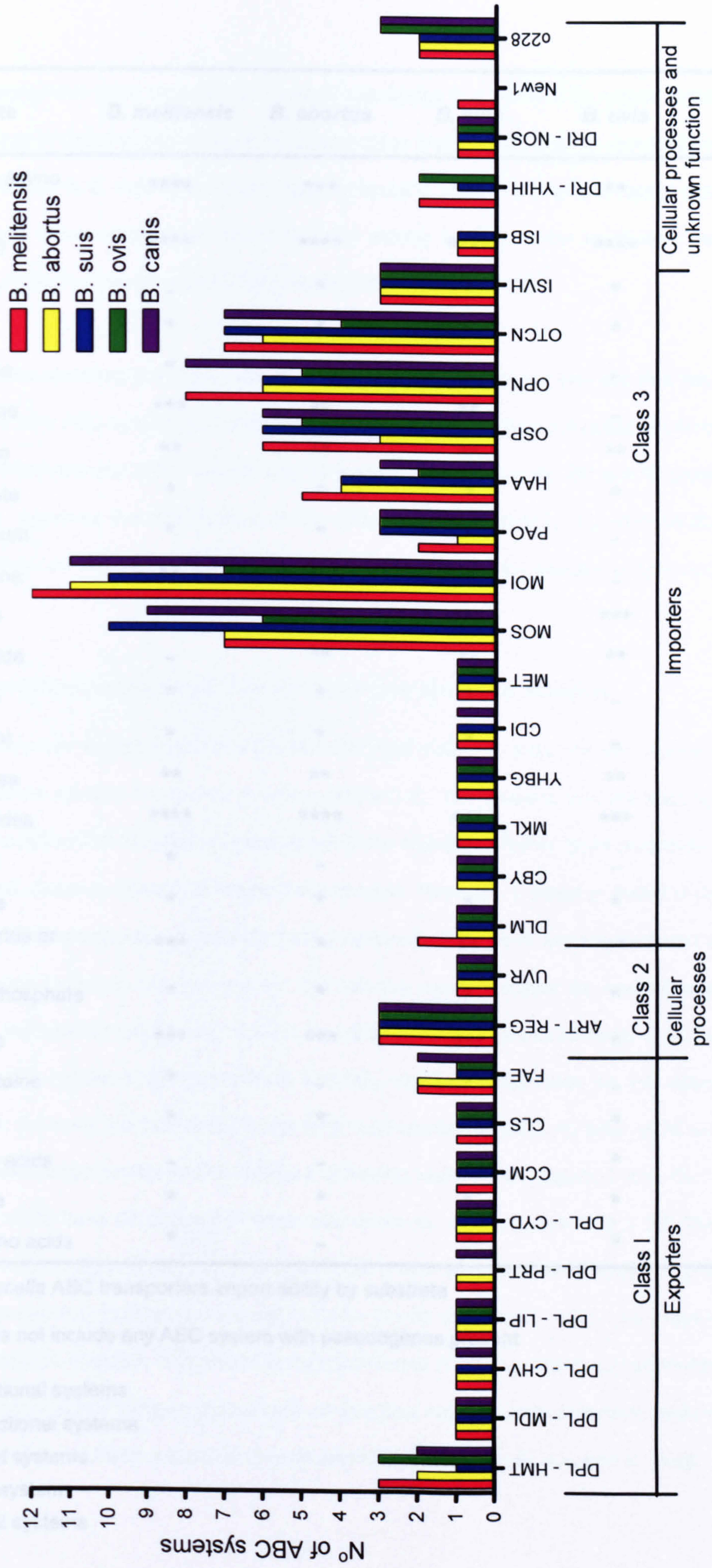
Numbers in brackets denote percentage of total ABC systems present on each chromosome or over both chromosomes

	<i>Brucella melitensis</i>	<i>Brucella suis</i>	<i>Brucella canis</i>	<i>Brucella abortus</i>	<i>Brucella ovis</i>
	Chromosome I				
Functional ABC systems (%)	38	34	34	29	29
N° of Importers (%)	20 (25)	20 (28)	19 (26)	16 (25)	16 (27)
N° of Exporters (%)	9 (11)	6 (8)	8 (11)	6 (9)	6 (10)
N° of Cellular process and unknowns (%)	9 (11)	8 (11)	7 (9)	7 (11)	8 (14)
	Chromosome II				
Functional ABC systems (%)	41	38	40	35	30
N° of Importers (%)	36 (46)	35 (49)	36 (49)	31 (48)	24 (41)
N° of Exporters (%)	3 (4)	2 (3)	3 (4)	3 (5)	3 (5)
N° of Cellular process and unknowns (%)	2 (4)	1 (1)	1 (1)	1 (2)	2 (3)
	Total				
Total functional ABC systems	79	72	74	64	59
Total N° of Importers (%)	56 (71)	55 (76)	55 (74)	47 (73)	40 (68)
Total N° of Exporters (%)	12 (15)	8 (11)	11 (15)	9 (14)	9 (15)
Total N° of Cellular process and unknowns (%)	11 (14)	9 (13)	8 (11)	8 (13)	10 (17)

and sub-families (figure 3.3) according to the functional classification system described by Dassa and Bouige [68]. The *Brucella* strains encode 8-12 class 1 systems, characterised by an ABC-IM domain fusion and comprising predicted export systems, and 5 class 2 systems, characterised by two fused ABCs and with predicted functions in antibiotic resistance and house-keeping. However, it has been observed that most of the ABC systems of *Brucella* species belong to class 3 with roles predicted in import processes. The further classification of *Brucella* ABC systems into families and sub-families shows that there are a high number of ABC systems of specific importer families, particularly the MOI (minerals and organic ions), MOS (monosaccharide), OPN (oligopeptides and nickel), OSP (oligosaccharides and polyols) and OTCN (osmoprotectants taurine cyanate and nitrate) families, all of which primarily function to acquire nutrients. Importing ABC transporters have also been shown to be important in nutrient acquisition by *Agrobacterium tumefaciens* and *Sinorhizobium meliloti*, two other α -proteobacteria, showing that ABC transporters are particularly important at nutrient acquisition for this genus [109,357].

Since these ABC systems have been identified by blast searches for similar systems, it is possible to assign each ABC system with a predicted substrate which it imports. This data can be used to assess the substrate-specific ABC transporter import ability for all of the *Brucella* strains. Table 3.3 shows the range of predicted substrates imported via ABC transporters present in the *Brucella* genomes. Overall, the results indicate that there is little difference in the import ability of the four strains of *Brucella* that are pathogenic to humans. However, an interesting observation that can be made from this data is that *B. ovis* is lacking the ability to import 8 of the 26 listed nutrients via ABC transporters. In fact, all of the 29 pseudogenes that are present within the *B. ovis* ABC system inventories occur within nutrient import systems. The nutrients that *B. ovis* is unable to import using ABC transporters include polyamines (specifically spermidine and putrescine), nickel, thiamine, glycine betaine, erythritol, xylose and molybdenum. It is possible that the defective uptake of these substrates by *B. ovis* may contribute to its likely lack of virulence in humans. For example, spermidine and putrescine are important organic polycationic molecules for maintaining conformation of nucleic acids, efficient DNA replication, transcription and translation. Polyamines have more recently been associated with bacterial virulence and pathogenicity in humans

Figure 3.3: Brucella ABC systems broken down by ABC family – subfamily and arranged by family type.



ABC system class/family - sub-family

Substrate	<i>B. melitensis</i>	<i>B. abortus</i>	<i>B. suis</i>	<i>B. ovis</i>	<i>B. canis</i>
Branch chain amino acids	****	***	***	**	***
Iron (III)	****	****	****	****	****
Cobalt	-	*	*	*	*
Zinc	*	*	*	*	*
Thiamine	*	*	*	-	*
Putrescine	***	**	**	-	**
Sulphate	**	**	**	**	**
Phosphate	*	*	*	*	*
Molybdenum	*	*	*	-	*
Spermidine	**	**	*	-	*
Ribose	***	***	***	***	***
Galactoside	-	**	**	**	*
Xylose	*	*	*	-	*
Erythritol	*	*	*	-	*
Dipeptides	**	**	**	**	***
Oligopeptides	****	****	***	***	****
Nickel	*	-	*	-	*
Maltose	*	*	*	*	*
Oligosaccharide or polyol	***	*	**	**	***
SN-glycerol-3-phosphate	*	*	*	*	-
Taurine	***	***	***	*	***
Glycine betaine	*	-	*	-	*
Nitrate	*	*	*	*	*
Polar amino acids	-	-	-	*	*
Cystine	*	*	*	*	*
General L amino acids	*	-	*	*	*

Table 3.3: *Brucella* ABC transporters import ability by substrate

This table does not include any ABC system with pseudogenes present.

**** \geq 5 functional systems

*** 3 or 4 functional systems

** 2 functional systems

* 1 functional system

- No functional systems

[296] and therefore have been targeted as vaccine candidates in a number of human pathogens [295,130]. The loss of nickel transport might not have a large impact on *B. ovis* as knockout mutants in the *nik* gene cluster in *B. suis* have shown little difference in intracellular growth rate to wild-type *Brucella*, although some enzymes do show reduced activity leading to the conclusion that the import of nickel could be compensated for by other bacterial systems [153].

This study has also identified one ABC system present in the *B. melitensis* genome that has been categorised as a new ABC system (currently labelled NEW1). The system comprises 4 proteins, 1 ABC protein, 1 IM protein and 2 BP. The BP and IM seem to be related to the BP and IM proteins of the MOS family. However, the ABC protein is too different to be related to the MOS family. It is unknown what this new ABC system is yet; experimental data would be needed to help define its function.

3.6 ABC systems/genes absent in at least one *Brucella* species

Although there is much similarity between the *Brucella* ABC systems, there are also systems that are present in some species but absent in others (table 3.4). The systems that are missing from each species are not critical for bacterial survival but these missing systems could contribute to the pathogenicity and virulence of each of the *Brucella* species. There are a range of systems missing from each of the *Brucella* species. *B. ovis* (5 missing systems), *B. canis* (4 missing systems) and *B. abortus* (4 missing systems) are the species that are the most disrupted by missing systems. Strikingly, all of the species are missing at least one o228 system. There is currently only one well characterised o228 system in *E. coli*, termed LolCDE, which is responsible for the release of lipoproteins from the inner cell membrane to the outer cell membrane [361]. All other o228 systems have as yet undiscovered functions. This makes it difficult to assess what impact, if any, the loss of these systems would have on *Brucella* lifestyle and virulence. The absence of the ISB (formally ABCX) system from the genomes of *B. ovis* and *B. canis* is a very interesting observation as the ISB systems are soluble complexes involved in labile [Fe-S] biogenesis, which are important in resistance to oxidative stresses. This indicates that *B. ovis* and *B. canis* reside in environments that are low in oxygen or high in oxygen reductants, or that they lack enzymes that need labile [Fe-S] centres [232,244] This could be a factor of the reduced virulence of both *B. ovis* and *B. canis*.

Table 3.4: ABC Systems/Genes absent in at least one species when compared to *B. melitensis*

#	Family	Subfamily	Substrate/Function	Type	<i>B. melitensis</i>	<i>B. abortus</i>	<i>B. suis</i>	<i>B. ovis</i>	<i>B. canis</i>
5	CCM		Possibly heme export	IM	BMEI1851	-	+	+	+
		IM		BMEI1852	-	+	+	+	
		ABC		BMEI1853	-	+	+	+	
6	CDI		Involved in cell division	IM	BMEI0073, fsx	+	+	-	+
		ABC		BMEI0072, fsE	+	+	-	+	
7	CLS		O antigen export system	ABC	BMEI1416, rfbB	-	+	+	+
		IM		BMEI1415, rfbD	-	+	+	+	
13	DPL	PRT	Proteases, lipase, S-layer protein export	OMP	BMEI1029	+	-	+	
14	DPL	CHV	Beta-(1->2) glucan export	IM-ABC	BMEI0984	+	+	+	
16	DPL	HMT	Involved in mitochondrial export systems	IM-ABC	BMEI1743	-	-	-	-
				IM-ABC	BMEI1742	-	-	+	-
22	FAE		Fatty acid export	IM-ABC	BMEI0976	+	+	+	
31	ISB (ABCX)		Iron/sulphur centre biogenesis	CYTP	BMEI1040	+	+	-	-
		CYTP		BMEI1042	+	+	-	-	
		ABC		BMEI1041	+	+	-	-	
36	MKL		Involved in toluene tolerance	ABC	BMEI0964	+	+	+	-
		IM		BMEI0965, tg2B	+	+	+	-	
		SS		BMEI0963, tg2C	+	+	+	-	
60	o228		Unknown	IM	BMEI0361	-	-	-	-
		MFP		BMEI0359	-	-	-	-	
		ABC		BMEI0360	-	-	-	-	
61	o228		Unknown	IM	-	BruAb10085	+	+	-
		MFP		-	-	-	-	BCAN_A1712	
		ABC		-	BruAb10084	+	+	+	
62	o228		Unknown	MFP	-	+	+	BOV_1617	
		IM-ABC		-	+	+	+	BCAN_A0087	

Excluding ABC systems involved in import.

- = gene absent in that *Brucella* species

+ = gene present in that *Brucella* species

+ = Non-functional gene present in that *Brucella* species

= refers to ABC system number in the full inventories/alignments of *Brucella* ABC systems.

The CDI system missing in *B. ovis* is comprised of two proteins FtsE (ABC protein) and FtsX (IM protein) [183]. This system has been extensively studied in *E. coli* and other bacteria including *Bacillus subtilis* [113] and *Mycobacterium tuberculosis* [220]. The CDI system is involved in cell division and it has been shown that mutants in the *ftsE* gene in *E. coli* have reduced growth capacity [286]. Of all of the *Brucella* species only *B. canis* is missing the MKL systems. It is unknown if not having the MKL system has any impact on the lifestyle of *B. canis*. However, research using *Pseudomonas putida* has shown that this system might have some link to toluene tolerance as Tn5 insertions within the *ttgA2* gene, which codes for the MKL ABC protein, elicits a toluene-sensitive phenotype [339].

3.7 Conclusions

In this study the ABC systems of *B. melitensis* 16M, *B. suis* 1330, *B. abortus* 9-941, *B. canis* RM6/66 and *B. ovis* 63/290 have been re-annotated using the ABCISSE database in order to provide new information and a uniform annotation and classification of ABC systems in these closely related species. The creation of ABC system inventories has been completed for a variety of organisms [31,69,191,268]. These inventories are all designed to assist in the understanding of the bacterial lifestyles and increase our knowledge of the bacterial survival and virulence. Previous analysis of the *Brucella* genomes has shown that there is over 90% genome similarity between the six traditionally recognised *Brucella* species [337,338]. Similarly, the ABC systems inventories compiled in this work reflects the close similarities of the *Brucella* species. However, despite the high genetic homology of *Brucella*, this work has highlighted differences in the predicted numbers and functions of the ABC systems encoded by each *Brucella* species. It is widely accepted that the four species that may cause human brucellosis are *B. melitensis*, *B. suis*, *B. abortus* and *B. canis*. This study has shown that these four species of *Brucella* have a larger set of ABC systems encoded within their genomes than *B. ovis*, which is not known to cause human disease. Although it is difficult to ascertain the exact effect the loss these ABC systems has on *B. ovis*, it is possible to hypothesise that, along with other genetic differences observed [54], they contribute to its overall reduced virulence in humans. Overall, the identified differences observed in the ABC system inventories of the *Brucella* strains studied should contribute to a greater understanding of differences in the lifestyles of the *Brucella* species.

Chapter 4 - Evaluation of vaccine candidates

4.1 Introduction

Current *Brucella* vaccines are generally live attenuated vaccines and although this type of vaccine does elicit a cellular immune response, they do have many disadvantages. *Brucella melitensis* Rev.1 is one of the most widely used animal vaccines but can still cause infections in vaccinated animals [29] and is also indistinguishable from a natural *Brucella* infection during diagnostic testing [291]. Additionally, Rev.1 can cause disease in humans and is therefore unsuitable as a human vaccine. A protective sub-unit vaccine would be an effective way of decreasing the problems associated with *Brucella* around the world. Such sub-unit vaccines often have fewer side effects than live attenuated vaccines and would be easily distinguished from naturally infected animals due to the specific immune responses generated.

4.1.1 Selection of vaccine candidates

Eight ABC transporter proteins were selected for evaluation as sub-unit vaccine candidates using literature searches to identify homologous protective ABC transporter proteins from other bacteria [316,130,18,295] and homologous ABC transporter proteins involved in virulence in *Brucella* species [164,168,94,277]. The down-selected proteins were PotD, PotF, Cgt, CydD, LolE, FbpA, OppA and ZnuA.

4.1.1.1 PotD (BMEI0923) and PotF (BMEI0411)

The Pot systems in *E. coli* are responsible for the import of polyamines (putrescine, spermidine and spermine). Polyamines are required for various cellular functions including nucleic acid and protein synthesis. They are part of the mineral and organic ions (MOI) import family of ABC transporters [107]. Polyamines are the cell's major sources of polycations which bind to intracellular polyanions such as nucleic acids and ATP to modulate their structure [142,68]. The crystal structure of both the *E. coli* PotF and PotD proteins have been resolved and, despite only having 35% amino acid homology, they are structurally very similar [331,310]. PotF is the binding protein of the putative potFGHI putrescine import system of *B. melitensis* 16M. This system is specific for putrescine and cannot bind other polyamines. In *E. coli* PotD is the binding protein of the putative potABCD spermidine import system. PotD preferentially binds spermidine although it can bind putrescine at a

lower affinity [142]. *Brucella melitensis*, *B. abortus*, *B. suis* and *B. ovis* all encode the putative potABCD system within their genomes. In comparison, all four species have the potFGHI system present but *B. abortus* and *B. ovis* have mutations in one or more of the system component proteins which may inactivate the system or reduce its functionality. There is now evidence in the literature that polyamines have roles in bacterial pathogenesis [296].

4.1.1.2 Cgt (BMEI0984)

Cgt is an ATP-binding cassette protein fused to an inner membrane protein (ABC-IM). Cgt forms part of the β (1-2) glucan (CHV) export subfamily which is a part of the drugs, peptides and lipids (DPL) ABC family [68]. Cgt is involved in β (1-2) glucan export in *Brucella* [277]. One study has shown that *B. abortus* mutants lacking the Cgt genes are able to infect HeLa cells as efficiently as wild-type bacteria, but intracellular replication in HeLa cells was significantly reduced indicating that the Cgt genes could be important for intracellular survival [277].

4.1.1.3 CydD (BMEI0762)

CydD is an ABC-IM fusion protein that is part of the DPL family, within the cytochrome bd biogenesis (Cyd) subfamily of ABC systems [68,266]. The Cyd genes are involved in oxidoreduction and form part of the *cydDCAB* operon, in which CydD assists in the construction of the cytochrome *bd* oxidase encoded by the *cydAB* genes [168]. The Cyd operon has been shown to be important for *Brucella* virulence, as *B. abortus* CydB knockout mutants are shown to have increased sensitivity to low pH and reactive oxygen intermediates [93].

4.1.1.4 LoIE (BMEI1139)

The Lol ABC systems are required for the release of lipoproteins from the inner membrane into the periplasm for sorting into the outer membrane [234]. These Lol systems comprise *lolCDE* and *lolAB* genes where *lolCDE* is responsible for the lipoprotein recognition and *lolAB* are binding proteins that assist in the movement of the lipoprotein across the periplasm [234]. LoIE is part of a putative *lolED* system in *Brucella* and it lies within the o228 ABC family [68]. Generally, the o228 ABC family is not well understood and a specific function has not yet been assigned. There are some o228

genes without a function assigned present within the *B. melitensis* 16M genome and it is possible that these are the missing lol system genes in *Brucella*.

4.1.1.5 FbpA (BMEII0584)

FbpA is an iron binding protein that is part of the MOI ABC family [68]. FbpA is responsible for the uptake of iron (III) [94]. Research has shown that the *B. abortus* FbpA is expressed in macrophages after 24 hrs growth [94], demonstrating that it could be important for *Brucella* survival in the intracellular environment.

4.1.1.6 OppA (BMEII0735)

OppA is the binding protein of the OppABCD ABC system. OppA is part of the oligopeptide and nickel (OPN) ABC importer family [68]. Studies of the *E. coli* and *Salmonella typhimurium* OppA have shown it to be one of the most abundant proteins in the periplasm of Gram-negative bacteria [134]. There are two putative sets of OppA genes in the *Brucella* genomes, each sharing a 70% sequence identity. This ensures that even though there may be a high concentration of OppA in the periplasm, there is still the ability to import a broad range of different oligopeptide molecules. OppA has been investigated as a vaccine candidate for *Yersinia pestis* [316]. Specifically, mice immunised with OppA adjuvanted with Alhydrogel showed a delayed time to death when challenged with *Y. pestis*.

4.1.1.7 ZnuA (BMEII0178)

Zinc is an important trace element for most organisms. There are a large number of enzymes or proteins that use zinc as a structural or catalytic cofactor [256]. ZnuA is the binding protein of the putative *znuABC* operon in *Brucella*, and it is part of the metal (MET) ABC import family [68]. Two studies have been completed on ZnuA in *B. abortus*. Kim *et al.* have shown that deletion of the *znuA* gene results in reduced intracellular survival both *in vitro* and *in vivo* [164]. Yang *et al.* showed that a *znuA* deletion mutant of *B. abortus* could be used as an attenuated live vaccine in mice [363]. The *B. abortus znuA* deletion mutant showed equivalent protection to *B. abortus* strain 19 and *B. abortus* RB51 vaccine.

4.2 Production of vaccine candidates

Eight ABC transporter proteins were selected as possible vaccine candidates (Table 4.1 and Section 4.1.1). The genes were located on the *B. melitensis* 16M annotated genome and visualised using the Artemis viewer. Membrane-spanning domains were identified using an internet-based programme called TMHMM version 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) [175] (Figure 4.1, section 2.1.2). PCR primers were designed to exclude membrane-spanning domains as they can cause problems in expression and purification processes. PCR was used to amplify the genes from *B. melitensis* 16M genomic DNA (section 2.3.1). PCR products were then ligated into the expression vectors pCR®T7/NT-TOPO® or pTrcHisA/B (section 2.3.3.1 and 2.3.3.2) (Invitrogen Ltd, Paisley, UK) and transformed into maintenance and expression strains of *E. coli*. Plasmid constructs were sequenced to check for PCR errors by Lark Technologies Inc (Takeley, Essex, UK) (section 2.3.3.4). Expression and solubility studies were carried out to assess the level of protein production and solubility of the protein produced (section 2.4.2.1 and section 2.4.2.2). Once optimal expression conditions were obtained, large scale expression of the vaccine candidates was started (section 2.4.2.3). Vaccine candidate purification was achieved using AKTA FPLC under the control of Unicorn Version 4.0 software (sections 2.4.2.6, 2.4.2.7 and 2.4.2.8). Examples of AKTA purification traces are shown in figures 4.2-4.9. An SDS-PAGE gel of all the purified vaccine candidate proteins are shown in figure 4.10.

Table 4.1: Candidates to be produced and evaluated as potential vaccines to *B. melitensis*

* = Inner membrane fused to an ATP-binding cassette

¶ = Geneart AG (Regensburg, Germany), constructs made by Geneart were codon optimised for use in *E. coli*

\$ = Membrane spanning domains

Protein	Full length or Truncated protein	Gene size (bp)	N° of MSD [‡]	Amino acid positions taken for truncates	Approximate size including 6xHis tag (kDa)	Vector plasmid used	Protein function (ABC family: Subfamily)
PotD	Truncated	972	1	25 - 349	39.8	PCR [¶] T7 NT-TOPO [¶]	Binding protein (MOI)
PotF	Truncated	1026	1	27 - 267	42.4	PCR [¶] T7 NT-TOPO [¶]	Binding protein (MOI)
Cgt	Truncated	933	5	290 - 599	38.7	PCR [¶] T7 NT-TOPO [¶]	IM-ABC (DPL:CHV)
CydD	Truncated	909	5	277- 578	35.8	PCR [¶] T7 NT-TOPO [¶]	IM-ABC (DPL:CYD)
LoIE	Truncated	684	5	52 – 278	28.8	PCR [¶] T7 NT-TOPO [¶]	Inner membrane protein (o228)
FbpA	Full length	1017	0	N/A	40.4	PCR [¶] T7 NT-TOPO [¶]	Binding protein (MOI)
ZnuA	Full length	987	0	N/A	39.3	pTrcHisB	Binding protein (MET)
OppA	Full length	1518	0	N/A	62.9	pTrcHisA	Binding protein (OPN)

Figure 4.1: Vaccine candidates membrane-spanning domains visualised using the web-based programme TMHMM v 3.0. Each red peak represents a putative membrane-spanning domain. X axis = number of amino acids

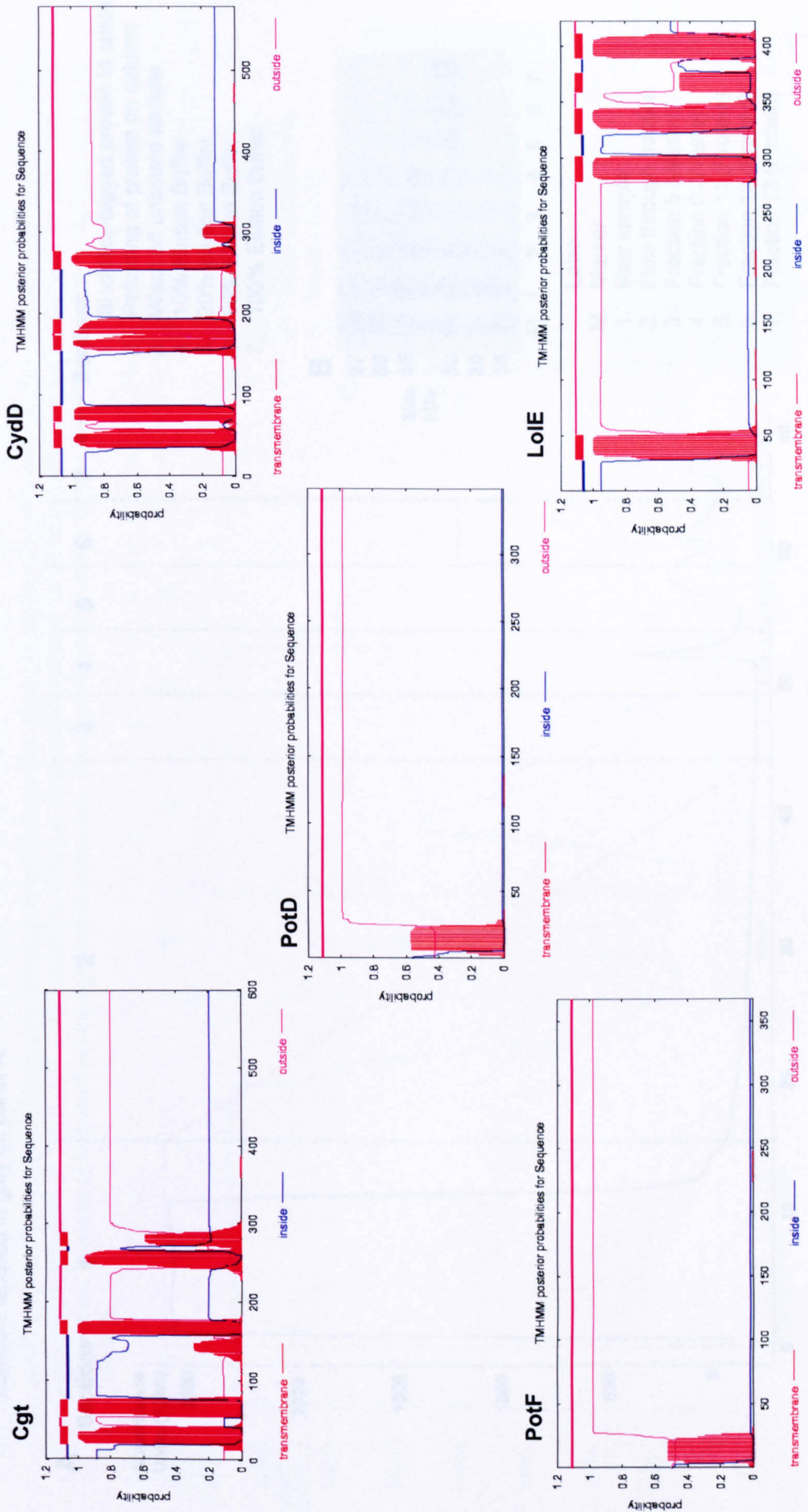


Figure 4.2: LolE purification
 FPLC trace showing fractions 1-23 of recombinant LolE purification (panel A) representing an insoluble protein purification from 4 litres of *E. coli* culture preparation. Sections 1-7 represent the different stages of the protein purification procedure. Panel B shows an SDS-PAGE gel of selected fractions of interest from the purification procedure. Fraction numbers in panel B represent fraction numbers labelled in grey on panel A.

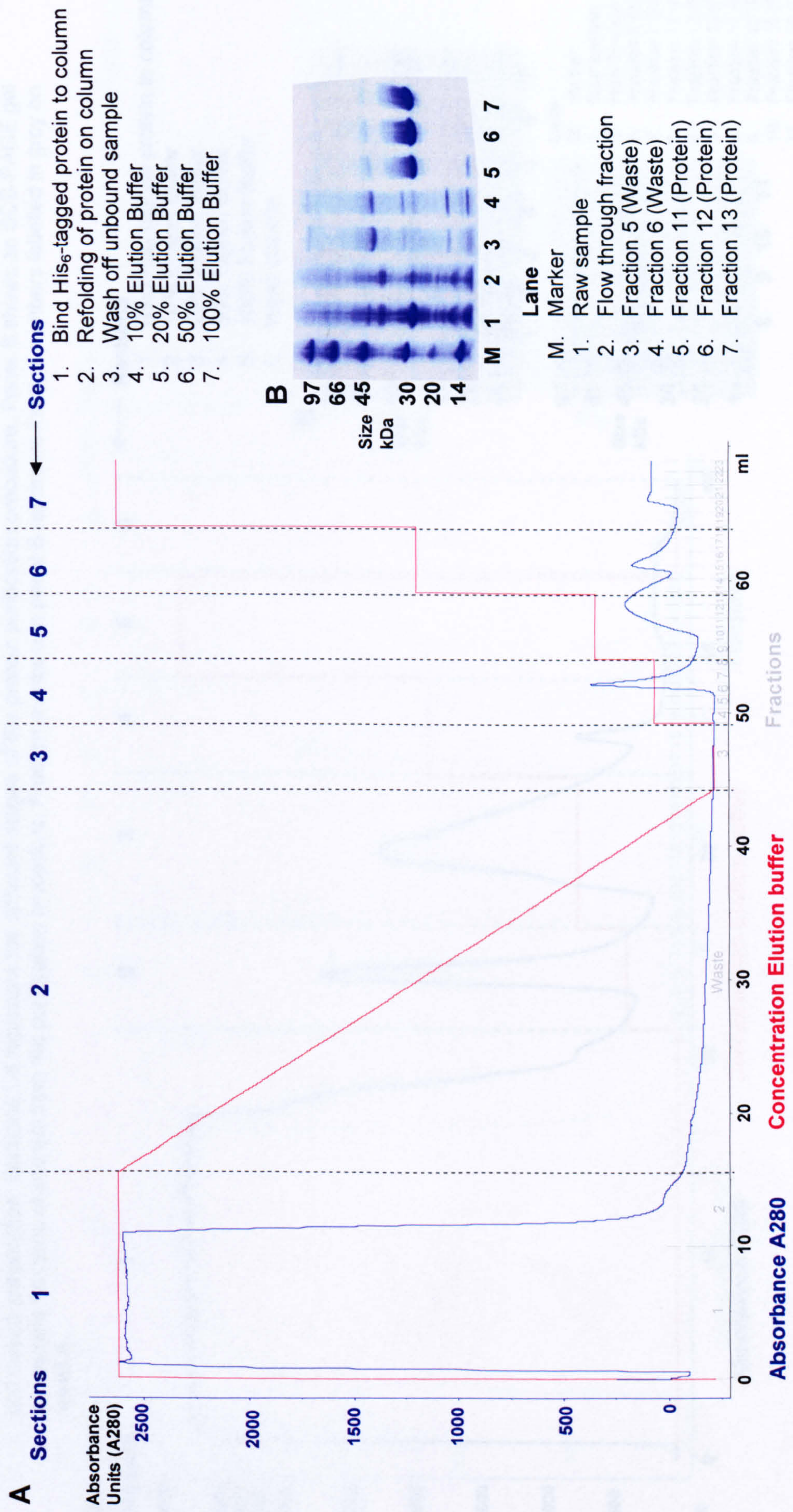


Figure 4.3: PotD Purification

FPLC trace showing fractions 1-26 of recombinant PotD purification (panel A) representing a soluble protein purification from 4 litres of *E. coli* culture preparation. Sections 1-6 represent the different stages of the protein purification procedure. Panel B shows an SDS-PAGE gel of selected fractions of interest from the purification procedure. Fraction numbers in panel B represent fraction numbers labelled in grey on panel A.

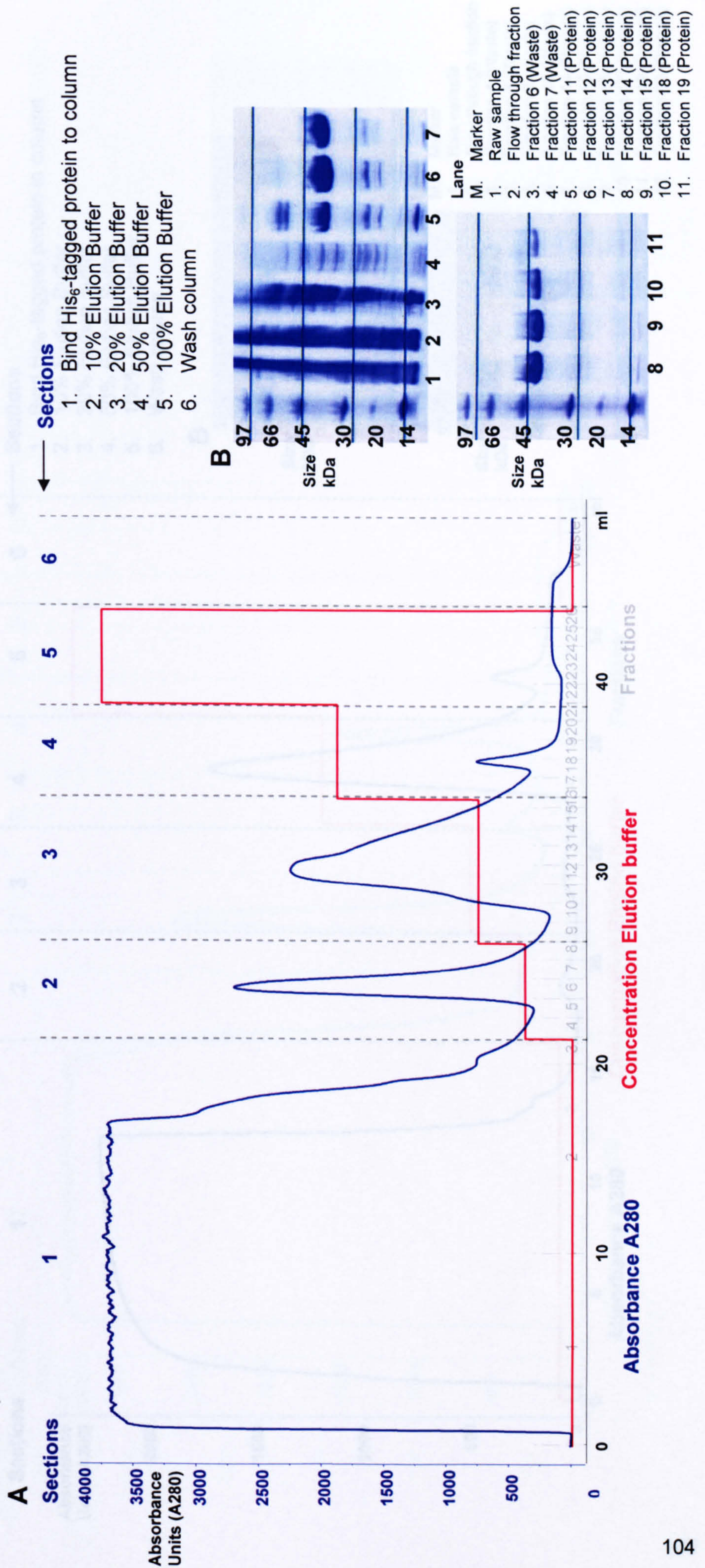


Figure 4.4: CydD Purification
 FPLC trace showing fractions 1-22 of recombinant CydD purification (panel A) representing a soluble protein purification from 4 litres of *E. coli* culture preparation. Sections 1-6 represent the different stages of the protein purification procedure. Panel B shows an SDS-PAGE gel of selected fractions of interest from the purification procedure. Fraction numbers in panel B represent fraction numbers labelled in grey on panel A.

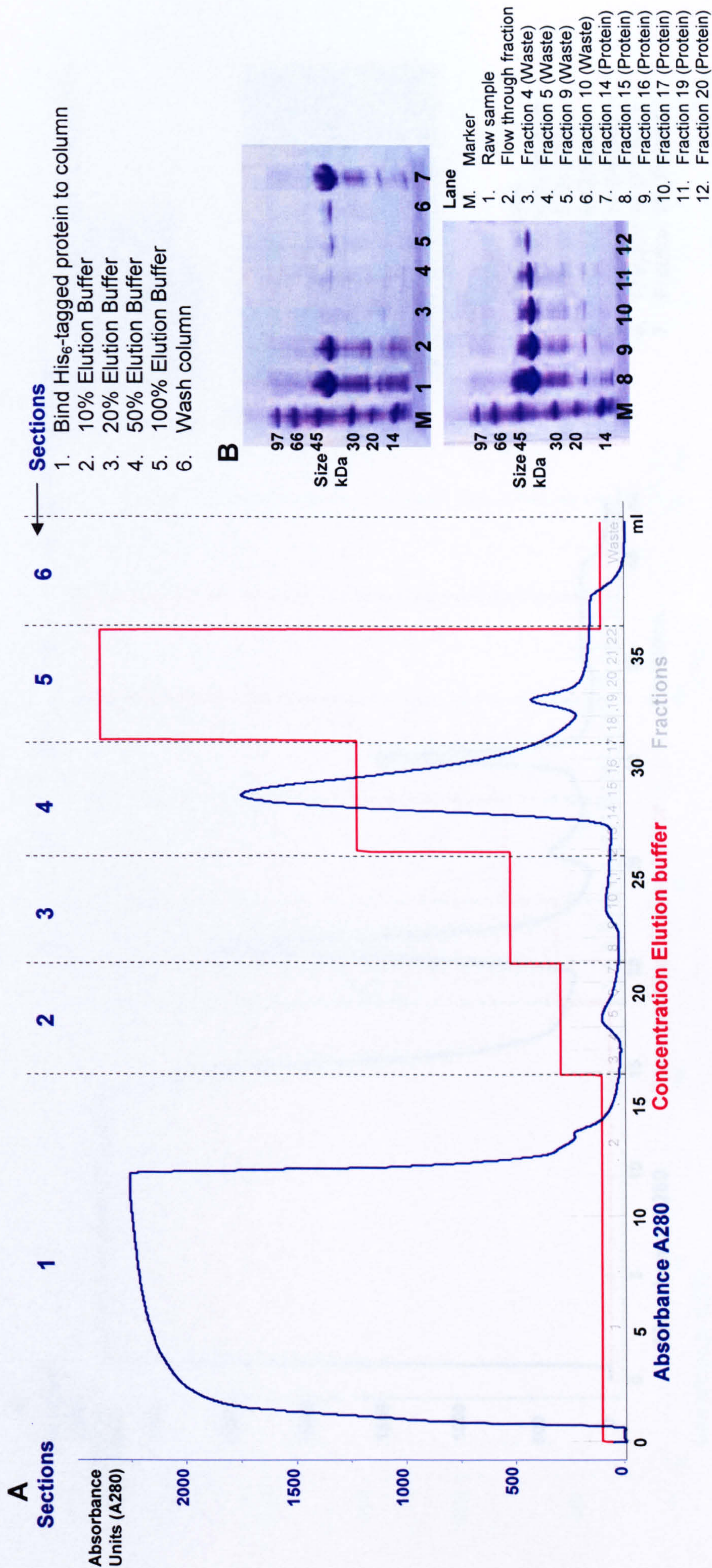


Figure 4.5: PotF Purification

FPLC trace showing fractions 1-22 of recombinant PotF purification (panel A) representing a soluble protein purification from 4 litres of *E. coli* culture preparation. Sections 1-6 represent the different stages of the protein purification procedure. Panel B shows an SDS-PAGE gel of selected fractions of interest from the purification procedure. Fraction numbers in panel B represent fraction numbers labelled in grey on panel A.

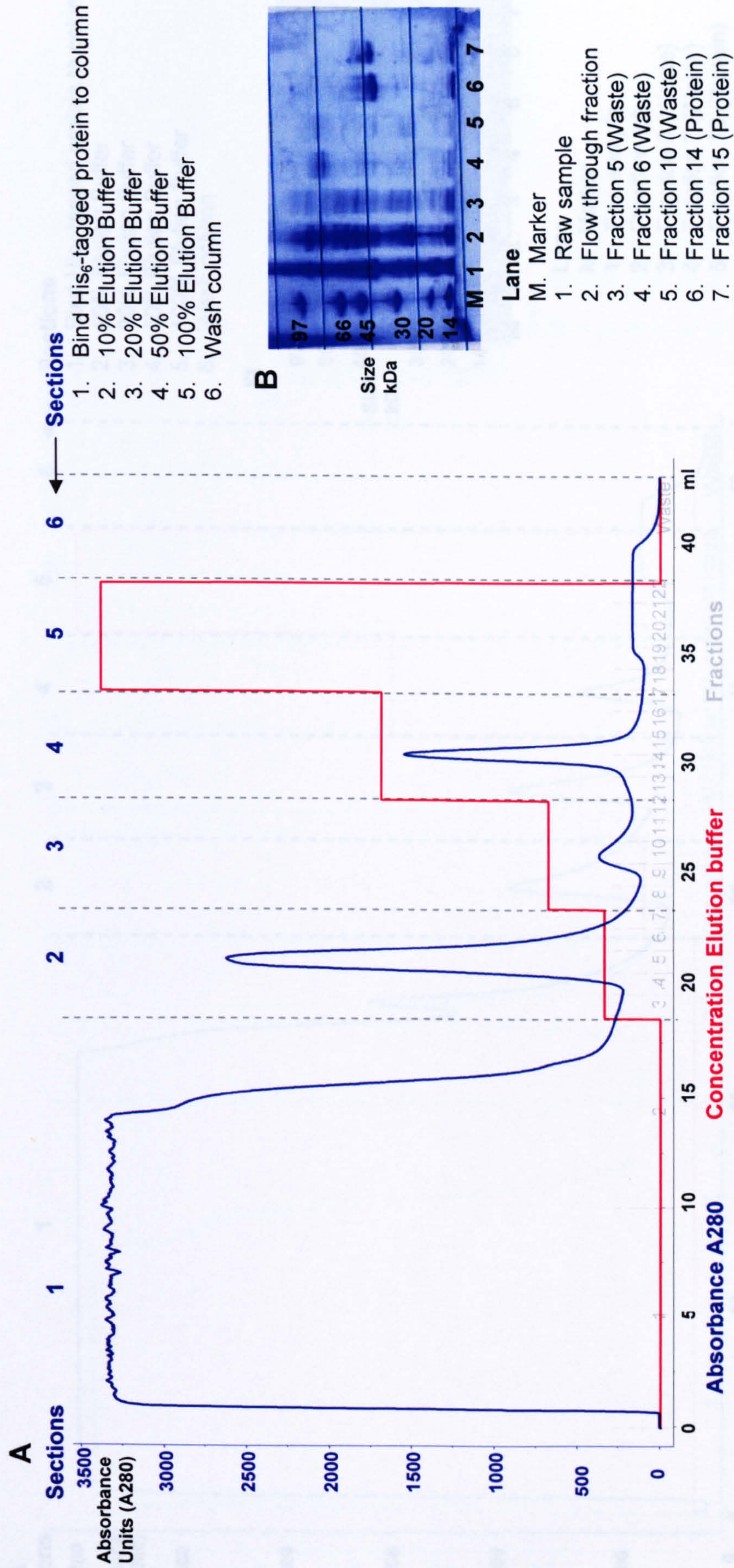


Figure 4.6: ZnuA Purification
 FPLC trace showing fractions 1-23 of recombinant ZnuA purification (panel A) representing a soluble protein purification from 4 litres of *E. coli* culture preparation. Sections 1-6 represent the different stages of the protein purification procedure. Panel B shows an SDS-PAGE gel of selected fractions of interest from the purification procedure. Fraction numbers in panel B represent fraction numbers labelled in grey on panel A.

