Identification and Delivery of Immunodominant Antigens of *Neospora caninum*

A Thesis submitted in part fulfilment of the requirements of the Degree of Doctor of Philosophy

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DECLARATION

The work presented in this thesis is the work of the author, except where stated. The assistance of others is gratefully acknowledged and is cited appropriately.

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ABSTRACT

Neospora caninum is an obligate intracellular protozoan parasite that infects a wide range of hosts. It is economically important in the cattle industries, since the pathologies include abortion and stillbirth of calves. It is primarily transmitted transplacentally, from dam to calf, and once it enters a herd it is difficult to treat. There is a need to develop a transmission-blocking vaccine that also prevents the acute pathologies associated with neosporosis. There are several vaccine strategies that may be useful including live delivery using attenuated organisms. The use of attenuated *Toxoplasma gondii* has been previously shown to be an efficacious delivery vector for heterologously expressed proteins. In this thesis, *T. gondii* tachyzoites are transfected with two genes from *N. caninum* and their expression studied. The specific immune response to *N. caninum* is measured when mice are inoculated with the transgenic *T. gondii*. The mouse model was carefully chosen to have minimum clinical symptoms after inoculation with the untransfected *T. gondii*.

Several immunodominant antigens of *N. caninum* have been identified using immune serum from infected animals. However, proteins that stimulate a cellular immune response – thought to be important in the generation of protection against *N. caninum* – have not been studied in detail. Proteins were separated using one- and two-dimensional SDS-PAGE and electroeluted from the gel for use in T-cell proliferation assays. Proliferation *in vitro* of T-cells from *N. caninum*-infected cattle is discussed. Protein fractions that stimulated a proliferative response were further analysed by mass spectrometry. One fraction was identified as superoxide dismutase from *N. caninum*. The potential of using this protein as a component of a vaccine against the acute pathology and vertical transmission of *N. caninum* is discussed.

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This thesis is dedicated to my parents.

"Each succeeds in reaching the goal by a different method"
Niccolo Machiavelli, "The Prince"

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ABBREVIATIONS

°C	degrees centigrade
γδ	gamma-delta
Δ	knockout
1D	one dimensional
2D	two dimensional
2-DE	two dimensional electrophoresis
AMP	adenosine monophosphate
APC	antigen presenting cell
ATP	adenosine triphosphate
BCG	Bacille Calmette-Guérin
bp	base pairs
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
cm	centimetre
cm^2	squared centimetre
CNS	central nervous system
ConA	concanavalin A
Cpm	counts per minute
CSP	circumsporozoite protein
d	day
Da	dalton
dCTP	deoxyribocytosine triphosphate
ddH_2O	double distilled water
dGTP	deoxyriboguanosine triphosphate
DH	definitive host
DHFR-TS	dihydrofolate reductase-thymidylate synthase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxyribothymidine triphosphate
EDTA.4Na	ethylene diamine tetra-acetic acid (sodium salt)
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
FACS	fluorescence activated cell sorting
FBS	foetal bovine serum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	force of gravity
GFP	green fluorescent protein
GMP	guanosine monophosphate
GPI	glycosylphosphatidylinositol
GRA	dense granule protein
h	hour
HBSA	hepatitis B surface antigen
HBSS	Hank's Balanced Salt Solution
HFFF	human foetal foreskin fibroblasts

HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HXGPRT	hypoxanthine-xanthine guanine phosphoribosyl transferase
IEF	isoelectric focussing
IFAT	immunofluorescent antibody test
IFNγ	interferon gamma
IgA	immunoglobulin A
IgG	immunoglobulin G
IL	interleukin
IH	intermediate host
IMDM	Iscove's modified Dulbecco Medium
IMN	intravacuolar membrane network
IMP	inosine monophosphate
IU	international unit
kb	kilobase
kDa	kilodalton
KMP-11	kinetoplast membrane protein-11
kV	kilovolt
µF	microFaraday
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
M	molar
Mab	monoclonal antibody
MALDI-ToF	Matrix assisted laser-desorption ionisation Time-of-Flight
min	minute
mg	milligram
MHC	major histocompatability complex
ml	millilitre
mm	millimetre
mM	millimolar
MPA	mycophenolic acid
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MudPIT	Multidimensional protein identification technology
NCDG1	*N. caninum* dense granule protein 1
NCDG2	*N. caninum* dense granule protein 2
NcGRA	*N. caninum* dense granule protein
Ncp38	*N. caninum* protein 38kDa
Ncp43	*N. caninum* protein 43kDa
NcSAG	*N. caninum* surface antigen
NcSRS2	*N. caninum* SAG-related surface protein-2
NK	Natural Killer
ng	nanogram
nm	nanometre
nt	nucleotide
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction

pg	picogram
p.i.	post inoculation
pI	isoelectric point
PRU	*T. gondii* Prugniaud strain
PRUΔHX	*T. gondii* Prugniaud strain HXGPRT knockout
PV	parasitophorous vacuole
RAPD	random amplification of polymorphic DNA
REMI	restriction enzyme mediated integration
RNA	Ribonucleic acid
ROP	rhoptry protein
RT-PCR	reverse transcription PCR
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SI	stimulation index
TBS-T	tris buffered saline – Tween 20
TCA	trichloro acetic acid
TgGRA	*T. gondii* dense granule protein
TgSAG	*T. gondii* surface antigen
TgSRS	*T. gondii* SAG-related surface antigen
TgTUB	*T. gondii* tubulin
Th1	T-helper 1
Th2	T-helper 2
TNFα	tumour necrosis factor alpha
ts-4	temperature-sensitive strain 4
U	enzyme units
UTR	untranslated region
UV	ultraviolet
V	volt
Vh	volt hours
v/v	volume per volume
w/v	weight per volume
xan	xanthine
XMP	xanthine monophosphate

CHAPTER 1:

INTRODUCTION

1. INTRODUCTION

1.1 Discovery of *Neospora caninum*

Neospora caninum is an obligate intracellular pathogen first isolated from the central nervous system of a dog in 1988, although a previously diagnosed neurological disease in dogs was later identified as neosporosis (Bjerkas *et* al, 1984; Dubey *et al*, 1988). It was thought for many years previously that the parasite, a coccidian, was *Toxoplasma gondii*, but the disease was not like that caused by *T. gondii*. *T. gondii* infects all warm-blooded animals and causes a wide spectrum of disease (Roberts and Janovy, 1996). *N. caninum* appears to be limited to canids as definitive hosts (DH) where the parasite undergoes sexual replication and a variety of intermediate hosts (IH) where the parasite replicates asexually (Dubey *et al*, 1990). Retrospective serological and morphological studies have indicated that misdiagnosis of cases of neosporosis in dogs span back several decades (Dubey *et al*, 1990).

1.2 Classification of *N. caninum*

Despite being morphologically similar, and irrespective of the fact that for many years they were mistaken as the same organism, *T. gondii* and *N. caninum* have been shown to be genetically and antigenically different (antigenic differences will be discussed in Section 1.8.1). In phylogenetic studies using ribosomal RNA sequences, *N. caninum* was placed in the Sarcocystidae family of the Apicomplexa alongside *T. gondii* (Holmdahl *et al*, 1994). Holmdahl and colleagues suggested that *N. caninum* was sufficiently close genetically not to warrant being a separate genus from *T. gondii* (Holmdahl *et al*, 1994). However, in further phylogenetic studies using full-length ribosomal RNA sequences, Mugridge and colleagues proposed that *N. caninum* was more closely related to *Hammondia heydorni* than to *T. gondii*

(Mugridge *et al*, 1999). Mehlhorn and Heydorn (2000) proposed that *N. caninum* should be considered to be a strain of *H. heydorni*, rather than a separate species, since they were morphologically inseparable (Mehlhorn and Heydorn, 2000).

The majority of the work done in trying to classify *N. caninum* was carried out using ribosomal RNA sequence comparison (Holmdahl *et al*, 1994; Mugridge *et al*, 1999). A study by Schock *et al* (2001) used genomic approaches to determine the extent of variation between *N. caninum* and other Apicomplexa. In this work, six strains of *N. caninum* were compared with three strains of *T. gondii* and a strain of *Sarcocystis* sp. using Random Amplification of Polymorphic DNA (RAPD) PCR. This technique uses random primers to amplify regions of DNA from the genome of the organisms. When the results were analysed, it was noted that the *N. caninum* strains clustered together and were distinct from the *T. gondii* and the *Sarcocystis* at 222 locations (Schock *et al*, 2001). There were an additional 54 markers that could be used to identify the individual *N. caninum* strains, indicating some intra-species variation within *N. caninum* (Schock *et al*, 2001).

In an attempt to reach a consensus, a consortium of *N. caninum* researchers proposed a redescription of *N. caninum* in relation to the other cyst-forming coccidian (Dubey *et al*, 2002) based on morphological, antigenic and genetic differences. In this paper, the authors identified differences between different cyst-forming coccidia, such as oocysts morphology and variations in the 16S ribosomal DNA, and sought to characterize each one according to its differences.