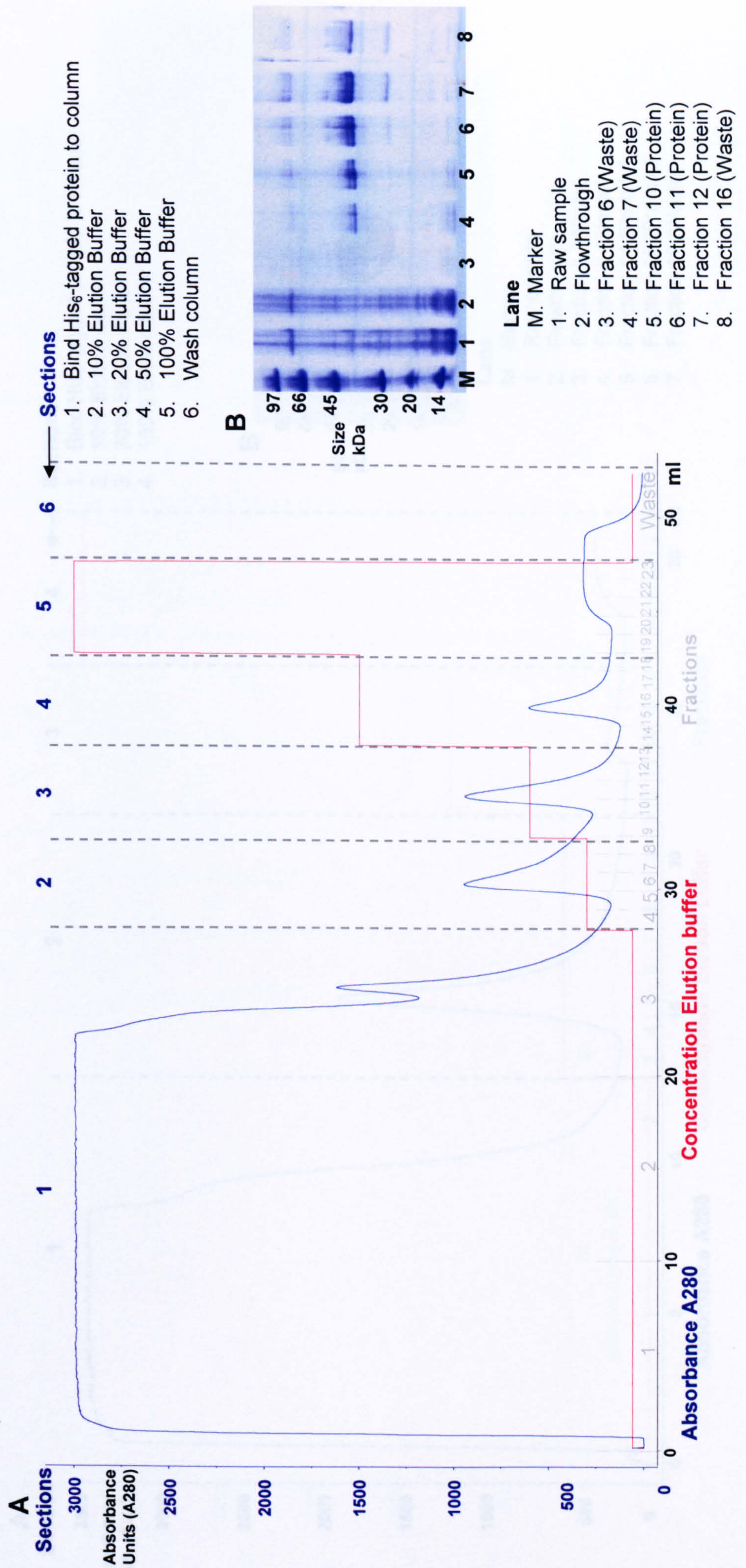


Figure 4.7: Cgt Purification
 FPLC trace showing fractions 1-21 of recombinant Cgt purification (panel A) representing a soluble protein purification from 4 litres of *E. coli* culture preparation. Sections 1-4 represent the different stages of the protein purification procedure. Panel B shows an SDS-PAGE gel of selected fractions of interest from the purification procedure. Fraction numbers in panel B represent fraction numbers labelled in grey on panel A.

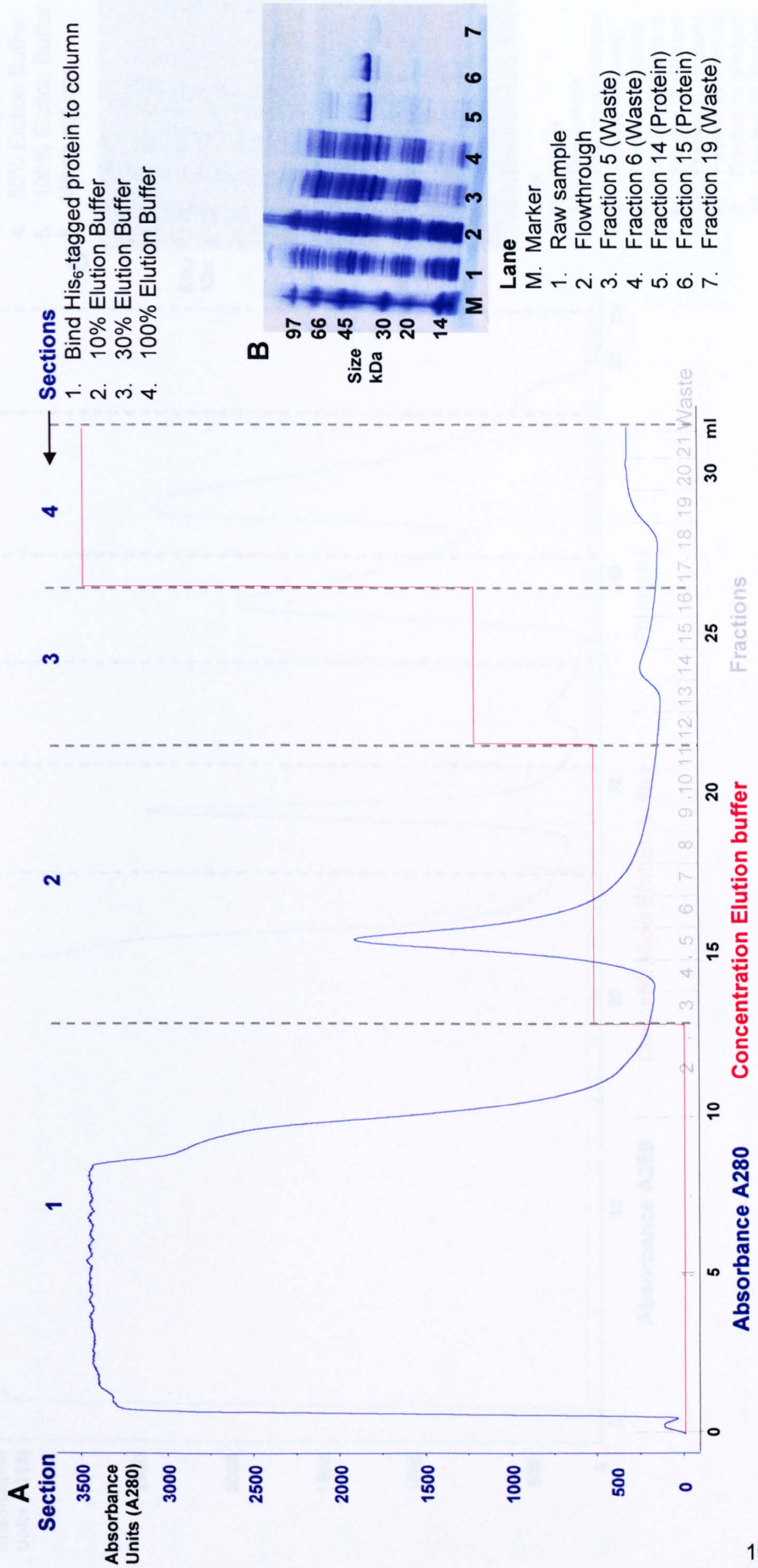


Figure 4.8: FbpA Purification

FPLC trace showing fractions 1-25 of recombinant FbpA purification (panel A) representing a soluble protein purification from 4 litres of *E. coli* culture preparation. Sections 1-4 represent the different stages of the protein purification procedure. Panel B shows an SDS-PAGE gel of selected fractions of interest from the purification procedure. Fraction numbers in panel B represent fraction numbers labelled in grey on panel A.

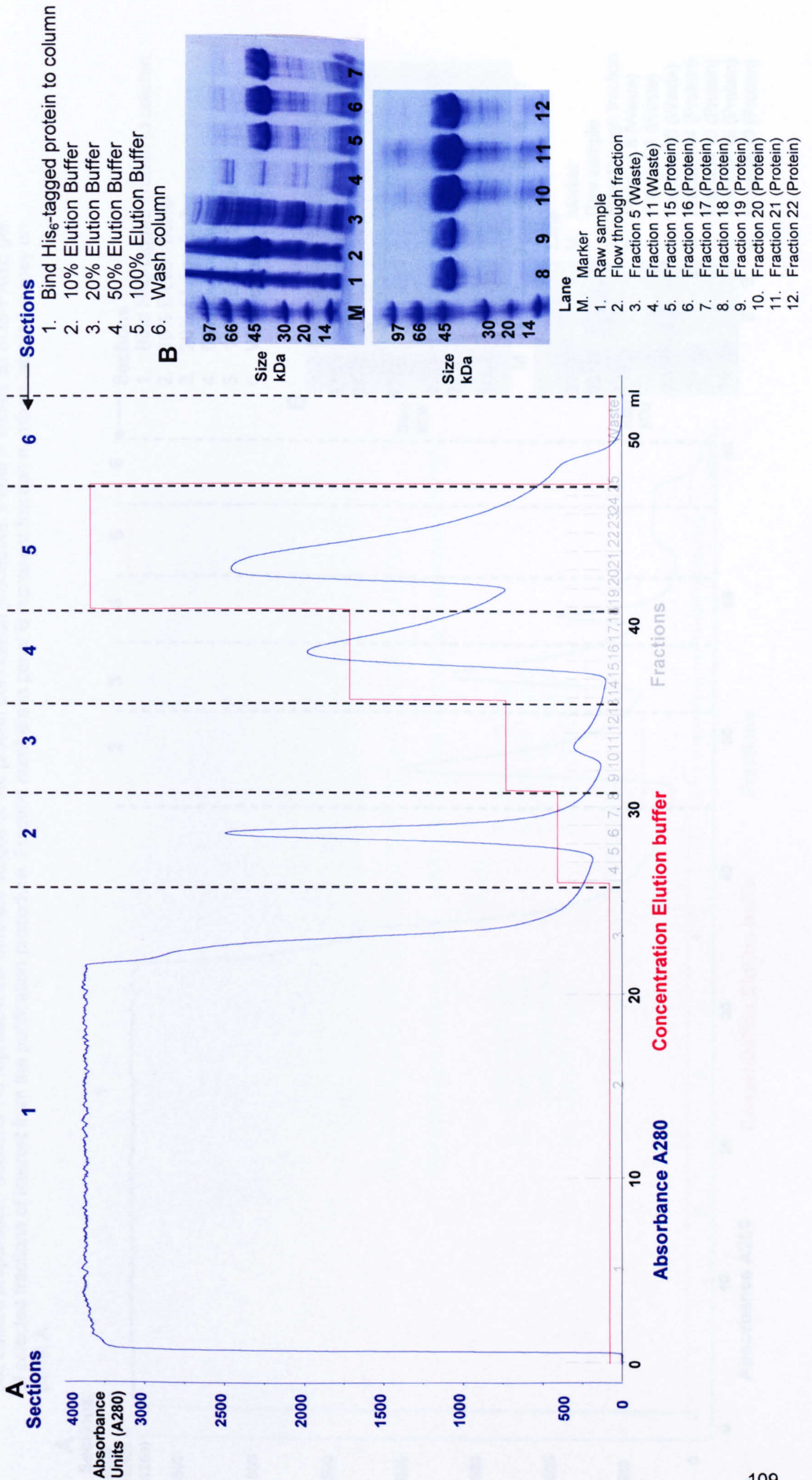
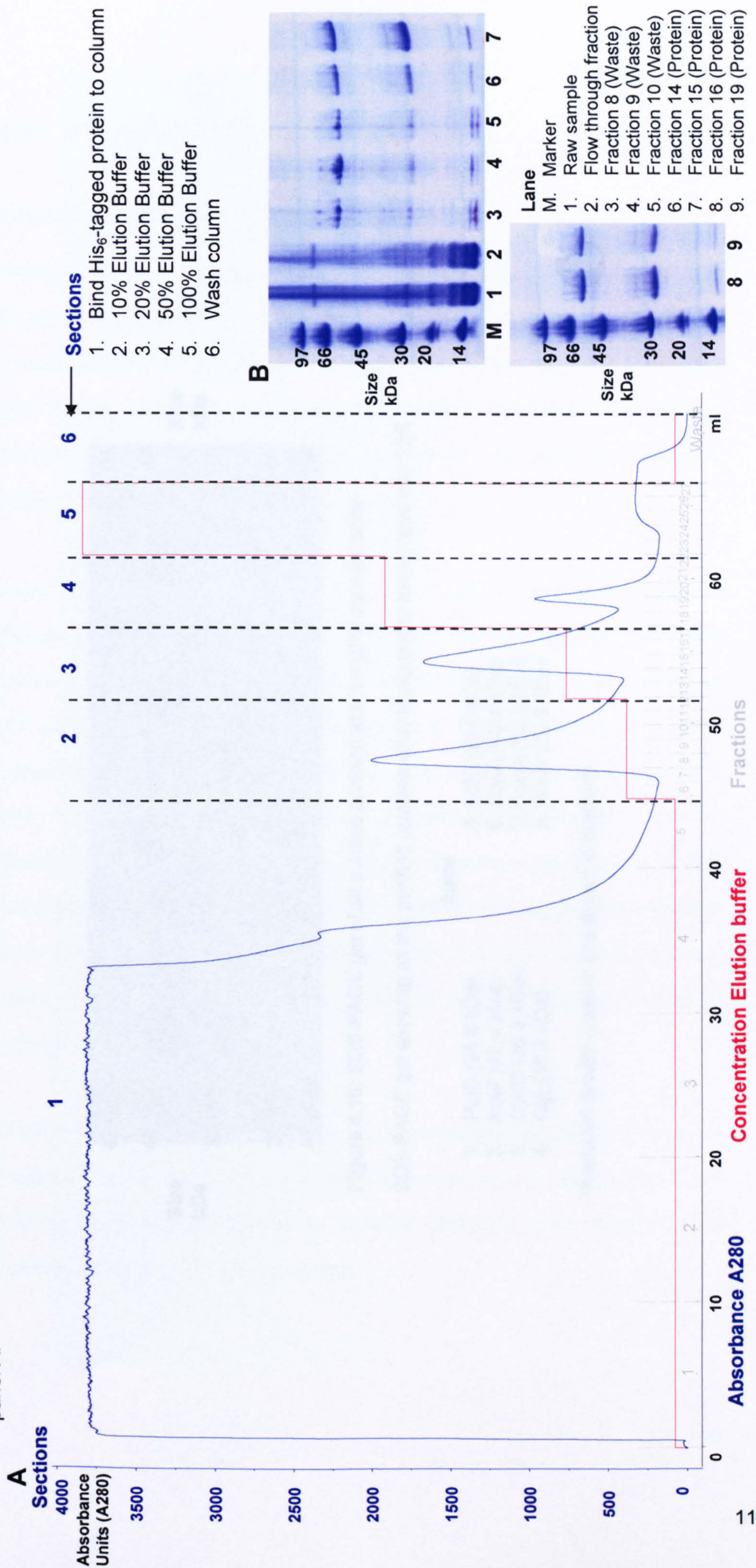


Figure 4.9: OppA Purification

FPLC trace showing fractions 1-27 of recombinant OppA purification (panel A) representing a soluble protein purification from 4 litres of *E. coli* culture preparation. Sections 1-6 represent the different stages of the protein purification procedure. Panel B shows an SDS-PAGE gel of selected fractions of interest from the purification procedure. Fraction numbers in panel B represent fraction numbers labelled in grey on panel A.



4.3 Evaluation of vaccine candidates

4.3.1 Expression of vaccine candidates

It is important to assess the expression of the vaccine candidates during growth to show the vaccine candidates genes are functional. To do this, genes encoding *B. melitensis* 1624, *B. sub* Thomsen and *B. abortus* 2302 produced during stationary phase growth was used to produce Western blots. These Western blots were probed using anti-*Brucella* specific monoclonal antibodies generated from the non-*Brucella* infected mice.

using ECL technology (Pierce and Warriner, 2004). The Western blot indicated the expression of the vaccine candidates.

The Western blot indicated the bands of the expected sizes for PotD (39.8 kDa), PotF (42.4 kDa), CydD (35.8 kDa), Cgt (38.7 kDa), 1060a, whereas expression of 1060a was only detected in two of the lanes.

expression is not detected in any of the lanes. The bands in the negative control lanes are of different sizes to the bands in the positive control lanes.

cell contaminants during the growth of the vaccine candidates. Although not all of the proteins were detected, the bands in the negative control lanes are of different sizes to the bands in the positive control lanes.

immunity. The immunized mice were then challenged with a single inoculum in the groin of the mice. Growth of vaccine candidates may not accurately reflect protein expression in a natural infection.

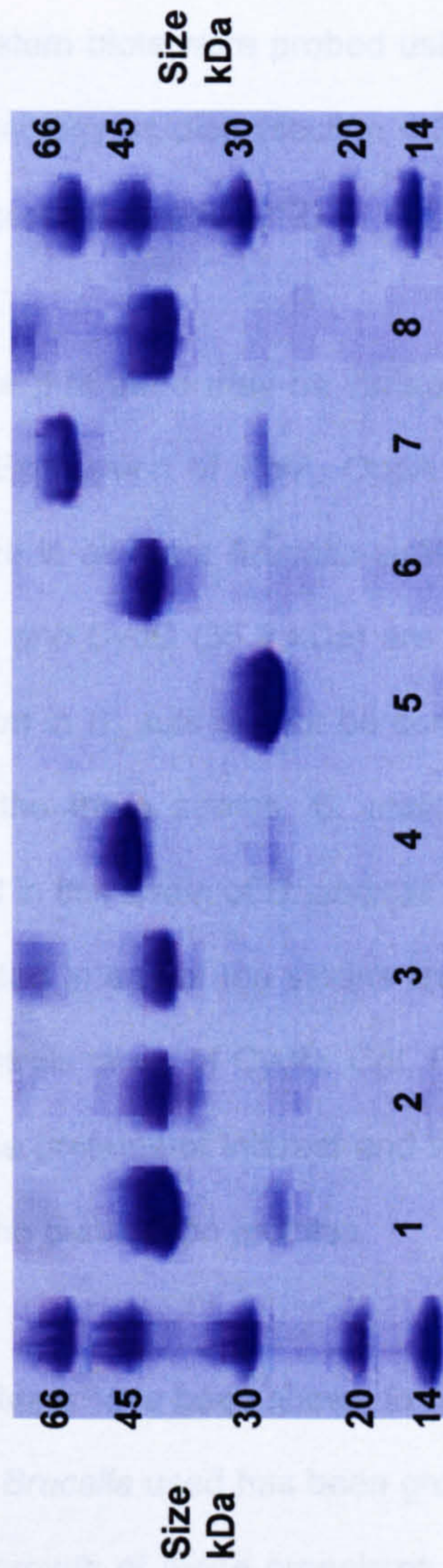


Figure 4.10: SDS-PAGE gel of all purified proteins at a 1 mg/ml concentration

SDS-PAGE gel showing all the purified proteins after being buffer exchanged into PBS.

- | Lane | |
|------|-----------------|
| 1. | PotD (39.8 kDa) |
| 2. | PotF (42.4 kDa) |
| 3. | CydD (35.8 kDa) |
| 4. | Cgt (38.7 kDa) |
| 5. | LoIE (28.8 kDa) |
| 6. | FbpA (40.4 kDa) |
| 7. | OppA (62.9 kDa) |
| 8. | ZnuA (39.3 kDa) |

Predicted protein sizes in are shown in brackets

4.3 Evaluation of vaccine candidates

4.3.1 Expression of vaccine candidates

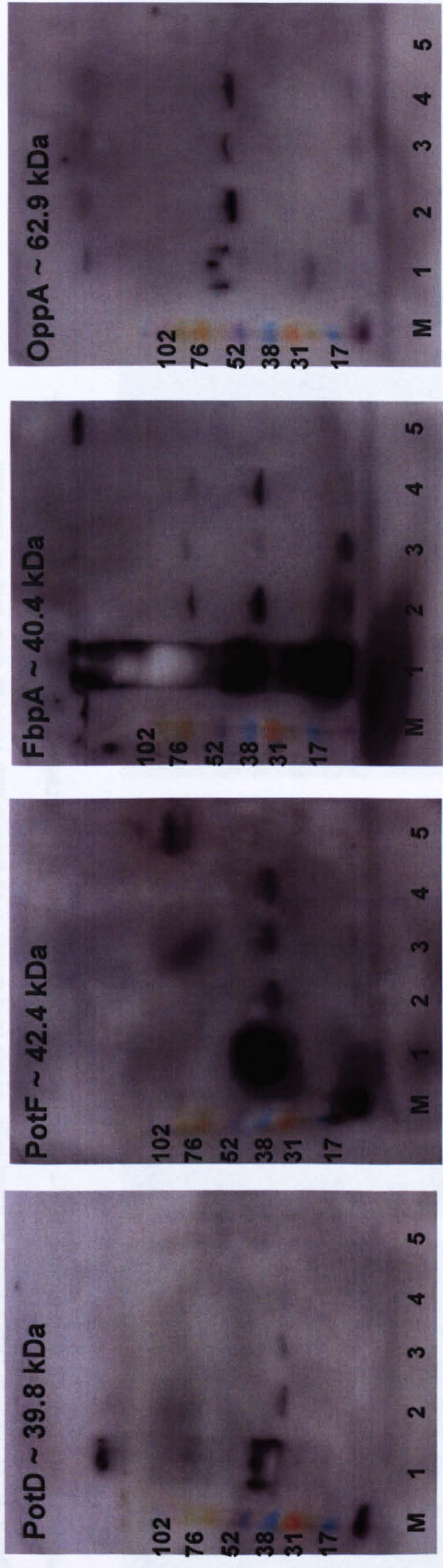
It is important to assess the expression of the vaccine candidates during growth to show the vaccine candidate genes are functional. To do this, gamma irradiated *B. melitensis* 16M, *B. suis* Thomsen and *B. abortus* 2308 produced during stationary phase growth was used to produce Western blots. These Western blots were probed using antigen specific polyclonal mouse antisera generated from the immunology studies (section 4.3.2). The Western blots were then developed using ECL technology (Figures 4.11 and 4.12).

The Western blots indicate that there may be variability between strains in expression of some of the vaccine candidates. Expression of PotF, OppA and LolE are the only candidates that yield bands of the expected size in all three *Brucella* strains tested. Bands of approximately the correct sizes for PotD (39.8 kDa) and CydD (35.9 kDa) are detected in *B. melitensis* 16M and *B. abortus* 10505, whereas expression in *B. suis* cannot be confirmed using this method. FbpA production is only detected in two of the three strains, *B. melitensis* 16M and *B. suis* Thomsen. However, expression is not detected in this strain of *B. abortus* during stationary phase growth. Cgt and ZnuA expression was not detected in any of the strains tested. The western blots show some additional bands in the negative controls lanes of CydD, Cgt, FbpA and PotF detection. However, the bands are of different sizes to the proteins of interest and so are likely to be caused by cross-reacting *E. coli* contaminants during the purification process.

Although not all of the proteins have been shown to be expressed, it is clear that this method has its limitations. The irradiated *Brucella* used has been grown to stationary phase in culture media. Thus a single timepoint in the growth of these organisms is reflected. Growth in culture media may not accurately reflect protein expression in a natural infection.

Figure 4.11: Natural expression of PotD, PotF, FbpA and OppA in *B. melitensis* 16M, *B. suis* and *B. abortus*

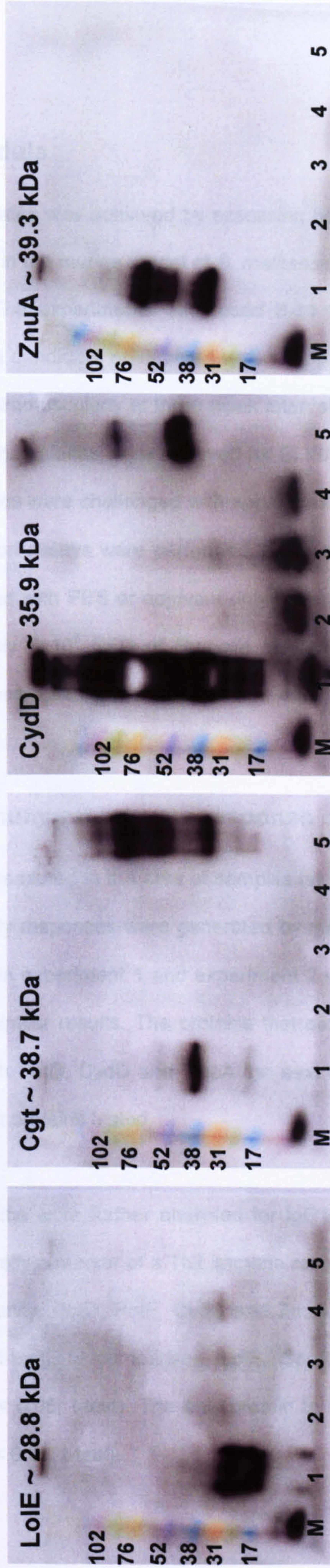
Western blots probed with mouse anti – vaccine candidate polyclonal sera raised during the immunology studies.



M = Full range rainbow markers (kDa)
 Lane 1 = Purified vaccine candidate protein
 Lane 2 = Irradiated *B. melitensis* 16M
 Lane 3 = Irradiated *B. abortus* 10505
 Lane 4 = Irradiated *B. suis* Thomsen
 Lane 5 = Negative control – random *E. coli* purified protein

Figure 4.12: Natural expression of LoIE, Cgt, CydD and ZnuA in *B. melitensis* 16M, *B. suis* and *B. abortus*

Western blots probed with mouse anti – vaccine candidate polyclonal sera raised during the immunology studies.



M = Full range rainbow markers (kDa)

Lane 1 = Purified vaccine candidate protein

Lane 2 = Irradiated *B. melitensis* 16M

Lane 3 = Irradiated *B. abortus* 10505

Lane 4 = Irradiated *B. suis* Thomsen

Lane 5 = Negative control – random *E. coli* purified protein

4.3.2 Immunisation schedule

Evaluation of the vaccine candidates was achieved by assessing both their protective efficacy and the immune response generated in the murine model of *B. melitensis* infection. Two different animal experiments were completed. The experiments both used 9-11 week old female Balb/c mice inoculated with 10 µg of vaccine candidate adjuvanted with 12.5 µg ISCOMs mixed with 6.25 µg CpG administered three times intramuscularly at three week intervals (section 2.5.2). On day 75 of experiment 1 mice were culled and spleens were removed for ELISPOT assays (section 2.6.2). On the same day in experiment 2 mice were challenged with approximately 1×10^4 CFU of *B. melitensis* 16M (i.p.) and splenic colonisation assays were performed 15 days later (section 2.5.4). Negative control groups of mice immunised with PBS or adjuvant only were also included. Mice immunised with a single dose approximately 2×10^5 CFU of *Brucella melitensis* Rev.1 were included as a positive control. Experiment 2 was split into three parts and each part contained the controls as listed above.

4.3.3 Antigen – specific humoral immune response

Total antigen-specific IgG was measured in the sera of samples taken on day 58 of the experiment via ELISA (Figure 4.13). Antibody responses were generated by mice immunised with each of the proteins. Responses generated in experiment 1 and experiment 2 were comparable, showing that independent studies produced similar results. The proteins that elicited the highest IgG response were PotF, FbpA and ZnuA, with PotD, CydD and OppA the next highest. Cgt elicited the lowest IgG response from all of the eight proteins tested.

The IgG responses to the proteins were further analysed for IgG1 and IgG2a responses (Figure 4.14). IgG1 antibodies are generally a marker of a Th2 immune response and IgG2a antibodies are used as a marker for Th1 immunity. PotD, PotF, CydD and ZnuA induced balanced IgG1:IgG2a immune responses ($p > 0.05$, t-test). In comparison, LolE, OppA and Cgt induced Th2-biased humoral immune responses ($p < 0.05$, t-test). The only protein to stimulate a Th1-biased cellular immune response was FbpA ($p < 0.05$, t-test).

Figure 4.13: Comparison of antigen-specific IgG responses generated by mice immunised with the individual vaccine candidates

This graphs show the total specific IgG response from mice immunised with the different ABC transporter proteins. Data shown is the average Total IgG concentration from six mice.

*** = significant increase of total IgG production from immunised mice compared to naïve mice ($p < 0.0001$, t-test)

** = significant increase of total IgG production from immunised mice compared to naïve mice ($p < 0.001$, t-test)

* = significant increase of total IgG production from immunised mice compared to naïve mice ($p < 0.01$, t-test)

Naïve and adjuvant only sera was included on each plate. However, concentrations obtained were below assay detection limits of 10 µg/ml

Error bars indicate the standard deviation of the data

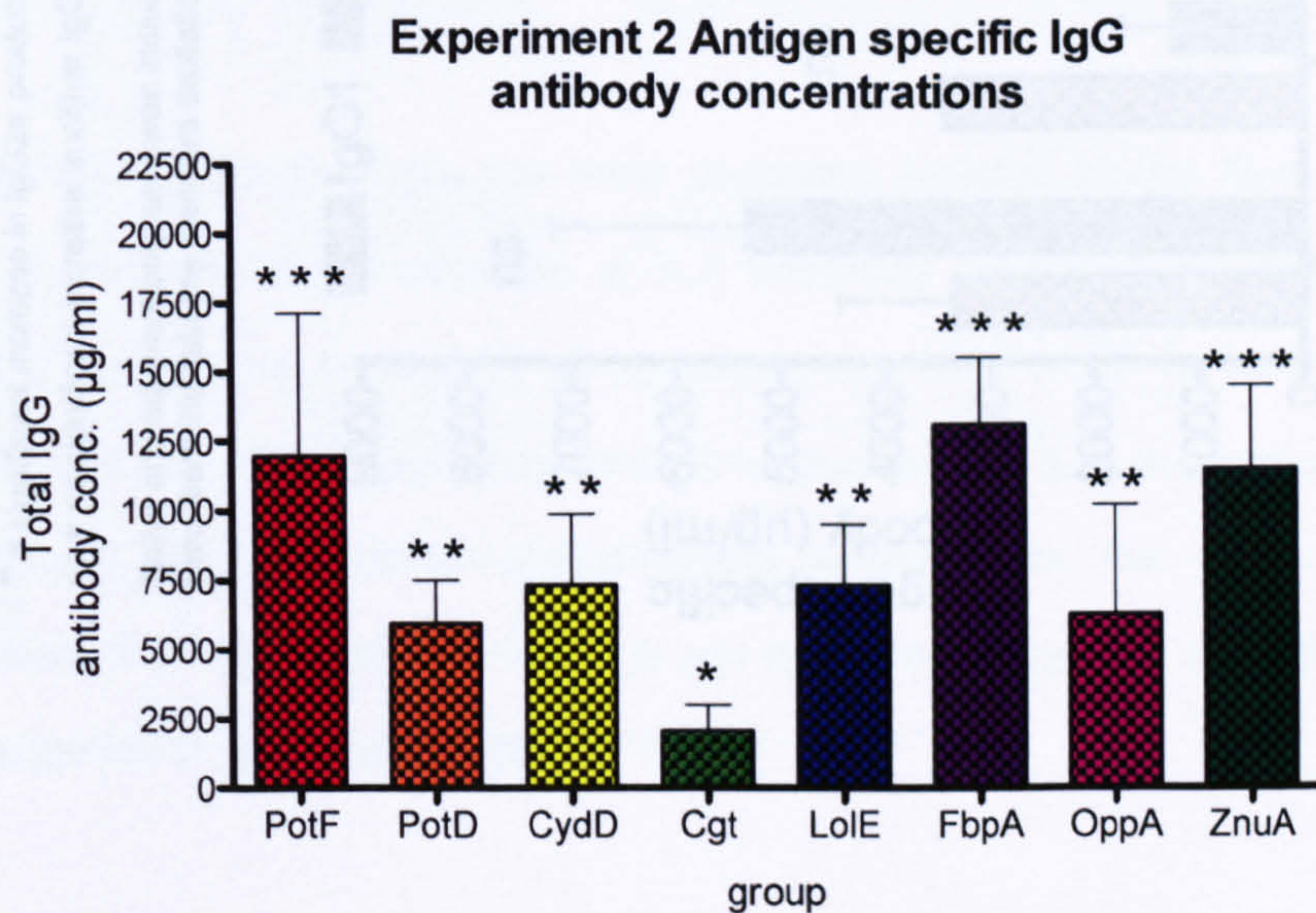
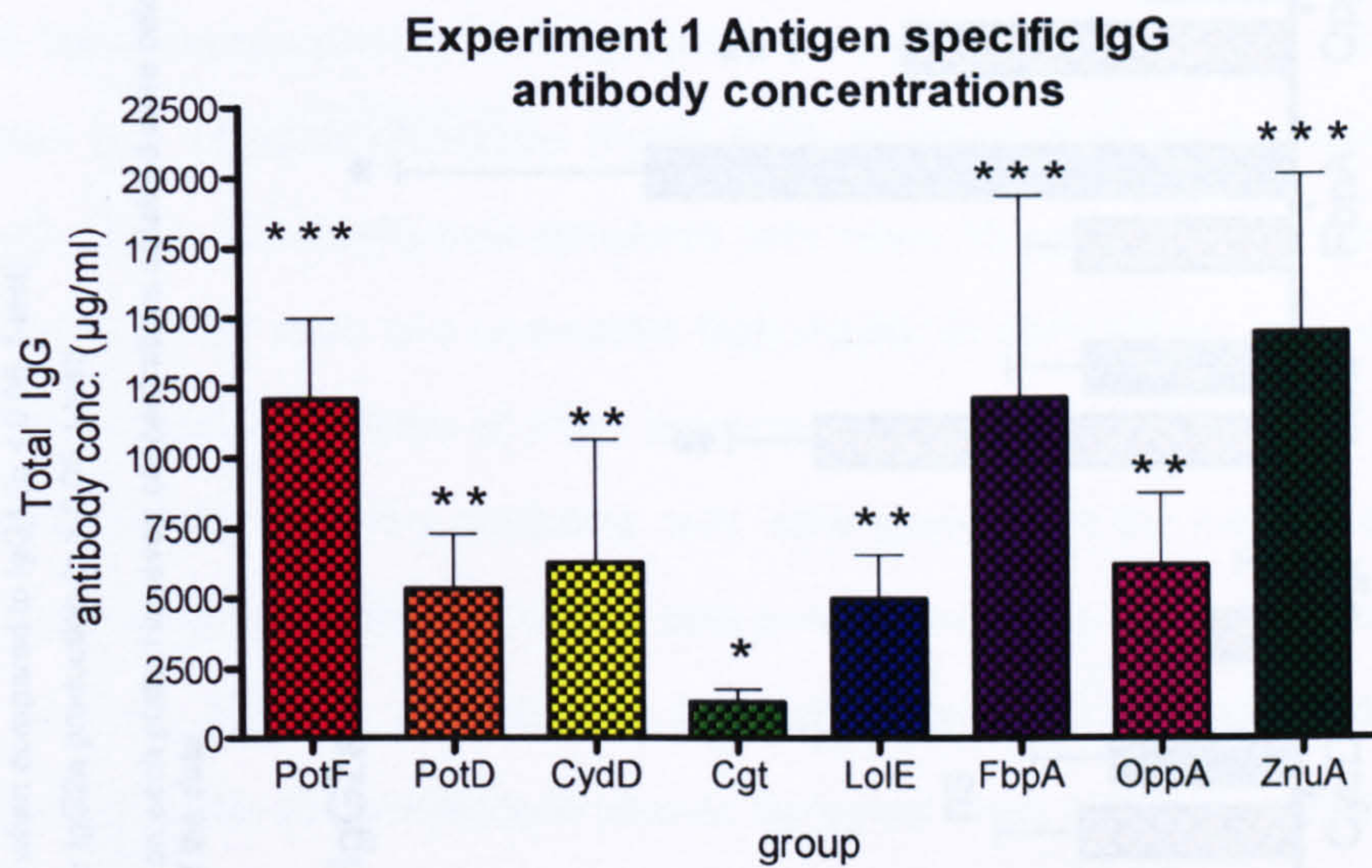


Figure 4.14: Comparison of cellular (IgG2a) vs humoral (IgG1) immune responses generated by mice immunised with individual vaccine candidates

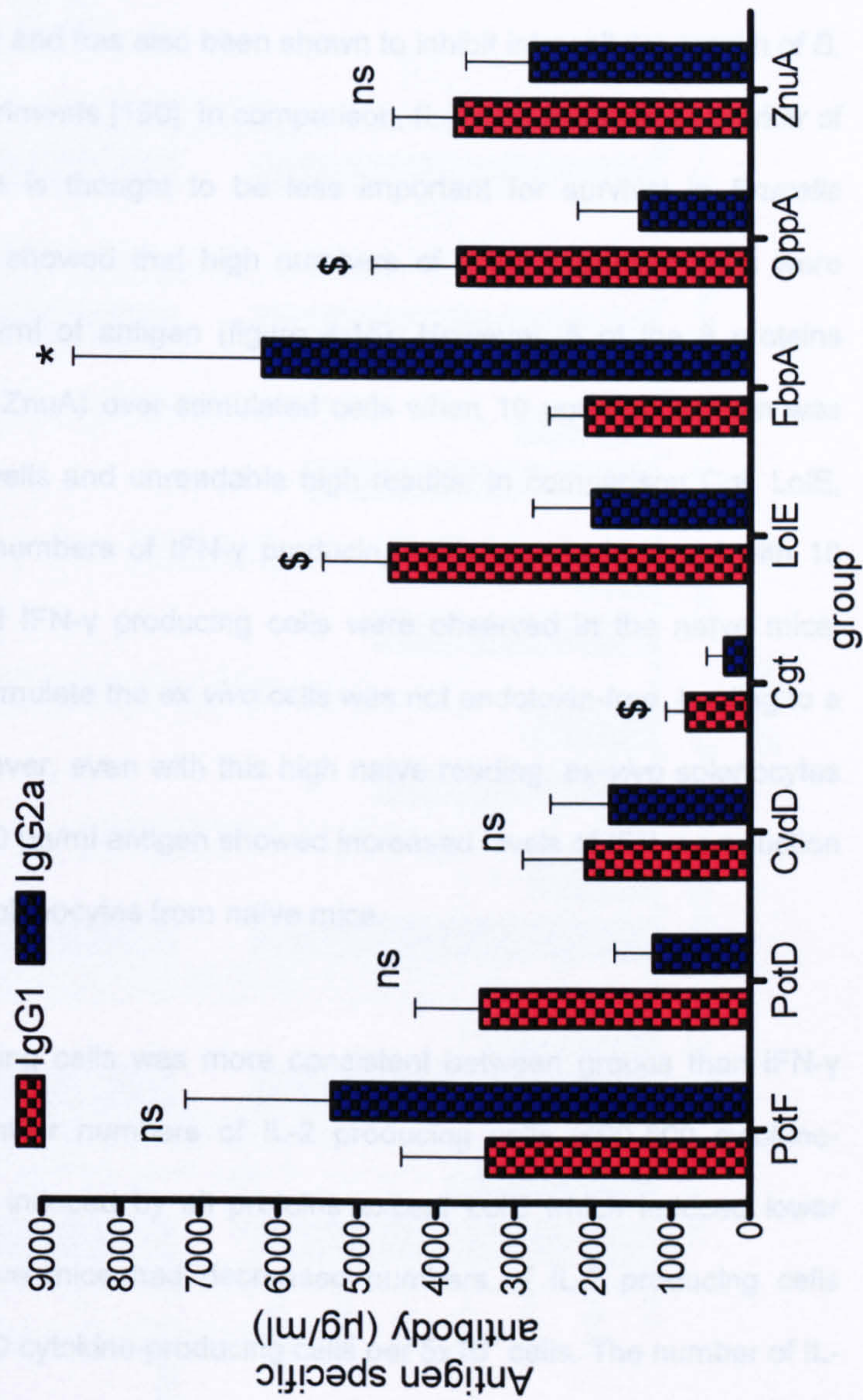
This graphs show the IgG1 and IgG2a response from mice immunised with the different ABC transporter proteins. Data shown is the average IgG1/IgG2a concentration from six mice.

\$ = significantly increase in IgG1 production when compared to IgG2a ($p < 0.05$, t-test)

* = significant increase in IgG2a production when compared to IgG1 ($p < 0.05$, t-test)

ns = no significant increase in either IgG1 or IgG2a production ($p > 0.05$, t-test)

Naïve and adjuvant only sera was included on each plate. However, concentrations obtained were below assay detection limits of 10 µg/ml
Error bars indicate the standard deviation of the data



4.3.4 Evaluation of antigen-specific memory response

Cytokine analysis on *ex-vivo* splenocytes taken from immunised mice was also completed using ELISPOT assays. The production of IFN- γ , IL-2 and IL-4 cytokines was assessed. IFN- γ is a crucial cytokine in *Brucella* resistance and it has been shown that mice that lack the IFN- γ gene will die of brucellosis, whereas wild-type mice will naturally clear a *Brucella* infection [229]. IL-2 is a good marker of the cellular immune response and has also been shown to inhibit intracellular growth of *B. abortus* in both *in vitro* and *in vivo* experiments [150]. In comparison, IL-4 was assayed as marker of the humoral immune response, which is thought to be less important for survival in *Brucella* infections. Results from initial assays showed that high numbers of IFN- γ produced cells were observed when stimulated with 10 $\mu\text{g/ml}$ of antigen (figure 4.15). However, 5 of the 8 proteins tested (PotD, PotF, CydD, OppA, and ZnuA) over-stimulated cells when 10 $\mu\text{g/ml}$ of antigen was used, leading to saturated ELISPOT wells and unreadable high results. In comparison Cgt, LolE, and FbpA proteins induced readable numbers of IFN- γ producing cells when stimulated with 10 $\mu\text{g/ml}$ of protein. Increased number of IFN- γ producing cells were observed in the naïve mice, possibly because the antigen used to simulate the *ex vivo* cells was not endotoxin-free, leading to a non-specific endotoxin response. However, even with this high naïve reading, *ex-vivo* splenocytes from immunised mice stimulated with 10 $\mu\text{g/ml}$ antigen showed increased levels of IFN- γ production when compared to stimulated *ex-vivo* splenocytes from naïve mice.