1.3 Classification of *T. gondii*

There is a significant body of literature examining the intra-species variability in *T. gondii* using genetic and genomic techniques (Lyons and Johnson, 1998; Appleford and Smith, 2000; Faezelli *et al*, 2000; Lehmann *et* al, 2000; Terry *et al*, 2001). These studies used differences in the sequence and expression of a *T. gondii* heat shock protein (Lyons and Johnson, 1998), intergenic spacer polymorphisms (Faezelli *et al*, 2000) and variations in genes encoding for antigens and housekeeping genes (Lehmann *et al*, 2000) to examine the differences in *T. gondii* strains. Terry and co-workers (2001) used a PCR-based method to identify differences in the mobile genetic elements within the *T. gondii* genome. Using specific primers, amplification of a virulence-associated fragment (only found in avirulent strains) occurred (Terry *et al*, 2001). All these studies suggested variability at a genetic level between different strains of *T. gondii.*

In an early study, Sibley and Boothroyd examined the genotypes of twenty-eight virulent and non-virulent strains of *T. gondii* (Sibley and Boothroyd, 1992). In this study, the authors noted that all the virulent strains tested appeared to be genotypically identical, suggesting a single clonal lineage, whereas the avirulent strains were polymorphic (Sibley and Boothroyd, 1992). A further study suggested that the *T. gondii* species comprised three distinct clonal lines (Howe and Sibley, 1995). These three clonal lineages – Types I, II and III – differed in their virulence. Type I *T. gondii* was considered to be highly virulent in mice and is characterised by the RH strain (Howe and Sibley, 1995). Types II and III *T. gondii* were considered to be avirulent in mice (Howe and Sibley, 1995). The Prugniaud (PRU) strain was considered to be a Type II strain.

1.4 Life Cycle of *N. caninum*

1.4.1 Definitive and Intermediate Hosts

The life cycle of *N. caninum* is very similar to that of *T. gondii*. Like *T. gondii*, *N. caninum* can infect many different animals (Table 1.1). Unlike *T. gondii*, however, *N. caninum* is not thought to be a human pathogen. No natural human infection has, as yet, been reported. A study by Tranas and colleagues (1999) showed that seroconversion in humans exposed to *N. caninum* was possible, although the anti-*Neospora* titre was low with no obvious pathology. There is currently no evidence to suggest that human infection with *N. caninum* causes abortion as it does in cattle (Petersen *et al*, 1999). However, other primates have been successfully infected with *N. caninum* in the laboratory (Barr *et al*, 1994). In this study, rhesus macaque foetuses were inoculated *in utero* with *N. caninum*, or pregnant macaques were inoculated intramuscularly with *N. caninum* (Barr *et al*, 1994). All foetuses were identified as being positive by immunohistochemistry and specific antibody titres for *N. caninum* when they were tested ten weeks after inoculation (Barr *et al*, 1994). This would suggest that primates are susceptible to infection by *N. caninum*.

Animal	**Host status** **IH: Intermediate Host** **DH: Definitive Host**	**Reference(s)**
Cattle	IH	(Anderson *et al*, 1994)
Sheep	IH	(Dubey and Lindsay, 1990)
Goats	IH	(Barr *et al*, 1992; Dubey *et al*, 1992)
Deer	IH	(Dubey *et al*, 1996)
Red Foxes	IH	(Buxton *et al*, 1997; Almeria *et al*, 2002)
Coyotes	IH/DH	(Lindsay *et al*, 1996; Gondim *et al*, 2004)
Water Buffalo	IH	(Dubey *et al*, 1998; Huong *et al*, 1998)
Camels	IH	(Hilali *et al*, 1998)
Dogs	IH/DH	(McAllister *et al*, 1998)

Table 1.1 Known intermediate and definitive host organisms of *N. caninum*

Table 1.1 shows a number of mammalian hosts can act as intermediate hosts and also some that are confirmed/suspected definitive hosts. It was not, however, until 1998, that McAllister and co-workers discovered that dogs shed the sexual form of the parasite, the oocyst, indicating its role as definitive host (McAllister *et al*, 1998). Wild canids, notably coyotes (*Canis latrans*) have also been identified as definitive hosts (Gondim *et al*, 2004), although others including foxes (*Vulpes vulpes*) have yet to be shown as definitive hosts. Initial experiments showed that when experimentally infected by ingestion of infected mouse placenta, dogs shed small numbers of oocysts (Lindsay *et al*, 1999). However, further experiments showed that when dogs were fed infected bovine placenta, they produced significantly more oocysts than when they were fed murine placenta (Gondim *et al*, 2002). This suggests that the cyclical transmission of parasites between dogs and intermediate hosts is feasible (Gondim *et al* 2004).

1.4.2 Life Cycle

The life cycle of *N. caninum* is shown in Figure 1.1. Oocysts sporulate within 24 hours of excretion (Lindsay *et al*, 1999) and are taken up by susceptible hosts following consumption of contaminated food/water (McAllister *et al*, 1998). A very important mode of transmission is vertically from mother to foetus, which will be discussed in more detail in Section 1.6.2. Once inside an intermediate host, the oocysts excyst and release tachyzoites. Some of the tachyzoites continue to multiply, while others differentiate to bradyzoites and tissue cysts. Switching from tachyzoites to bradyzoites is caused by a number of stimuli (Weiss *et al*, 1999). For example, in *T. gondii* an increase in pH to 8.1 causes bradyzoite switching in *T. gondii*, as does an increase in temperature to 43°C (Soete *et al*, 1994). The presence of nitric oxide,

an important immune effector molecule (reviewed in Bogdan *et al*, 2000) which is produced by interferon γ (IFN-γ) stimulation of macrophages and natural killer (NK) cells increases antigen switching from tachyzoite-specific to bradyzoite specific antigens, which indicates stage differentiation from tachyzoite to bradyzoites. It is the ingestion of bradyzoites that causes the infection of the definitive canine host.

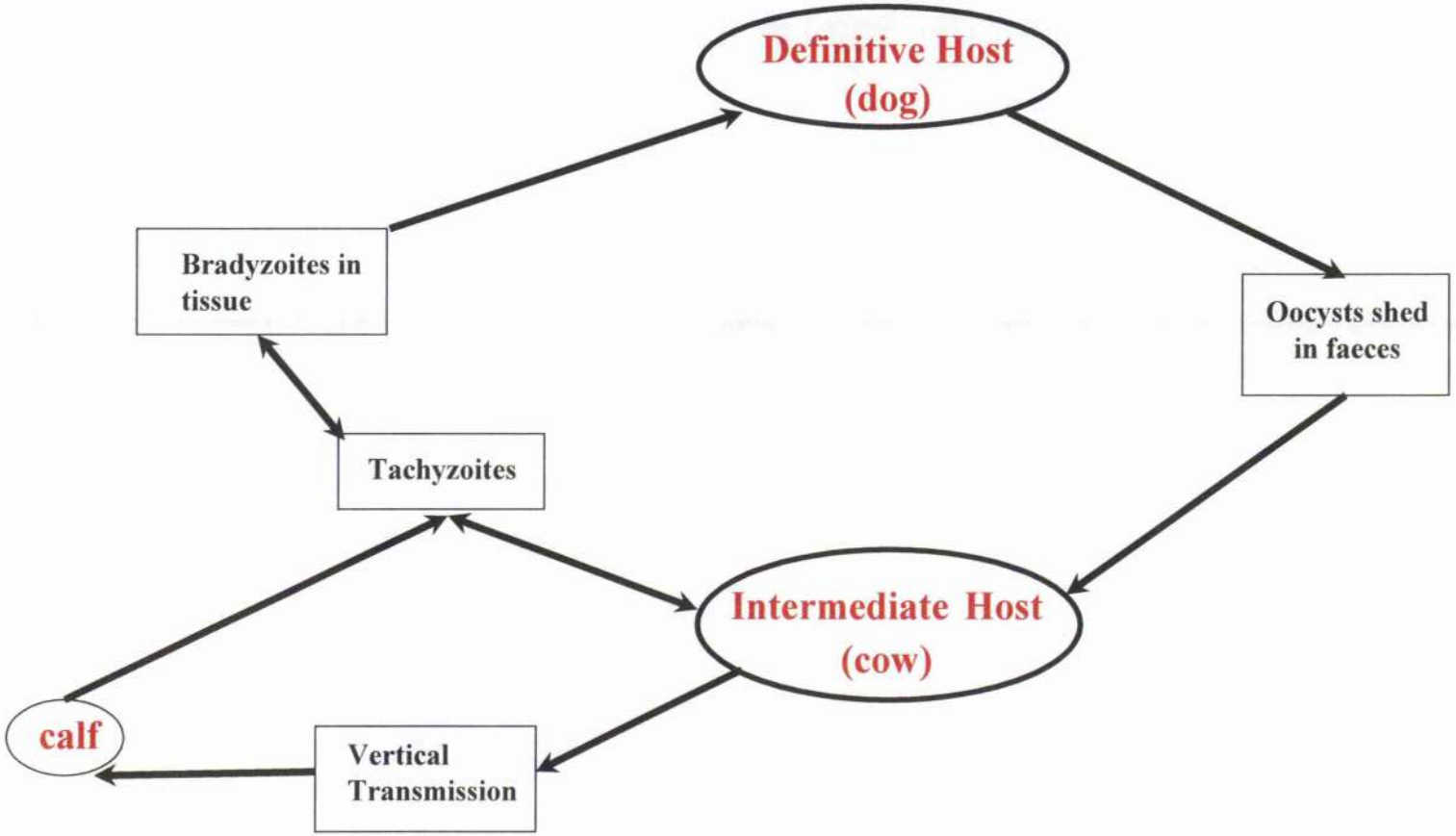


Figure 1.1 Life cycle of *N. caninum* (adapted from Dubey *et al*, 1999)

1.5 Neosporosis

1.5.1 Disease

Neosporosis is a worldwide disease, and as such the cost of the disease to agriculture is spread throughout the globe. The most important pathology attributed to neosporosis is abortion in cattle. Abortions due to neosporosis are caused in several ways. A point-source post-natal infection of cattle, often caused by the cohabitation of cattle and an infected definitive host, is a common cause of abortion (Dijkstra *et al*, 2001; Dijkstra *et al* 2002). Alternatively, the recrudescence of a latent infection can also produce an increased risk of abortion (Wouda *et al*, 1999). In this study, abortions within a herd occurred in seasonal "storms" caused by a recrudescence of a previous *N. caninum* infection in chronically infected cattle, although there was some evidence to suggest a concurrent point-source infection in some of the herds studied in this study (Wouda *et al,* 1999).

Abortions related to *N. caninum* cause approximately 25% of all bovine abortions and stillbirths in California that have known causes (Anderson *et al*, 1991). In other studies, aborted cattle foetuses from California were tested for the presence of *N. caninum* (Anderson *et al*, 1995). In this study, up to 42.5% of bovine abortions in California were attributed to *N. caninum* infection (Anderson *et al*, 1995). In a later study of a different herd in the United States, 40% of cattle within the herd aborted due to a recent previous infection with *N. caninum* (Jenkins *et al* 2000).

In the UK, neosporosis has been identified as a significant cause of pathology in cattle where up to 12.5% of all bovine abortions in England and Wales may be attributed to neosporosis (Davison *et al*, 1999). Similar results were obtained in

Scotland, where 15.9% of aborted foetuses contained antibodies to *N. caninum* (Buxton *et al*, 1997).

1.5.2 Economic Impact

It is evident that abortions due to neosporosis have the potential to be significant factors in the competitiveness of the dairy industry. Some studies suggest that infected cattle are three times more likely to abort than uninfected cattle (reviewed in Trees *et al*, 1998) In California, for example, the economic loss associated with *N. caninum* infection is estimated at US$35 million annually (Anderson *et al* 1991). This may be in part attributed to a decrease in milk production. In herds with abortion problems, cattle seropositive for *N. caninum* produce less milk (Hobson *et al*, 2002). The loss of foetuses and rebreeding costs, the cost of veterinary intervention and decreased milk yield are also important economic factors (Thurmond & Hietala, 1996; Thurmond & Hietala 1997a; Thurmond & Hietala 1997b).

It is therefore important to understand the biology of the parasite, and particularly how it is transmitted in herd animals. This knowledge could then be used to help farmers identify risk factors and reduce their economic losses due to *N. caninum*.

1.6 Transmission of *N. caninum*

1.6.1 Horizontal Transmission

Horizontal transmission of *N. caninum* is not a major route of natural infection (Davison *et al*, 1999; Bjorkman *et al*, 2003). In the study by Bjorkman and

colleagues, horizontal transmission was estimated to be 22% mean annual rate over three years (Bjorkman *et al,* 2003).

There is evidence to suggest that the presence of a *N. caninum*-infected dog on a farm increases the likelihood of horizontal transmission of *N. caninum* (Dijkstra *et al*, 2002). The rate of infection of cattle with *N. caninum* increases when the dog is fed placenta or consumes uterine discharge from a previously infected cow and is allowed to defecate near the housed cattle (Dijkstra *et al,* 2002). In addition to this, postnatal infection of calves by ingestion of oocysts may have a small role in horizontal transmission (Hietala and Thurmond, 1999). In this study, despite a high seroprevalence of *N. caninum* in the herd, horizontal transmission was estimated at less than 1% because of the high level of vertical transmission between the dam and foetus (Hietala and Thurmond, 1999).

A recent study of bull's semen has indicated the possibility of venereal transmission of *N. caninum* (Ortega-Mora *et al*, 2003). In this study, fresh and frozen semen was analysed by nested PCR, IFAT and immunoblot for the presence of *N. caninum* tachyzoites. Parasites were found in the cellular component of the semen, suggesting *N. caninum* infection of the sperm cells (Ortega-Mora *et al,* 2003). Whether this route is viable or important in the transmission of neosporosis has yet to be verified.

1.6.2 Vertical Transmission

One mode of infection, not directly dependent on oocysts, is vertical transmission. This is the infection of a foetus via the placenta. Vertical transmission has been seen in natural infections in cattle and sheep (Anderson *et al*, 1997; Bergeron *et al*, 2000;

Innes *et al*, 2001; Landmann *et al*, 2002; Kobayashi *et al*, 2001), as well as experimentally in dogs and mice (Cole *et al*, 1995a; Cole *et al* 1995b; Liddell *et al*, 1999; Omata *et al*, 2004). Vertical transmission is important in the maintenance of infection within a herd, since the parasite can be transmitted over several generations and in successive pregnancies (Davidson *et al*, 1999). Mathematical models of transmission have also shown that vertical transmission has a high probability of occurrence – 92.5% of seropositive dams gave birth to seropositive calves (Davidson *et al*, 1999). This would suggest that vertical transmission is an important mechanism by which the parasite maintains long-term infection of a herd.

Much of the work studying vertical transmission has been carried out using cattle. Since cattle are difficult and expensive to handle in an experimental situation, as well as having long gestation times, mice are also used to study vertical transmission of *N. caninum*. It could be argued, however, that mice are not ideal models for studies of this kind, due to differences in gestation times between cattle and mice.

1.6.2.1 Vertical Transmission in Mice

As mice are relatively easy to manipulate in laboratory conditions, they were among the first animals to be used as a model for vertical transmission in *N. caninum* studies (Cole *et al*, 1995b). In this study, vertical transmission of *N. caninum* in BALB/c mice was 85%. In a further study of murine congenital neosporosis, an outbred strain of mouse, the Quackenbush mouse was used (Quinn *et al*, 2002). The Quackenbush mouse displayed low pathology during infection and generated a partially protective response, sufficient to prevent vertical transmission (Quinn *et al* 2002). PCR could

not detect parasite DNA within foetal tissue derived from the infected pregnant Quackenbush mice (Quinn *et* al, 2002).

1.6.2.2 Vertical Transmission in Cattle

If a cow is infected with *N. caninum*, the parasite can be passed through the placenta to the foetus. As a result of vertical transmission of the parasite, foetuses can be dead *in utero*, resorbed, mummified, autolysed, stillborn or born persistently infected (Dubey, 1999). Persistent infection can be indicated by the calf having identifiable symptoms. For example, the calf may be born underweight and unable to rise. It may also have decreased patellar reflexes, ataxia and exopthalia (Dubey, 1999).

The rate of vertical transmission has been estimated using an IgG avidity ELISA at 85.5% (Bjorkman *et al* 2003). This result confirmed similar rates of vertical transmission that were estimated using another ELISA test that used Ncp38 as the antigen in a analogous assay (Schares *et al*, 2002).

While there are several proposed mechanisms for vertical transmission (discussed in Section 1.6.2), it is likely that the maternal immune response plays an important role in the progress of the infection by *N. caninum*.

1.7 Bovine Immunity to *N. caninum* Infections

Immune response to *N. caninum* infection in cattle is dependent on the pregnancy status of the dam. Therefore it is useful to separate discussion on the bovine immune response to neosporosis into the response in pregnant and non-pregnant cattle.

1.7.1 Immunity in Non-Pregnant Cattle

Immunity to *N. caninum* in non-pregnant cattle is associated with both cellular and humoral immune responses (Dubey *et* al, 1996; Lunden *et al*, 1998; De Marez *et al*, 1999; Staska *et al*, 2003).

Studies have indicated that protective cellular immunity to neosporosis does not develop in cattle following infection (Innes *et* al, 2002). However, later studies suggest that there is limited protection to further infection, since cattle experimentally infected with *N. caninum* and later challenged with tachyzoites demonstrated reduced pathology (Williams *et al*, 2003). However, this immunity does not protect against vertical transmission of the parasite from the dam to the calf, since parasite material was identified within foetal tissue by histological and molecular techniques (Williams *et al*, 2003).

1.7.1.1 Cell-Mediated Response

In the study by Lunden and co-workers (1998), the *in vitro* proliferative response of T-cells to *N. caninum* crude lysate was measured in calves inoculated with *N. caninum* tachyzoites. A high level of cellular proliferation and an increased IFNγ production in infected animals compared with the uninfected controls suggested a cell-mediated immune response was important in controlling *N. caninum* infection (Lunden *et al*, 1998).

Since cellular responses are known to be important in the immune response to *T. gondii* (Innes *et al*, 1995), it was extrapolated that they may also play a role in the immune response to *N. caninum* since the organisms were so similar in their mode of

infection (Marks *et al*, 1998). *N caninum* lysate was separated on a one-dimensional SDS-PAGE gel and blotted onto nitrocellulose before being used to stimulate $CD4^+$ T-cells from *N. caninum*-infected animals. A high level of proliferation was observed due to a number of protein fractions, and this was correlated with high levels of IFNγ production (Marks *et al*, 1998). T-cell proliferation and increased levels of IFNγ have previously been shown to be important in the development of a pro-inflammatory response against other intracellular pathogens including *T. gondii*, and it is possible that this is also the case in *N. caninum* infections (Denkers, 1999).

1.7.1.2 Humoral Response

The humoral response to *N. caninum* infection was further studied by De Marez *et al* (1999). In this study, calves were fed oocysts from dogs and the proliferative and humoral response examined by studying *N. caninum*-specific proliferation of T-cells and the levels of circulating antibodies specific to *N. caninum*. Although proliferation by *N-caninum*-specific T-cells *in vitro* was observed quickly (within 1 week post-inoculation), subsequently, *N. caninum*-specific IgG1 and IgG2 were observed in the blood of infected calves. The role of a humoral response to *N. caninum* is not full understood, but it is thought to be a mechanism by which the host animal prevents the tachyzoite, i.e. extracellular, stage of the parasite from invading the host cell (Hemphill, 1999).

1.7.2 Immunity in Pregnant Cattle

Pregnancy in mammals causes an immunomodulatory effect, down-regulating the Th1 response to protect the foetus from a deleterious maternal immune response (Raghupathy, 1997). In particular, there appears to be an up-regulation of IL-10

production, which in turn depresses the level of IFNγ (Wegmann *et al*, 1993) However, it is the Th1 response that has been shown to be important in protecting both the mother and foetus from infections by intracellular pathogens such as *N. caninum* (reviewed in Quinn *et al*, 2002).