The detection of IL-2 and IL-4 producing cells was more consistent between groups than IFN- γ (figures 4.16 & 4.17). Specifically, similar numbers of IL-2 producing cells (400-500 cytokine-producing cells per 5×10^5 cells) were induced by all proteins except LolE which induced lower numbers of IL-2 producing cells. Naïve mice had decreased numbers of IL-2 producing cells compared to immunised mice, with <100 cytokine-producing cells per 5×10^5 cells. The number of IL-4 producing cells was lower than the number of both IL-2 and IFN- γ producing cells with 200-350 cytokine-producing cells per 5×10^5 cells in all groups.

Figure 4.15: IFN- γ levels generated from immunised mouse *ex-vivo* splenocytes following stimulation with specific vaccine antigen

The number of IFN- γ producing cells was measured via ELISPOT assay from immunised and naïve mouse *ex-vivo* splenocytes stimulated for 20 hours with 10 $\mu\text{g/ml}$ of specific vaccine antigen. The data is the average of six individually assayed mouse spleens.

*** = significant increase of total IgG production from immunised mice compared to naïve mice ($p < 0.0001$, t-test)
 * = significant increase of total IgG production from immunised mice compared to naïve mice ($p < 0.01$, t-test)
 Error bars indicate the standard deviation of the data

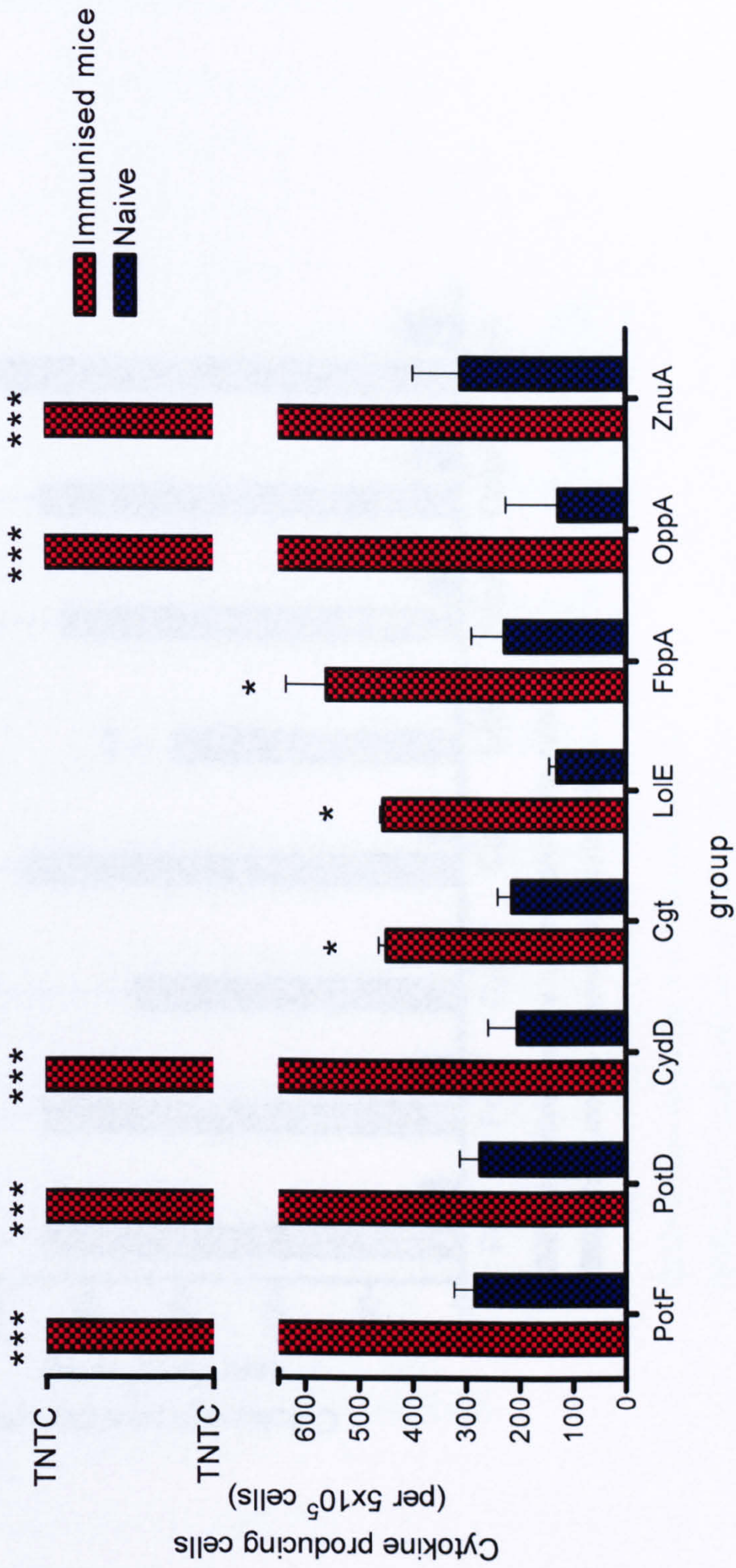


Figure 4.16: IL-2 levels generated from immunised mouse ex-vivo splenocytes following stimulation with specific vaccine antigen

The number of IL-2 producing cells was measured via ELISPOT assay from immunised and naïve mouse ex-vivo splenocytes stimulated for 20 hours with 10 µg/ml of specific vaccine antigen. The data is the average of six individually assayed mouse spleens.

** = significant increase of total IgG production from immunised mice compared to naïve mice ($p < 0.001$, t-test)
 Error bars indicate the standard deviation of the data

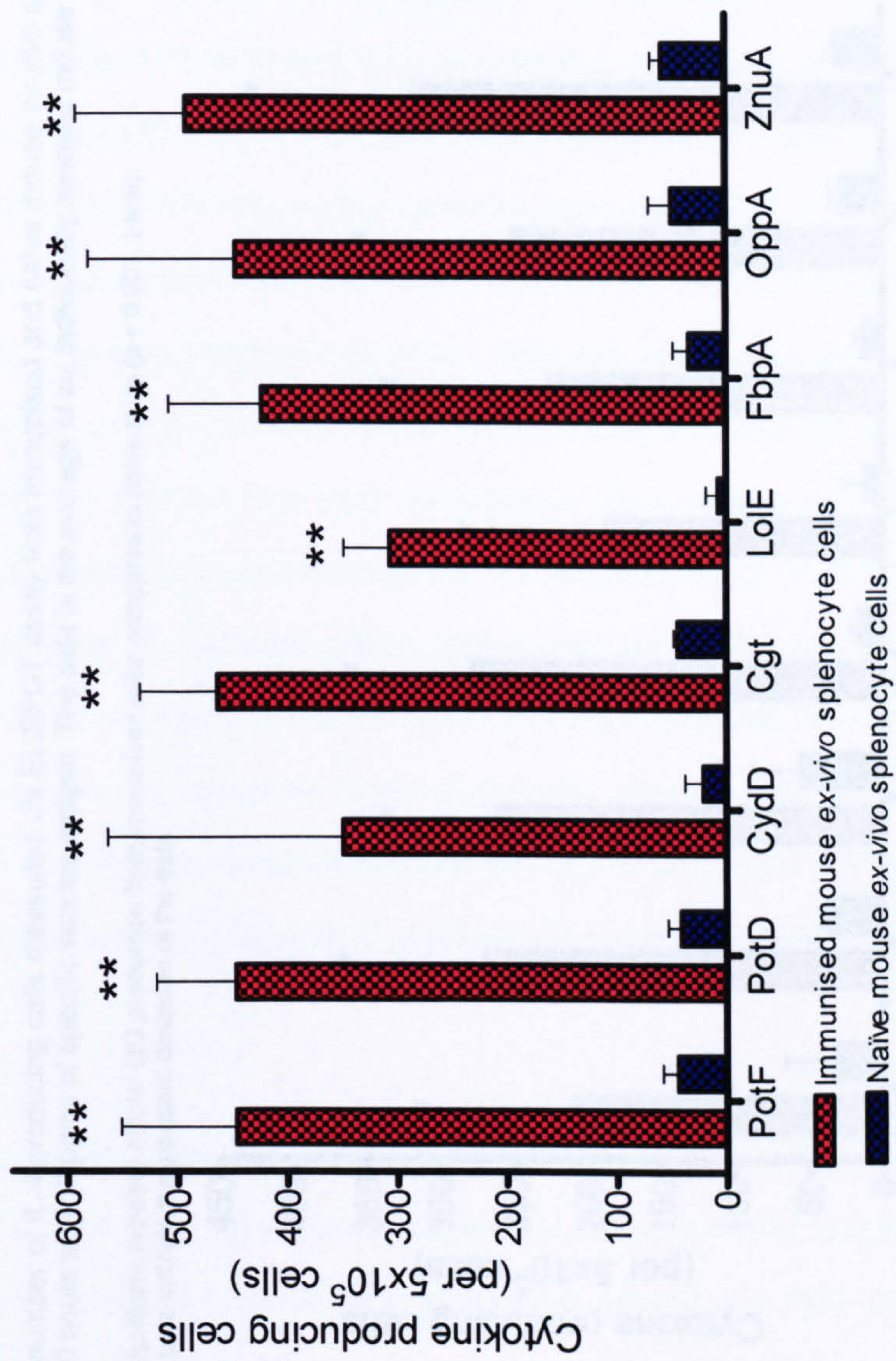
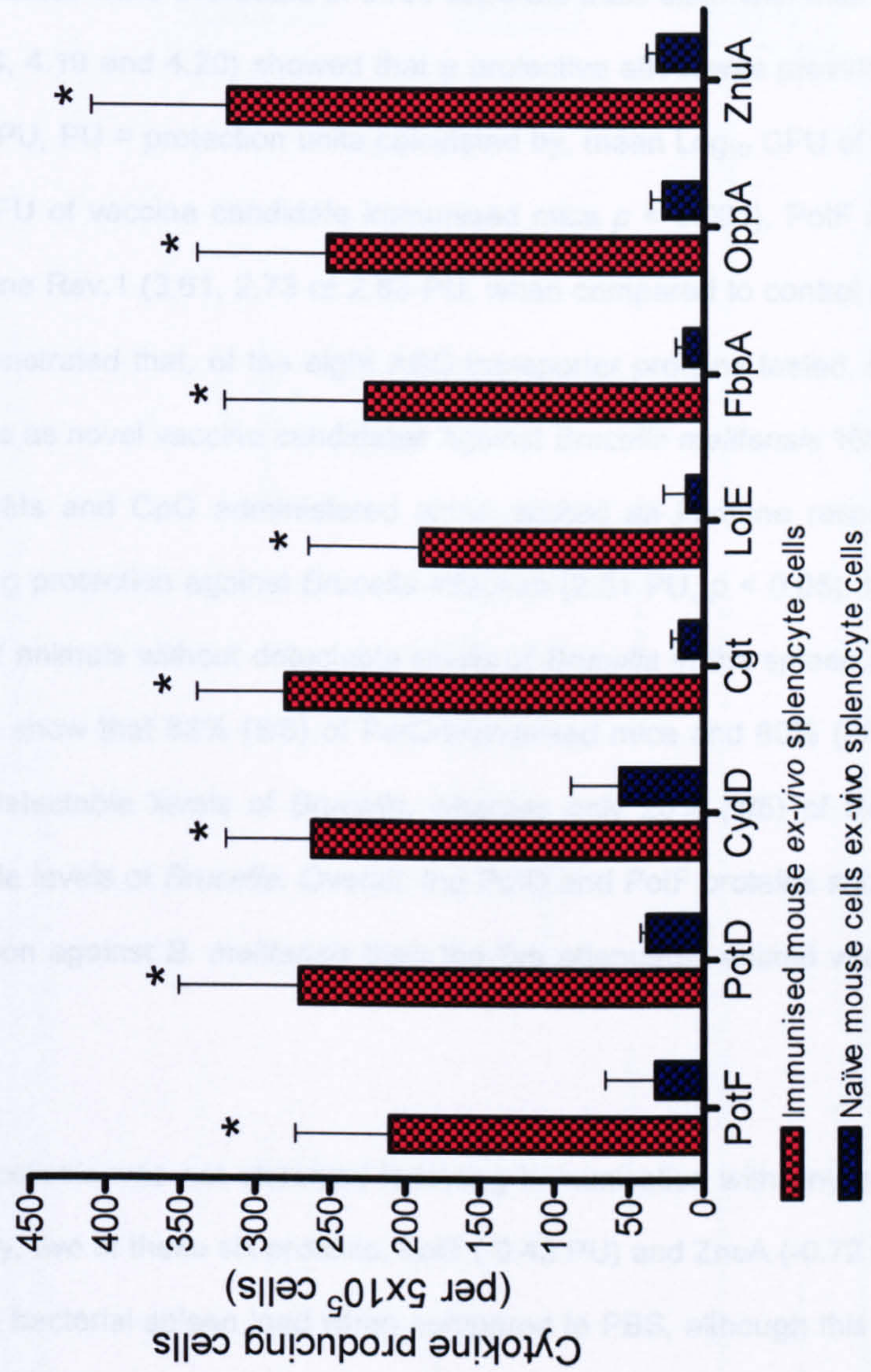


Figure 4.17: IL-4 levels generated from immunised mouse *ex-vivo* splenocytes following stimulation with specific vaccine antigen

The number of IL-4 producing cells measured via ELISPOT assay from immunised and naïve mouse *ex-vivo* splenocytes stimulated for 20 hours with 10 µg/ml of specific vaccine antigen. The data is the average of six individually assayed mouse spleens.

** = significant increase of total IgG production from immunised mice compared to naïve mice ($p < 0.001$, t-test)
 Error bars indicate the standard deviation of the data



4.3.5 Evaluation of protective efficacy of vaccine candidates

To determine whether the proteins might provide protection against brucellosis, inoculated mice (Section 4.3.2) were challenge with approximately 1×10^4 CFU of *B. melitensis* 16M and splenic colonisation assays were performed to determine the *Brucella* colonisation levels. For logistical purposes, the eight vaccine candidates were evaluated in three separate trials each with their own controls. The results (figures 4.18, 4.19 and 4.20) showed that a protective effect was provided to mice immunised with PotD (3.61 PU, PU = protection units calculated by, mean Log_{10} CFU of PBS inoculated mice – mean Log_{10} CFU of vaccine candidate immunised mice $p < 0.001$), PotF (2.46 PU, $p < 0.05$) or the control vaccine Rev.1 (3.61, 2.73 or 2.83 PU, when compared to control mice immunised with PBS). This demonstrated that, of the eight ABC transporter proteins tested, PotD and PotF provide the best promise as novel vaccine candidates against *Brucella melitensis* 16M. In one of the three trials the ISCOMs and CpG administered alone elicited an immune response capable of generating a controlling protection against *Brucella* infection (2.51 PU, $p < 0.05$). If the protection data is viewed as % of animals without detectable levels of *Brucella* in the spleen (less than 10 CFU per spleen), results show that 83% (5/6) of PotD-immunised mice and 60% (3/5) of PotF-immunised mice have no detectable levels of *Brucella*, whereas only 20% (1/5) of Rev.1-immunised mice had no detectable levels of *Brucella*. Overall, the PotD and PotF proteins showed comparable, if not better, protection against *B. melitensis* than the live attenuated animal vaccine Rev.1.

Significant protection against brucellosis was not observed following immunisation with any of the other six proteins and, interestingly, two of these six proteins, LolE (-0.43 PU) and ZnuA (-0.72 PU), appeared to cause an increase in bacterial spleen load when compared to PBS, although this was not significant. An interesting observation was found in *Brucella* recovered from mice immunised with LolE, since they exhibited abnormal colony morphology (personal communication, Dr Nicky Commander). Colonies were larger than normally expected and were mucoidal in appearance. Colonies were selected from these plates for 16S rDNA sequencing to confirm their *Brucella* identity, results of which identified the abnormal colonies as *B. melitensis*. This effect could be due to the fact that LolE is part of the ABC system responsible for the export of fatty acid to the outer

Figure 4.18: Comparison of protective efficacy of PotD, PotF and CydD potential vaccine candidates

The protective efficacy of PotD, PotF and CydD vaccine candidates after mice were administered with three doses at three week intervals of 10 µg protein adjuvanted with 12.5 µg ISCOMs and 6.25 µg CpG. Thirty days after their final immunisation mice were challenged via the intraperitoneal route with 1.28×10^4 CFU *B. melitensis* 16M. Fifteen days after challenge splenic colonisation assays were performed to determine the *Brucella* burden of each mouse. Results are displayed as Log₁₀ CFU per spleen.

* Significant reduction in bacterial load when compared to PBS immunised mice (1-way ANOVA + Bonferroni's post test)

Adjuvant = 12.5µg ISCOMs & 6.25µg 10103 CpG

Black bars represent the mean Log₁₀ *Brucella* CFU/spleen for each group

PU = Protection Units, calculated by [mean Log₁₀ cfu PBS inoculated mice – mean Log₁₀ cfu vaccine candidate immunised mice]

Zone 1 = Growth of *Brucella* above challenge dose meaning not protection has been observed

Zone 2 = Bacterial numbers less than the challenge dose but more than the Rev.1 mean indicating control of the *Brucella* infection

Zone 3 = A reduction in *Brucella* CFU below the mean of Rev.1 representing a protective effect.

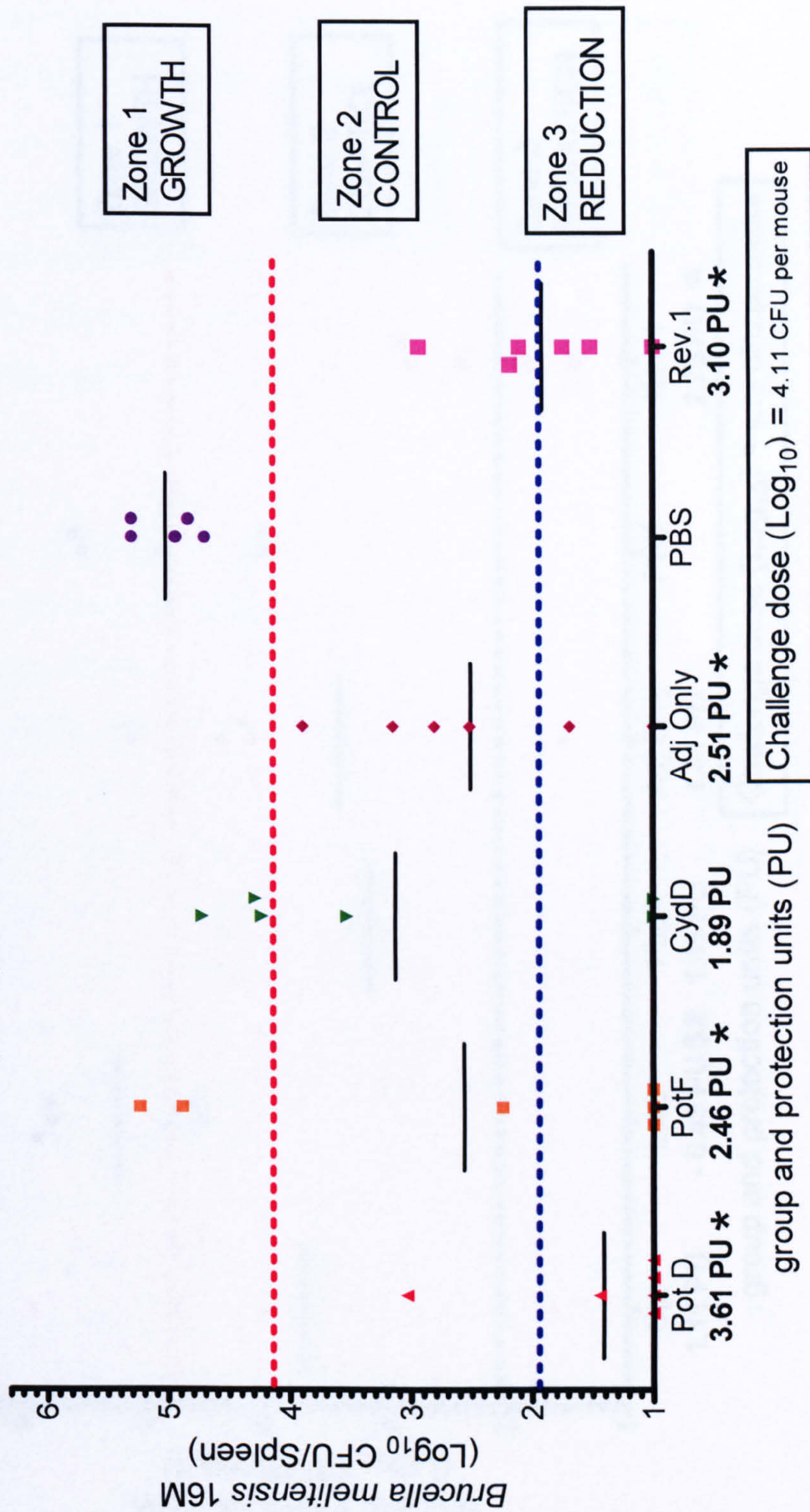


Figure 4.19: Comparison of protective efficacy of Cgt, LolE, and FbpA potential vaccine candidates

The protective efficacy of Cgt, LolE and FbpA vaccine candidates after mice were administered with three doses at three week intervals of 10 µg protein adjuvanted with 12.5 µg ISCOMs and 6.25 µg CpG. Thirty days after their final immunisation mice were challenged via the intraperitoneal route with 6.17×10^4 CFU *B. melitensis* 16M. Fifteen days after challenge splenic colonisation assays were performed to determine the *Brucella* burden of each mouse. Results are displayed as Log₁₀ CFU per spleen.

* Significant reduction in bacterial load when compared to PBS immunised mice (1-way ANOVA + Bonferroni's post test)
 \$ Significant increase in bacterial load when compared to PBS immunised mice (1-way ANOVA + Bonferroni's post test)
 # Unusual *Brucella* colony morphology seen
 Black bars represent the mean Log₁₀ *Brucella* CFU/spleen for each group
 Adjuvant = 12.5µg ISCOMs & 6.25µg 10103 CpG
 PU = Protection Units, calculated by [mean Log₁₀ cfu PBS inoculated mice – mean Log₁₀ cfu vaccine candidate immunised mice]
 Zone 1 = Growth of *Brucella* above challenge dose meaning not protection has been observed
 Zone 2 = Bacterial numbers less than the challenge dose but more than the Rev.1 mean indicating control of the *Brucella* infection
 Zone 3 = A reduction in *Brucella* CFU below the mean of Rev.1 representing a protective effect.

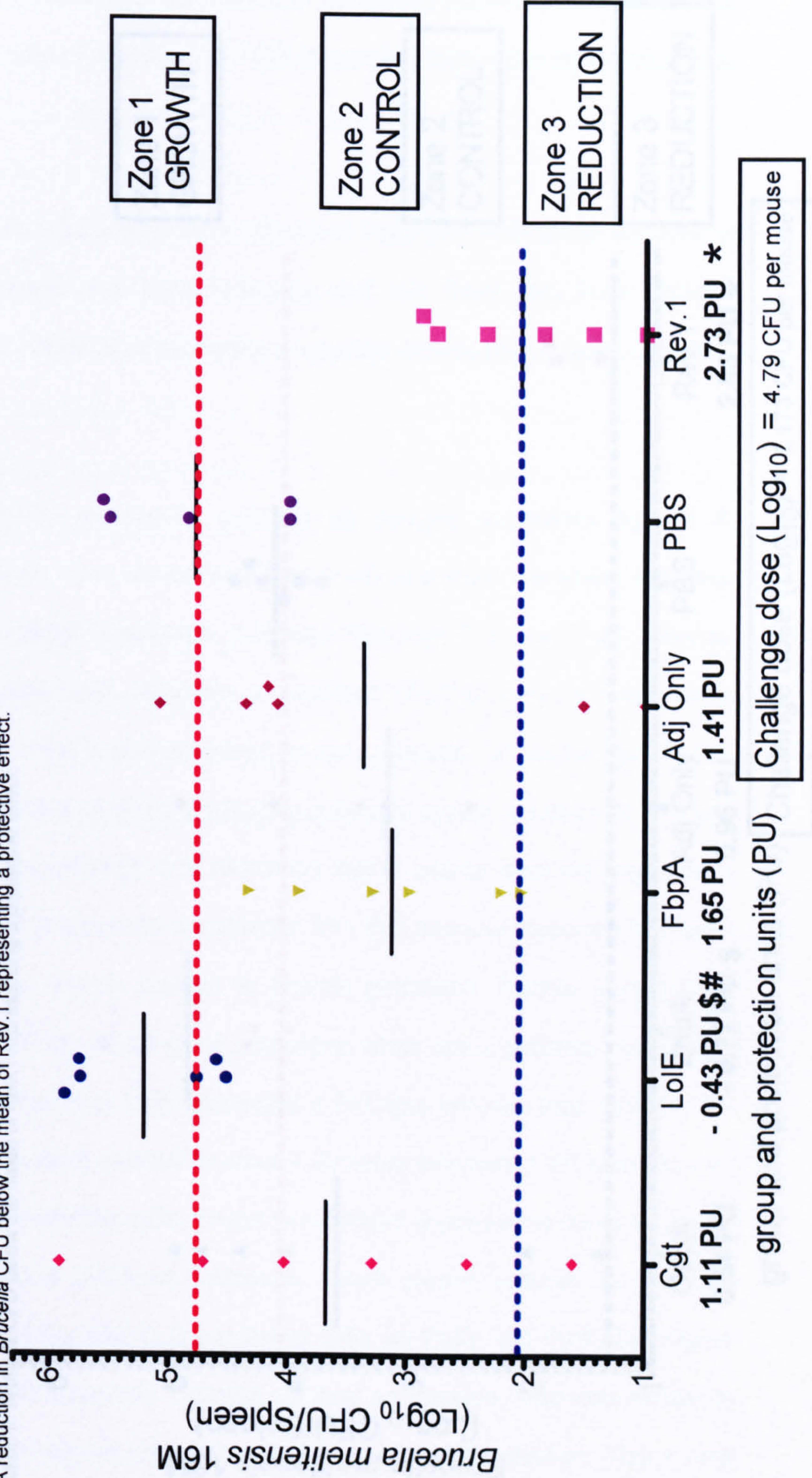


Figure 4.20: Comparison of protective efficacy of OppA and ZnuA potential vaccine candidates

The protective efficacy of Cgt, LolE and FbpA vaccine candidates after mice were administered with three week intervals of 10 µg protein adjuvanted with 12.5 µg ISCOMs and 6.25 µg CpG. Thirty days after their final immunisation mice were challenged via the intraperitoneal route with 1.48×10^4 CFU *B. melitensis* 16M. Fifteen days after challenge splenic colonisation assays were performed to determine the *Brucella* burden of each mouse. Results are displayed as Log₁₀ CFU per spleen.

* Significant reduction in bacterial load when compared to PBS immunised mice (1-way ANOVA + Bonferroni's post test)

\$ Significant increase in bacterial load when compared to PBS immunised mice (1-way ANOVA + Bonferroni's post test)

Adjutant = 12.5µg ISCOMs & 6.25µg 10103 CpG

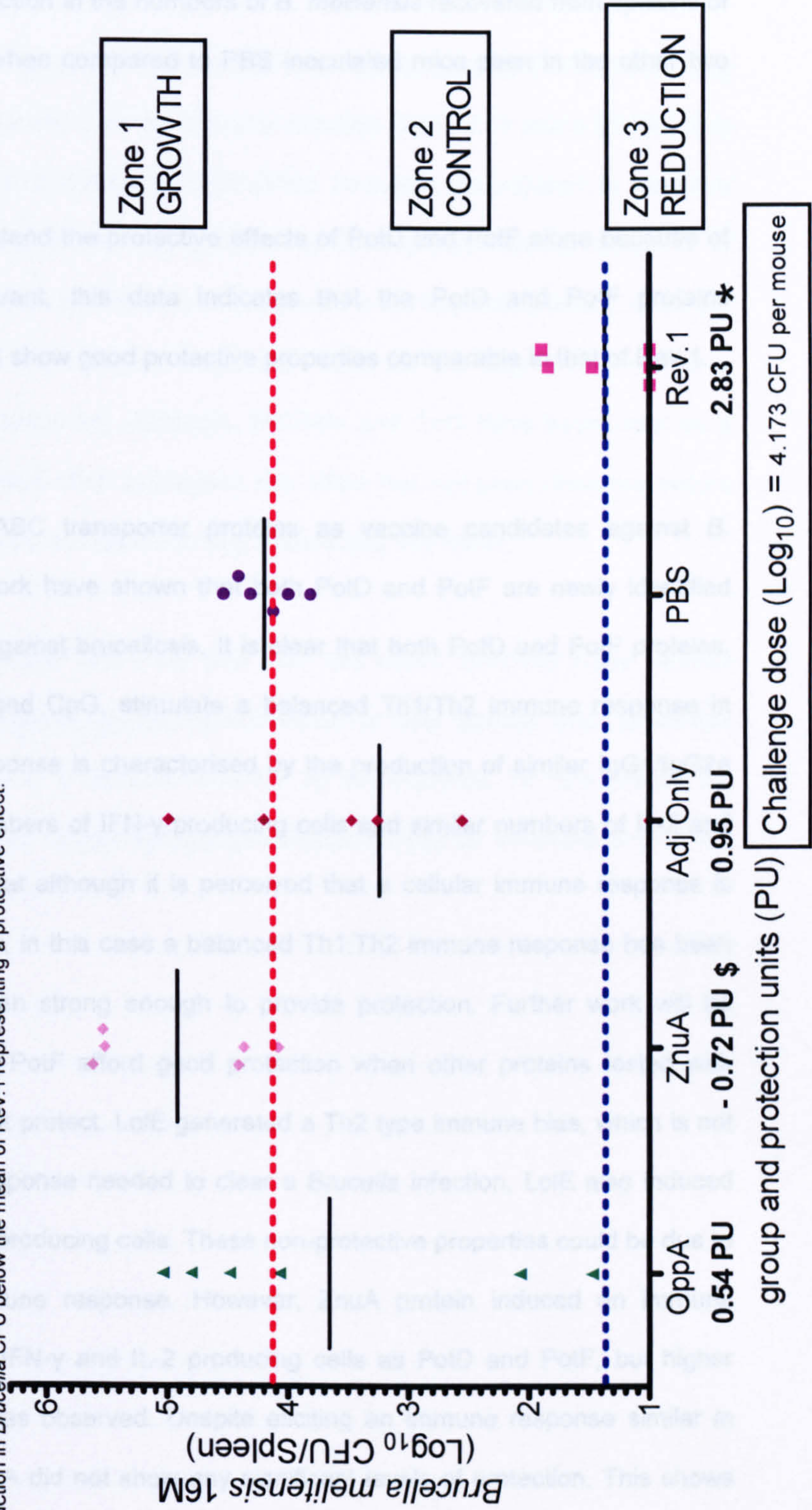
Black bars represent the mean Log₁₀ *Brucella* CFU/spleen for each group

PU = Protection Units, calculated by [mean Log₁₀ cfu PBS inoculated mice – mean Log₁₀ cfu vaccine candidate immunised mice]

Zone 1 = Growth of *Brucella* above challenge dose meaning not protection has been observed

Zone 2 = Bacterial numbers less than the challenge dose but more than the Rev.1 mean indicating control of the *Brucella* infection

Zone 3 = A reduction in *Brucella* CFU below the mean of Rev.1 representing a protective effect.



membrane of the cell [233]. Another interesting observation of this work was the protection observed by the ISCOMs and CpG alone. Although only significant in trial 1 (2.51 PU, $P < 0.05$), there is also at least a ten-fold reduction in the numbers of *B. melitensis* recovered from spleens of mice inoculated with the adjuvant when compared to PBS inoculated mice seen in the other two trials.

Although it is difficult to fully understand the protective effects of PotD and PotF alone because of the controlling effect of the adjuvant, this data indicates that the PotD and PotF proteins administered with ISCOMs and CpG show good protective properties comparable to that of Rev.1.

4.4 Conclusions

This study has evaluated eight ABC transporter proteins as vaccine candidates against *B. melitensis*. The results from this work have shown that both PotD and PotF are newly identified putative novel protective proteins against brucellosis. It is clear that both PotD and PotF proteins, when administered with ISCOMs and CpG, stimulate a balanced Th1/Th2 immune response in vaccinated mice. This immune response is characterised by the production of similar IgG1/IgG2a antibody levels along with high numbers of IFN- γ producing cells and similar numbers of IL-2 and IL-4 producing cells. This shows that although it is perceived that a cellular immune response is needed to clear a *Brucella* infection, in this case a balanced Th1:Th2 immune response has been generated that seems to have been strong enough to provide protection. Further work will be required to assess why PotD and PotF afford good protection when other proteins tested with similar immunological profiles do not protect. LolE generated a Th2 type immune bias, which is not considered the desired immune response needed to clear a *Brucella* infection. LolE also induced the lowest numbers IFN- γ and IL-2 producing cells. These non-protective properties could be due to LolE inducing a low cellular immune response. However, ZnuA protein induced an immune response with similar numbers of IFN- γ and IL-2 producing cells as PotD and PotF, but higher numbers of IL-4 producing cells was observed. Despite eliciting an immune response similar in nature to both PotD and PotF, ZnuA did not show any significant levels of protection. This shows that although IFN- γ production is important in the clearance of *Brucella*, this alone is not enough to

induce a protective immune response, an observation that has been made before in previous novel-vaccine approaches to brucellosis [59].

During the three protection studies presented here it has been observed that the adjuvant only control is able to induce a level of control over the *Brucella* infection (2.51, 1.41 and 0.95 PU). It is not known why ISCOMs and CpG induce this protective effect. However, the adjuvant is designed to elicit a Th1-type immune response which is considered the desired response for clearance of *Brucella*. The longevity of the protection provided by the adjuvant is an interesting factor, since the final immunisations were given 30 days before challenge. Thus, for the adjuvant to exhibit a controlling effect after this long is surprising. Although, ISCOMs and CpG have been used as a combination vaccine previously against other pathogens this effect has not been observed before [130,98]. Further studies will have to be completed to fully understand this effect.

It is clear from the work in this chapter that PotD and PotF are newly identified novel vaccine candidates against brucellosis. However, it has been difficult to fully assess their protective properties due to the varying levels of protection observed by the adjuvant. To fully assess the protective properties of PotD and PotF further studies will be required and an understanding of the adjuvant effect will be needed.

**Chapter 5 – Evaluation of ISCOMs and CpG
as an immunostimulant**

5.1 Introduction

The use of adjuvants to enhance the immune response when using recombinant sub-unit vaccines is commonplace. The use of dual adjuvants, however, is less common. Research using oil-based adjuvant systems and CpG has shown that the addition of CpG can enhance the immune response generated to vaccines [230,190]. The co-administration of ISCOMs and CpG as an adjuvant is less common but has been used successfully [130,98]. Immunisation with ISCOMS or CpG leads to a non-specific measurable cellular immune response [298,171]. In some cases immunisation with CpG alone can elicit a strong enough immune response to clear a subsequent infection [122,343,154].

Adjuvants are designed to either enhance all aspects of the immune response or to target specific branches of the immune system. ISCOMs and CpG, when used together or separately, are designed to elicit cellular immune responses [298,173]. The CpGs activate TLR9, an internal TLR that recognises short unmethylated DNA. Signalling through TLR9 causes the release of cytokines such as IFN- γ , IL-6, IL-12 and TNF- α [171].

ISCOMs are 30-40 nm cage-like structures derived from cholesterol, phospholipids and saponins [307]. Studies comparing ISCOMs to oil-based adjuvants have shown they induce similar humoral antibody responses but much higher CD8⁺ T cell responses [64]. The administration of ISCOMs to mice induces a strong inflammatory response starting with the recruitment of large numbers of neutrophils and mast cells, followed closely by macrophages, dendritic cells and lymphocytes [298].

In animal vaccination trials a negative control group consisting of adjuvant only is usually included to show that the adjuvant alone is not causing the protective effect observed by the adjuvanted vaccine candidates. In the protection studies detailed in Chapter 4, ISCOMs and CpG were used to adjuvant the vaccine candidates. Collation of the data from the chapter shows that ISCOMs and CpG alone provides a level of control over a *Brucella* infection in mice (figure 5.1). Although not as effective as Rev.1, mice immunised with ISCOMs and CpG and challenged with *B. melitensis*

Figure 5.1: Average bacterial load for all mice immunised with ISCOMs & CpG across all experiments completed in Chapter 4

Panel A represent all the data for the control groups of mice inoculated with ISCOMs and CpG, PBS or *B. melitensis* Rev.1 in Chapter 4. Mice were administered three doses of 12.5 µg ISCOMs and 6.25 µg CpG at three week intervals. Thirty days after their final inoculation mice were challenged via the intraperitoneal route with an average challenge dose of 2.57×10^4 CFU *B. melitensis* 16M. Fifteen days after challenge mice were culled and splenic colonisation assays were performed to calculate *Brucella* loads per spleen. Results are represented as average Log_{10} CFU/spleen \pm standard deviation (panel B) or as protection units.

* Significantly reduction in bacterial load when compared to PBS (1-way ANOVA + Bonferroni's post test)

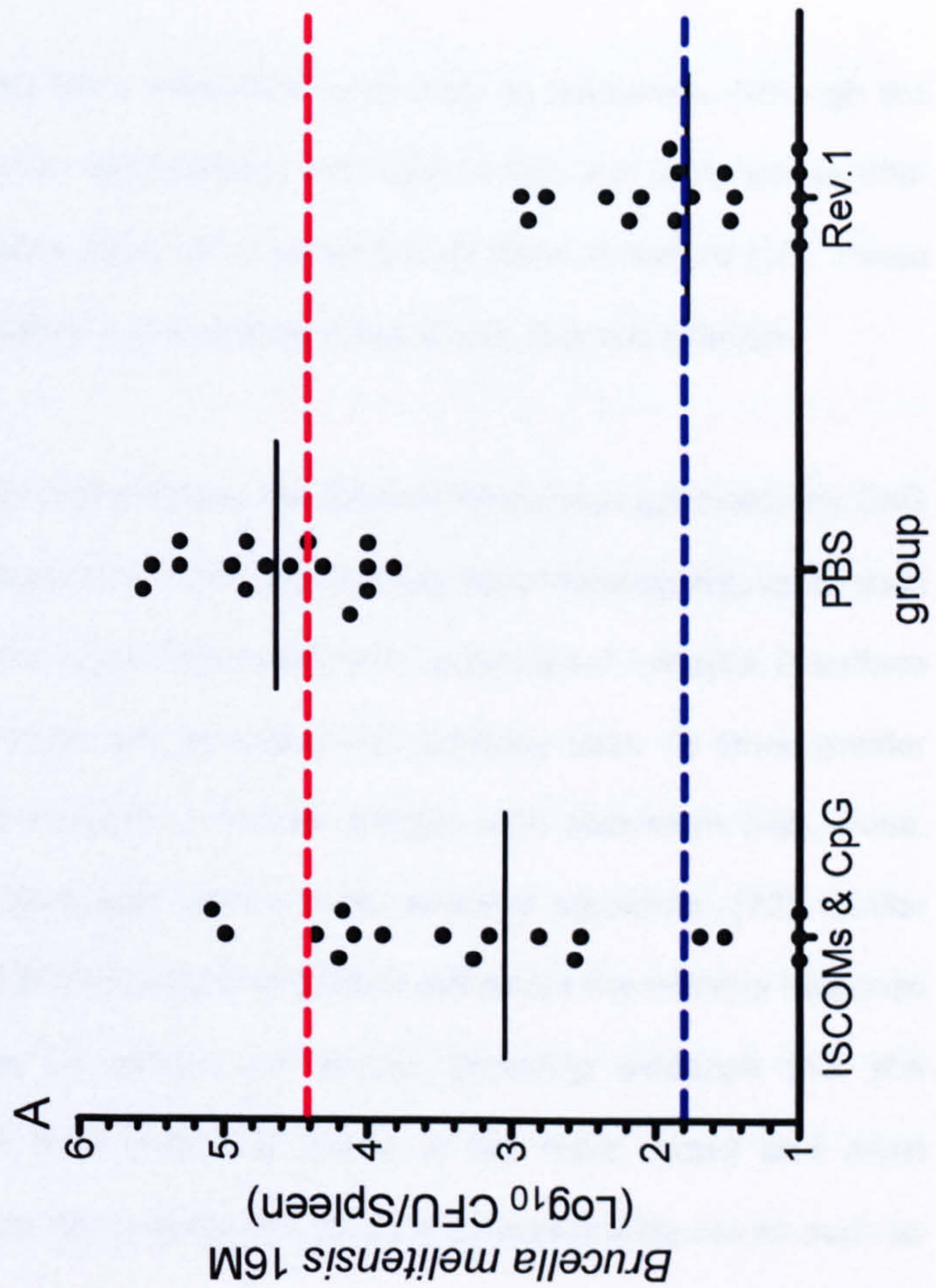
\$ Significantly reduction in bacterial load when compared to ISCOMs & CpG (1-way ANOVA + Bonferroni's post test)

Average challenge dose = 4.41 Log_{10} CFU (Indicated by red line)

Blue line indicates average bacterial load of mice immunised with *B. melitensis* Rev.1

Dose given - 12.5µg ISCOMs & 6.25µg CpG

Protection Units calculated by [mean Log_{10} CFU PBS immunised mice - mean Log_{10} CFU immunised mice]



B

Control	Bacterial Load (Log_{10} CFU/Spleen) Mean \pm SD	Protection Units
ISCOMs & CpG*	3.04 ± 1.36	1.59
PBS	4.64 ± 0.56	N/A
Rev.1**\$	1.78 ± 0.66	2.86

16M harboured significantly reduced *Brucella* loads in their spleen when compared to PBS-immunised mice. This protective effect of ISCOMs and CpG is an interesting observation as the *B. melitensis* challenge was administered 30 days after the final immunisation indicating that the protective immune response could remain after this time.

CpGs are an innate immune stimulator that can provide protection against various pathogens including viral, bacterial and parasitic pathogens, when administered to mice prior to exposure. For example, protection against malaria was provided to mice when administered with CpG one or two days prior to challenge [122]. Similarly, mice dosed with CpG two or six hours before exposure to *Mycobacterium tuberculosis* H37Rv subsequently had reduced H37Rv levels up to five weeks after the challenge [154]. Mice have also been shown to be protected from *Burkholderia pseudomallei* and *Burkholderia mallei* challenge when inoculated with CpG between two and ten days before challenge [343,356].

In one study, candidate *Brucella* vaccines were evaluated using CpG as adjuvants. Although the vaccine candidate P39 was protective when administered with CpG at four and eight weeks after challenge, mice immunised with CpG alone showed no protection at either timepoint [10]. These observations indicate that CpGs do not induce a long-lasting effect during *Brucella* infection.

Many strategies have been adopted to try and enhance the immune responses generated by CpG [231]. Early studies evaluated the incorporation of CpG into existing depot-forming adjuvants such as aluminium salt adjuvants. In one study, mice immunised with recombinant hepatitis B surface antigen adjuvanted with CpG and aluminium salt adjuvants had antibody titres 35 times greater than mice immunised with recombinant hepatitis B surface antigen with aluminium salts alone, indicating that together CpG and aluminium salts were a more effective adjuvants [72]. Similar studies have shown that combining CpG and emulsigen adjuvants enhances the immune response generated to herpesvirus glycoproteins in rabbits and sheep, providing evidence that this enhancement method is not limited to mice [144,143]. Some of the more recent and novel approaches to enhancing CpG activity include coupling of CpG to a polysaccharide carrier such as

a modified β -(1-3)-D-glucan schizophyllan (SPG, an extracellular polysaccharide produced by the fungus *Schizophyllum commune*) [221], binding of CpG to cationic carbon nanotubes [27], packaging of CpG to fusogenic liposomes (FL, derived from conventional liposomes with an inactivated Sendai virus-derived accessory protein attached) [366] and co-administration of CpG with polyphosphazenes (synthetic water-soluble, biodegradable polymers) [230]. There are also examples of CpG being packaged into liposomes to enhance their immunotherapeutic efficacy [354]. However, at the time of writing there is no known published literature describing the enhancement of CpG activity using ISCOMs. The results from Chapter 4 suggest that the co-administration of ISCOMs with CpG could enhance or prolonging the immune response generated by CpG. To investigate this further, this chapter describes a number of *in vitro* and *in vivo* studies designed to evaluate the stimulatory and properties of ISCOMs and CpG when used together as adjuvants. In all experiments the CpG 10103 (5'-TCGTCGTTTTTCGGTCGTTTT-3') was used, as in Chapter 4.

5.2 IFN- γ generation in ex-vivo splenocytes treated with ISCOMs & CpG

This experiment aimed to determine if the co-administration of ISCOMs with CpG may induce a greater immune response than administration of CpG alone. IFN- γ responses were measured in naïve *ex-vivo* mouse splenocytes stimulated with CpG \pm ISCOMs. The *ex-vivo* splenocytes were harvested from naïve mice and homogenised through a 40 μ M cell sieve, and the red blood cells were lysed using red cell lysis buffer (Sigma, UK). The cell populations were adjusted to 5×10^6 cell/ml and stimulated with 4 μ g ISCOMs \pm 2 μ g CpG. IFN- γ release was measured via ELISPOT assay after 20 hours stimulation at 37°C, 5% CO₂ (figure 5.2).

The results from this experiment indicate that *ex-vivo* splenocytes stimulated with ISCOMs and CpG or CpG alone produce high numbers of IFN- γ producing cells. However, cells stimulated with ISCOMs and CpG together produce significantly ($P < 0.05$) higher numbers of IFN- γ producing cells (300 IFN- γ producing cells per 5×10^5) than cells stimulated with CpG alone (280 IFN- γ producing cells per 5×10^5). Similarly, cells stimulated with CpG and ISCOMs or CpG alone both induce

significantly more IFN- γ producing cells ($P < 0.0001$) than unstimulated cells (58 IFN- γ producing cells per 5×10^5) or cells stimulated with ISCOMs alone (64 IFN- γ producing cells per 5×10^5).

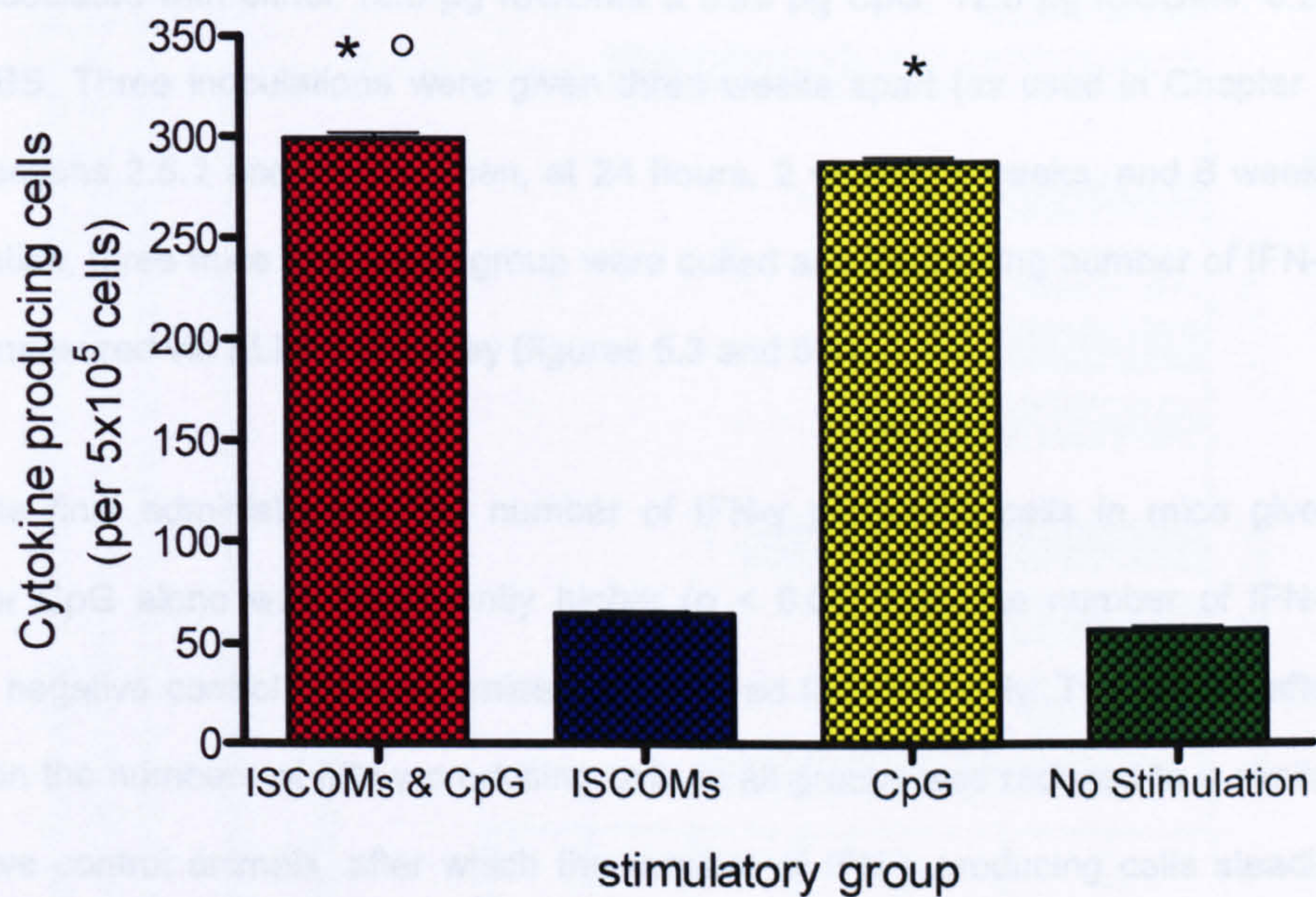
This finding could be responsible for increased macrophage activity, CD4⁺ T cell activation and NK cell activation, leading to an increased immune response that may result in an enhanced response to disease infection.

Figure 5.2: IFN- γ generated when *ex-vivo* splenocytes were stimulated with 4 μ g ISCOMs \pm 2 μ g CpG.

The number of IFN- γ producing cells was measured via ELISPOT assay generated by the stimulation of naïve mouse *ex-vivo* splenocytes with 4 μ g ISCOMs \pm 2 μ g CpG after 20 hours. The data shown was generated from five mice and at least twelve wells were mouse per stimulation group.

* = Significant increase in IFN- γ production when compared to no stimulation and ISCOMs group ($P < 0.0001$, t-test)

° = Significant increase in IFN- γ production when compared to CpG stimulation ($P < 0.05$, t-test)



significantly more IFN- γ producing cells ($P < 0.0001$) than unstimulated cells (56 IFN- γ producing cells per 5×10^5) or cells stimulated with ISCOMs alone (64 IFN- γ producing cells per 5×10^5).

This finding could be responsible for increased macrophage activity, CD8⁺ T cell activation and NK cells activation, leading to an increased immune response that may result in an enhanced response to *Brucella* infection.

5.3 Post inoculation IFN- γ production from ex-vivo splenocytes over a 6 week period

In order to understand the long-term anti-*Brucella* effects of ISCOMs and CpG treatment, IFN- γ production was observed over a six week period in inoculated mice. To evaluate the immune response to ISCOMs and CpG 30 days after final administration, four groups of 12 Balb/C mice (6 – 8 weeks old) were inoculated with either 12.5 μ g ISCOMs & 6.25 μ g CpG, 12.5 μ g ISCOMs, 6.25 μ g CpG or 100 μ l PBS. Three inoculations were given three weeks apart (as used in Chapter 4 protection studies, sections 2.5.2 and 2.5.3). Then, at 24 hours, 2 weeks, 4 weeks, and 6 weeks after the final vaccination, three mice from each group were culled and the resting number of IFN- γ producing cells was measured via ELISPOT assay (figures 5.3 and 5.4).

At 24 hours after the final administration, the number of IFN- γ producing cells in mice given ISCOMs and CpG or CpG alone was significantly higher ($p < 0.01$) than the number of IFN- γ producing cells from negative control mice and mice administered ISCOMs only. Two weeks after the final administration the numbers of IFN- γ producing cells in all groups was reduced to a similar number as in negative control animals, after which the number of IFN- γ producing cells steadily increased over the six week period tested. If the average number of IFN- γ producing cells for each group at 2, 4 and 6 weeks is plotted and an analysis of co-variance (ANCOVA) statistical analysis is completed, these data show that the number of IFN- γ producing cells in the animals administered ISCOMs and CpG group increases at a significantly greater rate ($P < 0.001$) than in any of the other treatment groups (figure 5.4). This steady increase in the number of IFN- γ producing cells after administration of ISCOMs and CpG is interesting as it is a pattern that is not known to have been reported before. However, the same pattern also occurs in animals inoculated with PBS only which

Figure 5.3: Resting IFN- γ production over a 6 week time period from ex-vivo splenocytes taken from immunised mice.

This graph shows the resting number of IFN- γ producing cells from mice inoculated with three doses of 12.5 μg ISCOMs \pm 6.25 μg CpG at three week intervals. At 24 hrs, 2, 4, or 6 weeks after their final inoculation mice were culled and their spleens removed, and the number of resting IFN- γ producing cells was measured via ELISPOT assay.

* = significantly more IFN- γ produce compared to mice inoculated with PBS or ISCOMs at 24 hrs ($p < 0.01$, t-test)

Error bars indicate the standard deviation of the data.

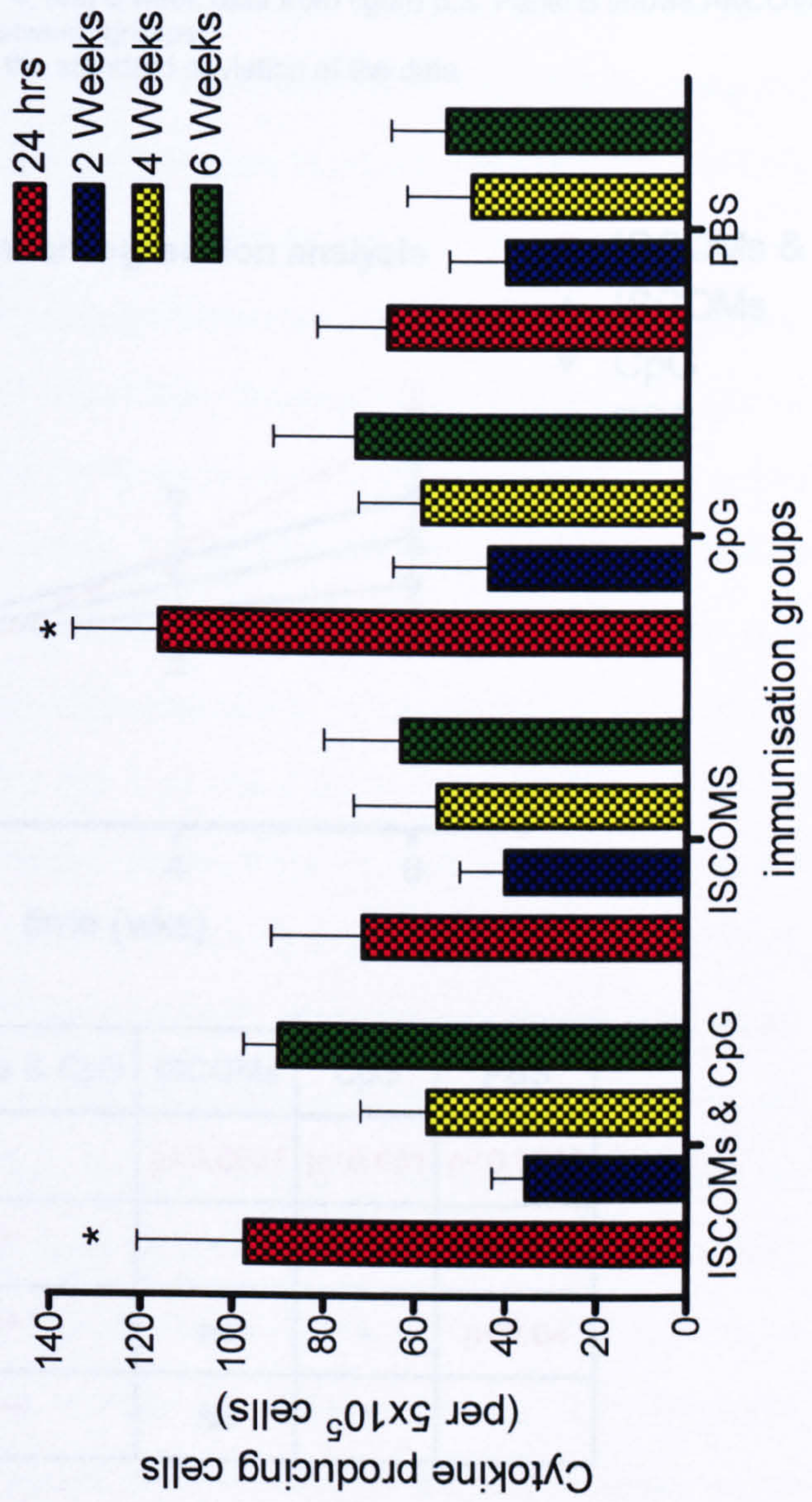
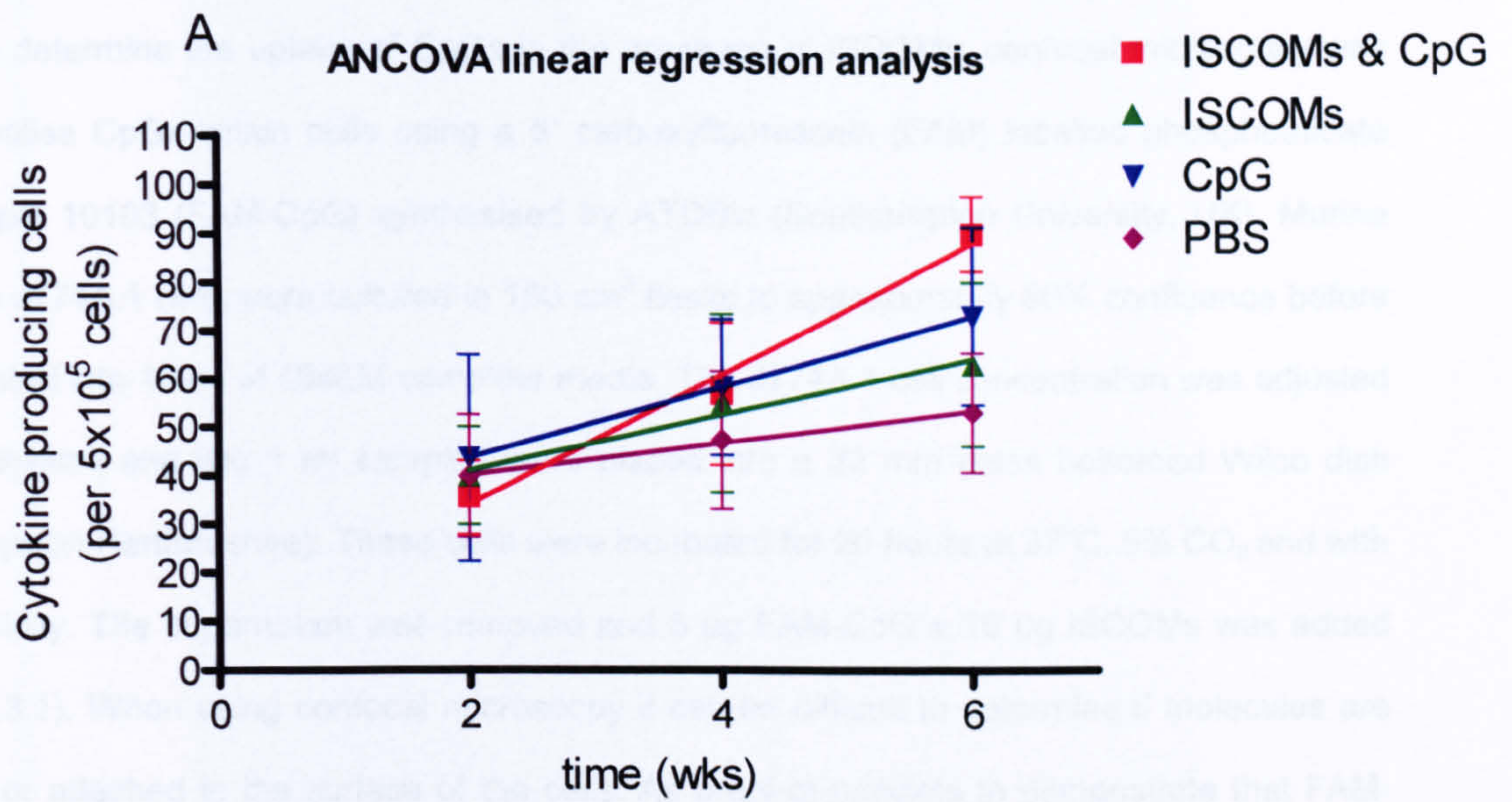


Figure 5.4: ANCOVA linear regression analysis of increasing IFN- γ production by *ex-vivo* splenocytes over six weeks.

ANCOVA linear regression analysis of the number of resting IFN- γ producing cells from inoculated mouse *ex-vivo* splenocytes. Panel A shows a graphical representation of the ANCOVA analysis of the 2, 4, and 6 week data from figure 5.3. Panel B shows ANCOVA p values comparing slopes between groups. Panel A error bars indicate the standard deviation of the data



B

	ISCOMs & CpG	ISCOMs	CpG	PBS
ISCOMs & CpG	-	p<0.0001	p<0.001	p<0.0001
ISCOMs	***	-	p=0.4	p=0.156
CpG	**	ns	-	p<0.04
PBS	***	ns	*	-

***** = p < 0.0001**

**** = p < 0.001**

*** = p < 0.04**

ns = not significant

is unexpected. Overall, the data generated in this experiment indicates that ISCOMs and CpG together lead to an increasing immune response over time. These data indicate that there could be a slow release of the CpG from the ISCOMs causing this increasing immune response.

5.4 Cellular uptake of CpGs

The internalisation of CpG is important in inducing an immune response as TLR9 (which is activated by CpG) is located inside the cell. It is possible that enhancing the rate of CpG uptake into host cells might play a role in inducing a more sustained immune response to the ISCOMs and CpG adjuvant. To determine the uptake of CpGs in the presence of ISCOMs, confocal microscopy was used to visualise CpGs within cells using a 5' carboxyfluorescein (FAM) labelled phosphothioate backbone CpG 10103 (FAM-CpG) synthesised by ATDBio (Southampton University, UK). Murine macrophage J774A.1 cells were cultured in 150 cm³ flasks to approximately 80% confluence before being harvested into 10 ml of DMEM complete media. The J774A.1 cell concentration was adjusted to 3.5x10⁶ cells/ml and two 1 ml samples were placed into a 22 mm glass bottomed Wilco dish (Intracel, Royston Hertfordshire). These cells were incubated for 20 hours at 37°C, 5% CO₂ and with relative humidity. The supernatant was removed and 5 µg FAM-CpG ± 10 µg ISCOMs was added (section 2.6.3.1). When using confocal microscopy it can be difficult to determine if molecules are internalised or attached to the surface of the cells. As proof-of-principle to demonstrate that FAM-CpGs were internalised, cells were stained with LysoTracker™ red (Invitrogen, Paisley, UK), which stains the lysosomes of the J774A.1 cells with a red fluorescent dye. If the FAM-CpGs are internalised into the lysosomes of the cells then the LysoTracker™ red and the FAM-CpG co-localise to produce an orange colour when viewed under the confocal microscope (figure 5.5). Images of cells that had been dosed with ISCOMs only were also taken to confirm that ISCOMs do not have any natural fluorescence that would interfere with measurements of the FAM-CpGs. LysoTracker™ red was not used during time course experiments as its intensity overpowered the fluorescence of the FAM-CpG in earlier time points. Following inoculation of the J774A.1 cells with ISCOMs and CpG, cells were imaged at 1, 2, 4, 6, 8 and 16 hours. At each timepoint eight random fields of view were imaged. Representative images of CpG uptake are shown in figure 5.6. Figure 5.7 shows the percentage of cells containing FAM-CpG over the 16 hour period studied.

Figure 5.5: Confocal microscopy images showing intracellular localisation of CpG

A/B: Split colour confocal microscopy images of FAM-CpG (green) uptake by J774A.1 macrophages (A) including LysoTracker™ (red) (B) to stain acid compartments within the cells. White arrows represent high concentrations of either CpG (A) or LysoTracker™ (B) inside cells.

Bar = 40 μm

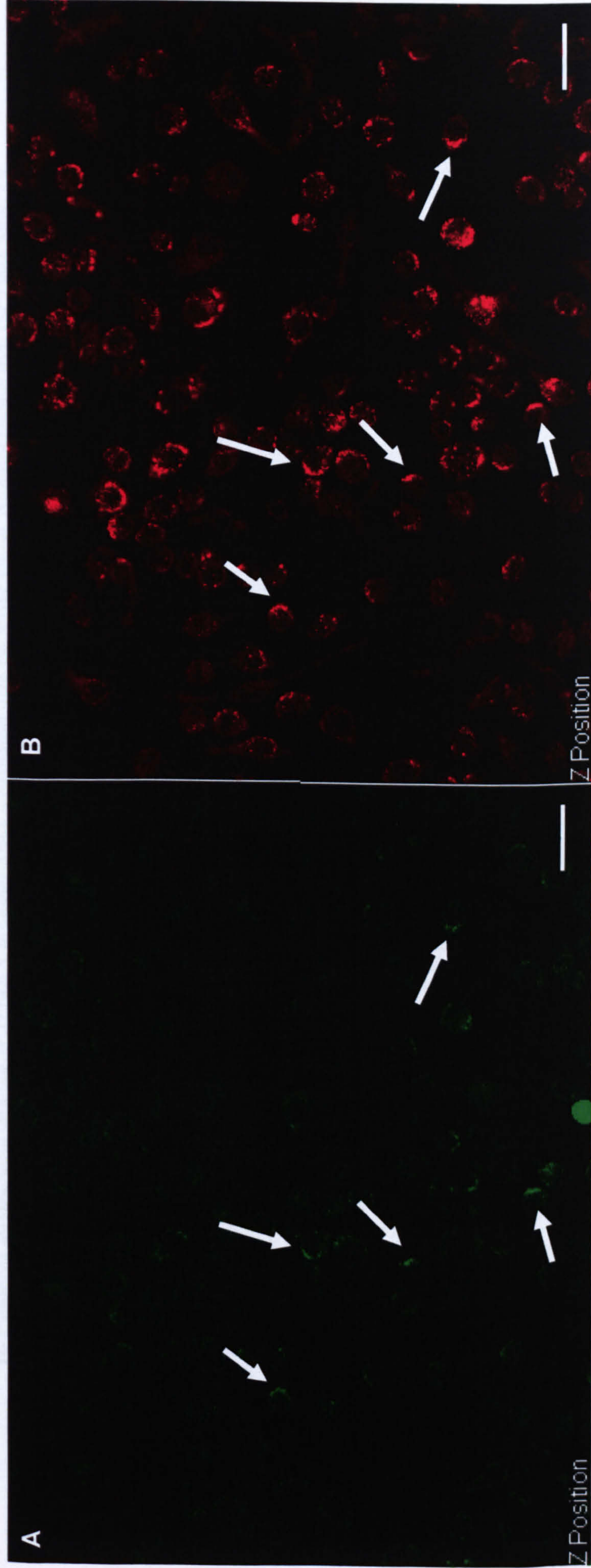


Figure 5.5: Confocal microscopy images showing intracellular localisation of CpG

C: Composite confocal microscopy image of panel A and B from page 134 of FAM-CpG (green) uptake by J774A.1 macrophages including LysoTracker™ (red) to stain acid compartments within the cells. This image shows that the FAM-CpG and LysoTracker™ co-localise inside the cells (orange) as indicated by the white arrows.

D: Confocal microscopy images of J774A.1 macrophages inoculated with ISCOMs alone, demonstrating that ISCOMs have no natural fluorescence that will interfere with the fluorescence of the FAM-CpGs.

Bar = 40 μ m

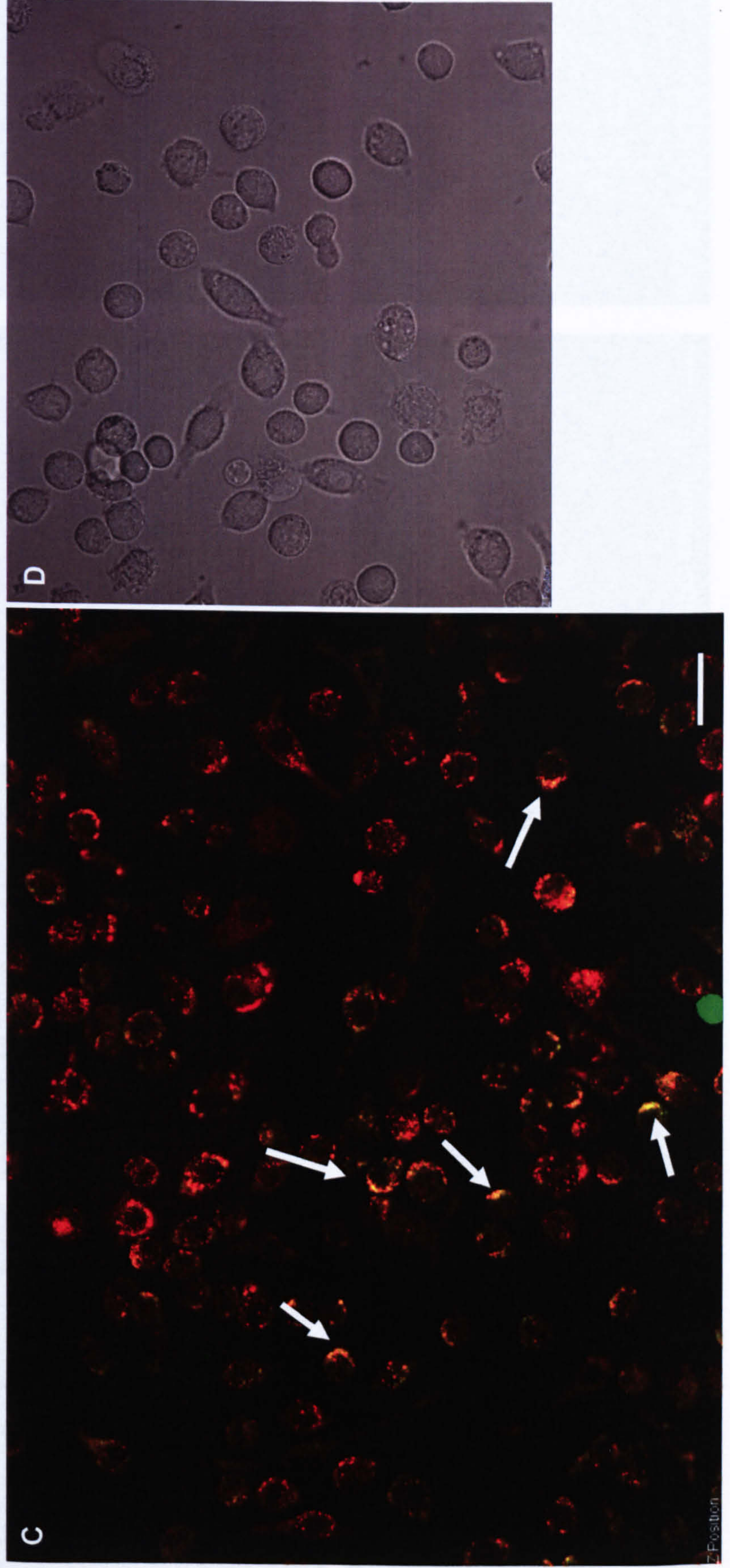


Figure 5.6: Increased uptake of FAM-CpG by J774A.1 in the presence of ISCOMs
 A = 1 h; B = 2 h; C = 4 h; D = 6 h; E = 8 h, F = 16 h.

These images demonstrate that a greater number of cells internalise FAM-CpG when mixed with ISCOMs. After around six hours J774A.1 cells seem to start dying in the presence of ISCOMs (determined by cell detachment from the glass slide) Images shown are representative of 8 images taken at each time point.

Bar = 40 μ m

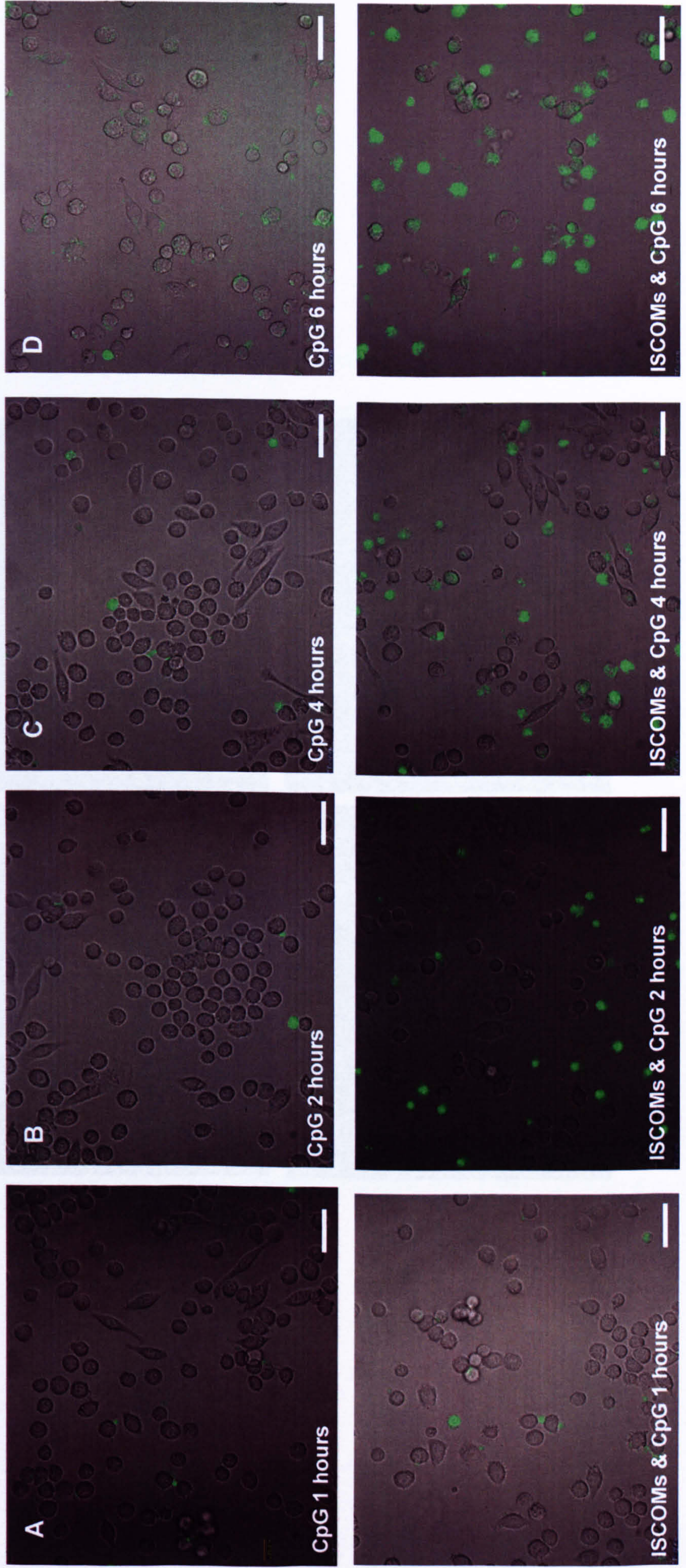


Figure 5.6: Increased uptake of FAM-CpG by J774A.1 in the presence of ISCOMs

A = 1 h; B = 2 h; C = 4 h; D = 6 h; E = 8 h, F = 16 h.
Bar = 40 μ m

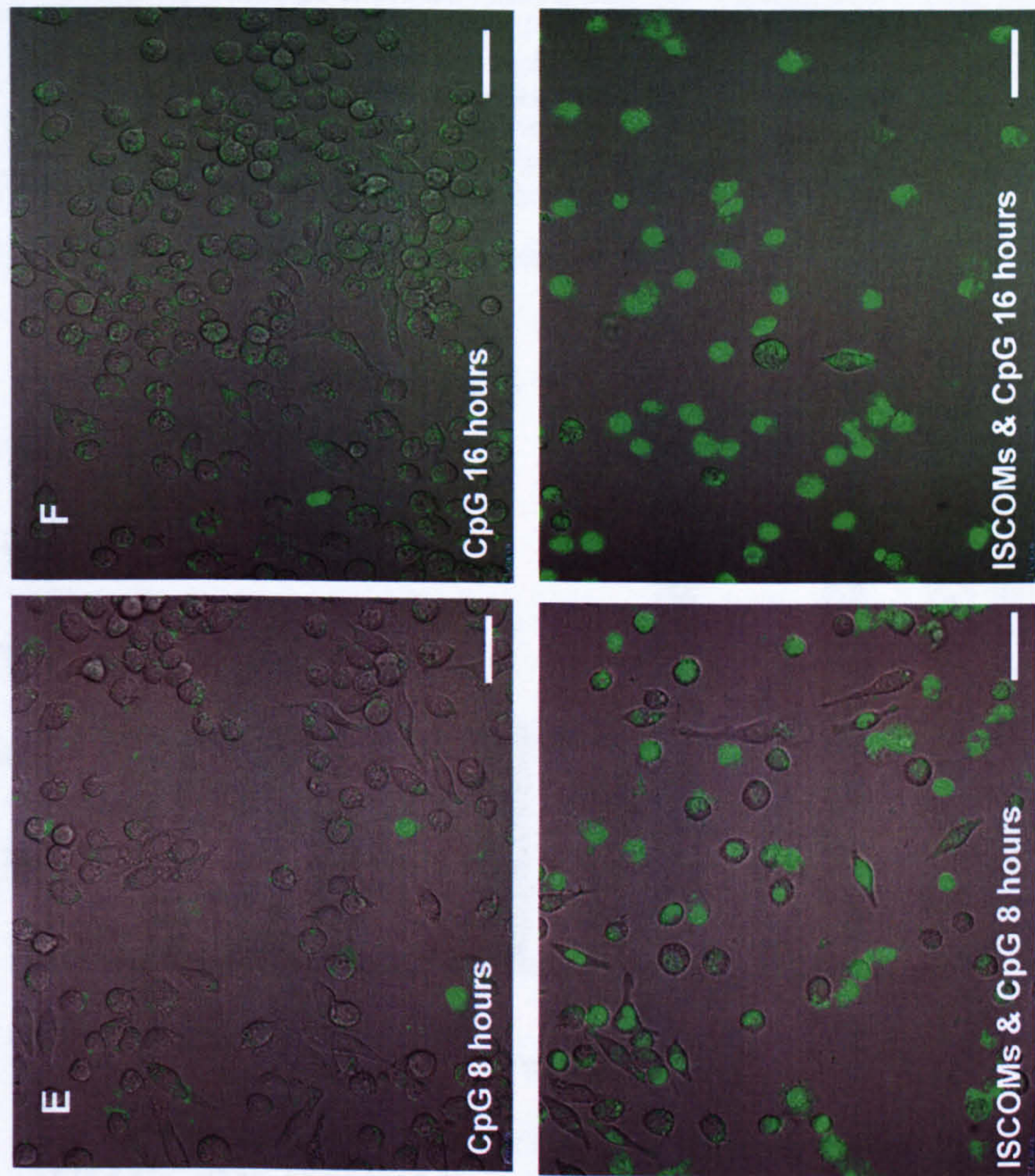
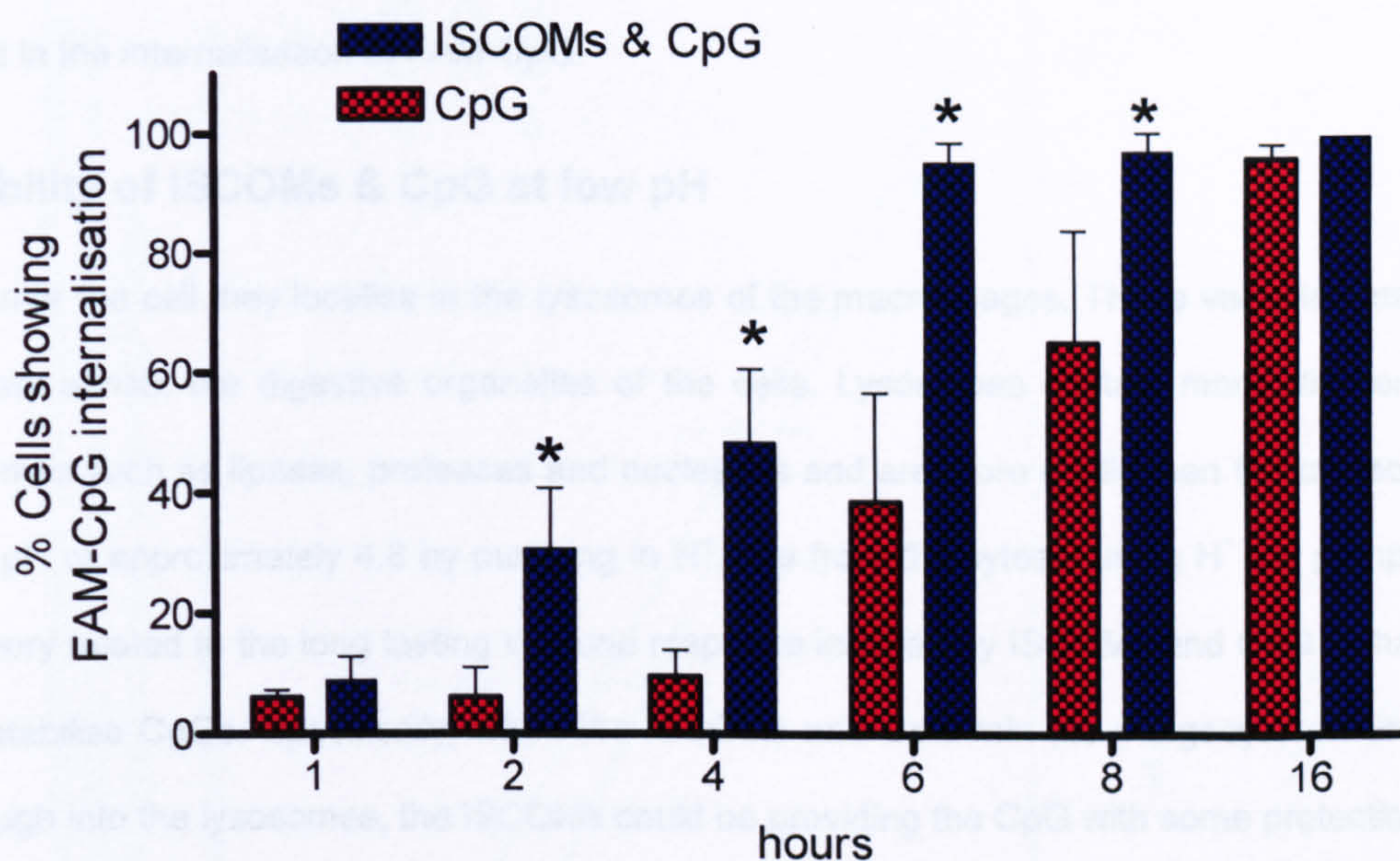


Figure 5.7: Uptake of FAM-CpG ± ISCOMs over a 16 hour period.

The graph shows the percentage of J774A.1 cells positive for internalisation of FAM-CpG. J774A.1 cells were administered 5 µg FAM-CpG ± 10 µg ISCOMs at 1, 2, 4, 6, 8 and 16 hours after the cells were visualised using confocal microscopy. Eight random fields of view were captured at each timepoint. The % of FAM-CpG positive cells was calculated as a percentage of the total cells on each image. Each bar on the graph represents the average % FAM-CpG positive cells over the eight fields of view captured. Although there is no significant difference at 16 hours cells administered both ISCOMs and CpG seem to have a greater uptake of CpG.

Error bar indicate the standard deviation of the data



* = significantly greater FAM-CpG in cells administered with ISCOMs and FAM-CpG when compared to cells administered with FAM-CpG only ($p < 0.0002$, t-test)

The results from this study indicate that FAM-CpG are internalised quicker in the presence of ISCOMs than they are when administered to cells alone. Within 2 hours of administration approximately 30 % of the cells inoculated with ISCOMs and CpG were positive for CpG uptake. In comparison, approximately 6% of cells inoculated with FAM-CpG only were positive for FAM-CpG. At time points 2, 4, 6 and 8 hours the cells inoculated with ISCOMs and CpG contain significantly ($P < 0.0002$) more internalised CpG than the cells dosed only with CpG showing that, in the presence of ISCOMs, FAM-CpG are more effectively internalised. After 6 hours approximately 95 % of the cells dosed with ISCOMs and FAM-CpG contained FAM-CpG. In comparison, it took 16 hours for approximately 96 % of cells that were dosed with FAM-CpG alone internalise FAM-CpG. After eight hours of incubation, cells administered with ISCOMs and CpG started to die, due to the toxicity of the saponin component of the ISCOMs. Overall, this data provides evidence to suggest that ISCOMs assist in the internalisation of FAM-CpG.