In cattle infected mid-gestation with *N. caninum*, it has been demonstrated that both a cell-mediated and humoral response is generated and can be detected in both mother and calf (Andrianarivo *et al*, 2001; Bartley *et al*, 2004). However, in a further study by Almeira *et al* (2003), it was proposed that increased levels of IFNγ together with the Th2 cytokine IL-4 that was observed in dams following infection at mid-gestation may increase the prevalence of transplacental transmission of *N. caninum* (Almeira *et al*, 2003). Increased IL-4 levels were also observed in a mouse model of neosporosis in pregnant mice (Quinn *et al*, 2004). Since IL-4 and the Th2 response are not generally protective against intracellular pathogen infections but are important with the maintenance of pregnancy, it may be that the increased Th2 responses in *N. caninum* infections in pregnancy facilitate the transmission of the parasite from mother to calf (Quinn *et al*, 2004).

1.7.3 Mechanisms of Vertical Transmission

Understanding the mechanisms of vertical transmission would aid the development of treatment to prevent such transmission. Several studies suggest that the maternal immune response may have a significant role to play in enabling the vertical transmission of *N. caninum* (Bjorkman *et al*, 1996; Guy *et al*, 2001; Innes *et al* 2001). Bjorkman and colleagues (1996) demonstrated that *N. caninum* could be transmitted from mother to calf without the presence of a definitive host.

Furthermore, the work by Guy and colleagues (2001) noted that an increase in the levels of *N. caninum*-specific antibodies in the second half of pregnancy coincided with increased foetal infection in persistently infected cattle. Innes and colleagues (2001) noted a general down-regulation of cell mediated immunity at mid-gestation in both infected and uninfected cattle. It was suggested that this reduction in cell-mediated immunity, and in particular the reduction in levels of IFNγ, might allow the recrudescence of *N. caninum* from tissue cysts. A reduction in the levels of IFNγ, a cytokine thought to be important in controlling multiplication of *N. caninum*, could allow the parasite to become more active and cause some of the pathology associated with *N. caninum* infections (Innes *et al*, 2001).

1.8 Comparison Between *T. gondii* and *N. caninum*

1.8.1 Antigenic Differences and Cross-Protective Immunity

Despite being very similar, *N. caninum* and *T. gondii* are antigenically diverse. It is true, however, that some proteins found in *N. caninum* have homologues in *T. gondii* (Hemphill *et al*, 1999). Nevertheless, this does not result in any cross-protective immunity in sheep that were immunised with *T. gondii* and challenged with *N. caninum* (Innes *et al*, 2001a). There is some evidence to suggest a level of protection against lethal *T. gondii* infection can be provided by vaccinating with live *N. caninum* (Lindsay *et al*, 1998). However, this protection is relatively limited and does not significantly reduce the parasite burden in an animal infected with highly virulent *T. gondii* (Lindsay *et al* 1998).

Several classes of antigens have been studied in *T. gondii* and *N. caninum*. However, for the purposes of this review, the focus will be on two: the surface proteins and the dense granule proteins.

1.8.2 T. gondii Surface Proteins

A number of proteins, the TgSAG (surface antigen) proteins, are found on the membranes of *T. gondii* tachyzoites. There are several of these proteins, which are anchored to the cell membrane by GPI (glycosylphosphatidylinositol) anchors (Hehl *et al*, 1997) as revealed by surface-labelling studies of laboratory reference strains (Couvreur *et al*, 1988; Tomavo *et al*, 1989; Tomavo *et al*, 1996). The dominant surface protein is TgSAG1, which is approximately 30kDa (Burg *et al*, 1988). It plays a role in attachment, mediating contact between the parasite and the host (Smith *et al*, 1995). TgSAG1 exists in a dimorphic state, one allele found in Type I virulent strains and the other found in Type II avirulent strains (Boothroyd *et al*, 1998). Indeed, there seems to be a higher level of TgSAG1 mRNA in virulent than in avirulent strains (Windeck and Gross, 1996). However, despite this dimorphism, TgSAG1 is remarkably well conserved. TgSAG3 (43kDa) (Cesbron-Delauw *et al*, 1994) is relatively homologous to TgSAG1, having a similar N-terminal signal peptide and glycosylphosphatidylinositol anchor attachment site (Nagel and Boothroyd 1989; Tomavo *et al*, 1992; Tomavo *et al*, 1993). TgSAG1 and TgSAG3 also have 24% overall amino acid identity and 12 conserved cysteine residues (Manger *et al*, 1998). TgSAG3 is thought to be a factor in the attachment of the parasite to the host (Tomavo *et al*, 1996).

TgSAG2 (22kDa) (Prince *et al*, 1990), however, shares no homology with TgSAG1 and TgSAG3. TgSAG2 has limited polymorphism, however, having 2 alleles that differ at 5 nucleotide positions and which generate 4 amino acid changes. These changes may account for the differences in antigenicity of TgSAG2 (Parmley *et al*, 1994). The role of TgSAG2 is thought to be in apical re-orientation and detachment once the tachyzoites have established contact with the host cell surface (Parmley *et al*, 1994). TgSAG3 may play a role in host cell attachment and invasion. When the gene for TgSAG3 is knocked out of tachyzoites of *T. gondii*, they are up to 50% less efficient in host cell invasion, reducing their virulence in mice (as demonstrated by decreased mortality) by 1000-fold (Tomavo *et al*, 1996).

There is another important family of surface proteins that may play a role in the immune response to *T. gondii* and of which there are homologues in *N. caninum*. This family is named the "SAG1-Related Sequences" or SRS's, of which 161 have been identified by homology searches of Genbank and other databases (Jung *et al*, 2004). The most abundant molecule of this family is TgSRS1. It is found on the surface of the tachyzoite and is a TgSAG1 homologue (Hehl *et al*, 1997). However, TgSRS1 is much less abundant than TgSAG1 (Boothroyd *et al*, 1998).

TgSRS2 shows high sequence homology to TgSRS1. The former is highly expressed in the avirulent type II and type III parasites, yet there is little expression of TgSRS2 in the virulent type I RH strain (Manger *et al*, 1998). Three other SRS genes have been fully characterised in *T. gondii* (Boothroyd *et al*, 1997). The members of the SRS family show a conserved molecular architecture, which includes conserved cysteine residues and hydrophobic regions (Manger *et al*, 1998). The strongly

hydrophobic C terminus suggests that, like TgSAG1 (Nagel and Boothroyd, 1989), the TgSRSs are GPI anchored (Manger *et al*, 1998).

1.8.3 N. caninum Surface Proteins

There is a homologue to TgSAG1 in *N. caninum*, which has been named NcSAG1, NcP29 (native PAGE) and NcP36 (in reducing SDS-PAGE) (Howe *et al*, 1998). It is, like TgSAG1 the dominant surface antigen and is 53% conserved with TgSAG1 (Howe, Crawford *et al*, 1998; Howe *et al*, 1998). There is, at present, no identified *N. caninum* homologue of TgSAG3, and there is only partial EST data on a putative NcSAG2 (Genbank Accession Numbers CF797894 and BF824534). TgSRS2 also has a homologue in *N. caninum*, NcSRS2 (Manger *et al*, 1998). NcSRS2 is also identified as NcP43 (Hemphill *et al*, 1996). NcSRS2 is a possible ligand during host-cell invasion in tachyzoites as well as cyst rupture and reactivation (Fuchs *et al*, 1998). NcSRS2 shows 44% conservation with TgSRS2 (Howe, Crawford *et al*, 1998), as well as homology to TgSAG1 (Kasper *et al*, 1992) and TgSAG3 (Cesbron-Delauw *et al*, 1994). Recombinant NcSRS2 has been localized to the extracellular surface of tachyzoites as well as in the posterior and anterior dense granules (Hemphill *et al*, 1997). Despite the similarity of NcSRS2 to proteins in *T. gondii*, anti-NcSRS2 antibodies failed to react with *T. gondii* (Hemphill and Gottstein, 1996). In immunisation studies, dogs were vaccinated with recombinant canine herpesvirus vector expressing NcSRS2 (Nishikawa et al, 2000). The study demonstrated that the dogs seroconverted to recognise the parasite protein. Mice immunized with recombinant vaccinia virus expressing NcSRS2 demonstrated splenic cell proliferation and a humoral and cellular immune response to NcSRS2 (Nishikawa *et al*, 2000). A further study, using recombinant vaccinia virus, also

expressing NcSRS2, prevented vertical transmission of *N. caninum* in BALB/c mice (Nishikawa, 2001). These studies suggest that NcSRS2 may play an important role in protection against *N. caninum* infection.

1.8.4 Dense Granule Proteins

Dense granules are spherical, electron dense organelles found in apicomplexa (reviewed in Blackman and Bannister, 2001). Unlike other apicomplexa-specific organelles such as the micronemes and rhoptries, the dense granules are not solely positioned in the apical complex, but rather located throughout the cell cytoplasm (Carruthers, 1999). The majority of studies of dense granules have been done in *T. gondii* rather than *N. caninum*.

1.8.4.1 T. gondii

Dense granule (GRA) proteins in *T. gondii* are, among other things, important for the establishment and proper function of the parasitophorous vacuole (PV) (Cesbron-Delauw *et al*, 1994). Some, for example TgGRA1, are secreted into the vacuolar space as soluble moieties (Mercier *et al*, 2001) while others, for example TgGRA2, TgGRA4, TgGRA6 and TgGRA9 are located in intravacuolar membranous tubules of the intravacuolar membranous network (IMN) (Mercier *et al*, 2001; Adjogble *et al* 2004). GRA proteins are also an important component of excretory/secretory antigens, which are thought to play a role in the generation of the immune response in the host animal (Cesbron-Delauw *et al* 1994) since they are shown to be protective in *in vivo* challenge experiments using *T. gondii* (Darcy *et al*, 1988; Duquesne *et al*, 1990). Indeed, most of the identified GRA proteins in *T. gondii* (TgGRA1, 2, 3, 4, 5,

7 and 8) are expressed at a lower level in an attenuated line of type I strain of *T. gondii* compared to the wild-type strain (Nischik *et al*, 2001).