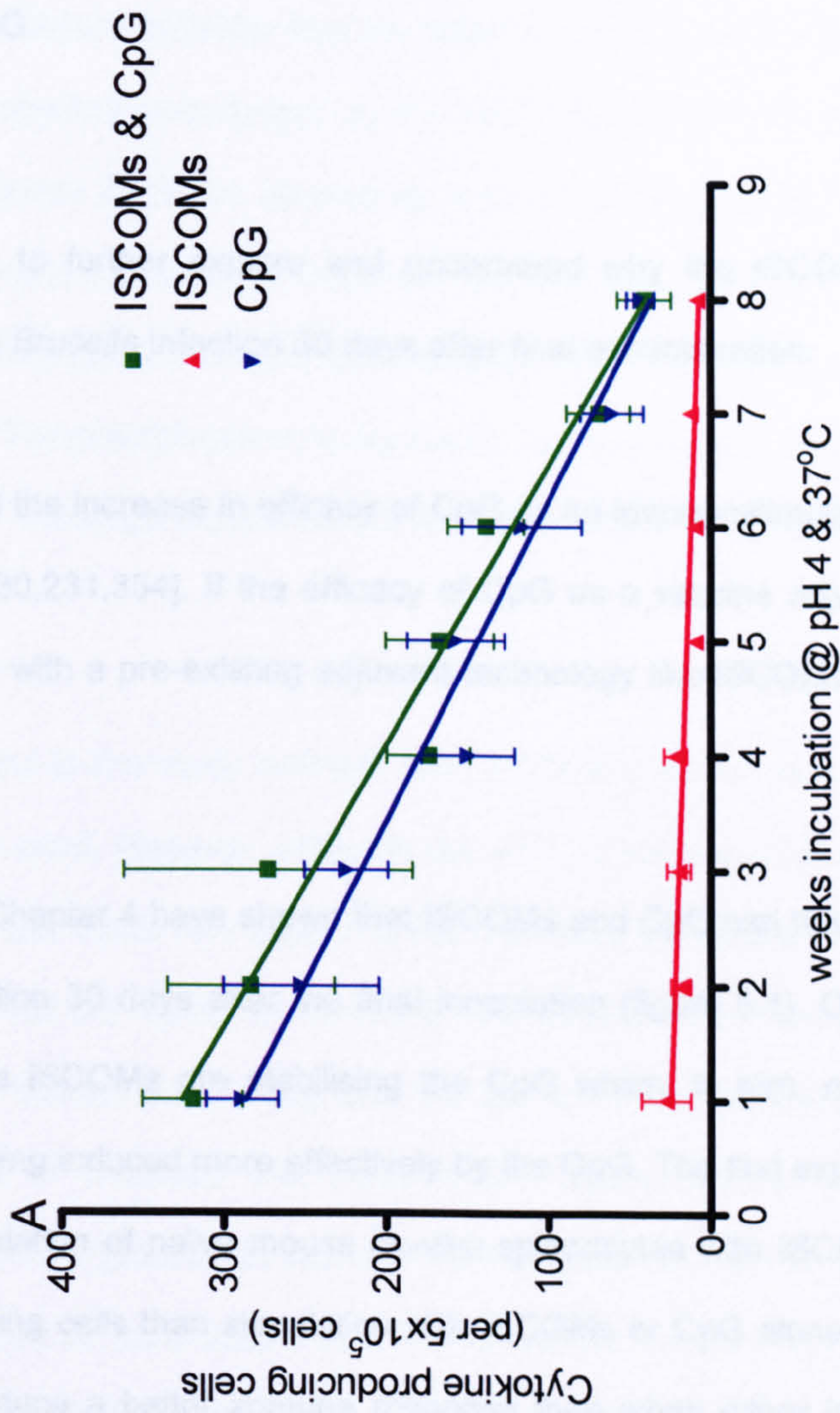
5.5 Stability of ISCOMs & CpG at low pH

When CpGs enter the cell they localise in the lysosomes of the macrophages. These vacuoles are, in a rudimentary sense, the digestive organelles of the cells. Lysosomes contain many different digestive enzymes such as lipases, proteases and nucleases and are more acidic than the cytosol, maintaining a pH of approximately 4.8 by pumping in H^+ ions from the cytosol using H^+ ion pumps [276]. One theory related to the long lasting immune response induced by ISCOMs and CpG is that the ISCOMs stabilise CpGs. Specifically, when the ISCOMs and CpG mix are phagocytosed and trafficked through into the lysosomes, the ISCOMs could be providing the CpG with some protection from the low pH and the enzymes present in the lysosomes. To assess the stability of CpG in the presence of ISCOMs, a pH 4 buffer was prepared from a mixture of sodium phosphate and citric acid buffers (section 2.6.3.2) and ISCOMs \pm CpG were incubated in the buffer for eight weeks at 37°C to simulate the low pH of a lysosome. At one week intervals the ISCOMs \pm CpG were diluted to working concentrations (40 $\mu\text{g/ml}$ ISCOMs and 20 $\mu\text{g/ml}$ CpG (section 2.6.3.2.2)) and 100 μl of the mixture (4 $\mu\text{g/ml}$ ISCOMs and 2 $\mu\text{g/ml}$ CpG) was used to simulate naïve mouse *ex-vivo* splenocytes. The number of IFN- γ producing cells was measured via ELISPOT assay to assess the activity of the ISCOMs and CpG (figure 5.8).

Figure 5.8: IFN- γ production from ex-vivo splenocytes with stimulated with 2 μg CpG \pm 4 μg ISCOMs incubated at pH 4 and at 37°C for an eight week period.

Panel A shows the ANCOVA linear regression analysis of the number of IFN- γ producing cells from naïve mouse ex-vivo splenocytes stimulated with 2 μg CpG \pm 4 μg ISCOMs incubated at pH 4 and at 37°C for an eight week period. Panel B shows the p values obtained from the ANCOVA linear regression analysis and their significance.

Panel A error bars indicate the standard deviation of the data



B

	ISCOMs & CpG	CpG	ISCOMs
ISCOMs & CpG	-	p = 0.1225	p < 0.0001
CpG	ns	-	p < 0.0001
ISCOMs	***	***	-

*** = p < 0.0001
ns = no significance

The results from this experiment show that *ex-vivo* splenocytes stimulated with ISCOMs and CpG or CpG alone both produce more IFN- γ producing cells than cells stimulated with ISCOMs alone ($p < 0.05$, figure 5.8). Similarly, the cells stimulated with ISCOMs and CpG appear to produce more IFN- γ producing cells than cells stimulated with CpG alone, although the difference is not significant ($P > 0.05$).

The results also indicate that the activity of both ISCOMs and CpG and CpG alone decreases over time when incubated at 37°C and pH 4. However, the decrease in activity over time is the same for both CpG alone and ISCOMs and CpG indicating that, in this assay, the ISCOMs do not seem to stabilise the CpG.

5.6 Conclusions

The aim of this work was to further explore and understand why the ISCOMs and CpGs provided protection during a *Brucella* infection 30 days after final administration.

Other studies have reported the increase in efficacy of CpG as an immunostimulant using many novel techniques [221,27,230,231,354]. If the efficacy of CpG as a vaccine adjuvant could be increased by administration with a pre-existing adjuvant technology like ISCOMs this would be very advantageous.

The observations made in Chapter 4 have shown that ISCOMs and CpG can have a controlling effect over a *Brucella* infection 30 days after the final inoculation (figure 5.1). One theory that may explain this is that the ISCOMs are stabilising the CpG which, in turn, means that the innate immune system is being induced more effectively by the CpG. The first experiment in this study has shown that stimulation of naïve mouse *ex-vivo* splenocytes with ISCOMs and CpG induced more IFN- γ producing cells than stimulation with ISCOMs or CpG alone, which shows that together they could induce a better immune response than when either is administered alone. The experiments performed in this study have demonstrated that, when administered together, ISCOMs and CpG could be a better adjuvant combination than when used separately. Specifically, these experiments have shown that ISCOMs can assist internalisation of CpG into cells at a faster rate than CpG alone, perhaps inducing a faster and/or longer immune response.

Although a number of experiments have been performed it has been difficult to demonstrate interactions between ISCOMs and CpG during an *in vitro* assay as cells start to die 6 to 8 hours after administration of ISCOMs. Thus, an animal experiment was completed to determine the resting IFN- γ response over a 6 week period after inoculation with CpG \pm ISCOMs. After three doses of ISCOMs and CpG the number of IFN- γ producing cells drops after the first 24 hours but increases slowly over a six week period to levels observed 24 hours after the final inoculation. Interestingly, all of the other inoculated groups also showed this pattern, although at a significantly lower rate than the animals given ISCOM and CpG. The high quantity of IFN- γ producing cells observed 24 hours after the final inoculation in the experimental groups is the initial stimulation of the innate immune system by the ISCOMs and/or CpG. However, in the PBS treated mice the response could be caused by the inoculation trauma which is not an uncommon response to a trauma event even in the human population [329]. What is clear from this data is that ISCOMs and CpG do cause a slow increase in the number of IFN- γ producing cells over a 6 week period. This 6 week period is equivalent to 42 days after final inoculation, so the data is applicable to the challenge experiments in chapter 4 where mice were challenged 30 days after final inoculation.

The stability assay carried out in this study indicated that ISCOMs were not able to stabilise the CpG in the pH 4 conditions used. However, although the pH 4 conditions used to incubate the CpG \pm ISCOMs are harsh and mirror one aspect of the lysosome environment, there are also other lysosomal conditions that are harder to effectively reproduce *in vitro*. This assay is limited to mimicking the pH of the lysosome but along with the low pH of the lysosome environment, there are also a plethora of enzymes that could attack CpGs causing breakdown and inactivation. Although it would be possible to incubate the ISCOMs and CpG at pH 4 along with enzymes, it would be difficult to accurately mimic the lysosome environment. Although this assay is limited, it does highlight that CpG 10103 \pm ISCOMs maintain their activity under very harsh conditions, retaining approximately 50 % of the activity after 5 weeks of incubation at pH 4 when compared to 1 week of incubation. The slow decrease in activity could be because CpG 10103 is a class B CpG which has a fully phosphorothioate backbone, which is inherently stable and resistant to breakdown [305].

Although the mechanism by which ISCOMs enhance CpG uptake into cells is not fully understood one theory may be that when the CpGs and ISCOMs are mixed the CpG are protected/stabilised by the ISCOM cages and the CpG and ISCOMs are internalised together. As the ISCOMs are larger than the CpG they are more likely to be phagocytosed as foreign antigens which would cause them to be trafficked to the lysosomes where TLR9 is present.

The data described in this chapter has shown that ISCOMs and CpG induce high numbers of IFN- γ producing cells which could assist in the clearance of a bacterial infection. It has also been demonstrated that ISCOMs increase the rate of internalisation of CpG into cells. Mice administered ISCOMs and CpG also produce a slowly increasing resting IFN- γ response up to six weeks post final inoculation. All these data suggest that ISCOMs and CpG could be better adjuvants when combined and administered together. There is also evidence to suggest that ISCOMs and CpG can produce a low level immune response 30 days after final inoculation which is strong enough to control a *Brucella* infection.

Chapter 6 - Optimisation of PotD and PotF as vaccine candidates

6.1 Introduction

The identification of the novel protective vaccine candidates PotD and PotF is a step forward in finding a suitable human and animal vaccine against brucellosis. During the course of this study it has been observed that, as well as being used to adjuvant PotD and PotF proteins, ISCOMs & CpG alone can be used to control a *Brucella* infection, making it difficult to fully assess the protective capabilities of PotD and PotF. It is possible that the CpGs are continuously being slowly released from the ISCOMs causing the immune response to be boosted. This slow release could mean that the CpGs are still present at 30 days after the final inoculation, the point at which inoculated mice are challenged.

Work presented in Chapter 5 has shown that mice inoculated with ISCOMs & CpG show higher resting IFN- γ production after 42 days than mice immunised with either ISCOMs or CpG alone. This provides some data to help explain the controlling effect that ISCOMs and CpG have on a *Brucella* infection. In this chapter, further studies have been designed to try to eliminate the controlling adjuvant effect in order to assess the potential of PotD and PotF as vaccine candidates against brucellosis.

The aim of this chapter is to fully explore the protective efficacy of PotD and PotF proteins observed in Chapter 4 using a number of animal trials designed to test PotD and PotF protective efficacy over longer time periods and in combination with different adjuvants. The creation and evaluation of PotD and PotF DNA vaccine is also presented in this chapter.

6.2 An evaluation of protective efficacy of PotD and PotF vaccine antigens after 60 days

During the course of this project, mice have been challenged 30 days after their final inoculation with proteins adjuvanted with ISCOMs and CpG. Results from Chapter 5 indicate that there could be an immune response still present in mice inoculated with ISCOMs and CpG at this timepoint. Extending the time of challenge from 30 days to 60 days after the final inoculation may enable a reduction in the immune response generated by ISCOMs and CpG in order to evaluate the protective efficacy of PotD and PotF.

6.2.1 Immunisation schedule

The protective efficacy of PotD and PotF was tested in the murine model of *B. melitensis* infection. Groups of 10 female Balb/C mice (9 – 11 weeks old) were inoculated with 10 µg protein adjuvanted with 12.5 µg ISCOMs and 6.25 µg CpG administered intramuscularly three times at three week intervals. Two positive control groups were included in this study; to assess *B. melitensis* Rev.1 protection after 60 days specifically, both groups of mice were immunised with a single sub-cutaneous dose of approximately 2×10^5 *B. melitensis* Rev.1 at either 30 days (Rev.1 30) or 60 days before challenge (Rev.1 60). Negative control groups of mice immunised with PBS or ISCOMs and CpG adjuvant only were also included. Sixty days after the final protein inoculation all mice were challenged with 1.41×10^4 of CFU *B. melitensis* 16M via the intraperitoneal route. Fifteen days after challenge all mice were culled and splenic colonisation assays were performed as previously described (section 2.5.4).

6.2.2 An evaluation of protective efficacy of PotD and PotF over 60 days

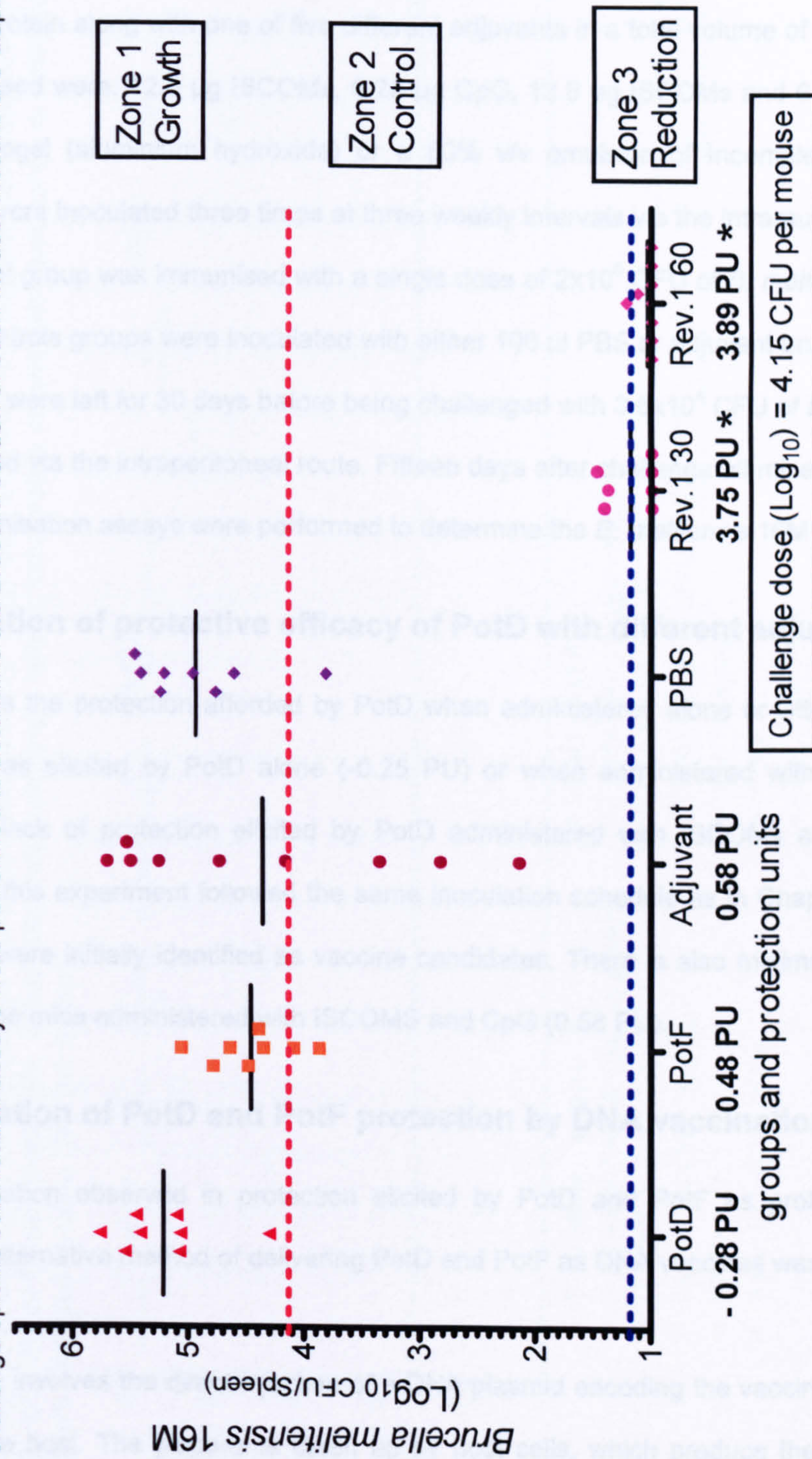
The protection provided by PotD and PotF in this experiment (figure 6.1) differs considerably from that observed in the previous experiment (Chapter 4, figure 4.18, page 120). Results show good protection induced by both Rev.1 30 and Rev.1 60 and, as expected, no protection from the PBS inoculated mice. However, there is no protection elicited by PotD (-0.28 PU) and PotF (0.48 PU) in this study. Increasing the time to challenge has reduced the protective effect of the adjuvant although, in this study, administration of the adjuvant alone caused significant variation in splenic *Brucella* load and has produced the highest PU of all the experimental groups.

6.3 An evaluation of adjuvants for PotD

Adjuvants are used to enhance the immune response to an antigen. There are different types of adjuvant designed to increase or prolong the immune response to an antigen, and the adjuvant used in these studies previously have been ISCOMs and CpGs. As mice immunised with ISCOMs and CpGs has controlled *Brucella* infection, confirmation of the protection afforded by PotD and PotF proteins administered alongside the ISCOMs and CpG has proven difficult. It is possible that the use of an alternative adjuvant may enable clarification or improvement of the

Figure 6.1: Protective efficacy of PotD and PotF in mice challenged 60 days after final inoculation

The protective efficacy of PotD and PotF vaccine candidates after mice were administered with three intramuscular doses at three week intervals of 10 µg protein adjuvanted with 12.5 µg ISCOMs and 6.25 µg CpG. Sixty days after their final immunisation mice were challenged via the intraperitoneal route with 1.41×10^4 CFU of *B. melitensis* 16M. Fifteen days after challenge splenic colonisation assays were performed to determine the *Brucella* burden of each mouse. Results are displayed as Log₁₀ CFU per spleen.



* Significant reduction in bacterial load when compared to PBS ($p < 0.001$, 1-way ANOVA + Bonferroni's post test)

Adjuvants = 12.5µg ISCOMs + 6.25µg CpG

Black bars represent the mean Log₁₀ *Brucella* CFU/spleen of each group

Protection Units calculated by [mean Log₁₀ CFU Naïve – mean Log₁₀ CFU vaccine candidates]

Rev.1 30 = Rev.1 administered 30 days before challenge. Rev.1 60 = Rev.1 administered 60 days before challenge.

Zone 1: denotes growth above the challenge dose administered

Zone 2: denotes control of *Brucella* growth

Zone 3: denotes a reduction of *Brucella* growth below the averages of the Rev.1 vaccine

protective efficacy of these proteins. Here PotD protein was evaluated in combination with a range of adjuvants for its protective efficacy against brucellosis.

6.3.1 Immunisation schedule

The efficacy of PotD administered with different adjuvants was tested in the murine model of *B. melitensis* infection. Groups of six female Balb/C mice (9 - 11 weeks old) were inoculated with 10 µg of PotD protein along with one of five different adjuvants in a total volume of 100 µl PBS. The adjuvants used were: 12.5 µg ISCOMs, 6.25 µg CpG, 12.5 µg ISCOMs and 6.25 µg CpG, 20% v/v Alhydrogel (aluminium hydroxide) or a 50% v/v emulsion of Incomplete Freund's Adjuvant. Mice were inoculated three times at three weekly intervals via the intramuscular route. A positive control group was immunised with a single dose of 2×10^5 CFU of *B. melitensis* Rev.1 and negative controls groups were inoculated with either 100 µl PBS or adjuvant only. After final inoculation mice were left for 30 days before being challenged with 3.6×10^4 CFU of *B. melitensis* 16M administered via the intraperitoneal route. Fifteen days after challenge all mice were culled and splenic colonisation assays were performed to determine the *B. melitensis* 16M load.

6.3.2 Evaluation of protective efficacy of PotD with different adjuvants

Figure 6.2 shows the protection afforded by PotD when administered alone or with adjuvants. No protection was elicited by PotD alone (-0.25 PU) or when administered with any of the adjuvants. The lack of protection elicited by PotD administered with ISCOMs and CpGs is surprising since this experiment followed the same inoculation schedule as in Chapter 4, when PotD and PotF were initially identified as vaccine candidates. There is also minimal protection observed from the mice administered with ISCOMS and CpG (0.58 PU).

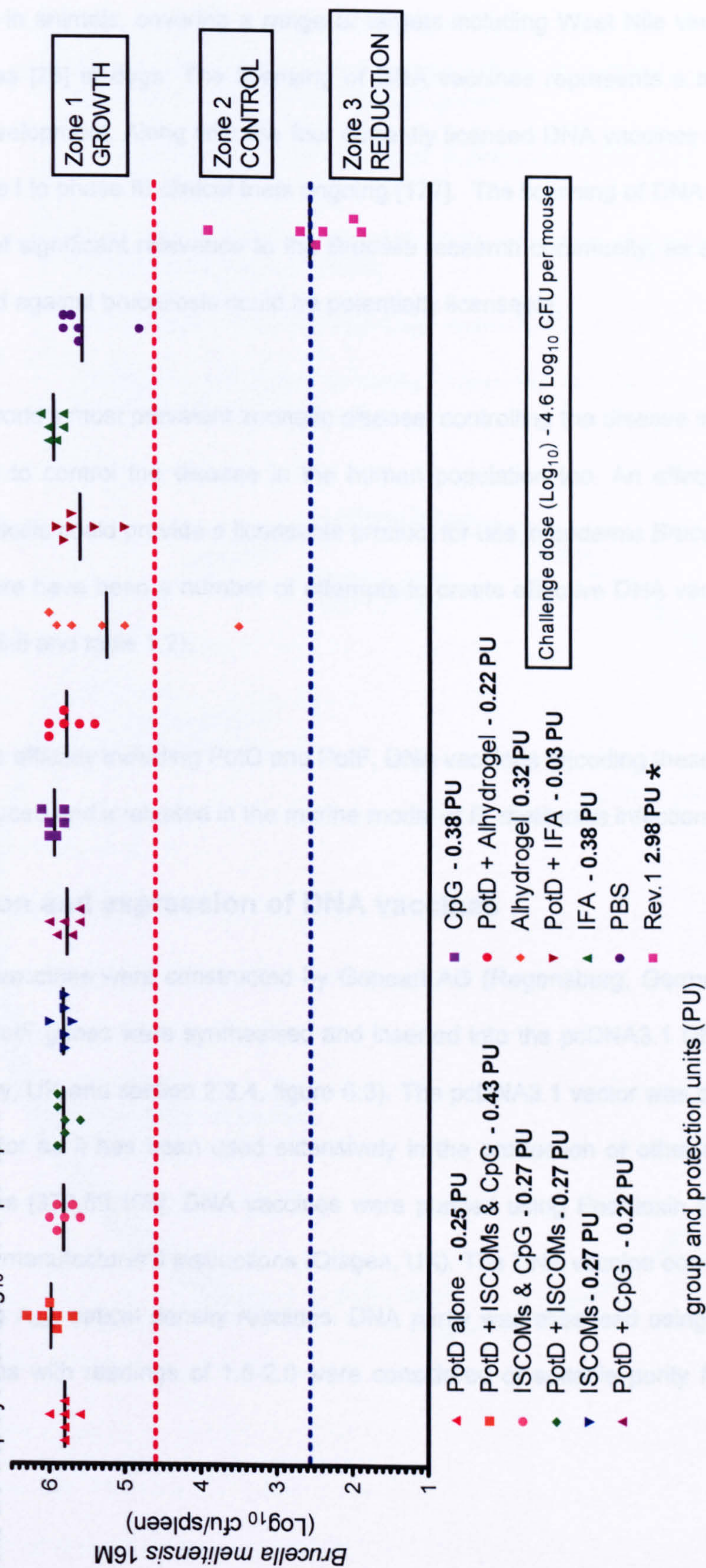
6.4 Evaluation of PotD and PotF protection by DNA vaccination

Due to the variation observed in protection elicited by PotD and PotF as protein vaccine candidates, an alternative method of delivering PotD and PotF as DNA vaccines was evaluated.

DNA vaccination involves the direct injection of a DNA plasmid encoding the vaccine candidate of interest, into a host. The plasmid is taken up by host cells, which produce the protein via transcription and translational mechanisms. Once produced, the protein is presented to the immune system. DNA vaccination has recently undergone a resurgence in popularity and, due

Figure 6.2: Protective efficacy of PotD administered with various different adjuvants

The protective efficacy of PotD vaccine candidates after mice were administered with three intramuscular doses at three week intervals of 10 µg protein adjuvanted with 12.5 µg ISCOMs ± 6.25 µg CpG, 20% Alhydrogel mix or 50% IFA emulsion. Thirty days after their final immunisation mice were challenged via the intraperitoneal route with 3.98×10^4 CFU of *B. melitensis* 16M. Fifteen days after challenge colonisation assays were performed to determine the *Brucella* burden of each mouse. Results are displayed as Log_{10} CFU per spleen.



* Significant reduction in bacterial load when compared to PBS ($p < 0.001$, 1-way ANOVA + Bonferroni's post test)

Black bars represent mean Log_{10} *Brucella* CFU/spleen of each group

Adjuvants = 12.5µg ISCOMs ± 6.25µg CpG, 20% Alhydrogel mixtures and 50% Freund's incomplete adjuvant emulsion

Protection Units calculated by [mean Log_{10} CFU PBS immunised mice – mean Log_{10} CFU vaccine candidate immunised mice]

Zone 1 = Growth of *Brucella* above challenge dose meaning not protection has been observed

Zone 2 = Bacterial numbers less than the challenge dose but more than the Rev.1 mean indicating control of the *Brucella* infection

Zone 3 = A reduction in *Brucella* CFU below the mean of Rev.1 representing a protective effect.

to improvements in molecular techniques and DNA vaccine understanding, they are becoming more effective at inducing strong immune responses. In recent years four DNA vaccines have been licensed for use in animals, covering a range of targets including West Nile virus [71] in horses and Melanomas [25] in dogs. The licensing of DNA vaccines represents a significant advancement their development. Along with the four currently licensed DNA vaccines there are over 90 different phase I to phase III clinical trials ongoing [177]. The licensing of DNA vaccines for use in animals is of significant relevance to the *Brucella* research community, as any novel DNA vaccine produced against brucellosis could be potentially licensable.

As brucellosis is the world's most prevalent zoonotic disease, controlling the disease in animals may be the best way to control the disease in the human population too. An effective DNA vaccine against brucellosis could provide a licensable product for use in endemic *Brucella* areas around the world. There have been a number of attempts to create effective DNA vaccines for brucellosis (section 1.6.5 and table 1.2).

In order to improve the efficacy including PotD and PotF, DNA vaccines encoding these vaccine candidates were produced and evaluated in the murine model of *B. melitensis* infection.

6.4.1 Construction and expression of DNA vaccines

PotD and PotF DNA vaccines were constructed by Genart AG (Regensburg, Germany). The truncated PotD and PotF genes were synthesised and inserted into the pcDNA3.1 DNA vector (Invitrogen Ltd, Paisley, UK and section 2.3.4, figure 6.3). The pcDNA3.1 vector was chosen as the DNA vaccine vector as it has been used extensively in the production of other candidate *Brucella* DNA vaccines [370,59,199]. DNA vaccines were purified using Endotoxin Free Giga prep kits according to manufacturer's instructions (Qiagen, UK). The DNA vaccine concentration was determined using A_{260} optical density readings. DNA purity was assessed using A_{260}/A_{280} ratios and preparations with readings of 1.8-2.0 were considered of suitable purity for further use.

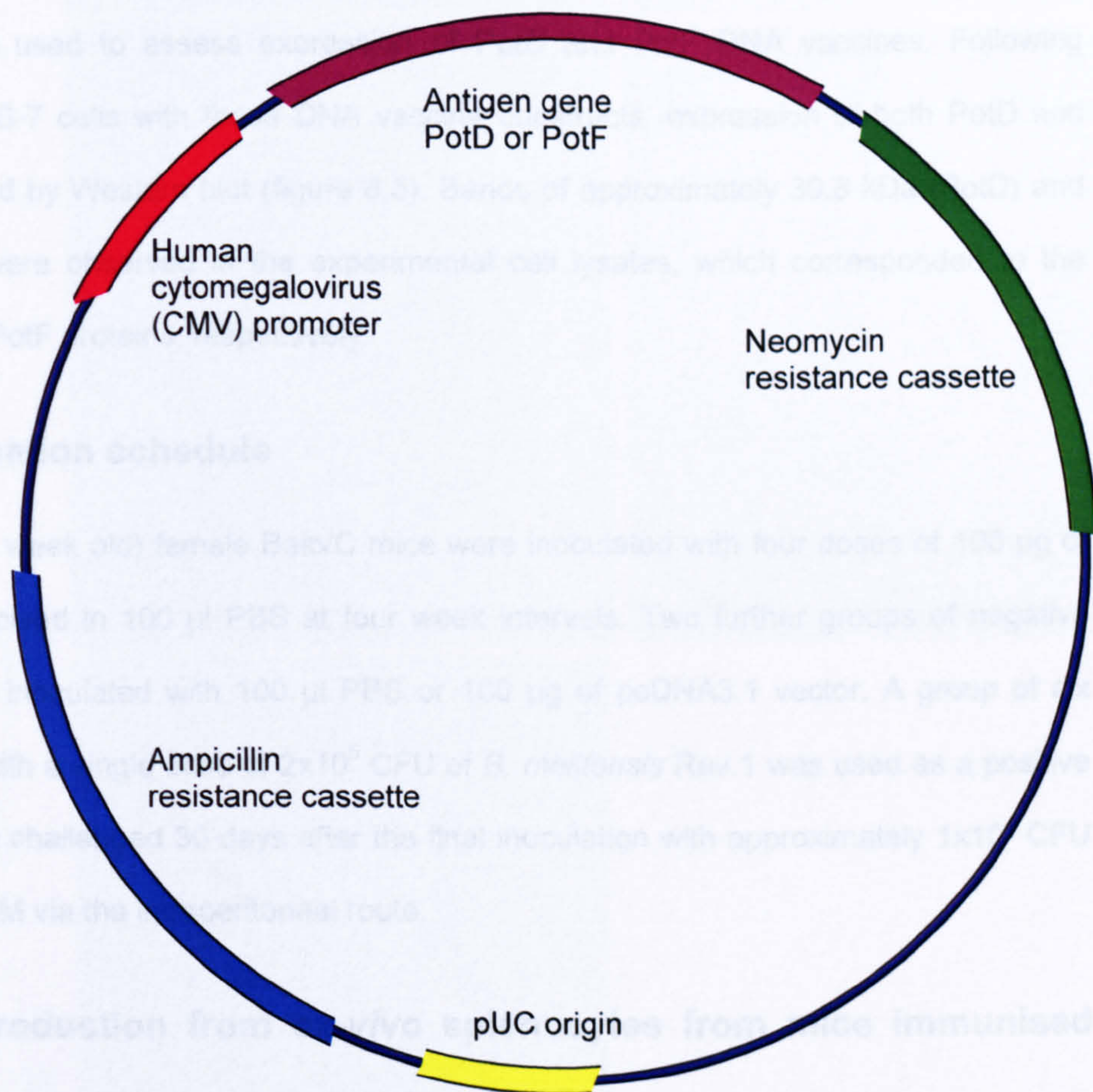
Figure: 6.3: Schematic representation of pcDNA3.1 vector containing PotD or PotF antigen genes

CMV promoter: Allow high level expression of the recombinant vaccine antigen

Neomycin resistance cassette: Allows selection of transfectants in mammalian tissue culture

pUC origin: Allows for high-copy replication in *E. coli*

Ampicillin resistance cassette: Allow for selection of stable transfectants in *E. coli*



Expression of PotD and PotF DNA vaccines was confirmed by transfection of COS-7 mammalian kidney cells with the DNA vaccines and subsequent evaluation by Western blotting (section 2.3.4.4 and 2.4.1.2). To assess the optimal transfection and expression conditions for the DNA vaccines two different transfection reagents (GeneJuice® or Polyfect®) were used. COS-7 cells were harvested and cultured in six well plates at a density of 4×10^5 cells, the plates were incubated overnight at 37°C and 5% CO₂. COS-7 cells were transfected with a plasmid expressing green fluorescent protein (GFP) using either GeneJuice® or Polyfect® transfection reagents and DNA to transfection reagent ratios recommended in the manufacturer's instructions. The cells were then incubated for 24 or 48 hours, after which the expression of GFP was assessed using confocal microscopy (figure 6.4, section 2.6.3.1.2). The optimal transfection reagent and expression length (GeneJuice® at a ratio of 3 µl to 1 µg DNA vaccine for 48 hours) was used to assess expression of PotD and PotF DNA vaccines. Following transfection of COS-7 cells with these DNA vaccine constructs, expression of both PotD and PotF was confirmed by Western blot (figure 6.5). Bands of approximately 39.8 kDa (PotD) and 42.4 kDa (PotF) were observed in the experimental cell lysates, which corresponded to the purified PotD and PotF proteins, respectively.

6.4.2 Immunisation schedule

Groups of six (6-8 week old) female Balb/C mice were inoculated with four doses of 100 µg of DNA vaccine dissolved in 100 µl PBS at four week intervals. Two further groups of negative control mice were inoculated with 100 µl PBS or 100 µg of pcDNA3.1 vector. A group of six mice immunised with a single dose of 2×10^5 CFU of *B. melitensis* Rev.1 was used as a positive control. Mice were challenged 30 days after the final inoculation with approximately 1×10^4 CFU of *B. melitensis* 16M via the intraperitoneal route.

6.4.3 IFN-γ production from ex-vivo splenocytes from mice immunised with DNA vaccines

The DNA vaccines were initially evaluated for their immunogenicity. Specifically, three mice were immunised using the schedule described in section 6.4.2 but, instead of receiving a challenge, mice were culled and their spleens removed and the number of IFN-γ producing cells

Figure 6.4: GFP expression from COS-7 cells transfected using GeneJuice® or Polyfect® transfection reagents

Confocal microscopy images representing COS-7 cells either untransfected (A) or transfected with a GFP expressing plasmid using two different transfection reagents, and different ratios of DNA: transfection reagent and different expression lengths (B-E)

Bar = 50 μ M

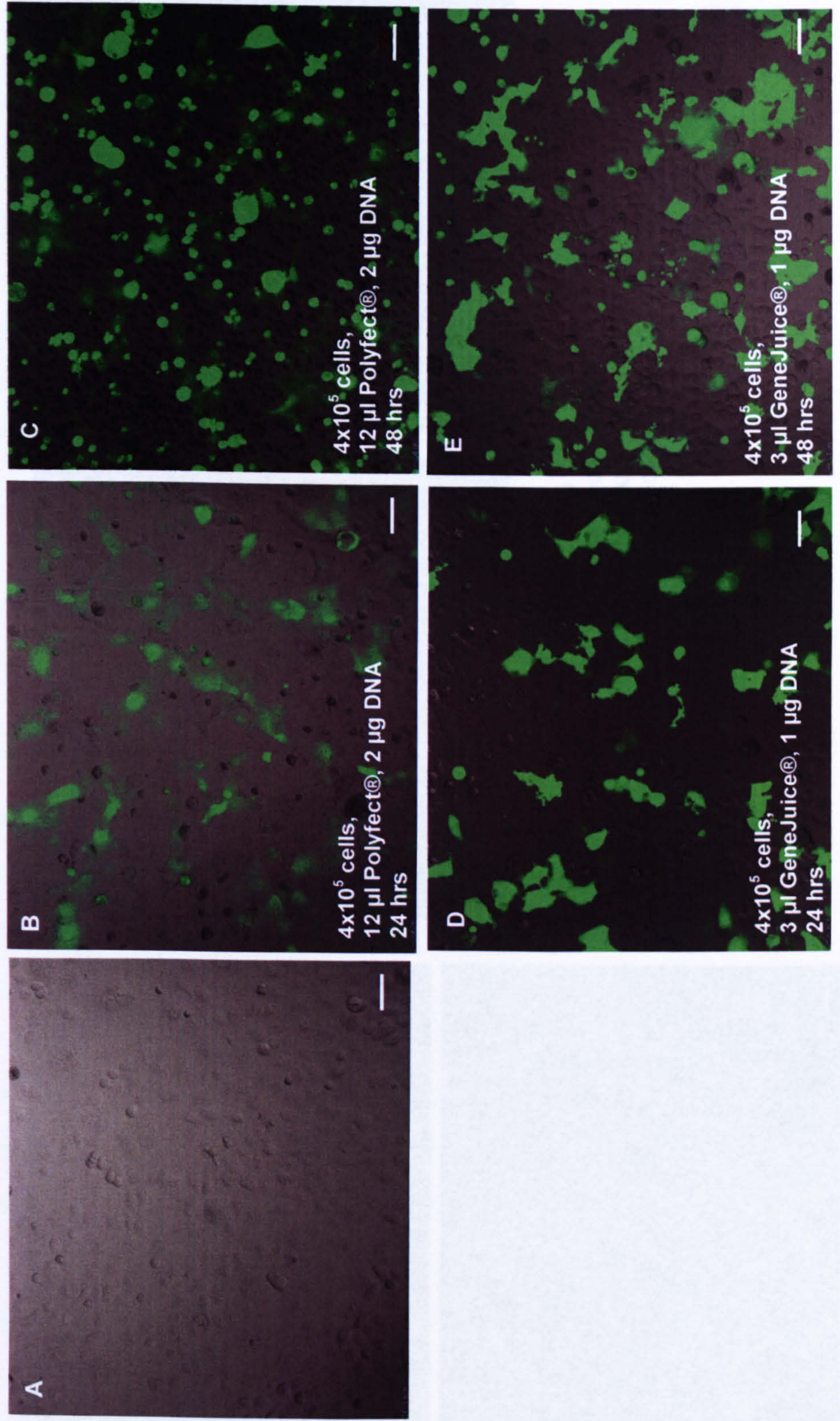
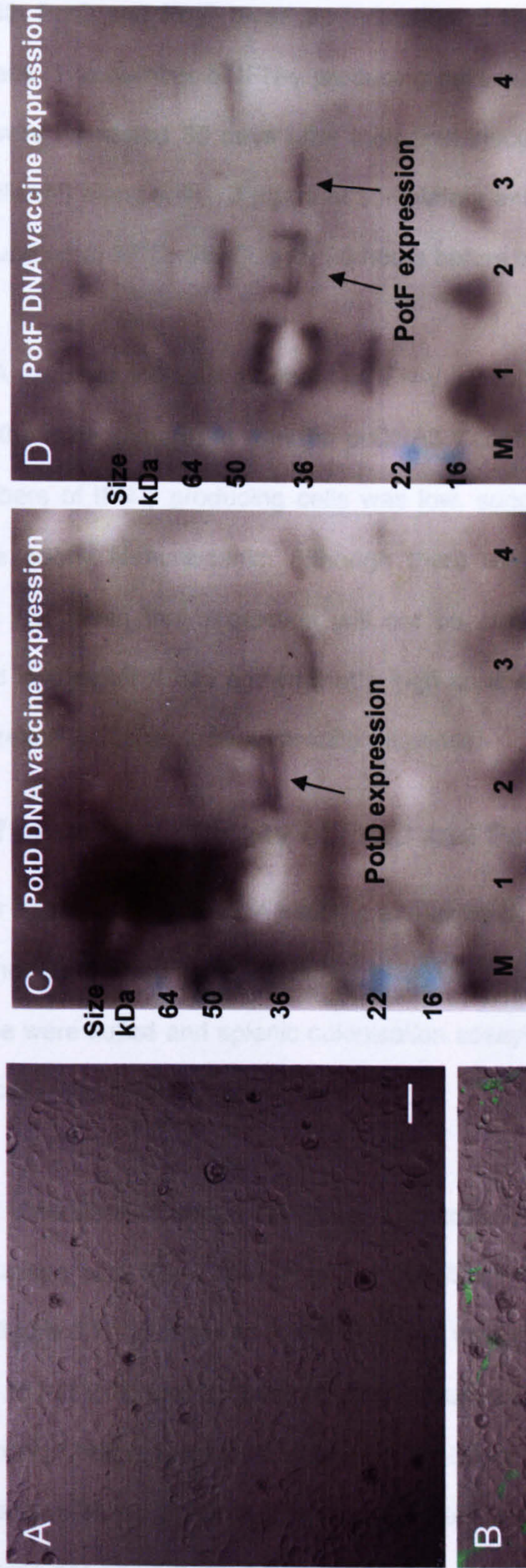


Figure 6.5 PotD and PotF DNA vaccine expression in COS-7 mammalian cells
 Confocal images (A and B) show examples of untransfected cells (A) and COS-7 cells transfected with the GFP expressing plasmid used previously as a positive control (B). Western blots (C and D) show expression from COS-7 cells transfected with the PotD (C) or PotF (D) DNA vaccines.



M = SeeBlue® marker (Invitrogen)
 Lane 1 = Purified PotD or PotF protein
 Lane 2 = PotD or PotF DNA vaccine pellet extract
 Lane 3 = PotD or PotF DNA vaccine supernatant extract
 Lane 4 = Blank pcDNA3.1 expression control

was quantified after stimulation with PotD and PotF antigens. IFN- γ has been established as an important cytokine in *Brucella* immunology [229]. Measuring the number of IFN- γ producing cells after stimulation with PotD and PotF gives an indication of the strength of the immune response to these antigens. The number of IFN- γ producing cells was measured by ELISPOT assay; mouse spleens were harvested 30 days after their final inoculation, homogenised, and plated out (at 5×10^6 cells/ml) along with 10 μ g/ml of stimulatory antigen (either PotD or PotF protein). Plates were incubated at 37°C, 5% CO₂ for 20 hours before being developed.

The PotD and PotF DNA vaccines induced numbers of IFN- γ producing cells in mice that was significantly higher than the mice immunised with the pcDNA3.1 control vector ($p < 0.05$ (figure 6.6). However, the numbers of IFN- γ producing cells was low, suggesting that the PotD and PotF DNA vaccines are poorly immunogenic. Although there was a low number of IFN- γ producing cells, it does not mean that protection will not be observed against brucellosis. Previous work presented in Chapter 4 has shown that a high number of IFN- γ producing cells does not necessarily correlate with a protective immune response.

6.4.4 Evaluation of protective efficacy of PotD and PotF DNA vaccines

Mice inoculated with four doses of 100 μ g DNA vaccine at four week intervals were challenged 30 days after their final inoculation with approximately 1×10^4 CFU of *B. melitensis* 16M. Fifteen days after challenge mice were culled and splenic colonisation assays were performed (section 2.5.4) to assess the *Brucella* load in the spleen.

There was a significant difference in splenic bacterial load mice inoculated with PBS when compared to mice immunised with Rev.1, showing that the Rev.1 vaccine provided effective protection as expected (figure 6.7). Vaccination with PotF DNA vaccine resulted in control of the *Brucella* infection with a PU of 2.76 and an average Log₁₀ *Brucella* CFU of 3.03, a significant reduction in *Brucella* burden when compared to naked pcDNA3.1 or PBS inoculated mice ($p < 0.05$). The PotD DNA vaccine had a PU of 2.08 which indicates a reduction in bacterial load. However, this reduction was not significantly different from the negative controls ($p > 0.05$), due to the amount of variation seen within the data set. These results indicated that PotF DNA vaccine can be considered novel protective antigens against brucellosis.

Figure 6.6: IFN- γ production from *ex-vivo* splenocytes of mice immunised with PotD and PotF DNA vaccines after 20 hours stimulation with 10 $\mu\text{g/ml}$ PotD and PotF proteins.