Nine dense granule proteins have been identified in *T. gondii*, the most recent TgGRA9 by Adjogble *et al* (2004). All dense granule (GRA) proteins of *T. gondii*, with the exception of TgGRA1, are associated with either the parasitophorous vacuole (PV) or the IMN or both (Carruthers, 1999).

Some TgGRA proteins are very abundant. For example, one study showed that 2% of Expressed Sequence Tags (EST) from *T. gondii* encoded for TgGRA1 (Carruthers, 1999) and TgGRA7 represents approximately 0.5% of total *T. gondii* protein and is expressed in strains of all three types of *T. gondii* (Jacobs *et al*, 1998). In addition, many of the TgGRA proteins have some level of antigenicity.

TgGRA2 is a molecule that is targeted to the IMN (Mercier *et al*, 1993) and is an important virulence factor. When it is knocked out of wild-type tachyzoites, the virulence in mice is reduced (Mercier *et al* 1998). TgGRA2 is a secreted antigen that contains at least three B-cell epitopes recognised by IgG from infected humans (Murray *et al*, 1993; Coughlan *et al* 1995).

TgGRA2 is not, however, the only molecule secreted from the dense granules that is recognised by the immune system. TgGRA4 is targeted to the IMN and is structurally similar to TgGRA2 (Odenthal-Schnittler *et al*, 1993; Mevelec *et al*, 1994). TgGRA4 elicits both a mucosal and systemic immune response following oral infection in mice (Mevelec *et al*, 1998). The epitopes of TgGRA4 are recognised by

both IgG and milk, intestinal and mucosal IgA (Mevelec *et al*, 1992). Additionally, when the DNA sequence of TgGRA4 is used to immunise mice, it elicits a Th1 response (Montgomery *et al*, 1997). This protocol involves the co-injection of TgGRA4 DNA and a plasmid encoding for GM-CSF, which seemed to enhance the immune response to the target antigen (Xiang *et al*, 1995). In mice, TgGRA4 vaccination caused a 62% reduction in mortality after lethal challenge, and induced specific anti-TgGRA4 IgG as well as increased IL-12 and IFNγ. This suggests that TgGRA4 induces a Th1 response (Desolme *et al*, 2001).

An anomaly in the TgGRA proteins is TgGRA7, which has very little homology with other proteins in Genbank, including with other dense granule proteins from *T. gondii*. TgGRA7 is 29kDa in size and its sequence contains N-terminal signal sequences and repeat motifs that suggests its function as a secreted molecule (Jacobs *et al*, 1998). The molecule possesses a putative transmembrane sequence in its primary amino acid sequence, similar to that found in TgGRA4, TgGRA5 and TgGRA6 (Schwab *et al*, 1994). TgGRA7 is secreted into the dense granule where it translocates to the PV membrane when the tachyzoite invades the host cell (Bonhomme *et al*, 1998). It has been postulated that a key role for TgGRA7 is, in concert with TgGRA4, 5 and 6, to form a "molecular sieve" on the membrane of the PV, allowing the free passage of small molecules between the cytosol of the host cell and the interior of the PV (Carruthers, 1999). TgGRA7 does however show some homology with a *N. caninum* dense granule protein, NCDG1 (Lally and Jenkins 1997).

1.8.4.2 N. caninum

There have been few dense granule proteins identified in *N. caninum*. The GRA proteins that have been studied in most detail in *N. caninum* are homologous to TgGRA6 and TgGRA7. NcDG1 (Lally *et al*, 1997) is 33kDa and is shown to have 42% similarity at the amino acid level to TgGRA7 (Fischer *et al*, 1998; Jacobs *et al*, 1998). Therefore, it has been recognised as NcGRA7 and will be referred to as such for the rest of this discussion. NcGRA7 is localised in tachyzoite dense granules on the tubovesicular network and the PV membrane of *N. caninum* tachyzoites (Hemphill *et al*, 1998). NcDG2 (Liddell *et al*, 1998) is smaller than NcGRA7 at 19kDa and has 34% similarity to TgGRA6, and this similarity meant that the molecule was renamed NcGRA6. NcGRA6 is associated with the PV membrane of *N. caninum* tachyzoites (Lecordier *et al*, 1995). NcGRA6 has a higher homology to TgGRA6 than TgGRA6 has to TgGRA5, which is further evidence for the close relationship between *N. caninum* and *T. gondii* (Lecordier *et al*, 1995).

The close phylogenetic relationship between *N. caninum* and *T. gondii* has allowed researchers to study the molecular biological characteristics of both organisms closely (Howe and Sibley, 1997). The ease by which *T. gondii* can be genetically manipulated has meant that it has become a useful tool in understanding the molecular biology of the Apicomplexa (Kim and Weiss, 2004).

1.9 Transfection of *Toxoplasma gondii*

Transfection technology has allowed the study of the expression of genes in *T. gondii* and their relation to the phenotype of the parasite. It has also allowed the possibility of heterologous expression in *T. gondii* of genes from other organisms. Because the

molecular biology of *T. gondii* is well characterised, it enables the study of genes from other less easily manipulated pathogens, for example *N. caninum* (Howe and Sibley, 1997). There are now several different strategies that can be used to transfect *T. gondii*, each of which requires different selection conditions. Transient transfection involves the electroporation of the parasite with a plasmid that encodes for a reporter gene. However, this plasmid is gradually lost from the parasite population by its lack of integration into the genetic material of the parasite (Soldati and Boothroyd, 1993). Stable transformation only occurs when the plasmid is inserted into the genome of the cell and is stably replicated (Kim and Boothroyd, 1995).

1.9.1 Transient Transfection

For many years, the ability to transfect an intracellular protozoan parasite was not thought possible. There were too many cell membranes to cross – that of the host cell and the parasite cell- and nuclear membrane. This was resolved using a two-step approach. Firstly, parasites were separated from the host cell and resuspended in an ionic solution that resembled the ionic composition of the cytoplasm of the host cell (as used by van den Hoff *et al*, 1992 when rat cells were transfected). Then, electroporation (the process by which an electrical discharge forms a reversible pore in the cell membrane, allowing macromolecules including DNA to pass through) was achievable (van den Hoff *et al*, 1992). These parasites were then used to infect cells in culture (Soldati and Boothroyd, 1993). Plasmids that contained the chloramphenicol acetyltransferase (CAT) gene with upstream and downstream sequences of the *T. gondii* gene encoding the major surface antigen TgSAG1 could

be transiently expressed for about 7 days following transfection by electroporation of the parasites (Soldati and Boothroyd, 1993).

1.9.2 Stable Transfection

1.9.2.1 CAT as Selectable Marker

CAT has been used successfully as a selectable marker in bacterial transformations (Burns *et al*, 2000) as an efficient and easily detected alternative to neomycin and hygromycin drug resistance markers. Chloramphenicol inhibits prokaryotic protein translation, which would suggest its mode of action in apicomplexans to be in inhibiting the prokaryotic-like elements in the plastid organelle (Fichera and Roos, 1997) or mitochondrion (Divo *et al*, 1985; Feagin *et al*, 1991). Chloramphenicol has a strong antiparasitic effect but with a delayed reaction. Also, neomycin and hygromycin resistance markers cannot be used since these drugs are toxic to the cell monolayer at concentrations that are inhibitory to *T. gondii* growth (Sibley *et al*, 1994). In the presence of chloramphenicol, however, *T. gondii* undergoes 2-3 cycles of replication before stopping growing (Kim *et al*, 1993). 10μM chloramphenicol in a parasite culture killed 90% of parasites with no noticeable effect on the host cell layer (Kim *et al*, 1993).

1.9.2.2 Dihydrofolate-Thymidylate Synthase (DHFR-TS) Selection

DHFR-TS is present in *T. gondii* as part of the folate metabolic pathway (Kovacs *et al*, 1990). The gene products are important targets for chemotherapy since blocking the folate metabolic pathway with the use of anti-folate drugs pyrimethamine and sulphonamide is an effective treatment for toxoplasmosis (Derouin, 2001). Mutations in the DHFR-TS gene have generated parasites resistant to this anti-folate treatment

(Donald and Roos, 1993). Double point mutations at amino acid Asn83, Ser36 or Phe245 induce *in vitro* resistance to pyrimethamine in *T. gondii* cultures containing 1μM pyrimethamine (Donald and Roos, 1993; Roos, 1993). The generation of resistance to the anti-folate treatments by *T. gondii* makes the use of the mutant DHFR-TS gene as a selection marker a viable option (Donald and Roos, 1993). The mutant gene may also be used as a marker for *T. gondii* transfections (Donald and Roos, 1994).

When *T. gondii* is electroporated with a plasmid containing a mutant DHFR-TS gene, the parasites exhibit transient resistance to pyrimethamine. It is therefore possible to select for stably transformed clones with pyrimethamine. By quickly killing any parasite that does not contain the mutant DHFR-TS with high levels of pyrimethamine, it is possible to isolate stably transfected clones at a relatively high frequency (Donald and Roos, 1994). The mutant DHFR-TS integrate throughout the genome, yet there is no sign of homologous recombination with either the DHFR-TS locus or the TgSAG1 (the promoter used to drive the expression of the mutant DHFR-TS) locus. This is due to the lack of long stretches of contiguous DNA for homologous targeting (Donald and Roos, 1994).

Despite the ease by which DHFR-TS can be used as a selection marker, it also has a major disadvantage. As introducing the mutant DHFR-TS into the genome of the parasite causes resistance to pyrimethamine, the generation of pyrimethamine-resistant *T. gondii* is undesirable from a clinical point of view. Pyrimethamine is one of few drugs available for use in acute toxoplasmosis and there is a risk, albeit small, that pyrimethamine-resistant *T. gondii* may be released into the environment and

cause infection that cannot be easily treated. In addition, pyrimethamine-resistant parasites represent a hazard for laboratory workers. However, since there are other transfection selection markers available, the need to use this particular approach should be limited.