* Significant increase ($p < 0.05$, t-test) in IFN- γ production from control mice

Error bars represent the standard deviation of the data

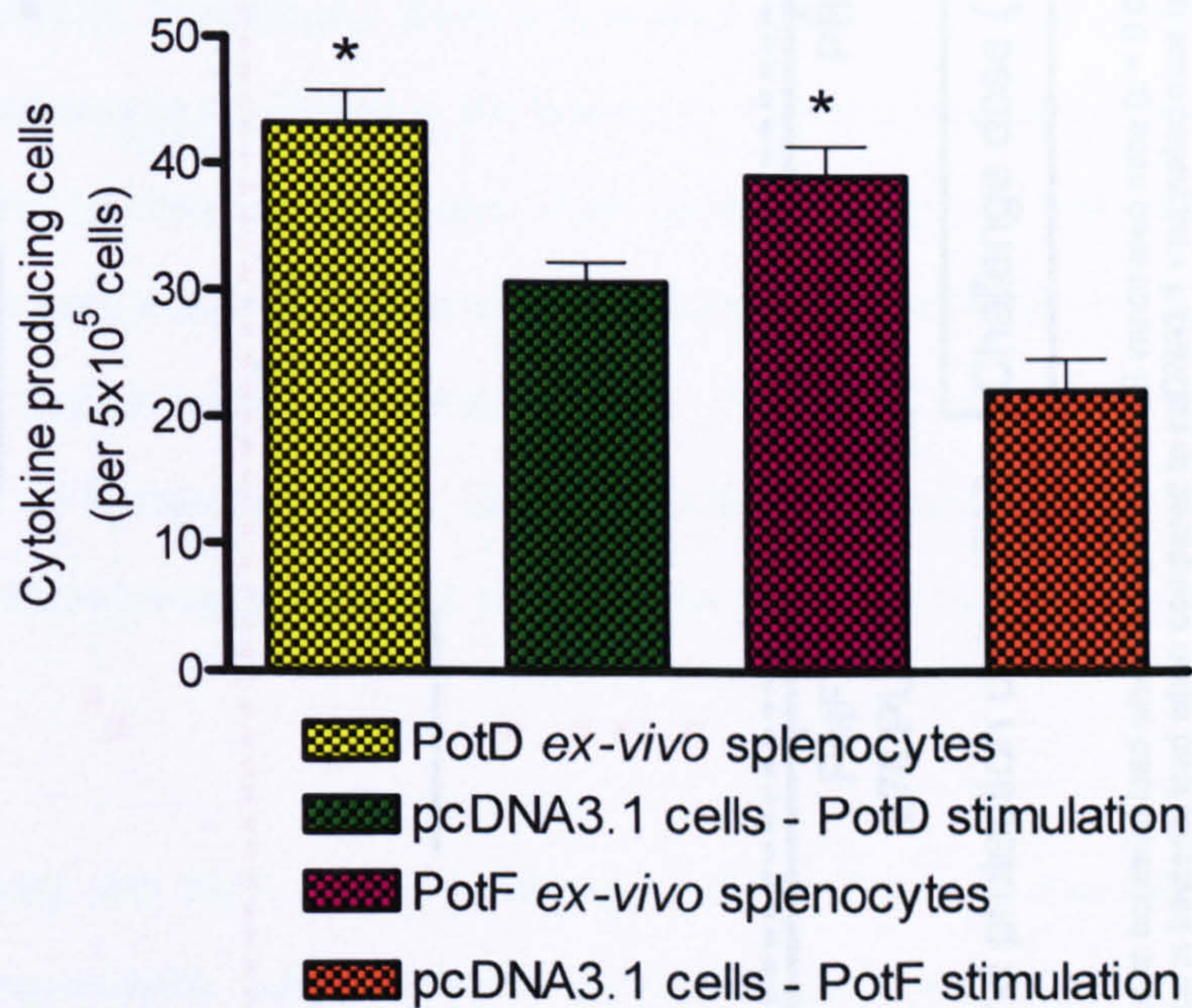
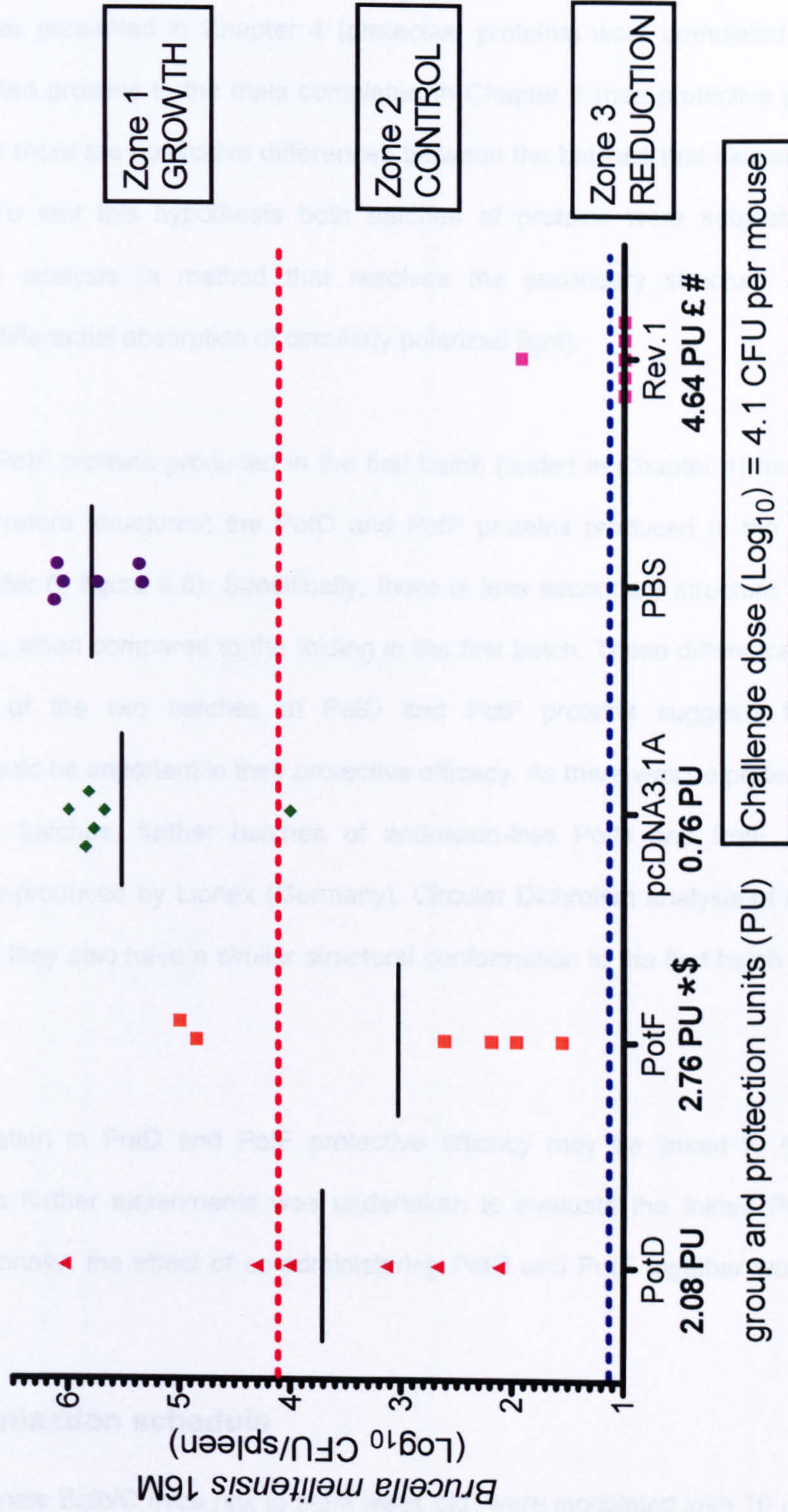


Figure 6.7: Comparison of protective efficacy of PotD and PotF DNA vaccines

The protective efficacy of PotD and PotF DNA vaccine candidates after mice were administered with four intramuscular doses at three week intervals of 10 µg DNA vaccine dissolved in 100 µl PBS. Thirty days after their final immunisation mice were challenged via the intraperitoneal route with 3.98×10^4 CFU of *B. melitensis* 16M. Fifteen days after challenge splenic colonisation assays were performed to determine the *Brucella* burden of each mouse. Results are displayed as Log_{10} CFU per spleen.



* Significant reduction in bacterial load when compared to PBS vaccinated mice ($p < 0.05$, 1-way ANOVA + Bonferroni's post test)
 \$ Significant reduction in bacterial load when compared to pcDNA3.1 vaccinated mice ($p < 0.05$, 1-way ANOVA + Bonferroni's post test)
 £ Significant reduction in bacterial load when compared to PBS vaccinated mice ($p < 0.001$, 1-way ANOVA + Bonferroni's post test)
 # Significant reduction in bacterial load when compared to pcDNA3.1 vaccinated mice ($p < 0.001$, 1-way ANOVA + Bonferroni's post test)
 Black bars represent mean Log_{10} *Brucella* CFU/spleen for each group
 Protection Units calculated by [mean Log_{10} CFU PBS immunised mice – mean Log_{10} CFU vaccine candidate immunised mice]
 Zone 1: denotes growth above the challenge dose administered (red dotted line)
 Zone 2: denotes control of *Brucella* growth
 Zone 3: denotes a reduction of *Brucella* growth below the averages of the Rev.1 vaccine (blue dotted line)

6.5 Re - evaluation of PotD and PotF in folded structural conformation

6.5.1 PotD and PotF protein structural analysis

The protective efficacy of PotD and PotF proteins showed significant variation. However, the challenge studies presented in Chapter 4 (protective proteins) were completed with different batches of purified proteins to the trials completed in Chapter 6 (non-protective proteins). This could mean that there are qualitative differences between the batches that caused the variation in protection. To test this hypothesis both batches of proteins were subjected to circular dichroism (CD) analysis (a method that resolves the secondary structure of protein by measuring the differential absorption of circularly polarized light).

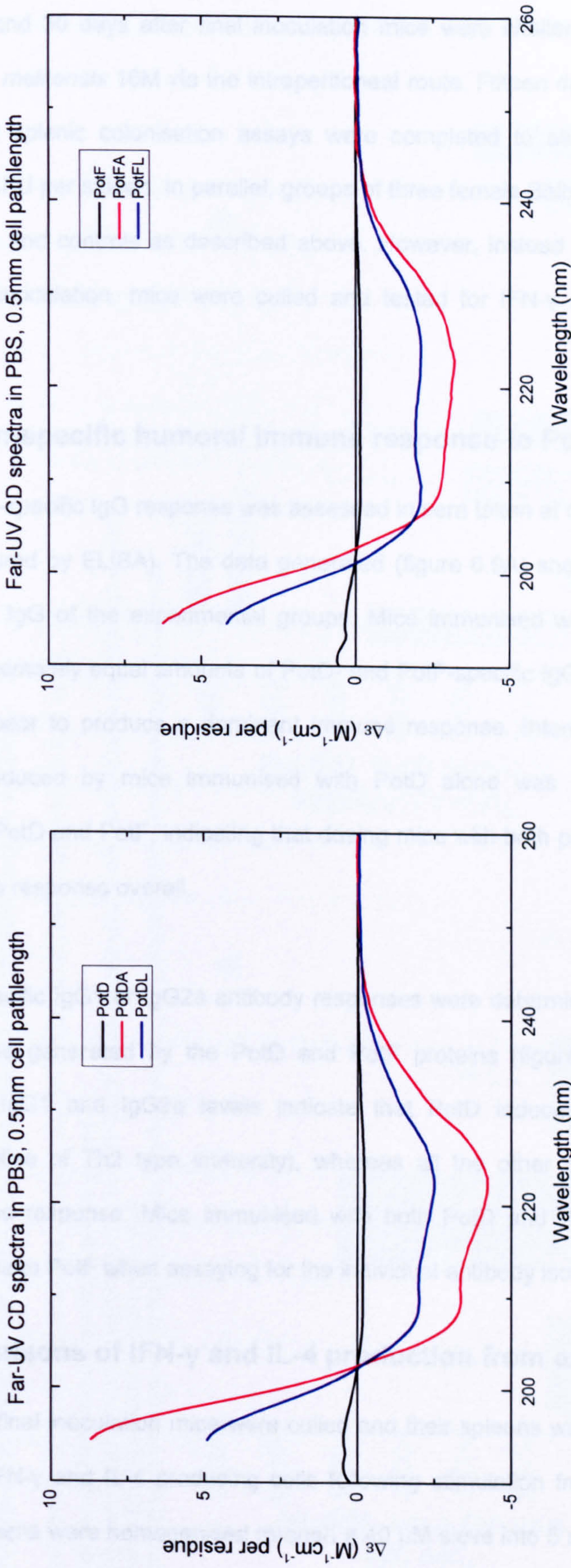
The PotD and PotF proteins produced in the first batch (tested in Chapter 4) had different CD traces (and therefore structures) the PotD and PotF proteins produced in the second batch (tested in Chapter 6, figure 6.8). Specifically, there is less secondary structure in the second batch of protein, when compared to the folding in the first batch. These differences observed in the structures of the two batches of PotD and PotF proteins suggests that structural conformation could be important in their protective efficacy. As there was no protein left from the original protein batches, further batches of endotoxin-free PotD and PotF proteins were commercially re-produced by Lionex (Germany). Circular Dichroism analysis of these proteins has shown that they also have a similar structural conformation to the first batch of (protective) protein.

Since the variation in PotD and PotF protective efficacy may be linked to their structural conformation, a further experiments was undertaken to evaluate the folded PotD and PotF proteins. Additionally, the effect of co-administering PotD and PotF together was assessed in this experiment.

6.5.2 Immunisation schedule

Groups of 6 female Balb/C mice (six to eight week old) were inoculated with 10 µg of PotD, 10 µg of PotF or 10 µg of PotD and 10 µg of PotF, adjuvanted with 12.5 µg ISCOMs and 6.25 µg CpG, via the intramuscular route. Further groups of mice were inoculated with a single sub-

Figure 6.8: Circular dichroism spectra for different batches of PotD and PotF protein



Red line (PotFA or PotDA) = protein batch 1 (0.3 mg/ml concentration)

Black line (PotF or PotD) = protein batch 2 (0.3 mg/ml concentration)

Blue line (PotFL or PotDL) = commercially produced protein (LPS free, 0.2 mg/ml concentration)

The circular dichroism (CD) traces indicate that the original batches of PotD and PotF (Red line) have a good secondary structure, whereas the second batches of protein (black lines) have no secondary structure at all. This difference may be the cause of the variation in protective efficacy observed between protein batches. The blue line indicates batches of protein produced commercially (Lionex, Germany), which has a similar secondary structure to the original batches.

cutaneous dose of 2×10^5 CFU of *B. melitensis* Rev.1 as a positive control or with 100 μ g PBS or 12.5 μ g ISCOMs and 6.25 μ g CpG as negative controls. Three inoculations were given at three week intervals, and 30 days after final inoculation mice were challenged with approximately 1×10^4 CFU of *B. melitensis* 16M via the intraperitoneal route. Fifteen days after challenge mice were culled and splenic colonisation assays were completed to assess the number of *B. melitensis* 16M CFU per spleen. In parallel, groups of three female Balb/C mice were inoculated with the proteins and controls as described above. However, instead of being challenged, 30 days after final inoculation, mice were culled and tested for IFN- γ and IL-4 production via ELISPOT assay.

6.5.3 Antigen-specific humoral immune response to PotD and PotF

The total antigen-specific IgG response was assessed in sera taken at day 58 of the vaccination schedule (measured by ELISA). The data generated (figure 6.9A) shows that PotF generated the highest total IgG of the experimental groups. Mice immunised with both PotD and PotF generated approximately equal amounts of PotD- and PotF-specific IgG showing that neither of the antigens appear to produce a dominant immune response. Interestingly, the total PotD-specific IgG produced by mice immunised with PotD alone was similar to that in mice immunised with PotD and PotF, indicating that dosing mice with both proteins may not produce a greater immune response overall.

Total antigen-specific IgG1 or IgG2a antibody responses were determined to clarify the type of immune response generated by the PotD and PotF proteins (figure 6.9B). Comparison of antigen-specific IgG1 and IgG2a levels indicate that PotD induced IgG1-biased antibody response (indicative of Th2 type immunity), whereas all the other proteins elicited a more balanced immune response. Mice immunised with both PotD and PotF showed a stronger antibody response to PotF when assaying for the individual antibody isotypes.

6.5.4 Comparisons of IFN- γ and IL-4 production from ex-vivo splenocytes

At 30 days post final inoculation mice were culled and their spleens were removed to evaluate the number of IFN- γ and IL-4 producing cells following stimulation from the specific vaccine antigen. The spleens were homogenised through a 40 μ m sieve into 5 ml of DMEM and the red cells

Figure 6.9: Humoral immune responses from mice immunised with the vaccine candidates

Graphs represent the humoral immune response generated from mice immunised with the folded PotD and PotF proteins. Panel A shows the amount of total antigen-specific IgG present on day 58 of the immunisation schedule and Panel B show the amounts of antigen-specific IgG1 vs IgG2a present on day 58 of the immunisation schedule. Data shows the average IgG concentrations from six mice.

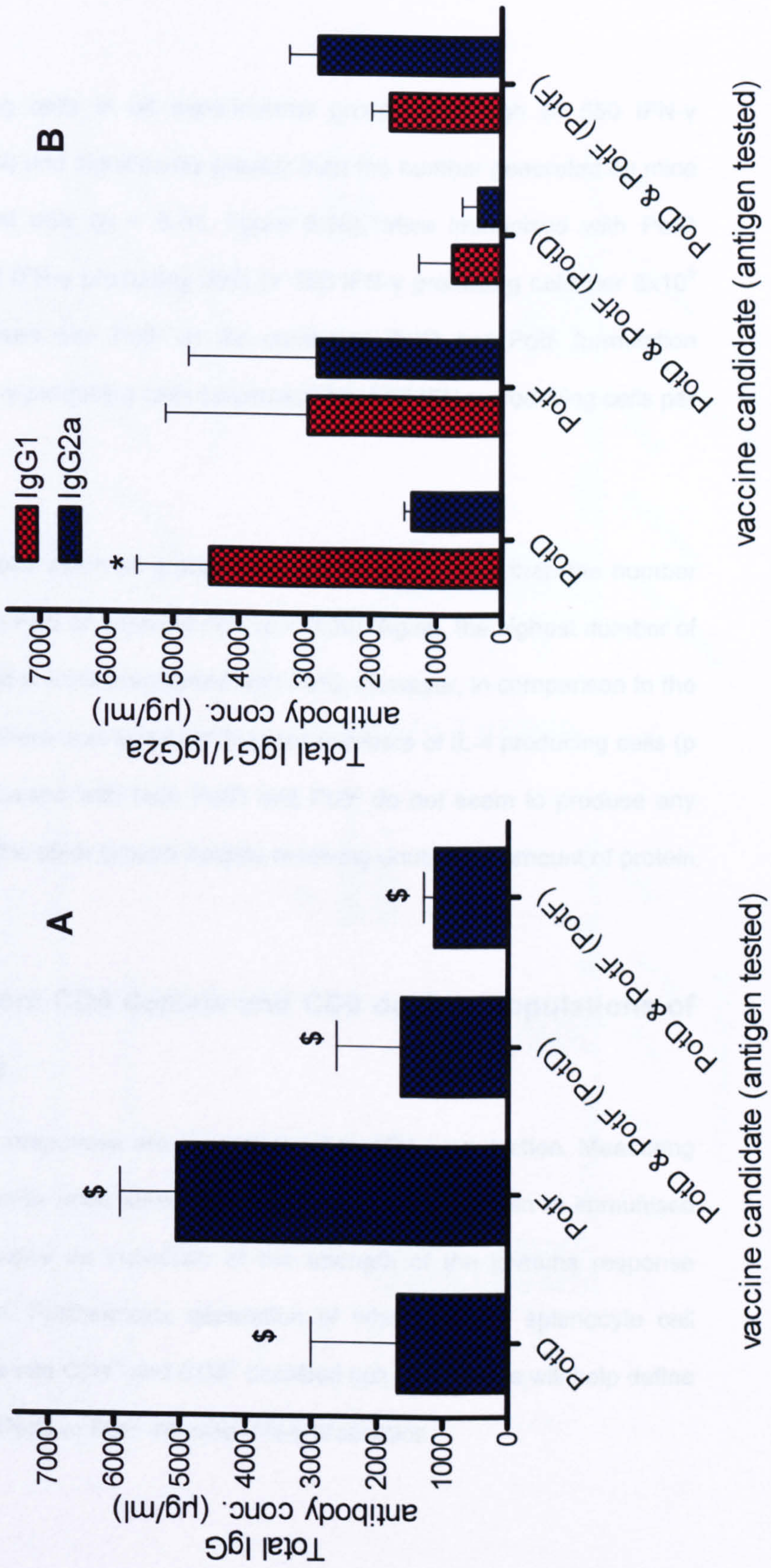
\$ = significant increase in IgG production when compared to PBS or adjuvant only immunised mice ($p < 0.01$, t-test)

* = significant IgG1 bias immune response ($p < 0.01$, t-test)

X axis represent the protein mice were immunised with and the brackets indicate which protein the antibodies are generated against

Error bars represent standard deviation of the data

Naïve and adjuvant only sera was included on each plate however, concentrations obtained were below assay detection limits.



blood were lysed by the addition of 10 ml red cell lysis buffer (Sigma, Paisley). The cells were counted and the density adjusted to 5×10^6 cells/ml, before 100 μ l of cell suspension was plated onto an anti-IFN- γ /IL-4 coated ELISPOT plate with 100 μ l of a 10 μ g/ml solution of vaccine candidate.

The number of IFN- γ producing cells in all experimental groups was high (> 550 IFN- γ producing cells per 5×10^5 cells/ml) and significantly greater than the number generated by mice inoculated with PBS or adjuvant only ($p < 0.01$, figure 6.10). Mice immunised with PotD produced the largest numbers of IFN- γ producing cells (> 750 IFN- γ producing cells per 5×10^5 cells/ml), whereas mice immunised with PotF or the combined PotD and PotF formulation produced similar numbers of IFN- γ producing cells (approximately 600 IFN- γ producing cells per 5×10^5 cells/ml).

The number of IL-4 producing cells within all groups was significantly higher than the number generated in mice inoculated with PBS or adjuvant only ($p < 0.05$). Again, the highest number of IL-4 producing cells was observed in mice immunised with PotD. However, in comparison to the number of IFN- γ producing cells there was significantly lower numbers of IL-4 producing cells ($p < 0.05$). Interestingly, mice immunised with both PotD and PotF do not seem to produce any more IFN- γ producing cells than the other groups despite receiving double the amount of protein during the inoculation.

6.5.5 IFN- γ production from CD4 deplete and CD8 deplete populations of *ex-vivo* splenocytes

Cell mediated Th1 type immune responses are characterised by IFN- γ production. Measuring the number of IFN- γ producing cells upon stimulation from the vaccine antigen in immunised mouse *ex-vivo* splenocytes provides an indication of the strength of the immune response induced by a vaccine candidate. Furthermore, separation of whole *ex-vivo* splenocyte cell populations from immunised mice into CD4 $^+$ and CD8 $^+$ depleted cell populations will help define which cell type is responsible for PotD or PotF induced IFN- γ production.

Figure 6.10: IFN- γ and IL-4 production from immunised mice ex-vivo splenocytes when stimulated with the specific vaccine antigen

A = 10 $\mu\text{g/ml}$ PotD stimulating antigen

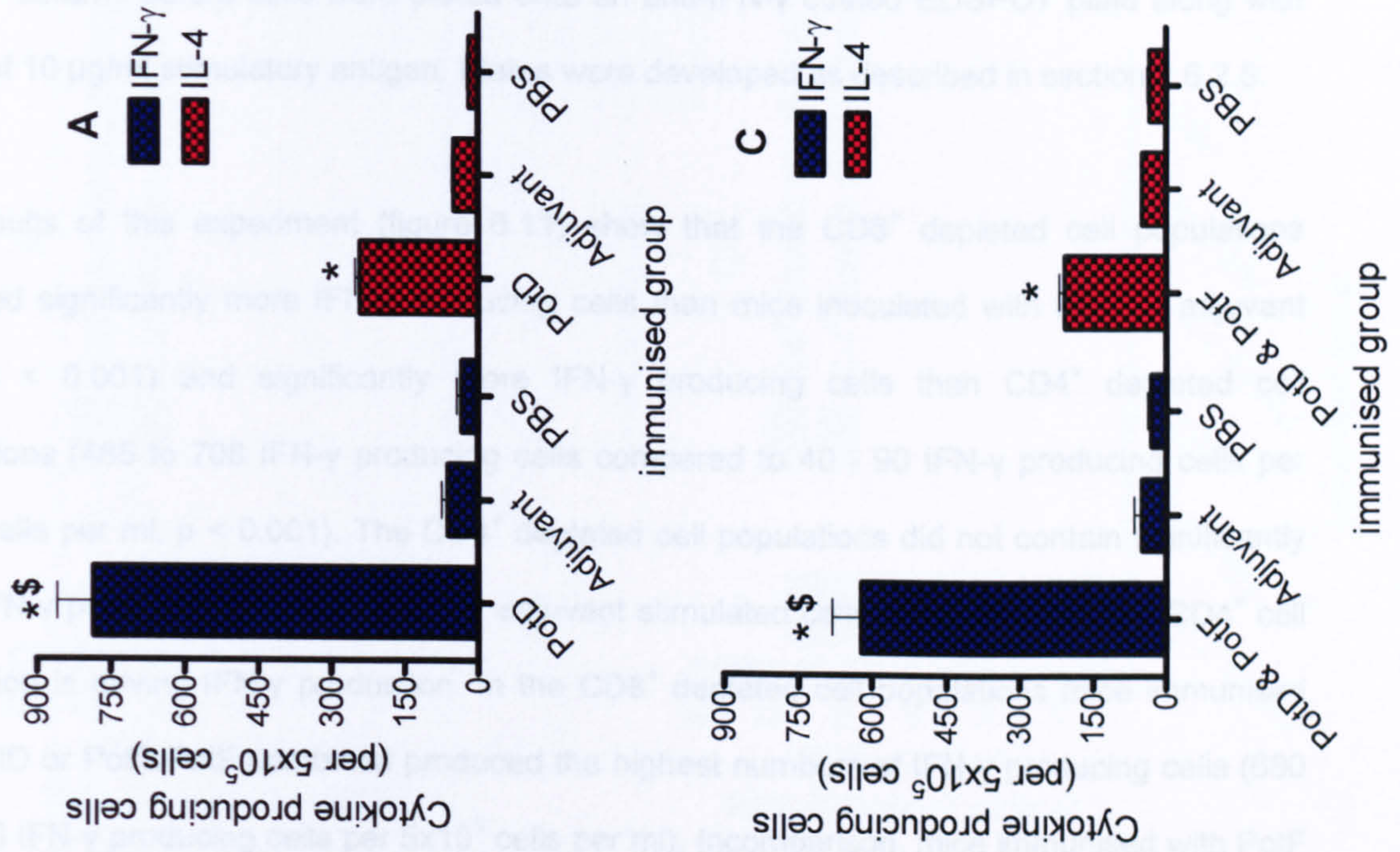
B = 10 $\mu\text{g/ml}$ PotF stimulating antigen

C = 10 $\mu\text{g/ml}$ PotD & 10 $\mu\text{g/ml}$ PotF stimulating antigen

* = Significant increase in cytokine production when compared to naïve and adjuvant controls ($p < 0.05$ for IL-4 and $p < 0.01$ for IFN- γ , t-test)

\$ = Indicates a significant increase in IFN- γ production when compared to IL-4 production ($p < 0.05$, t-test)

Error bars represent the standard deviation of the data



Mice inoculated with three doses (at three week intervals) of 10 µg PotD or PotF, or a PotD/PotF combination, were culled 30 days after their final immunisation and number of IFN-γ producing cells was measured as a memory response from their spleens via ELISPOT assay. Spleens were harvested and separated into CD4⁺ and CD8⁺ depleted populations using MACS separation columns (Miltenyi Biotech, Surrey UK, section 2.6.2.4). The cell density was adjusted to 5x10⁶ cells/ml before cells were plated onto an anti-IFN-γ coated ELISPOT plate along with 100 µl of 10 µg/ml stimulatory antigen. Plates were developed as described in section 2.6.2.5.

The results of this experiment (figure 6.11) show that the CD8⁺ depleted cell populations produced significantly more IFN-γ producing cells than mice inoculated with PBS or adjuvant only ($p < 0.001$) and significantly more IFN-γ producing cells than CD4⁺ depleted cell populations (465 to 708 IFN-γ producing cells compared to 40 - 90 IFN-γ producing cells per 5x10⁵ cells per ml, $p < 0.001$). The CD4⁺ depleted cell populations did not contain significantly more IFN-γ producing cells than PBS or adjuvant stimulated cells, indicating that the CD4⁺ cell population is driving IFN-γ production. In the CD8⁺ depleted cell populations mice immunised with PotD or PotD/PotF combined produced the highest numbers of IFN-γ producing cells (680 and 708 IFN-γ producing cells per 5x10⁵ cells per ml). In comparison, mice immunised with PotF had only an average of 465 IFN-γ producing cells per 5x10⁵ cells/ml, perhaps signifying that a more potent immune response was generated in mice immunised with PotD or PotD/PotF combined.

6.5.6 Protective efficacy of PotD and PotF when tested in the folded structural conformation

Mice inoculated with three doses (at three week intervals) of 10 µg PotD or PotF or a PotD/PotF combination were challenged 30 days after their final inoculation with 8.5x10³ CFU of *B. melitensis* 16M. Fifteen days after challenge mice were culled and their spleens removed for splenic colonisation assays (section 2.5.4). The results (figure 6.12) show that mice immunised with Rev.1 had significantly reduced *Brucella* loads (4.04 PU) in their spleens when compared to mice inoculated with PBS. Mice immunised with PotD (0.02 PU) or PotF (- 0.62 PU) did not show any significant signs of protection when compared to any of the negative control. However, mice immunised with 10 µg of PotD and 10 µg of PotF combined showed highly significant protection ($p < 0.001$) with a PU of 3.03. There was no significant difference the

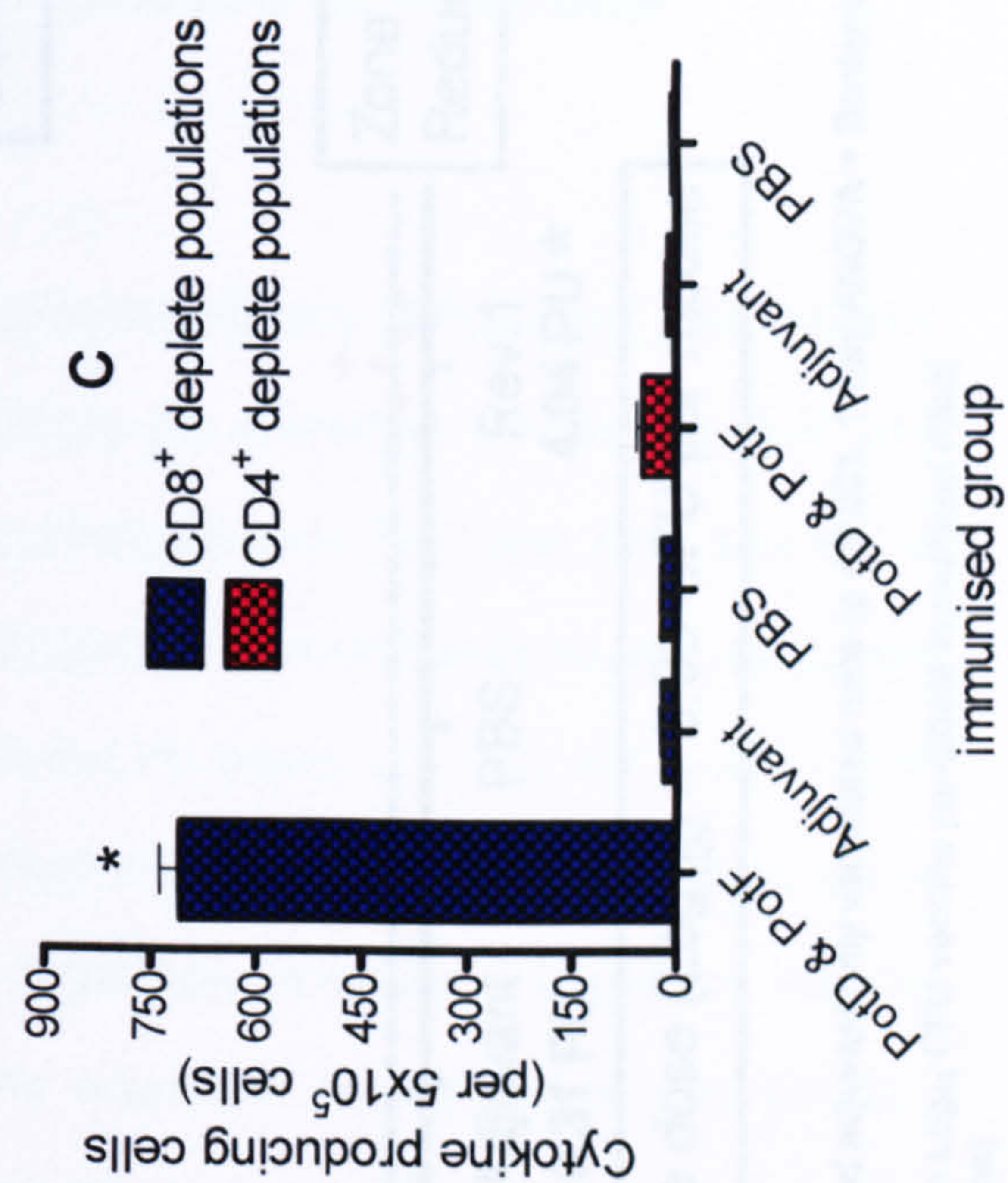
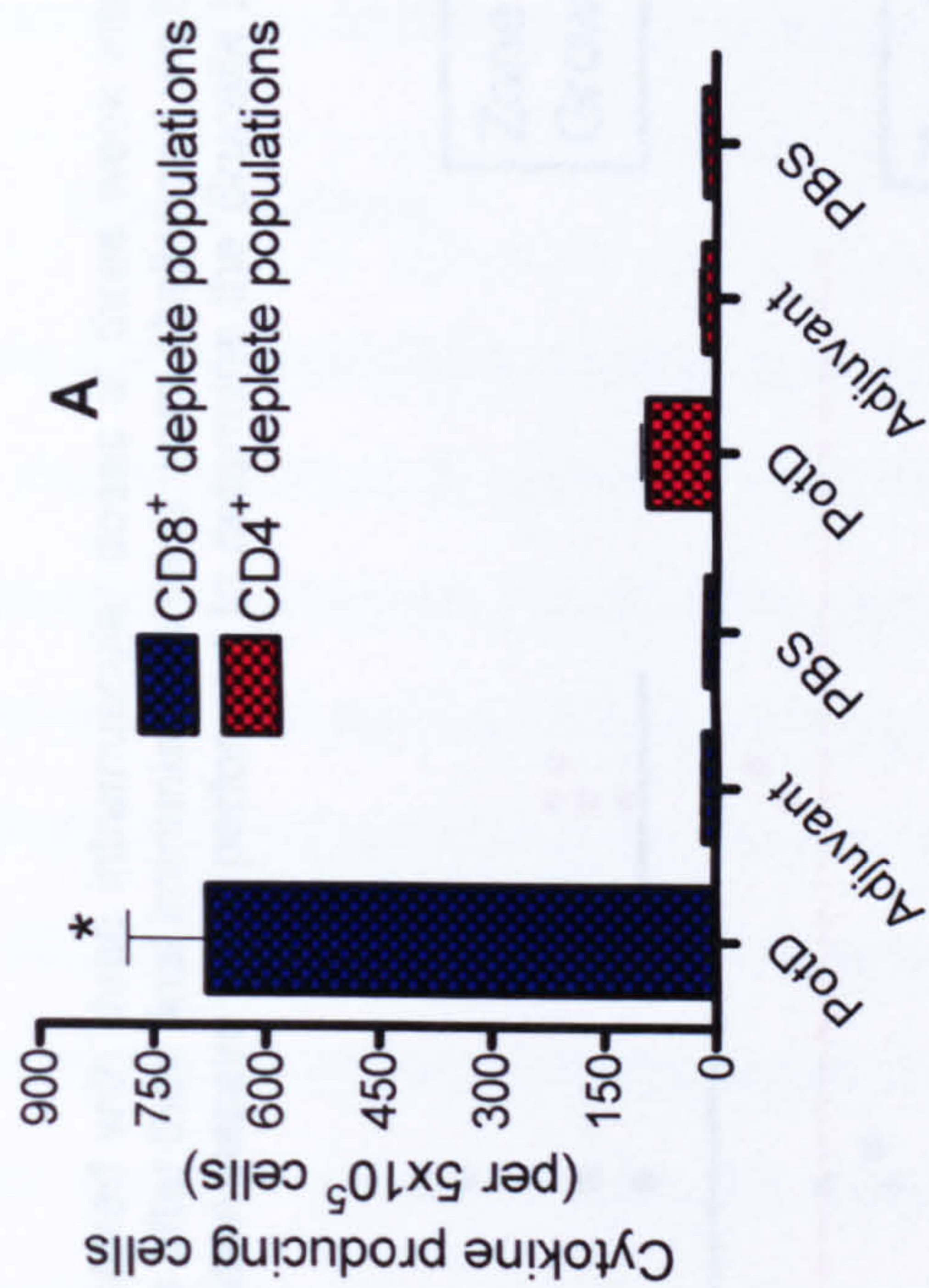


Figure 6.11: IFN- γ production from immunised mice *ex-vivo* splenocytes when whole, CD4⁺ and CD8⁺ depleted populations were stimulated with specific vaccine antigen

- A = 10 μ g/ml PotD stimulating antigen
- B = 10 μ g/ml PotF stimulating antigen
- C = 10 μ g/ml PotD & 10 μ g/ml PotF stimulating antigen

* = significant increase in IFN- γ production from CD8⁺ depleted population when compared to CD4⁺ deplete populations ($p < 0.001$, t-test).

Error bars represent the standard deviation of the data

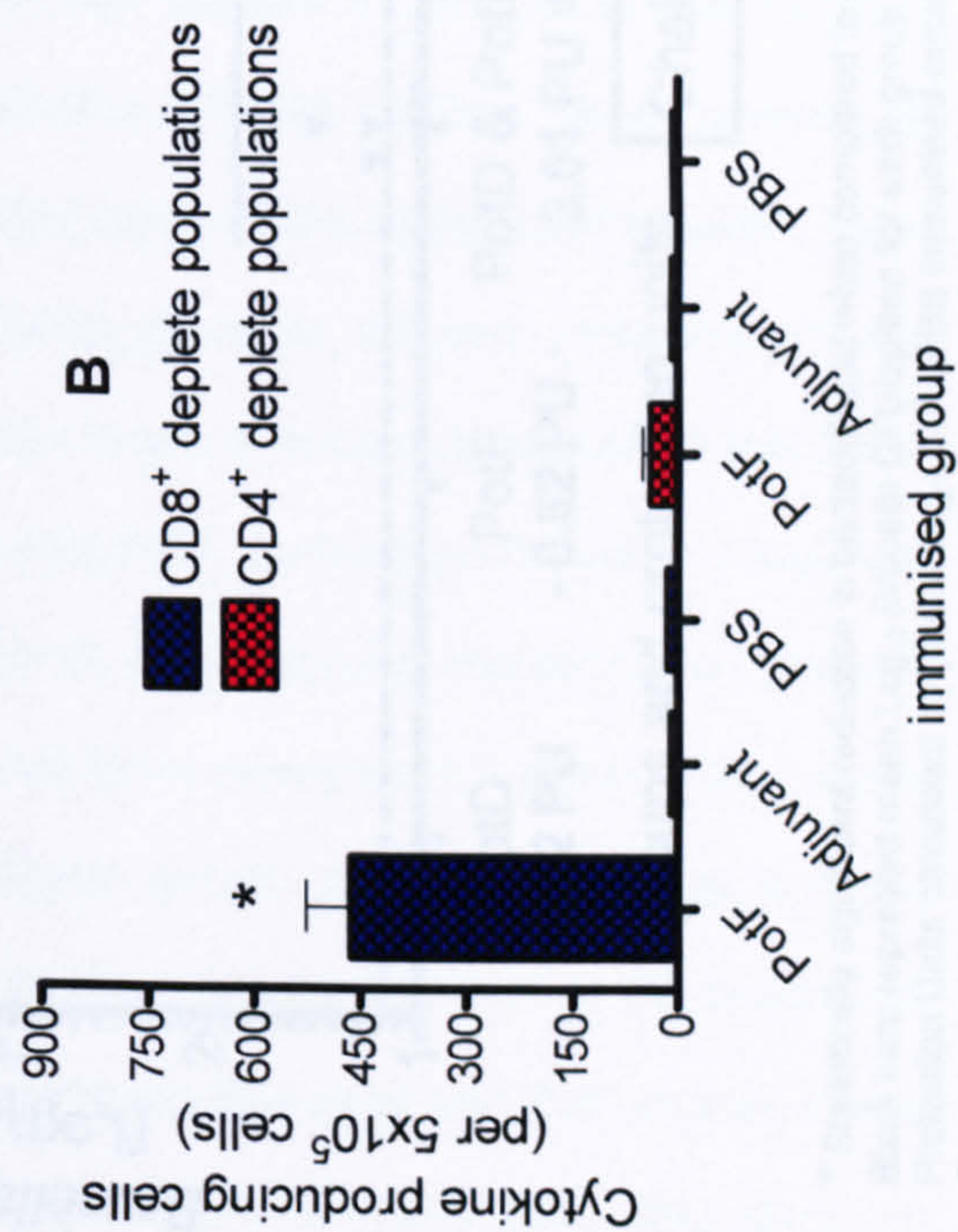
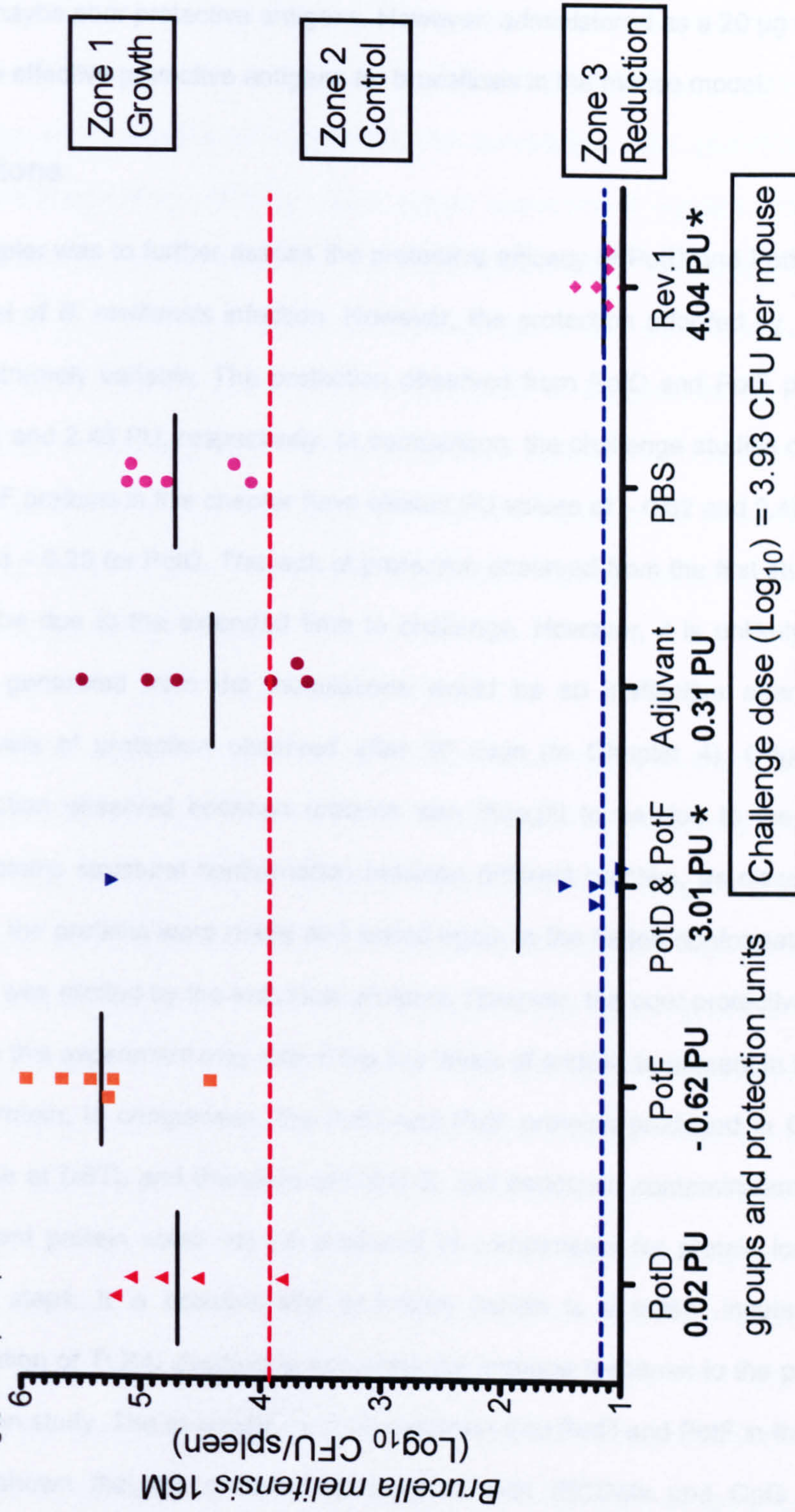


Figure 6.12: Protective efficacy of PotD and PotF in the folded structural conformation

The protective efficacy of PotD and PotF protein candidates after mice were administered with three intramuscular doses at three week intervals of 10 µg protein adjuvanted with 12.5 µg ISCOMs and 6.25 µg CpG dissolved in 100 µl PBS. Thirty days after their final immunisation mice were challenged via the intraperitoneal route with 3.98×10^4 CFU of *B. melitensis* 16M. Fifteen days after challenge colonisation assays were performed to determine the *Brucella* burden of each mouse. Results are displayed as Log_{10} CFU per spleen.



* Statistically significant reduction in bacterial load when compared to PBS and adjuvant only vaccinated mice ($p < 0.001$, 1-way ANOVA + Bonferroni's post test)

Black bars represent mean Log_{10} *Brucella* CFU/spleen for each group

Protection Units calculated by [mean Log_{10} CFU PBS immunised mice - mean Log_{10} CFU vaccine candidate immunised mice]

Zone 1: denotes growth above the challenge dose administered (red dotted line)

Zone 2: denotes control of *Brucella* growth

Zone 3: denotes a reduction of *Brucella* growth below the averages of the Rev.1 vaccine (blue dotted line)

protective efficacy of the PotD/PotF combined group and the mice immunised with Rev.1, indicating that a similar performance was observed in both groups.

Overall, these data suggest that 10 µg PotD and 10 µg PotF proteins in the folded conformation without endotoxin maybe poor protective antigens. However, administered as a 20 µg combined formulation they are effective protective antigens for brucellosis in the mouse model.

6.6 Conclusions

The aim of this chapter was to further assess the protective efficacy of PotD and PotF proteins in the murine model of *B. melitensis* infection. However, the protection afforded by PotD and PotF proteins is extremely variable. The protection observed from PotD and PotF proteins in Chapter 4 was 3.61 and 2.46 PU, respectively. In comparison, the challenge studies completed using PotD and PotF proteins in this chapter have elicited PU values of – 0.62 and 0.48 for PotF and 0.02, - 0.28 and – 0.25 for PotD. The lack of protection observed from the first study in this chapter trial could be due to the extended time to challenge. However, it is unlikely that the memory response generated from the inoculations would be so ineffective after 60 day, considering the levels of protection observed after 30 days (in Chapter 4). Originally the difference in protection observed between proteins was thought to be due to the variation observed in the proteins structural conformation between different batches, as discovered by CD analysis. When the proteins were made and tested again in the folded conformation, there no protective effect was elicited by the individual proteins. However, the poor protective efficacy of PotD and PotF in this experiment may reflect the low levels of endotoxin present in this batch of highly purified protein. In comparison, the PotD and PotF proteins produced in Chapter 4 were made in-house at DSTL and therefore removal *E. coli* endotoxin contamination was not achieved as sufficient protein could not be produced to compensate for protein loss during endotoxin removal steps. It is possible that endotoxin (which is a potent innate immune stimulator via activation of TLR4) assisted in activating the immune response to the proteins in the original protection study. The re-assessment of endotoxin-free PotD and PotF in their folded conformation has shown that the proteins administered with ISCOMs and CpG gave no protection. However, the results from this experiment also indicated that administered together, PotD and PotF induce a highly significant reduction in bacterial spleen load compared to control

mice, highlighting that the combined vaccination approach has an application against brucellosis.

Bacterial pathogens are complex prokaryotic organisms that have many different survival techniques, including multiple mechanisms for similar processes. PotD and PotF are both polyamine periplasmic binding proteins in *E. coli* [142], where the potFGHI system is involved in the specific binding and importing of putrescine. In comparison, the potABCD system preferentially imports spermidine, but it can also import putrescine if needed. Thus, if the potFGHI system is damaged or inactivated, the potABCD system might be able to compensate for its loss. When PotD and PotF are administered alone the generated immune response may be targeting one of these systems, perhaps allowing *Brucella* to compensate via a different mechanism. However, when the proteins are administered together the immune response should target both systems, causing a deficiency in polyamine import for the *Brucella*. Many of the more promising novel vaccine candidates developed against *Brucella* are also a combination of two antigens [47,75,199,370].

PotD and PotF were also tested as DNA vaccines to see if the antigens were protective when delivered by an alternative approach. The truncated PotD and PotF genes were inserted into the pcDNA3.1 DNA vaccine vector and their protective efficacy and immunogenicity was tested using the murine model of *B. melitensis* infection. The PotD and PotF DNA vaccines are poorly immunogenic since *ex-vivo* splenocytes from DNA immunised mice, when stimulated with PotD and PotF protein, produced very low levels of IFN- γ . There was also no detectable antigen-specific antibody in serum removed from DNA-immunised mice. However, despite the fact that these DNA vaccines are poorly immunogenic, they provided some protective efficacy over a *Brucella* infection with PU of 2.08 (PotD) and 2.76 (PotF), although the protective efficacy of PotD was not significant when compared to the negative controls. With a 100-fold drop in bacterial load it is difficult to dismiss the PotD DNA vaccine as a potential DNA vaccine candidate without further analysis. When comparing the protective efficacy of PotD and PotF DNA vaccines to other DNA vaccine previously reported in the literature it is clear that they are very good candidates (Chapter 1, table 1.2, page 40). However, a direct comparison to other DNA vaccines is difficult due to differences in experimental techniques and *Brucella* species or

strains used. When comparing PotD and PotF to *B. melitensis* Omp25 and IalB DNA vaccines (with PU of 2.54 and 2.70, respectively [59]), the data indicates that PotD and PotF are viable DNA vaccine candidates against *B. melitensis*, confirming PotD and PotF as novel sub-unit vaccine candidates against brucellosis.

During the immunology studies completed in this chapter the number of IFN- γ producing cells has been used as a measure of the strength of the immune response. This is because IFN- γ production is a good indicator of a Th1 type immune response, which is considered the appropriate immune response for clearance of intracellular pathogens [292]. It has also been demonstrated that IFN- γ production is crucial for survival of mice infected with *Brucella* as in its absence mice will die of brucellosis [229]. In both the PotD and PotF protein and DNA vaccine studies there have been different numbers of IFN- γ producing cells generated. Mice immunised with PotD and PotF individually produced increased numbers of IFN- γ producing cells and yet showed no protection, whereas mice immunised with the PotD and PotF DNA vaccine produced low but significant higher numbers of IFN- γ producing cells and induced controlling levels of protection. This difference in the number of IFN- γ producing cells reinforces the point that, although protective immunity is dependant upon the generation of IFN- γ , the production of IFN- γ alone does not always correlate with a protective immune response. A similar observation was made in Chapter 4 with the ZnuA vaccine candidate, where high numbers of IFN- γ producing cells was observed and yet no protection was elicited. It has previously been demonstrated that other vaccine candidates are able to stimulate a strong IFN- γ responses but elicit no protection [19,10,335]. The reason for the high immunogenicity of some of these candidates and yet their inability to elicit a protection is not fully understood. However, it could be that these antigens are not presented to the immune systems during *Brucella* infection or that they are not critical to *Brucella* survival, so if disabled by an immune response the *Brucella* are still infectious.

The data generated in this chapter indicates that, in the correct protein formulation or as DNA vaccines, PotD and PotF should be considered as novel vaccine candidates against *Brucella melitensis* 16M.

Chapter 7 – General Discussion

7.1 General discussion

Brucella species are the causative agents of brucellosis and are endemic to the Middle East, Mexico, Asia, South America and the Mediterranean [251,130]. As brucellosis is classed as the world's most prevalent zoonotic disease, management of animal brucellosis is the best way to reduce the prevalence of human brucellosis. However, management of animal brucellosis requires strict control procedures and regulation of diagnostic testing, which in certain endemic areas is difficult to achieve. Although the use of live attenuated animal vaccines such as *B. abortus* RB51, *B. melitensis* Rev.1 and *B. abortus* S19 is widespread in endemic areas these vaccines have drawbacks. Firstly, they can interfere with diagnostic testing, making it difficult to distinguish between vaccinated animals and naturally infected animals. Second, as these are live attenuated strains it has been shown that they can occasionally cause symptomatic infections in vaccinated animals [291,130]. Additionally these vaccines are still able to cause infection in humans, making their use as human vaccines impossible. It is clear from this that there is no vaccine for both animal and human brucellosis. The development of a novel vaccine against brucellosis would be of great use in endemic areas of the world. However, a new vaccine should ideally enable vaccinated animals to be distinguishable from naturally infected animals using current diagnostic tests. There should be no chance of the vaccine reverting to full virulence or causing infection and the vaccine should be licensable for human use. A novel sub-unit vaccine candidate offers the most potential to meet these criteria.

The import and export of molecules across cell membranes is essential for bacterial survival, and ABC transporters are responsible for much of this movement [68]. ABC systems generally comprise two hydrophilic ABC domains associated with two hydrophobic membrane-spanning domains (inner membrane (IM) proteins). Import systems are only found in prokaryotic organisms and contain both ABC domains and IM domains, along with extra-cytoplasmic binding proteins (BP) adapted to bind the specific allocrite of that ABC system [68]. In Gram-negative bacteria, the BP are located in the periplasm whereas, in Gram-positive bacteria, they are anchored to the outer membrane of the cell via N-terminal lipid groups [261,370]. Research into the roles of ABC systems has indicated their potential as virulence factors or as protective antigens [130,316,295,112,160,115,218].

The creation of inventories of all ABC systems within several genome sequenced strains of *Brucella* was described in Chapter 3. These inventories have identified potentially interesting differences between the *Brucella* species. Of the five *Brucella* strains studied, the four strains that are known to infect humans (*B. melitensis*, *B. abortus*, *B. suis* and occasionally *B. canis*) all have more ABC systems than *B. ovis* which is not known to infect humans. Some of the ABC systems absent in *B. ovis* but present in the other species have been studied in other bacterial species. For example, it has been demonstrated in *E. coli* that disruption of the CDI system (comprised of the FtsE, the ABC protein component, and FtsX, the inner membrane protein component) leads to a reduced growth capacity [286]. As *B. ovis* is the only *Brucella* strain studied that lacks the CDI system it is possible to theorise that it could be related to its reduced virulence, and therefore could be targeted in the other *Brucella* species as a potential virulence factor.

After the completion of this work the *B. melitensis* 16M genome has been re-annotated. Thus, it is possible that this re-annotation might slightly change the ABC system inventory for *B. melitensis* 16M produced in this study. It should also be noted there that four further *Brucella* strains have been sequenced since this work was completed: *B. melitensis* ATCC 23475, *B. abortus* 2308, *B. abortus* S19 and *B. suis* biovar 2. It would be interesting to compile ABC systems inventories of these strains to see if any further differences can be identified. Among the newly sequenced strains is a strain from *B. suis* biovar 2, which is not known to cause disease in humans, and *B. abortus* S19, a vaccine strain. ABC system inventories of these strains would be of particular interest since they are considered less pathogenic than the wild-type strains and yet the reasons for this lack of pathogenicity are currently unknown.

In this thesis the compilation of ABC system inventories of *Brucella* (Chapter 3) has provided a framework from which eight potential vaccine candidates (Chapter 4) could be selected and evaluated. In turn the preliminary evaluation led to further studies of the adjuvant system used and the protection demonstrated. Figure 7.1 summarises a schematic flow of the work in this thesis. This work contributes to studies in the *Brucella* field and in the wider field of sub-unit vaccine development (summarised in figure 7.2).

Figure 7.1: A visual representation of the results generated in this thesis

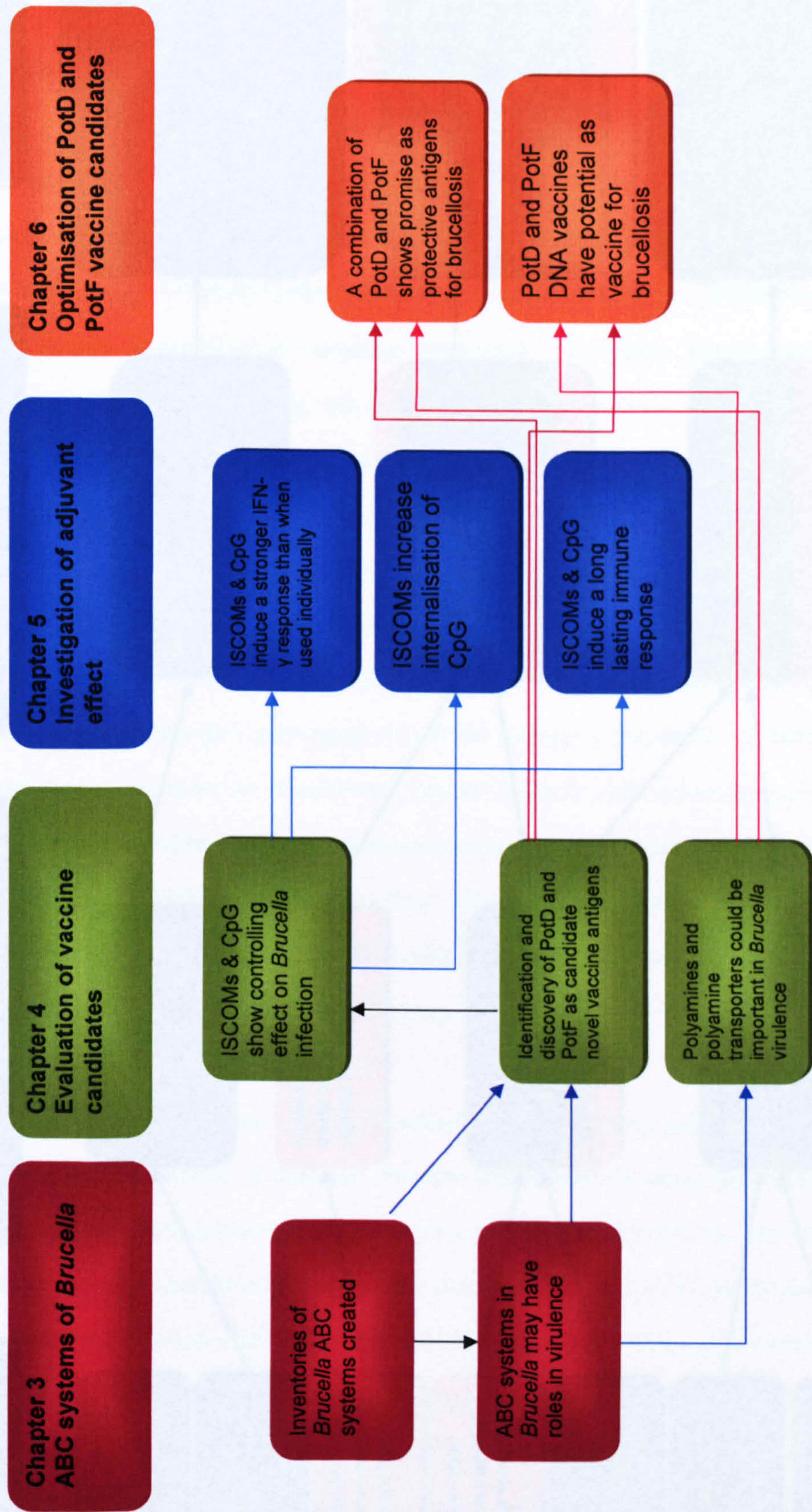
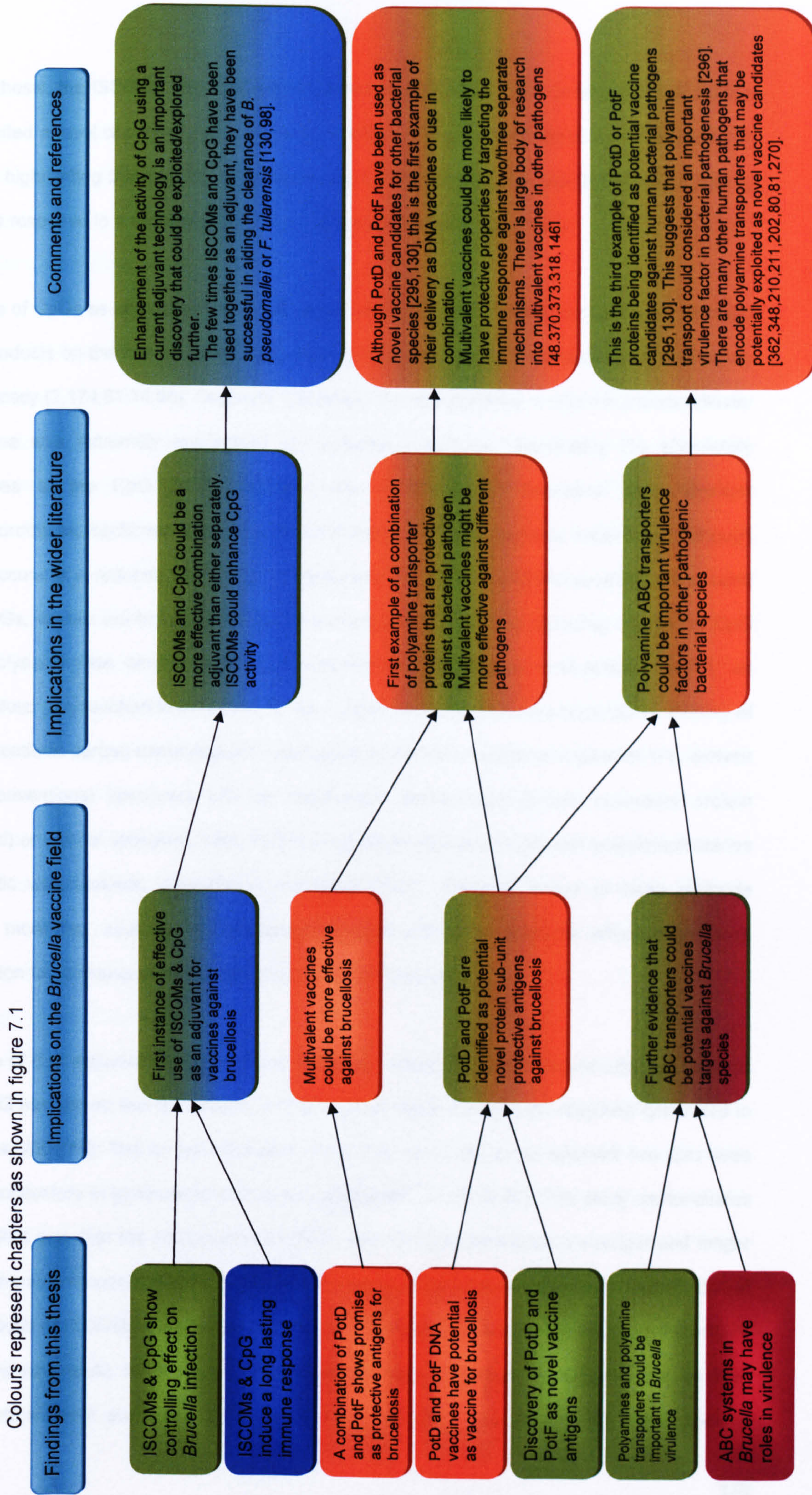


Figure 7.2: Implications of the work in this thesis on the *Brucella* field and in the wider literature



In this thesis, the ISCOMs and CpG combination used as an adjuvant in all protection studies has elicited a level of control over a *Brucella* infection. Chapter 5 attempted to understand this finding, highlighting that the use of ISCOMs and CpG in combination leads to a longer lasting immune response to a vaccine antigen than when administered separately.

The use of CpGs as an adjuvant is well established and, although there are no directly licensed CpG products on the market, there have been a number of clinical trials evaluating their safety and efficacy [2,174,61,14,60]. Originally CpGs had a major limitation in that the phosphodiester backbone was extremely susceptible to nuclease breakdown, inactivating the stimulatory properties of the CpG [297]. Changing the phosphodiester backbone to a stronger phosphorothioate backbone has decreased the susceptibility to nuclease breakdown, although it still occurs at a reduced rate [187]. To overcome the pharmacokinetic problems associated with CpGs, studies are being carried out to enhance their properties, including coupling of CpG to a polysaccharide carrier such as a modified β -(1-3)-D-glucan schizophyllan (SPG, an extracellular polysaccharide produced by the fungus *Schizophyllum commune*) [221], binding of CpG to cationic carbon nanotubes [27], packaging of CpG into fusogenic liposomes (FL, derived from conventional liposomes with an inactivated Sendai virus-derived accessory protein attached) or normal liposomes [366,354] and co-administration of CpG with polyphosphazenes (synthetic water-soluble, biodegradable polymers) [230]. However, many of these methods involve modifying, coupling or packaging the CpG with other products, which might have implication for licensing and/or production of the CpG preparation.

The use of dual adjuvants is not common. However, research using oil-based adjuvant systems with CpG has shown that the addition of CpG can enhance the immune response generated to vaccines [230,190]. The co-administration of ISCOMs and CpG as an adjuvant has also been used successfully to generate responses to vaccine antigens [130,98]. This study demonstrates for the first time that the combination of CpGs with ISCOMs stimulates a stronger and longer lasting immune response than CpG alone. The data generated shows that the administration of three doses of ISCOMs and CpG to mice leads to a strong immune response after the first 24 hours, which would be expected from both of these adjuvants individually due to their immunostimulatory properties [173,298,7]. However, after two weeks this initial immune

response decreases before a further slow increase until six weeks after the final dose (Chapter 5, section 5.3, pages 129 to 131). There is no known description of this pattern of immune response elicited by any other adjuvant. The ability to combine two current adjuvant technologies to generate a greater and longer immune response may be a particularly useful feature for the development of new combination adjuvants.

Although ISCOMs and CpG have previously been used in combination as an adjuvant [130,98] this is the first demonstration of their use in combination with a vaccine candidate for brucellosis. Since this adjuvant is able to decrease the *Brucella* load in an immunised mouse by approximately 1 Log₁₀ without the need for any other antigens, the use of ISCOMs and CpG adjuvant with novel vaccine candidates could help to increase their protective efficacy for brucellosis. For example, one of the most efficacious novel vaccine candidates against brucellosis in the literature is P39 (a putative periplasmic binding protein) [10]. Three doses of 20 µg P39 protein were administered to Balb/C mice adjuvanted with 20 µg of CpG at three week intervals. Three weeks after their final inoculation mice were challenge with 5x10⁴ CFU of *B. abortus* 544, and then at four weeks and eight weeks later mice were culled and the *Brucella* burden was assessed by splenic colonisation assays. The adjuvanted vaccine candidate P39 + CpG elicited a PU of 2.48 at four weeks and 1.21 at 8 weeks [10]. It is possible that a combination of ISCOMs and CpG used as an adjuvant might significantly enhance the PU value at four weeks, since Chapter 5 showed that mice had increased immune responses at six weeks post final inoculation with ISCOMs and CpG. It is possible that, at eight weeks post challenge, mice immunised with P39 might also have improved PU values.

Vaccine development in the *Brucella* field focuses mainly on sub-unit vaccine development or the creation of attenuated mutants as potential vaccines. The problems associated with newly created attenuated mutants are similar to those of the current live attenuated vaccines so, unless they are well characterised and have multiple gene knockouts, then they are considered unlikely to be licensable for human use. Due to the limitations of attenuated vaccines the aim of this project was to select, produce and evaluate ABC transporter proteins as potential sub-unit vaccines against *B. melitensis*. The *Brucella* ABC system inventories generated in Chapter 3,

coupled with published literature enabled the identification of potential sub-unit vaccine candidates.

The selection of the vaccine candidates was achieved through the identification of ABC transporter proteins with homologies to already identified ABC transporter vaccine candidates for other pathogens (PotD [295], PotF [130], LolE [130] and OppA [316]) or ABC transporter proteins identified in *Brucella* that might be involved in virulence (Cgt [277], ZnuA [164,363], CydD [93] and FbpA [94]). All eight of the ABC transporter proteins were evaluated using the murine model of *B. melitensis* infection and, of these, two proteins (PotD 3.61 PU and PotF 2.51 PU) were identified as candidate novel protective antigens against *B. melitensis* challenge. Further testing of the PotD and PotF proteins yielded results indicating that PotD (-0.28 PU and - 0.43 PU) and PotF (0.48 PU) might not be protective antigens when administered alone. CD analysis confirmed that there were structural differences between the protein batches, which was thought could be the reason for the differing protection results. Due to the structural differences of the protein batches, the PotD and PotF antigens were tested as DNA vaccines, the results of which indicated that both PotD and PotF DNA vaccine induced at least a 2-log reduction in *Brucella* spleen load (2.08 PU PotD DNA vaccine and 2.76 PU PotF DNA vaccine). Subsequently, a re-evaluation of PotD and PotF in the folded conformation revealed that, when administered alone, PotD and PotF induce no protective immunity (PotD 0.02 PU and PotF - 0.62 PU) whereas, when administered as a multivalent vaccine combination, PotD and PotF induce significant protection (3.01 PU).

Due to the variation observed in all the animal studies it is difficult to fully understand the potential of PotD and PotF as novel vaccine candidates. The variation in protective efficacy observed between the PotD and PotF protein experiments in Chapter 4 (protective) and Chapter 6 (non-protective) could be attributed to the *E. coli* endotoxin present in the original batches of protein used in Chapter 4. Endotoxin is a potent stimulator of the immune system via TLR4 activation, which can start a non-specific inflammatory immune response. The endotoxin could have provided a boost to the immune response needed to induce a protective immune response when mice were administered the PotD and PotF proteins in Chapter 4. The intergroup variation observed when PotD and PotF were used as DNA vaccine could be attributed to the

intramuscular injections. When administering a dose of 50 μ l i.m. to a mouse it is possible that a large proportion of the volume (< 30 μ l) does not go into the muscle tissue. During DNA vaccination it is imperative that the DNA vaccine goes into the muscle as it needs to be taken up by myocytes for effective expression/presentation to occur. Without effective expression/presentation any immune response generated will be very weak, as seen with PotD and PotF DNA vaccines. Perhaps a gene gun delivery of the PotD and PotF DNA vaccine might increase their efficacy as it is direct inoculation into cells via the skin. The combination of PotD/PotF protein works well as a multivalent vaccine which is potentially due to there being twice as much protein present than when the proteins are administered individually. However, the data generated from the immune response studies did not indicate that a stronger immune response was being generated by immunisation with twice as much protein. The reason for the protection observed from the PotD/PotF combination could be due to the generation of a memory response to two different *Brucella* antigens meaning that during a *Brucella* infection a second exposure to either PotD or PotF would result in a strong memory immune response. It is clear that a repeat study is needed to confirm the PotD/PotF combination protection but this work clearly indicates these proteins have potential as novel vaccine candidates to *Brucella*.

This is the first example of the use of ABC transporter proteins as a novel vaccine candidates for brucellosis. Although there has been conflicting data generated in this study, PotD (2.08 PU) and PotF (2.76 PU) DNA vaccines and the PotD/PotF protein combination (3.01 PU) have been shown to reduce bacterial burdens after an experimental *B. melitensis* 16M challenge. A comparison of the data generated from these trials against other protein or DNA vaccine candidates described in the open literature (Chapter 1, table 1.1 and 1.2, pages 32 and 37) reveals that PotD and PotF should be considered among the best newly identified potential vaccine candidates against brucellosis. In fact, a comparison of the PotD/PotF protein combination to the other proteins tested against *B. melitensis* indicates that the PotD/PotF protein combination is the best candidate currently identified [46].

The PotD and PotF proteins have been identified as novel vaccine candidates for other pathogens, specifically *S. pneumoniae* [295,294] and *B. pseudomallei* [130]. Sub-cutaneous inoculation of CBA/CaHN-*Brk*^{xid} mice with three doses of 5 μ g of *S. pneumoniae* PotD protein

adjuvanted with 1 mg/ml alum provided protection from experimental challenge of 10^4 CFU of *S. pneumoniae* WU2 [295]. More recently, the *S. pneumoniae* PotD was shown also elicit protection when administered via the intranasal route [294]. Similarly, mice inoculated via the intraperitoneal route with three doses of 10 µg of *B. pseudomallei* PotF adjuvanted with ISCOMs and CpG were protected from experimental challenge with 4×10^4 CFU of *B. pseudomallei* K96243. Thus, this study describes a third example of the use of putative polyamine transporters as potential sub-unit vaccines against human and animal pathogens [295,130], indicating that polyamine transporters could be considered globally important virulence factors for bacteria.

There are four microbial polyamines, putrescine, spermidine, spermine and cadaverine, which are essential small molecules that have diverse functions. For example, the roles of polyamines include binding to RNA in conjunction with magnesium ions to stabilise higher order structures such as ribosomes (which increases the accuracy of codon translation during protein synthesis) [145] and the interactions with outer membrane porins, resulting in porin closure which decreases outer membrane permeability [147]. The addition of putrescine to growth media can also resolve virulence gene expression in *Shigella flexneria* mutants unable to make nucleosides needed for translational RNA synthesis [86]. The acquisition of polyamines by bacteria takes place via two main channels: *de novo* synthesis via the breakdown of amino acids L-arginine, L-ornithine, L-lysine and L-methionine or import from the environment using the two ABC transporters *potABCD* or *potFGHI* [296]. The *de novo* synthesis of polyamines in *E. coli* is complex. The synthesis of putrescine from L-arginine requires the enzymes SpeA (arginine decarboxylase) and SpeB (agmatine ureohydrolase). The synthesis of putrescine from L-ornithine requires the SpeC (ornithine decarboxylase) enzyme. Spermidine can be synthesised directly from putrescine using the SpeE (spermidine synthase) enzyme or from L-methionine using SpeD (S-adenosylmethionine decarboxylase) and MetK (methionine adenosyltransferase). Production of cadaverine from L-lysine uses the CadA (lysine decarboxylase) enzyme [312,313,296,226]. A search for these enzymes in the *B. melitensis* 16M genomes (using <http://patric.vbi.vt.edu>) reveals that *B. melitensis* 16M has only four of these enzymes, SpeA, SpeC, MetK and CadA, meaning *B. melitensis* 16M is likely to be able to synthesis cadaverine from L-lysine and putrescine from L-ornithine. Studies in *E. coli* have

shown that mutants in *de novo* synthesis grow slower than wild-type *E. coli* [314,126] and, similarly, mutants in polyamine transport systems also grow at slower rates than their wild-type strains *in vitro* [161]. These findings indicate that *E. coli* might depend upon both *de novo* synthesis and the transport of polyamines for optimal growth. It has also been demonstrated that *Agrobacterium tumefaciens* (a relative of *Brucella* in the α -proteobacteria) requires the *potB*, *potH*, *potC* and *potI* genes for effective virulence in dicotyledonous plants [208]. This indicates that *Brucella* might require both the *de novo* synthesis pathways and ABC transporter systems for optimal growth. Thus, when one or both of the polyamine ABC transporter systems in *Brucella* is targeted by the immune response there may be deficient import of polyamines. In turn, this may lead to a reduced growth rate and therefore increased susceptibility to the immune system. Polyamine transport systems have been identified in other human pathogens such as *Shigella* species [348,362], *Salmonella* species [210,211], *Treponema pallidum* [202], *Yersinia pestis* [80], *Bacillus anthracis* [270] and many others [296]. It is possible that these organisms have both the *de novo* synthesis pathways and the ABC transporter systems. It would be interesting to evaluate their ABC transporter proteins as vaccine candidates based on the findings in this study and other published literature [295,294,130].

7.2 Further studies with PotD and PotF proteins or DNA vaccines

The construction and evaluation of DNA vaccine expressing PotD or PotF is described in Chapter 6. The basic pcDNA3.1 mammalian DNA vaccine expression vector [59,199,370] was used to enable protein expression in mammalian host cells. However, more advanced DNA vaccine vectors may be more effective at inducing strong immune responses. For example, DNA vaccines are being developed that enhance MHC class I or MHC class II antigen processing and therefore influence the type of immune response generated by DNA vaccines [351,184]. Targeting the DNA vaccine products to different intracellular destinations also leads to different MHC class processing which, in turn, leads to different types of immune response. For example, fusing DNA vaccine products with a signal sequence such as tissue plasminogen activator (TPA) [374], endosomal targeting of DNA vaccine products via LAMP-1 signalling [349] proteosomal targeting using murine ubiquitin A76 [74] or endoplasmic reticulum targeting [360] are all approaches that enhance or alter the type of immune response generated. Endosomal

targeting can increase MHC class II presentation leading to an increased CD4⁺ T cell mediated immune response, whereas proteosomal targeting enhances entry to the degradation pathway leading to an increase in MHC class I presentation, therefore increasing the activity of CD8⁺ T cells [351]. It is possible that developing PotD and PotF DNA vaccines that are targeted to the endosome may increase MHC class II presentation, leading to increased Th1 or Th2 cell activation which could increase cellular and humoral immune responses. In comparison, PotD and PotF DNA vaccines targeted to the proteosome might increase MHC I presentation, which increases the CD8⁺ T cell activation and therefore killing of infected cells. Both of these strategies could lead to an increased immune response, in turn leading to an improved protective efficacy.

Another approach to increasing the protective efficacy of the PotD and PotF DNA vaccines would be to construct a multivalent DNA vaccine encoding both the PotD and PotF genes. Luo *et al.* created a divalent DNA vaccine for brucellosis encoding two known partially protective antigens, L7/L12 and Omp16 [199]. In their study mice immunised intramuscularly with three doses of 100 µg of L7/L12, Omp16 or L7/12-Omp16 DNA vaccines all had significantly less *Brucella* in their spleens at four weeks after challenge. Importantly, mice immunised with the divalent L7/L12-Omp16 DNA vaccine showed greater protection than mice dosed with the individual DNA vaccines. However, the creation of divalent DNA vaccines can be complicated and some studies have shown that simply inoculating with a mixture of DNA vaccines encoding different antigens can also achieve increased levels of protective efficacy. For example, Yu *et al.* mixed three DNA vaccines encoding the partially protective antigens L7/L12 (1.26 PU) [176], superoxide dismutase (SOD, 1.52 PU) [227] and a 31 kDa *Brucella* protein (BCSP31, 1.30 PU) [50], and achieved 3.58 PU against experimental *B. abortus* 2308 challenge. This indicates that a divalent DNA vaccine or a mixture of the PotD and PotF DNA vaccines might be more effective than the DNA vaccines administered separately. This may correlate to the finding that when PotD and PotF proteins are administered together, high levels of protection against brucellosis are observed.

Due to the variation observed in some of the animal studies in this work further studies would include a repeat evaluation of the PotD/PotF combined protein vaccine candidate to confirm its

protective efficacy. Subsequently, studies could aim to optimise/improve the protective efficacy of the PotD/PotF combined vaccine. There are several examples of novel multivalent sub-unit vaccines described [33,141,209,318,373], including the novel plague vaccine comprising the F1 and V antigens of *Y. pestis* [321]. Approaches used to increase the protective efficacy of the F1 and V antigens could be transferred to PotD and PotF to improve upon their protective efficacy. One of the methods used to optimise the protective efficacy of the vaccine was to evaluate different adjuvants including Incomplete Freund's adjuvant [352], the Ribi adjuvant system [91] and Alhydrogel [353]. However, for the *Y. pestis* vaccine, the choice of adjuvant had no effect on its protective efficacy, and therefore Alhydrogel was chosen as it is the only widely licensed adjuvant for human use. Although the use of different adjuvants was initiated in Chapter 6 (using the PotD and PotF proteins in the incorrect conformational), and it would be useful to repeat this study with the PotD/PotF protein combination vaccine. Another approach that could be advantageous for the PotD and PotF vaccine candidates would be a DNA vaccine prime, protein boost study. The data in this study has shown that both the PotD and PotF DNA vaccines and the PotD and PotF proteins have potential as novel vaccine candidates. If administered in a prime boost strategy this might increase the protective efficacy observed from the DNA vaccines or proteins when inoculated individually.

Another vaccine technology that would be interesting to explore with PotD and PotF would be the creation of a *Salmonella*-based delivery system. This approach offers a number of advantages. Firstly, *Salmonella*-based delivery systems can be administered orally (a natural route of infection for both *Salmonella* and *Brucella*). Second, since this type of vaccine uses live attenuated bacterial strains of *Salmonella* for expression of heterologous antigens, cellular immune response can be induced. Additionally, there are live attenuated strains of *Salmonella typhi* being developed for use as oral vaccine delivery systems, meaning that the development of safe licensable *Salmonella* vaccines is possible [116,111]. This approach has been shown to work with other well developed vaccine antigens, including the Protective Antigen (PA) of *B. anthracis* [309] and the F1 and V antigens of *Y. pestis* [38,320].

Using the approaches described above it may be possible to improve the protective efficacy elicited by PotD or PotF based vaccines. Subsequently, an assessment of optimised vaccine

candidates in different models of infection could be possible. Animal models of infection with injected or aerosolised *Brucella* have been developed making it possible to test vaccines against infection by different routes [156,299]. This would be important in the development of a PotD and PotF vaccine, as it would be desirable to achieve protection via different natural infection routes. In addition to *B. melitensis*, it would also be important to test the efficacy of the vaccines against the other economically important species of *Brucella* such as *B. abortus* and *B. suis*. A further goal for any developed vaccine candidate would be to test its protective efficacy in a larger natural brucellosis host such as the goat [89,157].

Once a sub-unit vaccine has been developed for brucellosis then the search for accurate correlates of protection could start. A correlate of protection is a measurable sign of immunity to a pathogen. For example, immunisation with the PA vaccine for anthrax elicits a strong antibody response, and measurement of the concentration and neutralisation titre of the antibody produced provides a measure of the protection that an individual may have [271]. As *Brucella* are intracellular pathogens, antibody responses are likely not to be useful as a correlate of protection, as cellular response are likely to contribute to the protection against brucellosis.

The cytokine IFN- γ is often used as a measure of the immune response to vaccine candidates for brucellosis because IFN- γ is critical in the *Brucella* immune response [229]. Although the presence of IFN- γ is important in brucellosis, this work confirms other studies that show that the presence of IFN- γ alone does not serve as a correlate of protection [59,19,10]. Parnavitana *et al.* have studied cytokine and chemokine profiles of mice orally immunised with *Brucella* in search of a correlate of protection for brucellosis and, although their data showed an interesting range of cytokines and chemokines are generated, no clear correlates of protection were found [254]. A novel approach that could be used to create a fingerprint of a protective immune response to brucellosis would be microarray technology. Using a whole mouse genome microarray [45] to profile the genes that are switched on or off during a protective immune response might provide a way of finding an accurate correlate of protection. Studies to profile the genes that are activated in mouse J774A.1 and RAW264.7 macrophages upon infection with *Brucella* represent a start for this type of protective profile [95,133]. The search for a correlate of protection for a disease like brucellosis might be extremely difficult due to its

complex nature. It may be appropriate, therefore, to initiate correlate of protection studies once a next generation sub-unit vaccine has been developed that provides consistent protection.

In summary, this work has identified the first known putative ABC transporter proteins (PotD and PotF) as novel vaccine candidates for *B. melitensis* 16M. It has also reinforced the idea that polyamines and polyamine transporters might have roles in bacterial virulence and pathogenicity [296,295,294,130] and are targets for vaccine development. Additionally, this work has evaluated a combination adjuvant in ISCOMs and CpG that could prove to be a more effective vaccine adjuvant for intracellular pathogens. Overall, there are a number of further studies that should be completed with PotD and PotF to fully understand their protective efficacy, but this work describes the first step in exploiting them as potential sub-unit vaccines for *Brucella*.