1.9.2.3 Complementation of Tryptophan Auxotrophy as A Selectable Marker

T. gondii is naturally auxotrophic for tryptophan (Sibley *et al*, 1994). In other words, the parasite is unable to survive without an exogenous supply of tryptophan. Auxotrophy can be used to select for parasites transfected with the *Escherichia coli* trpB gene (Sibley *et al*, 1994). This gene encodes the β-subunit of tryptophan synthase which catalyses the conversion of serine and indole to tryptophan (Hartman and Mulligan, 1988). *T. gondii* transfected with this construct, under the control of TgSAG1 promoter sequences, can survive in tryptophan-depleted environments (Sibley *et al* 1994). Naturally occurring tryptophan was depleted by interferon γ (IFNγ) treatment and this normally inhibits wild-type parasites (Pfefferkorn, 1984). $TrpB^+$ *T. gondii* (transfected with a plasmid encoding trpB) produce enough tryptophan to resist IFNγ treatment (Sibley *et al*, 1994).

When the tryptophan complementation was carried out, a majority of the transformants had more than 10 copies of the trpB gene (Sibley *et al*, 1994). The IFNγ treatment selected against those parasites that could not produce sufficient levels of tryptophan, so perhaps having low numbers of trpB genes did not give high enough levels of tryptophan for survival. The level of tryptophan present is also dependent on the level of expression the transfected genes. The trpB gene was under the control of the TgSAG1 promoter that has previously been shown to be a

relatively weak promoter (Soldati and Boothroyd, 1993). Had a stronger promoter, for example the TgTUB promoter, been used, selection against parasites with low levels of transfected trpB may not have been as strong (Sibley *et al*, 1994)

The advantage of using tryptophan complementation as a selection marker is that there are none of the toxicity problems associated with drug resistance markers. Wild-type parasites, ones with no predefined genetic background, can be used unlike in hypoxanthine-guanine phosphoribosyltransferase selection (Sibley *et al*, 1994).

1.9.2.4 Hypoxanthine-Guanine Phosphoribosyltransferase (HXGPRT) Selection

The use of selection markers that have little or no deleterious effect on the ability of the parasite to survive are of course desirable. In this sense, metabolic markers may be the most desirable to use, particularly if they do not appear in the host cell. HXGPRT is one such selection marker.

HXGPRT is a key enzyme in the parasite purine salvage pathway (Chaudhary *et al*, 2004). Since *T. gondii* is auxotrophic for purines, the parasite needs to obtain all its purines from exogenous sources (Chaudhary *et al*, 2004). HXGPRT is a multifunctional enzyme, catalyzing both the production of inosine monophosphate (IMP) and guanosine monophosphate (GMP) from hypoxanthine and guanine, and the conversion of xanthine to xanthine monophosphate (XMP) (Nakaar *et al*, 2000). However, the parasite is not solely reliant on HXGPRT for these products. The parasite can utilise the adenosine monophosphate (AMP) deaminase/IMP dehydrogenase pathway which catalyses the conversion of AMP to GMP via IMP and XMP. Therefore, if the HXGPRT pathway is disrupted, *T. gondii* shows no apparent phenotype change, since the adenosine pathway provides the adenine

nucleotides (Donald *et al*, 1996). ΔHXGPRT knockouts of *T. gondii* (i.e. not containing a functional copy of the HXGPRT gene) can therefore be created with no lethality or change in phenotype. The RH strain of *T. gondii* had a 1.4kb deletion at the HXGPRT locus to produce the knockout strain (Donald *et al*, 1996). These knockouts can be used as the basis for the selection of transfected parasites as described below.

HXGPRT selection can either be positive, that is selecting for the presence of HXGPRT, or negative. Negative selection selects for parasites that do not contain the functional gene by supplementing the growth medium with 6-thioxanthine, a xanthine analogue (Donald and Roos, 1998). Positive selection, which for the purposes of a transfection marker is more valid (co-transfecting a functional HXGPRT gene and the gene of interest and then selecting for the presence of the HXGPRT gene product), requires the use of mycophenolic acid and xanthine. Mycophenolic acid inhibits the action of IMP dehydrogenase thereby blocking the adenosine pathway for adenine nucleotide production (Donald and Roos, 1998). This, along with the addition of an excess of xanthine in the growth medium, ensures that the only way in which the parasite can obtain GMP is via the HXGPRT pathway. Only parasites with a functional HXGPRT, and transfected with the gene of interest, can survive.

It should be noted that HXGPRT selection gives lower frequency of transformation than by using the pyrimethamine-resistant DHFR-TS alleles (Donald and Roos, 1993). However, there are no clinical disadvantages to this system unlike the use of mutant DHFR-TS. The frequency of stable transformation can be enhanced 10-100

fold by using flanking sequences derived from DHFR-TS, since this allows for stable homologous recombination of the HXGPRT gene into the DHFR-TS locus (Donald *et al*, 1996).

Selection using HXGPRT using normal transfection vectors is a relatively simple and effective procedure, yet there is one major disadvantage. A ΔHXGPRT mutant, it could be argued, may be less adapted to its environment and so may not be as evolutionarily competitive compared with wild-type parasites. One way around this is to use anti sense HXGPRT RNA. This would block the expression of endogenous HXGPRT (Nakaar *et al*, 2000) in wild-type parasites. Stably transfected *T. gondii* expressing anti-sense *hxgprt* reduces dramatically the levels of endogenous HXGPRT. Parasites attenuated in this way can be recovered by selecting with 6-thioxanthine, a subversive substrate of HXGPRT (Pfefferkorn and Borotz, 1994).

1.9.2.5 Stable Episomal Shuttle Vector

The development of a transfection vector that is designed to self-replicate is a somewhat different manner of studying heterologous gene expression in *T. gondii.* This episomal shuttle vector would require sequences that allow independent replication and stabilizing of *T. gondii* episomes. In other eukaryotic cells, autonomous replicating sequences have been isolated (Stinchcomb, 1980; Clyne and Kelly, 1997) but these have not been found in *T. gondii*. In a system designed by Black and Boothroyd (1998), a 500 base pair fragment that allowed for stable transformation without drug selection pressure was used in complementation experiments (Black and Boothroyd, 1998).

The advantage of using an episomal shuttle vector as opposed to vectors that recombine in the genome of the organism is that the probability of inducing a mutation is reduced (Black and Boothroyd, 1998). It also allows for relatively easy recovery of the construct, which is important if there is association between phenotype and genotype. However, for long-term stably transformed cells, recombination of the construct is probably more favourable.

1.10 Vaccine Development Strategies

Vaccines are used to protect humans and animals, and indeed plants, against diseases caused by viruses, bacteria and parasites. There are several methods used to develop vaccines. For the majority of the 200 years since vaccination began (by Edward Jenner in 1796), vaccines have consisted of killed or live, attenuated pathogens. Examples of killed vaccines include the vaccine against Pertussis and the inactivated polio vaccine, while live attenuated pathogens have been used as vaccines against measles, mumps and rubella (Plotkin, 1993).

1.10.1 Killed vaccines

Killed vaccines, as the name suggests, consist of dead organisms and thus cannot revert to wild-type or replicate. The organisms are inactivated either by heat treatment or by chemical inactivation by, for example, formalin or phenol (Murdin *et al*, 1996). Inactivated viral vaccines, for example the killed poliomyelitis vaccine (LaForce, 1990) have shown stronger protective responses than killed bacterial vaccines (Werzberger *et al*, 1992). This may be due to the immune response generated by dead bacterial vaccines being different to that generated due to a live bacterial infection. For example, in a study by Babu *et al* (2003) the cellular response

to killed and live *Salmonella enteritidis* vaccines was examined *in vitro*. When splenocytes from chickens vaccinated with either the killed or live vaccine were exposed to *S. enteritidis* antigen *in vitro*, the cells from the chicken vaccinated with the live vaccine showed highest proliferation (Babu *et al*,2003). This may suggest that live vaccines induce a better cell-mediated response to homologous challenge than killed vaccines (Babu *et al*, 2003).

1.10.2 Live Vaccines

Live vaccines are generally cell culture attenuated bacteria or viruses, when the pathogen is passaged through *in vitro*-cultivated cells many times to attenuate the pathogenicity of the organism. This was the method used to develop the attenuated live vaccine used extensively against polio (Sabin and Boulger, 1973) and the vaccine against measles, mumps and rubella (Stokes *et al*, 1971). Attenuated *Mycobacterium bovis* - Bacille Calmette-Guérin (BCG) – was culture attenuated, being passaged 231 times in *in vitro* culture (Weill-Halle, 1957) and has, until recently, been highly successful in controlling tuberculosis (reviewed in Kumar *et al*, 2003). However there are other attenuated vaccines that are not culture attenuated. A temperature-sensitive respiratory syncytial virus vaccine, which used a strain of virus that is adapted to growing in higher-than-physiological temperatures (thus being attenuated at physiological temperatures) and is therefore attenuated compared to its wild-type original, has been developed (McKay *et al*, 1988). Similarly non-culture-attenuated is the typhoid vaccine strain, Ty21a, of *Salmonella typhi*, which was chemically attenuated (Germanier and Fuer, 1975).

Despite the success of these attenuated pathogens as vaccines, the advances made in molecular biology over the past 20 years have enabled vaccine researchers to attenuate pathogens genetically for use as vaccines. By manipulating the genetic structure of the pathogen, it is possible to delete partial or full sequences of genes that encode for virulence factors. For example, a strain of herpes simplex virus, which lacks essential glycoprotein H, generates the same humoral and cellular immune responses of the wild type virus, yet cannot self-replicate so has no pathogenic potential (Farrell *et al*, 1994). Similarly, an attenuated strain of *Vibrio cholerae* in which the gene for the cholera toxin has been deleted has been used to vaccinate against cholera (Tacket *et al*, 1992).

None of these attenuation methods was used in the most successful vaccine developed to date – the smallpox vaccine. In this vaccine, the vaccinia virus that caused the cattle version of the disease was used (Baxby, 1965). The virus was sufficiently similar to confer protection against the human form of the disease, while not being pathogenic (Henderson, 1977). There are problems, however with using animal pathogens to combat human diseases, especially if the vaccine strain is obtained from *in vivo* cultures. There may be a risk of mutation of the non-pathogenic animal strain into a human pathogen for which there is no natural immunity. It has been suggested that the human immunodeficiency virus is a strain of the simian version of the virus that crossed the species divide when monkeys were being used to trial oral poliovirus (de Cock, 2001). The non-pathogenic simian strain mutated and proliferated in a host with no natural immunity. However, this has yet to be verified (de Cock, 2001).

Perhaps a safer method of developing vaccines is not to use the whole pathogenic organism: rather to use the antigens that stimulate the immune response in the event of a challenge to the host animal. One way of achieving this is to use purified bacterial, viral or parasite proteins, polysaccharides or other pathogen-derived molecules identified either through immunological screening using cellular proliferation or antibody assays (Marks *et al*, 1998; Hemphill *et al*, 1999), or through homology to similar known antigens. These molecules can either be obtained by purification of cultures or by being expressed as recombinant molecules (Liljeqvist and Stahl, 1999). Non-recombinant sub-unit vaccines are not generally used commercially due to the high manufacturing costs, as well as the difficulties of culturing vast quantities of pathogenic organisms (Liljeqvist and Stahl, 1999). This is the main reason that recombinant sub-unit vaccines have become increasingly popular.

1.10.3 Recombinant Sub-Unit Vaccines

The first commercially available recombinant sub-unit vaccine was against the hepatitis B surface antigen (HBSA), which was first released in 1986 (Liljeqvist and Stahl, 1999). This vaccine consisted of the gene encoding for HBSA that was inserted into *Saccharomyces cerevisiae*, which is normally non-pathogenic in humans (Valenzuela *et al*, 1982). The advantages of using recombinant sub-unit vaccines are many. In general, they are cheaper and easier to manufacture, they do not require mass cultivation of potentially lethal pathogens and it negates the risk of a reversion to wild-type or an incomplete attenuation of the pathogen (Liljeqvist and Stahl, 1999). Also, the delivery can be tailored to ensure the immune response is

optimal, for example by using the appropriate delivery of the antigen, whether it is a live delivery system, one using particulate delivery, or a killed delivery.

The optimisation of recombinant protein expression is essential if the vaccine is to be as effective as possible and many tools have been developed to achieve this (Makrides, 1996). The manufacturing of recombinant protein for vaccines has also been optimised, to include the use of fusion proteins to increase the immunogenicity of the target peptide (Sjölander *et al*, 1997) and the engineering of the target protein to increase solubility (Murby *et al*, 1995). The final optimisation step produces strongly immunogenic peptides which, when delivered with an adjuvant or other carrier molecule, shows strong protection against the pathogenic organism (Hsu *et al*, 1996; Simard *et al*, 1997)

Recombinant protein vaccines can be produced in many different cell types (Liljeqvist and Ståhl, 1999). As mentioned earlier, the recombinant hepatitis B vaccine is produced in *S. cerevisiae* (Valenzuela *et al*, 1982). There are other eukaryotic expression systems available including mammalian cell lines to transgenic plants and animals (Geisse *et al*, 1996). These eukaryotic expression systems are not as commercially viable as using yeast or prokaryotic expression vectors.

By far the easiest and most commercially viable method of producing recombinant protein is in bacteria. This is because of a good understanding of bacterial genetics and the associated tools for genomic manipulation, as well as the ease of culturing large quantities of organisms (Makrides, 1996). For these reasons, *E. coli* is by far the most utilised bacterial production vector (Makrides, 1996). Nevertheless, other

bacteria have been used to produce recombinant protein for vaccines. These include *Salmonella typhimurium*, which expressed the G glycoprotein from human syncitial virus (Martin-Gallardo *et al.*, 1993) and *Bacillus brevis* that was used as an alternative production vector to the transgenic potato and expressed cholera toxin B (Ichikawa *et al.*, 1993).

Despite the efficacy of recombinant protein sub-unit vaccines in combating many infectious diseases such as cholera (Ichikawa *et al*, 1993) and other *Vibrio* sp. (Toranzo *et al*, 1997), *S. typhimurium* (Martin-Gillardo *et al*, 1993), lyme disease (Fawcett *et al*, 2004) and *Tritrichomonas foetus* and *Campylobacter foetus* (Cobo *et al*, 2004), there was still a problem of optimising the immune response against, primarily, intracellular organisms. As stated previously, the best method of presenting immunogenic proteins to the immune response was in a way similar to the presentation during infection, and that is by using a live delivery vector.

1.10.4 Live Delivery Systems

Live delivery systems are similar to live attenuated vaccines in that they utilise whole live organisms as a fundamental part of the vaccine. The main difference between these two methods of vaccine presentation is that the live delivery system involves the presentation of a heterologously expressed protein, and in that sense they are similar to the production vectors of recombinant sub-unit vaccines. Unlike the production vectors, however, the organisms used as heterologous expression systems are an integral part of the vaccine. There are two main categories of live antigen delivery systems used in commercial vaccines at present – bacterial and viral.

1.10.4.1 Bacterial

The use of live bacterial vectors has the advantage that, along with generating an immune response against the heterologously expressed proteins, they can infect cells of the immune system (macrophages and other dendritic cells) and are thus able to prime naïve T cells, as in natural infection (Drabner and Guzmán, 2001). This is essential, since the generation of the wrong type of immunity could be damaging to the vaccinated animal, perhaps even worse than the disease vaccinated against. For example, when a Th1 response is required, an intracellular delivery vehicle such as *Toxoplasma gondii* can be used (Ramirez *et al*, 2001). However, if a Th2 response were needed, using such a delivery system would be counter-productive, since generation of the wrong T-cell response would, in itself, be detrimental. This is due to the differences in which antigens are processed and presented in the animal.

There are several species and strains of bacteria that are currently used either in commercially available vaccines (for example BCG) or as models for delivery of heterologously expressed antigens such as *Salmonella* sp. These include *Mycobacterium* species, *Salmonella* spp. and *Streptococcus* spp.

BCG has been used previously as an attenuated live vaccine against *M. tuberculosis* (Weill-Halle, 1957). It generates a strong Th1 response, indicated by an increase in levels of IFNγ, and the stimulation of cytotoxic CD8+ T lymphocytes (Drabner and Guzman, 2001). BCG has also been used as a recombinant live delivery vector against viral, bacterial, protozoal and metazoal pathogens (Hanson *et al*, 1995). Antigens from HIV (Cirillo *et al*., 1995), *Borrelia burgdorferi* (Miller *et al*, 1999), *Leishmania* spp. (Flynn *et al*, 1994) and *Schistosoma mansoni* (Kremer *et al*, 1996)

have all been expressed in BCG with varying degrees of immunity against the pathogen shown.

Streptococcus gordonii was one of the first Gram-positive bacteria to be used as a live delivery vector. It was transformed with an engineered M6 surface protein from *Streptococcus pyogenes* (Fischetti *et al*, 1989) which itself expressed the E7 oncoprotein from human papillomavirus (Pozzi *et al*, 1992). The transformation, utilising homologous recombination into the bacterial chromosome, resulted in a stably transfected line of *S. gordonii*, which expressed the oncoprotein (Pozzi *et al*, 1992). This heterologous expression allowed the non-pathogenic species *S. gordonii* to present gene products from the pathogenic papillomavirus to the immune system of a mouse and for a specific immune response to occur (Pozzi *et al*, 1992).

Despite the use of Gram-positive *S. gordonii* as a live delivery vector, it is Gram-negative bacteria that are used commonly as delivery vehicles for heterologous antigens (Spreng *et al*, 2000). A common example is that of the intracellular bacterium *Salmonella typhimurium*. *S. typhimurium* can be engineered to contain a eukaryotic expression vector, and has the ability to transport the vector into the cytosol of the host cell (Gentschev *et al*, 2001). This particular system is perhaps of most relevance when vaccinating against parasitic protozoa, for example *T. gondii* or *N. caninum*, since *S. typhimurium* is also an intracellular pathogen. The delivery and presentation of heterologous parasite antigens will be discussed in more detail later.

1.10.4.2 Viral

Viral vectors can also stimulate humoral and cellular immunity, and are easier to genetically manipulate to present multiple antigens for presentation to the immune system than more complex organisms such as bacteria or protozoa (Flexner *et al*, 1988). The main advantage viral vectors have over extracellular bacterial delivery systems is the intracellular expression of the virally-delivered antigen, which is processed via the MHC class I pathway (Liljeqvist *et al*, 1999). Extracellular bacteria phagocytosed by the host cell are processed via the MHC class II pathway (Liljequist *et al*, 1999).

The earliest, and most widely used, system for delivering heterologous antigens used the vaccinia virus as a vector (Mackett *et al*, 1982). The vaccinia virus has been used as a vector for delivery of vaccine antigens in commercially available vaccines as well as a vector for delivery of candidate antigens in pathogen research. A recombinant vaccinia virus expressing rabies antigens was used in large-scale eradication programmes with good effect (Brochier *et al*, 1991).