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Appendix

ABC system families/subfamilies found in *Brucella* species and their respective function

Name		Description and Function
Family	Sub-Family	
Exporters (Predicted and Experimental)		
<i>DPL, Drugs, Peptides, Lipids</i>	HMT	Mitochondrial and bacterial transporters II
	CHV	Beta(1---2) Galcan export
	MDL	Mitochondrial and bacterial transporters I
	LIP	Lipid A or glycerophospholipid export
	PRT	Proteases, Lipases, S-Layer protein export
	CYD	Cytochrome bd biogenesis
CCM		Cytochrome C biogenesis
CLS		Capsular polysaccharide, lipopolysaccharide and teichoic acids
FAE		Fatty Acid Export
Importers		
DLM		D- L-Methionine and derivatives
CBY	CBU	Cobalt uptake, putative
MKL		Related to MOI family but unknown substrate
YHBG		Related to HAA family, but unknown substrate
CDI		Cell division
MET		Metals
MOS		Monosaccharides
MOI		Mineral and Organic ions
PAO		Polar amino acids and Opines
HAA		Hydrophobic amino acids and amides
OSP		Oligosaccharides and polyols
OPN		Oligopeptides and Nickel
OTCN		Osmoprotectants Taurine Cyanate and Nitrate
ISVH		Iron-Siderophores VitaminB-12 and Hemin
Cellular Process (Experimental)		
ISB		Iron-sulphur centre biogenesis
<i>ART, Antibiotic resistance and translation regulation</i>	REG	Translation regulation
UVR		DNA repair and drug resistance
Unknown		
<i>DRI, Drug resistance, bacteriocin and lantibiotic immunity</i>	YHIH	Drug resistance, putative
	NOS	Possible nitrous oxide reduction
NO		Unclassified Systems
o228		Unknown

Reconstruction and comparative inventories of Brucella ABC systems

#	Family	Subfamily	Substrate/Function	Type	<i>B. melitensis</i>	<i>B. abortus</i>	<i>B. suis</i>	<i>B. ovis</i>	<i>B. canis</i>
1	ART	REG	Involved in gene expression regulation	ABC2	BMEI0288	BruAb11738	BR1753	BOV_1692	BCAN_A1791
2	ART	REG	Involved in gene expression regulation	ABC2	BMEI0553	BruAb11451	BR1456	BOV_1411	BCAN_A1491
3	ART	REG	Involved in gene expression regulation	ABC2	BMEI1258	BruAb10711	BR0692	BOV_0683	BCAN_A0704
4	CBY	CBU	Cobalt import	ABC	BMEI0635	BruAb11365	BR1368	BOV_1324	BCAN_A1395
	CBY	CBU	Cobalt import	IM	BMEI0637	BruAb11364	BR1367	BOV_1323	BCAN_A1394, CbiQ
5	CCM		Possibly heme export	IM	BMEI1851		BR0096, ccmC	BOV_0094	BCAN_A0098, ccmC
	CCM		Possibly heme export	IM	BMEI1852		BR0095, ccmB	BOV_0093	BCAN_A0097, ccmB
	CCM		Possibly heme export	ABC	BMEI1853		BR0094, ccmA	BOV_0092	BCAN_A0096, ccmA
6	CDI		Involved in cell division	IM	BMEI0073, ftsX	BruAb11971	BR1996		BCAN_A2042
	CDI		Involved in cell division	ABC	BMEI0072, ftsE	BruAb11972, ftsE	BR1997, ftsE		BCAN_A2043, ftsE
7	CLS		O antigen export system	ABC	BMEI1416, rfbB		BR0519, rfbE	BOV_0523	BCAN_A0531, rfbB
	CLS		O antigen export system	IM	BMEI1415, rfbD		BR0520, rfbD	BOV_0524	BCAN_A0532, rfbD
8	DLM (ABCY)		D-L-Methionine and derivatives import	LPP	BMEI1954				
9	DLM (ABCY)		D-L-Methionine and derivatives import	IM	BMEI0336	BruAb20271	BRA0962	BOV_A0903	BCAN_B0983
	DLM (ABCY)		D-L-Methionine and derivatives import	ABC	BMEI0337	BruAb20272	BRA0961	BOV_A0902	BCAN_B0982
	DLM (ABCY)		D-L-Methionine and derivatives import	LPP	BMEI0338	BruAb20273	BRA0960		
10	DPL	CYD	Cytochrome bd biogenesis and cysteine export	IM-ABC	BMEI0761, cydC	BruAb20713	BRA0509	BOV_A0443	BCAN_B0508
	DPL	CYD	Cytochrome bd biogenesis and cysteine export	IM-ABC	BMEI0762, cydD	BruAb20714, cydD	BRA0508, cydD	BOV_A0442	BCAN_B0507, CydD
11	DPL	MDL	Involved in mitochondrial export systems	IM-ABC	BMEI0323, msbA	BruAb11700	BR1715	BOV_1657	BCAN_A1753
12	DPL	HMT	Involved in mitochondrial export systems	IM-ABC	BMEI0472	BruAb11533	BR1545	BOV_1493	BCAN_A1581
	DPL	HMT	Involved in mitochondrial export systems	IM-ABC	BMEI0471	BruAb11534	BR1544	BOV_1494	BCAN_A1582
13	DPL	PRT	Proteases, lipase, S-layer protein export	OMP	BMEI1029, TolC	BruAb10954		BOV_0449	BCAN_A0957
14	DPL	CHV	Beta-(1-->2) glucan export	IM-ABC	BMEI0984	BruAb11004	BR0998		BCAN_A1015
15	DPL	HMT	Heavy metal tolerance protein	IM-ABC	BMEI1492	BruAb10321	BR0442		BCAN_A0446
16	DPL	HMT	Involved in mitochondrial export systems	IM-ABC	BMEI1743				
	DPL	HMT	Involved in mitochondrial export systems	IM-ABC	BMEI1742				
17	DPL	LIP	Involved in lipid A or polysaccharide export	IM-ABC	BMEI0250	BruAb20990	BRA1050	BOV_0198	BCAN_B1071
18	DRI	YHIH	Unknown	IM	BMEI0656	BruAb11347	BR1349	BOV_1307	BCAN_A1377
	DRI	YHIH	Unknown	IM	BMEI0655				
	DRI	YHIH	Unknown	ABC2	BMEI0654	BruAb11348	BR1350 (ABC2-IM)	BOV_1308	BCAN_A1378
	DRI	YHIH	Unknown	MFP	BMEI0653	BruAb11349	BR1351	BOV_1309	BCAN_A1379
19	DRI	YHIH	Unknown	IM	BMEI0801	BruAb20757	BRA0465	BOV_A0403	BCAN_B0467
	DRI	YHIH	Unknown	ABC	BMEI0802, drrA	BruAb20758	BRA0464	BOV_A0404	BCAN_B0466
	DRI	YHIH	Unknown	MFP	BMEI0803	BruAb20759	BRA0463	BOV_A0254	BCAN_B0465
20	DRI	NOS	Nitrous oxide reduction	IM	BMEI0970, nosY	BruAb20902, nosY	BRA0278, nosY	BOV_A0253	BCAN_B0280
	DRI	NOS	Nitrous oxide reduction	ABC	BMEI0971, nosF	BruAb20903, nosF	BRA0277, nosF	BOV_A0253	BCAN_B0279
	DRI	NOS	Nitrous oxide reduction	SS	BMEI0972	BruAb20904, nosD	BRA0276, nosD	BOV_A0252	BCAN_B0278
21	FAE		Fatty acid export	IM-ABC	BMEI0520	BruAb11484	BR1490		BCAN_A1528
22	FAE		Fatty acid export	IM-ABC	BMEI0976	BruAb20908		BOV_A0247	BCAN_B0273

Reconstruction and comparative inventories of Brucella ABC systems

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23	HAA		Branched-chain amino acids	IM	BMIE0258, LivH	BruAb11771	BR1790	BOV_1725	BCAN_A1829
	HAA		Branched-chain amino acids	IM	BMIE0259, LivM	BruAb11772	BR1791	BOV_1724	BCAN_A1828
	HAA		Branched-chain amino acids	ABC	BMEI0260, braF	BruAb11770	BR1788	BOV_1723	BCAN_A1827
	HAA		Branched-chain amino acids	ABC	BMEI0261, braG	BruAb11769	BR1789	BOV_1722	BCAN_A1826
	HAA		Branched-chain amino acids	BP	BMEI0263	BruAb11765	BR1785	BOV_1720	BCAN_A1823
	HAA		Branched-chain amino acids	BP	BMEI0264	BruAb11767	BR1782		BCAN_A1820
	HAA		Branched-chain amino acids	BP	BMEI0265			BOV_1719	
	HAA		Branched-chain amino acids	BP	BMEI1930		BR0014	BOV_0012	BCAN_A0014
	HAA		Branched-chain amino acids	ABC	BMEI0065, livF	BruAb20027	BRA0028	BOV_A0025	BCAN_B0030
	HAA		Branched-chain amino acids	ABC	BMEI0066, livG	BruAb20028	BRA0027	BOV_A0024	BCAN_B0029
24	HAA		Branched-chain amino acids	IM	BMEI0067, livM	BruAb20025	BRA0026	BOV_A0023	BCAN_B0028
	HAA		Branched-chain amino acids	IM	BMEI0068, livH	BruAb20026	BRA0025	BOV_A0022	BCAN_B0027
	HAA		Branched-chain amino acids	BP	BMEI0069	BruAb20024	BRA0024	BOV_A0021	BCAN_B0026
	HAA		Branched-chain amino acids	ABC	BMEI0098	BruAb21132	BRA1197	BOV_A1099	BCAN_B1227
	HAA		Branched-chain amino acids	ABC	BMEI0099	BruAb21133	BRA1196	BOV_A1098	BCAN_B1226
	HAA		Branched-chain amino acids	IM	BMEI0101	BruAb21131	BRA1194	BOV_A1097	BCAN_B1225
	HAA		Branched-chain amino acids	IM	BMEI0102	BruAb21130	BRA1195	BOV_A1096	BCAN_B1224
	HAA		Branched-chain amino acids	BP	BMEI0103	BruAb21129	BRA1193	BOV_A0195	BCAN_B1223
	HAA		Branched-chain amino acids	ABC	BMEI0119	BruAb21111	BRA1176	BOV_A1079	BCAN_B1207
	HAA		Branched-chain amino acids	IM-ABC	BMEI0120	BruAb21112	BRA1175	BOV_A1078	BCAN_B1206
25	HAA		Branched-chain amino acids	IM	BMEI0121	BruAb21110	BRA1174	BOV_A1205	BCAN_B1205
	HAA		Branched-chain amino acids	BP	BMEI0122	BruAb21109	BRA1173	BOV_A1076	BCAN_B1204
	HAA		Branched-chain amino acids	IM	BMEI0340	BruAb20276	BRA0957	BOV_A0897	BCAN_B0977
	HAA		Branched-chain amino acids	IM	BMEI0341	BruAb20277	BRA0956	BOV_A0897	BCAN_B0978
	HAA		Branched-chain amino acids	ABC	BMEI0342	BruAb20278	BRA0955	BOV_A0896	BCAN_B0976
	HAA		Branched-chain amino acids	ABC	BMEI0343	BruAb20279	BRA0954	BOV_A0895	BCAN_B0975
	HAA		Branched-chain amino acids	BP	BMEI0344	BruAb20280	BRA0953	BOV_A0894	BCAN_B0974
	HAA		Branched-chain amino acids	ABC	BMEI0628	BruAb20574	BRA0652	BOV_A0613	BCAN_B0652
	HAA		Branched-chain amino acids	ABC	BMEI0629	BruAb20575	BRA0651	BOV_A0614	BCAN_B0651
	HAA		Branched-chain amino acids	IM	BMEI0630	BruAb20577	BRA0650	BOV_A0611	BCAN_B0649
26	HAA		Branched-chain amino acids	IM	BMEI0632	BruAb20576	BRA0649	BOV_A0612	BCAN_B0650
	HAA		Branched-chain amino acids	BP	BMEI0633	BruAb20578	BRA0648	BOV_A0610	BCAN_B0648
	HAA		Branched-chain amino acids	BP	BMEI0875	BruAb20801	BRA0392	BOV_A0343	BCAN_B0398
	HAA		Branched-chain amino acids	BP	BMEI0868	BruAb20809	BRA0400	BOV_A0338	BCAN_B0395
	HAA		Branched-chain amino acids	ABC	BMEI0874	BruAb20806	BRA0395	BOV_A0337	BCAN_B0389
	HAA		Branched-chain amino acids	ABC	BMEI0873	BruAb20807	BRA0394	BOV_A0337	BCAN_B0396
	HAA		Branched-chain amino acids	IM	BMEI1040	BruAb20808	BRA0393	BOV_A0336	BCAN_B0397
	ISB (ABCX)		Iron/sulphur centre biogenesis	CYTP	BMEI1040	BruAb10941	BR0931		
	ISB (ABCX)		Iron/sulphur centre biogenesis	CYTP	BMEI1042	BruAb10940	BR0933		

Reconstruction and comparative inventories of Brucella ABC systems

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	ISB (ABCX)		Iron/sulphur centre biogenesis	ABC	BMEI1041	BruAb10942	BR0932		
32	ISVH		Iron-siderophores, VB12 and Hemin import	ABC	BMEI0660	BruAb11342	BR1344	BOV_1302	BCAN_A1371
	ISVH		Iron-siderophores, VB12 and Hemin import	IM	BMEI0659	BruAb11343	BR1345	BOV_1304	BCAN_A1372
	ISVH		Iron-siderophores, VB12 and Hemin import	OMR	BMEI0657	BruAb11344	BR1347	BOV_1306	BCAN_A1374
	ISVH		Iron-siderophores, VB12 and Hemin import	BP	BMEI0658	BruAb11345	BR1346	BOV_1305	BCAN_A1373
33	ISVH		Iron(III) dicitrate import	BP	BMEI0535	BruAb20476	BRA0756	BOV_A0705	BCAN_B0763
	ISVH		Iron(III) dicitrate import	IM	BMEI0536, fecD	BruAb20477	BRA0755	BOV_A0704	BCAN_B0764
	ISVH		Iron(III) dicitrate import	ABC	BMEI0537, fecE	BruAb20478	BRA0754	BOV_A0703	BCAN_B0762
34	ISVH		Iron(III) import	ABC	BMEI0604	BruAb20550	BRA0678	BOV_A0635	BCAN_B0677
	ISVH		Iron(III) import	IM	BMEI0605, fatC	BruAb20551	BRA0676	BOV_A0634	BCAN_B0675
	ISVH		Iron(III) import	IM	BMEI0606, fatD	BruAb20552	BRA0677	BOV_A0633	BCAN_B0676
	ISVH		Iron(III) import	BP	BMEI0607	BruAb20553	BRA0675	BOV_A0632	BCAN_B0674
35	MET		Zinc import	IM	BMEI0176, ZnuB	BruAb21061, ZnuB	BRA1124, ZnuB	BOV_A1029	BCAN_B1152
	MET		Zinc import	ABC	BMEI0177, ZnuC	BruAb21060, ZnuC	BRA1123, ZnuC	BOV_A1028	BCAN_B1151
	MET		Zinc import	BP	BMEI0178, ZnuA	BruAb21059, ZnuA	BRA1122, ZnuA	BOV_A1027	BCAN_B1150
36	MKL		Involved in toluene tolerance	ABC	BMEI0964	BruAb11025	BR1020	BOV_0987	
	MKL		Involved in toluene tolerance	IM	BMEI0965, tfg2B	BruAb1024	BR1019	BOV_0986	
	MKL		Involved in toluene tolerance	SS	BMEI0963, tfg2C	BruAb11026	BR1021	BOV_0988	
37	MOI		Thiamine import	ABC	BMEI0283, thiQ	BruAb11744	BR1759	BOV_1698	BCAN_A1798
	MOI		Thiamine import	IM	BMEI0284, thiP	BruAb11743, thiP	BR1758, thiP	BOV_1696	thiP, BCAN_A1797
	MOI		Thiamine import	BP	BMEI0285	BruAb11744, thiB	BR1757, thiB	BOV_1695	thiB, BCAN_A1796
38	MOI		Putrescine import	BP	BMEI0411, potF	BruAb11599	BR1612	BOV_1556	BCAN_A1649
	MOI		Putrescine import	ABC	BMEI0412	BruAb11598	BR1611	BOV_1555	BCAN_A1648
	MOI		Putrescine import	IM	BMEI0413	BruAb11596	BR1609	BOV_1554	BCAN_A1647
	MOI		Putrescine import	IM	BMEI0414	BruAb11597	BR1610	BOV_1553	BCAN_A1646
39	MOI		Sulphate import	IM	BMEI0675, cysW	BruAb11328, cysW2	BR1328, cysW2	BOV_1288	CysW, BCAN_A1353
	MOI		Sulphate import	IM	BMEI0674, cysT	BruAb11329	BR1329	BOV_1289	CysT, BCAN_A1354
	MOI		Sulphate import	BP	BMEI0673	BruAb11330	BR1330	BOV_1290	BCAN_A1355
40	MOI		Sulphate import	ABC	BMEI1838 cysA	BruAb10107	BR0110	BOV_0107	CysA, BCAN_A0113
	MOI		Sulphate import	IM	BMEI1839, cysW	BruAb10106	BR0109, cysW1	BOV_0106	CysW, BCAN_A0112
	MOI		Sulphate import	IM	BMEI1840, cyst	BruAb10105, cysT	BR0108	BOV_0105	CysT, BCAN_A0111
	MOI		Sulphate import	BP	BMEI1841	BruAb10104	BR0107	BOV_0104	BCAN_A0110
41	MOI		Phosphate import	ABC	BMEI1986, pstB	BruAb12116, pstB	BR2141, pstB	BOV_2056	BCAN_A2185, pstB
	MOI		Phosphate import	IM	BMEI1987, pstA	BruAb12114, pstC	BR2139, pstC	BOV_2055	BCAN_A2184, pstA
	MOI		Phosphate import	IM	BMEI1988, pstC	BruAb12115, pstA	BR2140	BOV_2054	BCAN_A2183, pstC
	MOI		Phosphate import	BP	BMEI1989	BruAb12113	BR2138	BOV_2053	BCAN_A2128
42	MOI		Molybdenum import	ABC	BMEI0003, modC	BruAb20090	BRA0090, modC	BOV_A0084	BCAN_B0093, ModC
	MOI		Molybdenum import	IM	BMEI0004, modB	BruAb20089	BRA0089, modB	BOV_A0083	BCAN_B0092, ModB
	MOI		Molybdenum import	BP	BMEI0005	BruAb20088	BRA0088, modA	BOV_A0082	BCAN_B0091

Reconstruction and comparative inventories of Brucella ABC systems

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43	MOI		Spermidine/putrescine import	ABC	BMEI10193, potA	BruAb21046	BRA1107		BCAN_B1129
	MOI		Spermidine/putrescine import	IM	BMEI10194, potB	BruAb21044	BRA1106		BCAN_B1128
	MOI		Spermidine/putrescine import	IM	BMEI10195, potC	BruAb21045	BRA1105		BCAN_B1127
	MOI		Spermidine/putrescine import	BP	BMEI10196	BruAb21043	BRA1104		BCAN_B1126
44	MOI		Unknown	BP	BMEI10479	BruAb20422	BRA0810	BOV_A0760	BCAN_B0824
	MOI		Unknown	ABC	BMEI10481	BruAb20423	BRA0809	BOV_A0759	BCAN_B0823
	MOI		Unknown	IM	BMEI10483	BruAb20424	BRA0807	BOV_A0758	BCAN_B0822
	MOI		Unknown	IM	BMEI10484	BruAb20425	BRA0808	BOV_A0757	BCAN_B0821
45	MOI		Iron(III) import	BP	BMEI10565	BruAb20510	BRA0720	BOV_A0676	BCAN_B0726
	MOI		Iron(III) import	IM2	BMEI10566	BruAb20511	BRA0719	BOV_A0675	BCAN_B0274
	MOI		Iron(III) import	ABC	BMEI10567	BruAb20512	BRA0718	BOV_A0674	BCAN_B0725
46	MOI		Iron(III) import	ABC	BMEI10583	BruAb20529	BRA0701	BOV_A0656	BCAN_B0702
	MOI		Iron(III) import	BP	BMEI10584	BruAb20530	BRA0700	BOV_A0655	BCAN_B0703
	MOI		Iron(III) import	IM2	BMEI10585	BruAb20531	BRA0699	BOV_A0654	BCAN_B0701
47	MOI		Spermidine/putrescine import	IM	BMEI10920, potC	BruAb20852	BRA0328	BOV_A0303	BCAN_B0331
	MOI		Spermidine/putrescine import	IM	BMEI10921, potB	BruAb20853	BRA0329	BOV_A0302	BCAN_B0330
	MOI		Spermidine/putrescine import	ABC	BMEI10922, potA	BruAb20855	BRA0327	BOV_A0301	BCAN_B0329
	MOI		Spermidine/putrescine import	BP	BMEI10923, potD	BruAb20854	BRA0326	BOV_A0300	BCAN_B0328
48	MOI		Iron(III) import	BP	BMEI1120	BruAb20113	BRA0115	BOV_A0105	BCAN_B0119
	MOI		Iron(III) import	IM	BMEI1121, sufB	BruAb20111	BRA0114	BOV_A0104	BCAN_B0118
	MOI		Iron(III) import	IM	BMEI1122, sufB	BruAb20112	BRA0113	BOV_A0103	BCAN_B0117
	MOI		Iron(III) import	ABC	BMEI1123, sufC	BruAb20110	BRA0112	BOV_A0102	BCAN_B0116
49	New1		Unknown	IM	BMEI0013	BruAb12030	BR2055	BOV_1975	BCAN_A2101
	New1		Unknown	ABC	BMEI0012	BruAb12031	BR2056		BCAN_A2102
	New1		Unknown	BP	BMEI0014				
	New1		Unknown	BP	BMEI0015				
50	MOS		Ribose import	ABC2	BMEI0391	BruAB11620, rbsA-2	BR1632, rbsA-2	BOV_1576	BCAN_A1669
	MOS		Ribose import	IM	BMEI0392	BruAB11619, rbsC-2	BR1631, rbsC-2	BOV_1575, rbsC2	BCAN_A1668
	MOS		Ribose import	BP	BMEI0393	BruAB11618	BR1630	BOV_1574	BCAN_A1667
51	MOS		Ribose Import	ABC	BMEI0665	BruAb11337	BR1339	BOV_1299	BCAN_A1367
	MOS		Ribose Import	IM	BMEI0664	BruAb11338	BR1340	BOV_1300	BCAN_A1368
	MOS		Ribose Import	BP	BMEI0663	BruAb11340	BR1342	BOV_1301	BCAN_A1369
	MOS		Ribose Import	BP	BMEI0662	BruAb11335			
52	MOS		Ribose import	BP	BMEI1390	BruAb10566, rbsB1	BR0544, rbsB1	BOV_0546 rbsB1	BCAN_A0557
	MOS		Ribose import	IM	BMEI1391, rbsC	BruAb10565, rbsC1	BR0543, rbsC1	BOV_0545 rbsC1	BCAN_A0555
	MOS		Ribose import	ABC2	BMEI1392, rbsA	BruAb10564, rbsA1	BR0542, rbsA1	BOV_0544 rbsA1	BCAN_A0554, rbsA
53	MOS		Possibly galactoside	BP	BMEI10083	BruAb20010	BRA0010	BOV_A0007	
	MOS		Possibly galactoside	ABC2	BMEI10085, mgIA	BruAb20009	BRA0009	BOV_A0006	
	MOS		Possibly galactoside	IM	BMEI10086, mgIC	BruAb20007	BRA0007	BOV_A0005	

Reconstruction and comparative inventories of Brucella ABC systems

#	Family	Subfamily	Substrate/Function	Type	<i>B. melitensis</i>	<i>B. abortus</i>	<i>B. suis</i>	<i>B. ovis</i>	<i>B. canis</i>
54	MOS		Possibly galactoside	IM	BMEI10087	BruAb20008	BRA0008	BOV_A0004	BCAN_B1181
	MOS		Xylose import	IM	BMEI10144, xyIH	BruAb21089, xyIH	BRA1152, xyIH	BOV_A1057	BCAN_B1180, xyIG
	MOS		Xylose import	ABC2	BMEI10145, xyIG	BruAb21088, xyIG	BRA1151, xyIG	BOV_A1056	BCAN_B1179, xyIF
	MOS		Xylose import	BP	BMEI10146, xyIF	BruAb21087, xyIF	BRA1150, xyIF	BOV_A1055	
55	MOS		Ribose import	ABC2	BMEI10300, rbsA	BruAb20239,rbsA4	BRA0995, rbsA4	BOV_A0937	BCAN_B1014
	MOS		Ribose import	IM	BMEI10301 rbsC	BruAb20240,rbsC5	BRA0993, rbsC5		BCAN_B1013
	MOS		Ribose import	IM	BMEI10302 rbsC	BruAb20239, rbsC4	BRA0994, rbsC5	BOV_A0935	BCAN_B1012
	MOS		Ribose import	BP		BruAb20238	BRA0996, rbsB3	BOV_A0938	BCAN_B1015
56	MOS		Monosaccharide import	BP	BMEI10360, chvE	BruAb20296	BRA0937	BOV_A0879	BCAN_B0957
	MOS		Monosaccharide import	ABC2	BMEI10361	BruAb20297	BRA0936	BOV_A0878	BCAN_B0956
	MOS		Monosaccharide import	IM	BMEI10362	BruAb20298	BRA0935	BOV_A0877	BCAN_B0955
57	MOS		Erythritol import	ABC2	BMEI10432, rbsA	BruAb20371, rbsA3	BRA0860, rbsA3	BOV_A0807, rbsA3	BCAN_B0877
	MOS		Erythritol import	IM	BMEI10433, rbsC	BruAb20372, rbsC3	BRA0859, rbsC3	BCAN_B0876	BCAN_B0875
	MOS		Erythritol import	BP	BMEI10435	BruAb20373, rbsB2	BRA0858, rbsB2	BOV_A0533	BCAN_B0570
58	MOS		Galactoside/Ribose import	ABC2	BMEI10698	BruAb20654	BRA0570	BOV_A0534	BCAN_B0567
	MOS		Galactoside/Ribose import	IM				BOV_A0535	
	MOS		Galactoside/Ribose import	IM	BMEI10700	BruAb20655	BRA0568	BOV_A0532	BCAN_B0568
	MOS		Galactoside/Ribose import	IM	BMEI10701	BruAb20656	BRA0569	BOV_A0242	BCAN_B0567
	MOS		Galactoside/Ribose import	BP	BMEI10702		BRA0567	BOV_A0241	BCAN_B0267
59	MOS		Monosaccharide import	IM	BMEI10981	BruAb20913	BRA0267	BOV_A0240	BCAN_B0266
	MOS		Monosaccharide import	ABC2	BMEI10982	BruAb20914	BRA0266		
	MOS		Monosaccharide import	BP	BMEI10983	BruAb20916	BRA0265		
60	o228		Unknown	IM	BMEI0361				
	o228		Unknown	MFP	BMEI0359				
	o228		Unknown	ABC	BMEI0360				
61	o228		Unknown	IM		BruAb10085	BR0087	BOV_0085	
	o228		Unknown	MFP					BCAN_A1712
	o228		Unknown	ABC		BruAb10084	BR0086	BOV_0084	BCAN_A1711
62	o228		Unknown	MFP		BruA11658	BR1671	BOV_1617	
	o228		Unknown	IM-ABC		BruA11657	BR1670	BOV_1616	BCAN_A0087
63	o228		Lipoprotein release system	ABC	BMEI1138, LoID	BruAb10838,LoID	BR0824, LoID	BOV_0818	BCAN_A0839
	o228		Lipoprotein release system	IM	BMEI1139, LoIE	BruAb10837, LoIE	BR0823, LoIE	BOV_0817	BCAN_A0838
64	OPN		Dipeptide import	ABC	BMEI0438, dppF	BruAb11569	BR1582	BOV_1527	BCAN_A1617
	OPN		Dipeptide import	ABC	BMEI0437, dppD	BruAb11570	BR1583	BOV_1528	BCAN_A1618
	OPN		Dipeptide import	IM	BMEI0435, dppC	BruAb11571	BR1584	BOV_1530	BCAN_A1620
	OPN		Dipeptide import	IM	BMEI0436, dppC	BruAb11572	BR1585	BOV_1529	BCAN_A1619
	OPN		Dipeptide import	BP	BMEI0433, dppA	BruAb11573	BR1586	BOV_1531	BCAN_A1621
65	OPN		Oligopeptide import	ABC2	BMEI1938, oppD	BruAb10006	BR0006	BOV_0006	BCAN_A0006
	OPN		Oligopeptide import	BP	BMEI1934	BruAb10007	BR0007	BOV_0009	BCAN_A0010

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#	Family	Subfamily	Substrate/Function	Type	<i>B. melitensis</i>	<i>B. abortus</i>	<i>B. suis</i>	<i>B. ovis</i>	<i>B. canis</i>
66	OPN		Oligopeptide import	BP	BMEI1935	BruAb10008	BR0008	BOV_0010	BCAN_A0009
	OPN		Oligopeptide import	IM	BMEI1936, oppB	BruAb10009	BR0009	BOV_0008	BCAN_A0008
	OPN		Oligopeptide import	IM	BMEI1937, oppC	BruAb10010	BR0010	BOV_0007	BCAN_A0007
	OPN		Oligopeptide import	ABC	BMEI199, oppF	BruAb21039	BRA1100		BCAN_B1123
	OPN		Oligopeptide import	ABC	BMEI10200, oppD	BruAb21040	BRA1101		BCAN_B1122
	OPN		Oligopeptide import	IM	BMEI10201, oppC	BruAb21037	BRA0099		BCAN_B1121
67	OPN		Oligopeptide import	IM	BMEI10202, oppB	BruAb21038	BRA0098		BCAN_B1119
	OPN		Oligopeptide import	BP	BMEI101203	BruAb21036	BRA0097		BCAN_B1117
	OPN		Dipeptide import	ABC	BMEI10205, dppF	BruAb21033	BRA1095	BOV_A0950	BCAN_B1116
	OPN		Dipeptide import	ABC	BMEI10206, dppD	BruAb21034	BRA1094	BOV_A0951	BCAN_B1115, dppC
	OPN		Dipeptide import	IM	BMEI10207, dppC	BruAb21031	BRA1093		
	OPN		Dipeptide import	IM	BMEI10209, dppB	BruAb21032	BRA1092	BOV_A0952	
68	OPN		Dipeptide import	IM	BMEI10210			BOV_A0953	BCAN_B1114
	OPN		Dipeptide import	BP	BMEI10217	BruAb21030	BRA1090	BOV_A0954	BCAN_B1113
	OPN		Dipeptide/ Oligopeptide import	BP	BMEI10217	BruAb21024	BRA1084		BCAN_B1107
	OPN		Dipeptide/ Oligopeptide import	IM	BMEI10220	BruAb21020	BRA1081		BCAN_B1104
	OPN		Dipeptide/ Oligopeptide import	IM	BMEI10221	BruAb21021	BRA1080		BCAN_B1103
	OPN		Dipeptide/ Oligopeptide import	ABC	BMEI10222	BruAb21018	BRA1079		BCAN_B1102
69	OPN		Dipeptide/ Oligopeptide import	ABC	BMEI10223	BruAb21019	BRA1078		BCAN_B1101
	OPN		Dipeptide import	BP	BMEI10284	BruAb20952	BRA1012	BOV_A0504	BCAN_B1032
	OPN		Dipeptide import	IM	BMEI10285	BruAb20950	BRA1009	BOV_A0501	BCAN_B1031
	OPN		Dipeptide import	IM	BMEI10286	BruAb20951	BRA1008	BOV_A0502	BCAN_B1030
	OPN		Dipeptide import	ABC	BMEI10287	BruAb20948	BRA1011	BOV_A0500	BCAN_B1029
	OPN		Dipeptide import	ABC	BMEI10288	BruAb20949	BRA1010	BOV_A0501	BCAN_B1028
70	OPN		Nickel import	BP	BMEI10487	BruAb20428	BRA0804	BOV_A0754	BCAN_B0818, NiKA
	OPN		Nickel import	IM	BMEI10488, nikB	BruAb20429, nikB	BRA0802, nikC	BOV_A0752	BCAN_B0817, NiKB
	OPN		Nickel import	IM	BMEI10489, nikC	BruAb20430, nikV	BRA0803, nikB	BOV_A0753	BCAN_B0816, NiKC
	OPN		Nickel import	ABC	BMEI10490, nikD	BruAb20431, nikD	BRA0800, nikE	BOV_A0751	BCAN_B0815, NiKD
	OPN		Nickel import	ABC	BMEI10491, nikE	BruAb20432, nikE	BRA0801, nikD		BCAN_B0814, NiKE
	OPN		Oligopeptide import	BP	BMEI10504	BruAb20446	BRA0783	BOV_A0737	BCAN_B0800
71	OPN		Oligopeptide import	IM	BMEI10505	BruAb20447	BRA0788	BOV_A0736	BCAN_B0799
	OPN		Oligopeptide import	IM	BMEI10506	BruAb20448	BRA0787	BOV_A0735	BCAN_B0798
	OPN		Oligopeptide import	ABC	BMEI10507	BruAb20449	BRA0786	BOV_A0734	BCAN_B0797
	OPN		Oligopeptide import	ABC	BMEI10508			BOV_A0733	BCAN_B0796
	OPN		Oligopeptide import	BP	BMEI10691	BruAb20648	BRA0576	BOV_A0542	
	OPN		Oligopeptide import	BP	BMEI10734	BruAb20684	BRA0538	BOV_A0468	BCAN_B0538
73	OPN		Oligopeptide import	BP	BMEI10735, oppA	BruAb20685	BRA0537	BOV_A0467	BCAN_B0537
	OPN		Oligopeptide import	IM	BMEI10736	BruAb20686	BRA0536	BOV_A0466	BCAN_B0535
	OPN		Oligopeptide import	IM	BMEI10737	BruAb20687	BRA0535	BOV_A0465	BCAN_B0536

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#	Family	Subfamily	Substrate/Function	Type	<i>B. melitensis</i>	<i>B. abortus</i>	<i>B. suis</i>	<i>B. ovis</i>	<i>B. canis</i>
74	OPN		Oligopeptide import	ABC2	BMEI10738	BruAb20688	BRA0534	BOV_A0464	BCAN_B0534
	OPN		Oligopeptide import	BP	BMEI10859	BruAb20792	BRA0409	BOV_A0352	BCAN_B0412
	OPN		Oligopeptide import	IM	BMEI10860		BRA0408	BOV_A0351	BCAN_B0411
	OPN		Oligopeptide import	IM	BMEI10861	BruAb20794	BRA0407	BOV_A0350	BCAN_B0410
	OPN		Oligopeptide import	ABC	BMEI10863	BruAb20796	BRA0405	BOV_A0347	BCAN_B0408
75	OPN		Oligopeptide import	ABC	BMEI10864	BruAb20797	BRA0404	BOV_A0348	BCAN_B0407
	OSP		Maltose import	ABC	BMEI1713, malK	BruAb10233	BR0238	BOV_0231	BCAN_A0241
	OSP		Maltose import	IM	BMEI1714, malG	BruAb10231	BR0237	BOV_0230	BCAN_A0240
	OSP		Maltose import	IM	BMEI1715, malF	BruAb10232	BR0236	BOV_0229	BCAN_A0239
	OSP		Maltose import	BP	BMEI1716	BruAb10230	BR0235	BOV_0228	BCAN_A0238
76	OSP		Oligosaccharide or polyol import	ABC	BMEI10112, ugpC	BruAb21119	BRA1183	BOV_A1086	BCAN_B1214
	OSP		Oligosaccharide or polyol import	IM	BMEI10113, ugpA	BruAb21118	BRA1181	BOV_A1085	BCAN_B1213
	OSP		Oligosaccharide or polyol import	IM	BMEI10114, ugpE	BruAb21117	BRA1182	BOV_A1084	BCAN_B1212
	OSP		Oligosaccharide or polyol import	BP	BMEI10115	BruAb21116	BRA1180		BCAN_B1211
	OSP		Oligosaccharide or polyol import	IM	BMEI10541	BruAb20483	BRA0749	BOV_A0700	BCAN_B0757
77	OSP		Oligosaccharide or polyol import	IM		BruAb20482	BRA0750	BOV_A0699	BCAN_B0756
	OSP		Oligosaccharide or polyol import	BP	BMEI10542	BruAb20484	BRA0748	BOV_A0698	BCAN_B0755
	OSP		Oligosaccharide or polyol import	ABC	BMEI10544	BruAb20487	BRA0745	BOV_A0696	BCAN_B0753
	OSP		Oligosaccharide or polyol import	BP	BMEI10590	BruAb20537	BRA0693	BOV_A0648	BCAN_B0691
	OSP		Oligosaccharide or polyol import	IM	BMEI10591	BruAb20538	BRA0691	BOV_A0647	BCAN_B0690
78	OSP		Oligosaccharide or polyol import	IM	BMEI10592	BruAb20539	BRA0692	BOV_A0646	BCAN_B0689
	OSP		Oligosaccharide or polyol import	ABC	BMEI10593	BruAb20540	BRA0690	BOV_A0645	BCAN_B0688
	OSP		SN-glycerol-3-phosphate import	ABC	BMEI10621, ugpC	BruAb20568, ugpC	BRA0658, ugpC	BOV_A0620	BCAN_B0658
	OSP		SN-glycerol-3-phosphate import	IM	BMEI10622, ugpE	BruAb20569, ugpE	BRA0657, ugpE	BOV_A0619	BCAN_B0657
	OSP		SN-glycerol-3-phosphate import	IM	BMEI10623, ugpE	BruAb20570, ugpA	BRA0656, ugpA	BOV_A0618	BCAN_B0656
79	OSP		SN-glycerol-3-phosphate import	IM	BMEI10624, ugpA				
	OSP		SN-glycerol-3-phosphate import	BP	BMEI10625	BruAb20571, ugpB	BRA0655, ugpA	BOV_A0617	BCAN_B0655
	OSP		Oligosaccharide or polyol import	ABC	BMEI10750	BruAb20702	BRA0521	BOV_A0454	BCAN_B0520
	OSP		Oligosaccharide or polyol import	IM	BMEI10752	BruAb20704	BRA0519	BOV_A0452	BCAN_B0518
	OSP		Oligosaccharide or polyol import	IM	BMEI10753	BruAb20705	BRA0518	BOV_A0451	BCAN_B0517
80	OSP		Oligosaccharide or polyol import	BP	BMEI10754	BruAb20706	BRA0516	BOV_A0449	BCAN_B0516
	OSP		Oligosaccharide or polyol import	BP	BMEI10755				
	OSP		Maltose import	ABC	BMEI10940	BruAb20874	BRA0307	BOV_A0282	BCAN_B0308
	OSP		Maltose import	IM	BMEI10942	BruAb20875	BRA0306	BOV_A0281	BCAN_B0307
	OSP		Maltose import	IM	BMEI10943	BruAb20876	BRA0305	BOV_A0280	BCAN_B0306
81	OSP		Maltose import	BP	BMEI10944			BOV_A0279	
	OSP		Maltose import	BP	BMEI10945	BruAb20877	BRA0304		BCAN_B0305
	OTCN		Glycine betaine/L-proline import	ABC	BMEI0439, proV	BruAb11568	BR1581	BOV_1526	BCAN_A1616
	OTCN		Glycine betaine/L-proline import	IM	BMEI0440, proW	BruAb11567	BR1580	BOV_1525	BCAN_A1615

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83	OTCN		Glycine betaine/L-proline import	BP	BMEI0441, proX	BruAb11566	BR1579	BOV_1524	BCAN_A1614
	OTCN		Choline S ³ -dependent regulation of <i>yehZYXW</i>	BP	BMEI1725	BruAb10220	BR0225	BOV_0216	BCAN_A0228
	OTCN		Choline S ³ -dependent regulation of <i>yehZYXW</i>	IM	BMEI1726, proW	BruAb10217	BR1222	BOV_0215	BCAN_A0227
	OTCN		Choline S ³ -dependent regulation of <i>yehZYXW</i>	IM	BMEI1728, proW	BruAb10219	BR0224	BOV_0213	BCAN_A0225
	OTCN		Choline S ³ -dependent regulation of <i>yehZYXW</i>	ABC	BMEI1727, proV	BruAb10218	BR0223	BOV_0214	BCAN_A0226
84	OTCN		Osmoprotectants, Taurine, Cyanate & Nitrate	BP	BMEI1737	BruAb10207	BR0211	BOV_0204	BCAN_A0215
	OTCN		Osmoprotectants, Taurine, Cyanate & Nitrate	IM	BMEI1739	BruAb10206	BR0213	BOV_0202	BCAN_A0213
85	OTCN		Osmoprotectants, Taurine, Cyanate & Nitrate	ABC		BruAb10208			
	OTCN		Taurine import	BP	BMEI10109	BruAb21122	BRA1186	BOV_A1089	BCAN_B1218
	OTCN		Taurine import	IM	BMEI10107, tauC	BruAb21124	BRA1188	BOV_A1091	BCAN_B1219
86	OTCN		Taurine import	ABC	BMEI10108, tauB	BruAb21123	BRA1187	BOV_A1090	BCAN_B1217
	OTCN		Glycine betaine/L-proline import	ABC	BMEI10548	BruAb20492	BRA0740	BOV_A0692	BCAN_B0748
	OTCN		Glycine betaine/L-proline import	IM	BMEI10549	BruAb20493	BRA0739	BOV_A0691	BCAN_B0747
	OTCN		Glycine betaine/L-proline import	BP	BMEI10550	BruAb20494	BRA0738	BOV_A0690	BCAN_B0746
	OTCN		Nitrate import	BP	BMEI10797	BruAb20753	BRA0469	BOV_A0406	BCAN_B0471
87	OTCN		Nitrate import	ABC	BMEI10798, nrtC	BruAb20755	BRA0467	BOV_A0407	BCAN_B0470
	OTCN		Nitrate import	IM	BMEI10799, nrtB	BruAb20755	BRA0468	BOV_A0408	BCAN_B0469
	OTCN		Taurine import	ABC	BMEI10961	BruAb10894	BRA0286	BOV_A0262	BCAN_B0288
	OTCN		Taurine import	IM	BMEI10962	BruAb10895	BRA0285	BOV_A0261	BCAN_B0287
	OTCN		Taurine import	BP	BMEI10963	BruAb10896	BRA0284	BOV_A0260	BCAN_B0286
89	PAO		Polar amino acid import	ABC	BMEI10108	BruAb11932	BR1959	BOV_A0336	BCAN_A2004
	PAO		Polar amino acid import	ABC	BMEI10111	BruAb11935	BR1956	BOV_1885	BCAN_A2001
	PAO		Polar amino acid import	IM	BMEI10112	BruAb11931	BR1955	BOV_1882	BCAN_A2000
	PAO		Polar amino acid import	IM	BMEI10113	BruAb11930	BR1954	BOV_1081	BCAN_A1999
	PAO		Polar amino acid import	BP	BMEI10114	BruAb11929	BR1953	BOV_1880	BCAN_A1998
90	PAO		Polar amino acid import	BP		BruAb11929	BR1953	BOV_1879	
	PAO		Arginine/Ornithine binding protein precursor	BP		BruAb20594		BOV_A0594	
	PAO		Arginine/Ornithine binding protein precursor	BP	BMEI1022	BruAb20595	BRA0632	BOV_A0593	
91	PAO		Arginine/Ornithine binding protein precursor	BP		BruAb10874	BRA0631	BOV_0945	BCAN_A0967
	PAO		General L-amino acid import	ABC	BMEI1208, appP	BruAb10762	BR0745	BOV_A0890	BCAN_A0760
	PAO		General L-amino acid import	IM	BMEI1209, appM	BruAb10758	BR0744	BOV_0739	BCAN_A0759
	PAO		General L-amino acid import	IM	BMEI1210, appQ	BruAb10760	BR0743	BOV_0737	BCAN_A0758
	PAO		General L-amino acid import	BP	BMEI1211, appJ	BruAb10761	BR0741	BOV_0738	BCAN_A0756
92	PAO		General L-amino acid import	BP	BMEI10349, appJ	BruAb20285	BRA0948	BOV_0736	BCAN_B0969
	PAO		Arginine	BP	BMEI1627	BruAb10321	BR0295	BOV_0308	
93	PAO		Cystine import	ABC	BMEI10599	BruAb20545	BRA0684	BOV_A0640	BCAN_B0682
	PAO		Cystine import	IM	BMEI10600	BruAb20546	BRA0683	BOV_A0639	BCAN_B0681
	PAO		Cystine import	BP	BMEI10601	BruAb20547, fltY	BRA0682, fltY	BOV_A0638, fltY	BCAN_B0680
94	PAO		Polar amino acid import	IM		BruAb20547, fltY	BR0952		BCAN_A0964

Reconstruction and comparative inventories of Brucella ABC systems

#	Family	Subfamily	Substrate/Function	Type	<i>B. melitensis</i>	<i>B. abortus</i>	<i>B. suis</i>	<i>B. ovis</i>	<i>B. canis</i>
	PAO		Polar amino acid import	IM			BR0953		BCAN_A0965
	PAO		Polar amino acid import	BP	BMEI1104		BR0955	BOV_0854	
95	PAO		Polar amino acid import	BP			BR0862	BOV_A0903	
96	UVR		DNA repair	ABC2	BMEI0878	BruAb1110, UvrA	UvrA	BOV_1063	BCAN_A1124
97	YHBG		Possible LPS transport to outer membrane	ABC	BMEI1790	BruAb10153	BR157	BOV_0152	BCAN_A0162
	YHBG		Possible LPS transport to outer membrane	SS	BMEI1791	BruAb10152	BR156	BOV_0151	BCAN_A0161
KEY									
	ABC		ATP-Binding Cassette						
	IM		Inner membrane protein						
	BP		Binding protein						
	IM-ABC		Inner membrane protein-ATP binding cassette fusion						
	ABC2		2 ABC proteins fused together						
	OMP		Outer membrane protein						
	MFP		Membrane fusion protein						
	SS		Signal sequence						
	LPP		Extracytoplasmic protein with a lipoprotein type signal sequence						
	BM		<i>Brucella melitensis</i>						
	BA		<i>Brucella abortus</i>						
	BS		<i>Brucella suis</i>						
	Red Text		Indicates a frame shift mutation or a premature stop codon in that gene						