1.10.5 Nucleic Acid Vaccines

A more recent addition to the vaccine armoury against pathogens is the development of the nucleic acid vaccine. In one of the first studies of efficacy, a protective immune response against influenza A virus was detected in animals previously immunised with the DNA encoding for a gene from the pathogenic virus (Montgomery *et al*, 1993). The use of DNA to vaccinate against pathogens has been widely used (reviewed in Clarke and Johnson, 2001) while the use of RNA, an intrinsically less stable molecule than its deoxyribose counterpart is still relatively

novel (Liljeqvist *et al*, 1999). This may be due to the fact that, because RNA is so transient and unstable the ability to generate a long-term immune response is limited (Liljeqvist *et al*, 1999). However, Ying and colleagues have demonstrated the effectiveness of using self-replicating RNA molecules as a vaccine to protect against tumour challenge in mice (Yine *et* al, 1999). DNA vaccines are also thought to induce a Th1 response, which is important in intracellular pathogen infections such as *Leishmania major* (Piedrafita *et al*, 1999)

1.11 Vaccines Against *T. gondii* and *N. caninum*

There are currently limited numbers of vaccines against protozoan parasites, all of which are for veterinary use only, and are primarily against apicomplexan parasites (reviewed in Jenkins, 2001). Several strategies have been used to vaccinate animals against *T. gondii* and *N. caninum*, with varying degrees of success.

1.11.1 T. gondii

Several different strategies have been employed to produce a successful vaccine against *T. gondii* (reviewed in Bhopale, 2003). Early studies suggested that a live attenuated vaccine was more efficacious than a killed vaccine, since a live vaccine was more likely to be processed by the MHC I pathway and presented to the immune system in an efficacious manner, and recommended the study of the attenuated ts-4 strain of *T. gondii* as a potential vaccine strain (Waldeland and Frenkel, 1983).

1.11.1.1 Attenuated Vaccines

The only commercially available *T. gondii* vaccine (Toxovax®) is based on an incomplete strain of *T. gondii* (S48) that does not produce oocysts (Buxton and

Innes, 1995). This vaccine has been shown to provide protection against abortion in sheep (Buxton *et al,* 1993) after oral challenge with oocysts of *T. gondii*. The vaccine enabled the development of a specific protective humoral and cellular response to *T. gondii* challenge (Buxton *et al,* 1994; Wastling *et al,* 1995). The protection to sheep that is provided by vaccination by this method appears to be relatively specific. Sheep that were vaccinated with Toxovax® were challenged with *N. caninum* tachyzoites but, despite demonstrating a cellular immunological cross-reactivity and an increase in IFNγ production, there was no protective immunity to *N. caninum* (Innes *et al,* 2001). Other methods of immunisation were therefore required for *N. caninum* infections.

However, Toxovax® is limited to veterinary use, because it is based on a live pathogen and therefore would not be eligible for licensing for medical use. Other vaccine strategies need to be considered. Among these strategies is the use of recombinant sub-unit vaccines and DNA vaccines.

1.11.1.2 Recombinant Sub-Unit Vaccines against T. gondii

Several antigens have been suggested as potential vaccine candidates against *T. gondii* (reviewed in Jenkins, 2001). Among these are the major surface antigen (SAG1), which when administered with recombinant IL-12 gave significant protection against *T. gondii* challenge, reducing the parasite load in the brain, compared to the non-vaccinated control mice, by 40% (Letscher-Bru *et al,* 1998). Recombinant SAG1, when used as a vaccine with alum as the adjuvant generates a significant protective response against tachyzoite challenge (Petersen *et al,* (1998).

SAG1 has also been used as the antigen in DNA vaccination studies (Nielsen *et al*, 1999) (see Section 1.11.1.3).

1.11.1.3 DNA Vaccines

DNA vaccination with SAG1 has had varying degrees of success (Nielsen *et al*, 1999; Angus *et al*, 2000). In the work by Nielsen and colleagues, mice were immunized with plasmids containing the gene sequence for SAG1 before being challenged by the Type I virulent RH strain of *T. gondii*. The mice vaccinated with the SAG1 plasmid were almost completely protected against challenge (80-100% protection), compared to the 80% mortality seen in the control mice that were immunized with empty plasmid (Neilsen *et al*, 1999). In contrast to this, the study by Angus and co-workers (2000) showed that there was no protection against challenge by RH strain *T. gondii* in mice vaccinated with a plasmid containing SAG1 (Angus *et a*, 2000). Variations in mouse strain (Neilsen *et al* used BALB/c mice; Angus *et al*, used C57Bl/6 mice) and virulence of different cultures of RH may be the cause of these divergent results.

Other antigen genes used in DNA vaccination against toxoplasmosis have been tried, including dense granule proteins GRA1 and GRA2 and rhoptry protein-2 (ROP2) (Vercammen *et al*, 2000). Variation in the mouse strains used appears to play a role in the development of a protective immune response when vaccinated with plasmids encoding GRA1, GRA2 or ROP2. Both BALB/c and C57Bl/6 mice did not show signs of protective immunity, whereas C3H mice immunized with these plasmids had protection against challenge with oocysts of two strains of *T. gondii* (Vercammen *et al*, 2000).

1.11.2 N. caninum

Vaccination against neosporosis is primarily aimed at preventing abortion and also vertical transmission in cattle. The stimulation of immunity by different adjuvants has been studied to try and prevent vertical transmission in cattle (Andrianarivo *et al*, 1999), though the experiments by Andrianarivo and colleagues (1999) failed to generate protection against challenge by *N. caninum*. Sheep were vaccinated with whole *N. caninum* tachyzoite lysate and challenged with live parasites and stimulated a partially protective humoral response (O'Handley *et al*, 2003).

The procedure described by O'Handley *et al* (2003) was also used to vaccinate mice (Liddell *et al*, 1999; Nishikawa *et al*, 2001; Liddell *et al*, 2003). Liddell and colleagues (1999) immunised BALB/c mice with crude *N. caninum* lysate along with a commercial adjuvant and then challenged with live parasites. There appeared to be complete protection against vertical transmission (Liddell *et al*, 1999).

Nishikawa and co-workers (2001) used recombinant vaccinia virus expressing NcSRS2 as a delivery vector for immunising BALB/c mice to protect against challenge by live parasites. This produced a protective cellular and humoral response and was effective in preventing vertical transmission (Nishikawa *et al*, 2001). Liddell and colleagues vaccinated BALB/c mice with a DNA vaccine encoding for NcGRA7 and NcHSP33 (Liddell *et al*, 2003). This generated partial protection against vertical transmission. Both NcSRS2 and NcGRA7 are discussed in more detail in Sections 2.1.2.5.1 and 2.1.2.5.2.

It should be noted that there is a commercially available vaccine for *N. caninum* (Neoguard® (Intervet)), a vaccine made of whole killed *N. caninum* tachyzoites in conjunction with a proprietory adjuvant. There are data to suggest that the vaccine does generate some immunity against challenge with *N. caninum* that prevents abortion in cattle (Choromanski *et al*, 2001). A further large scale field trial using 876 cattle in Costa Rica suggested that the use of the vaccine reduced the abortion rate two-fold (Romero *et al*, 2004).

1.12 Aims and Objectives

The work in the following chapters seeks to generate a stable transfection of *T. gondii* with *N. caninum* genes. These were then used to investigate immunodominant antigens of *N. caninum*, and in particular study the immune response in small animal models to these antigens, when the antigens are delivered using a live delivery vector, transgenic *T. gondii*. In addition to this, novel antigens that stimulate a proliferative response *in vitro* by T-cells from *N. caninum*-infected cattle are identified by mass spectrometry. Thus, the aims and objectives of the work presented in this thesis are:

- Produce and characterise transgenic *T. gondii* that express proteins of *N. caninum*.
- Assess the efficacy of transgenic *T. gondii* as a delivery vehicle for *N. caninum* proteins for stimulation of a specific immune response to *N. caninum* in a small animal model.
- Use a combination of proteomic technologies and immunological assays to identify potentially immunodominant proteins of *N. caninum* that may have potential as vaccine candidates.

CHAPTER 2:

TRANSGENIC EXPRESSION OF *NEOSPORA CANINUM* DENSE GRANULE PROTEIN-7 (*NcGRA7*) AND SAG-1 RELATED SURFACE PROTEIN-2 (NcSRS2) GENES IN *TOXOPLASMA GONDII*

- Clone two *N. caninum* gene sequences (*NcGRA7* and NcSRS2) into a transfection vector that will enable the expression of the *N. caninum* protein in tachyzoites of *T. gondii*.
- Transfect *T. gondii* with the transfection construct and generate stable transfectants.
- Analyse and characterise these transfectants for stability and presence of the transgene by molecular and immunological techniques.

2.1 INTRODUCTION

2.1.1 Transfection of *T. gondii*

T. gondii has been used previously to study the expression of genes from other Apicomplexa, such as *Plasmodium sp.* (Cristina *et al.*1999) and other parasitic protozoa such as *Leishmania sp.* (Ramirez *et al*, 2001). In the case of *Plasmodium*, the use of *T. gondii* for transgenic studies was initiated due to the difficulty of transfecting *Plasmodium sp.* and the relative ease by which *T. gondii* can be transfected, though advances in *Plasmodium* transfection have been made recently (Wang *et al.* 2002). There are several methods available to transfect *T. gondii* tachyzoites with genes from other organisms, including the complementation of tryptophan auxotrophy (Sibley *et al* 1994); the utilisation of a drug-resistance phenotype at the dihydrofolate reductase-thymidylate synthase (DHFR-TS) locus (Donald & Roos, 1993; Donald & Roos, 1994); the use of chloramphenicol acetyl transferase (CAT) as a selectable marker of transfection (Soldati & Boothroyd 1993; Kim, *et al* 1993); and selection based on the presence or absence of an active hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) gene (Donald, *et al.* 1996; Donald & Roos 1998; Nakaar *et al.* 2000).

It was this latter strategy, based on the presence of an active HXGPRT gene that was used in the following studies. A diagrammatic representation of this transfection strategy is show in Figure 2.1.

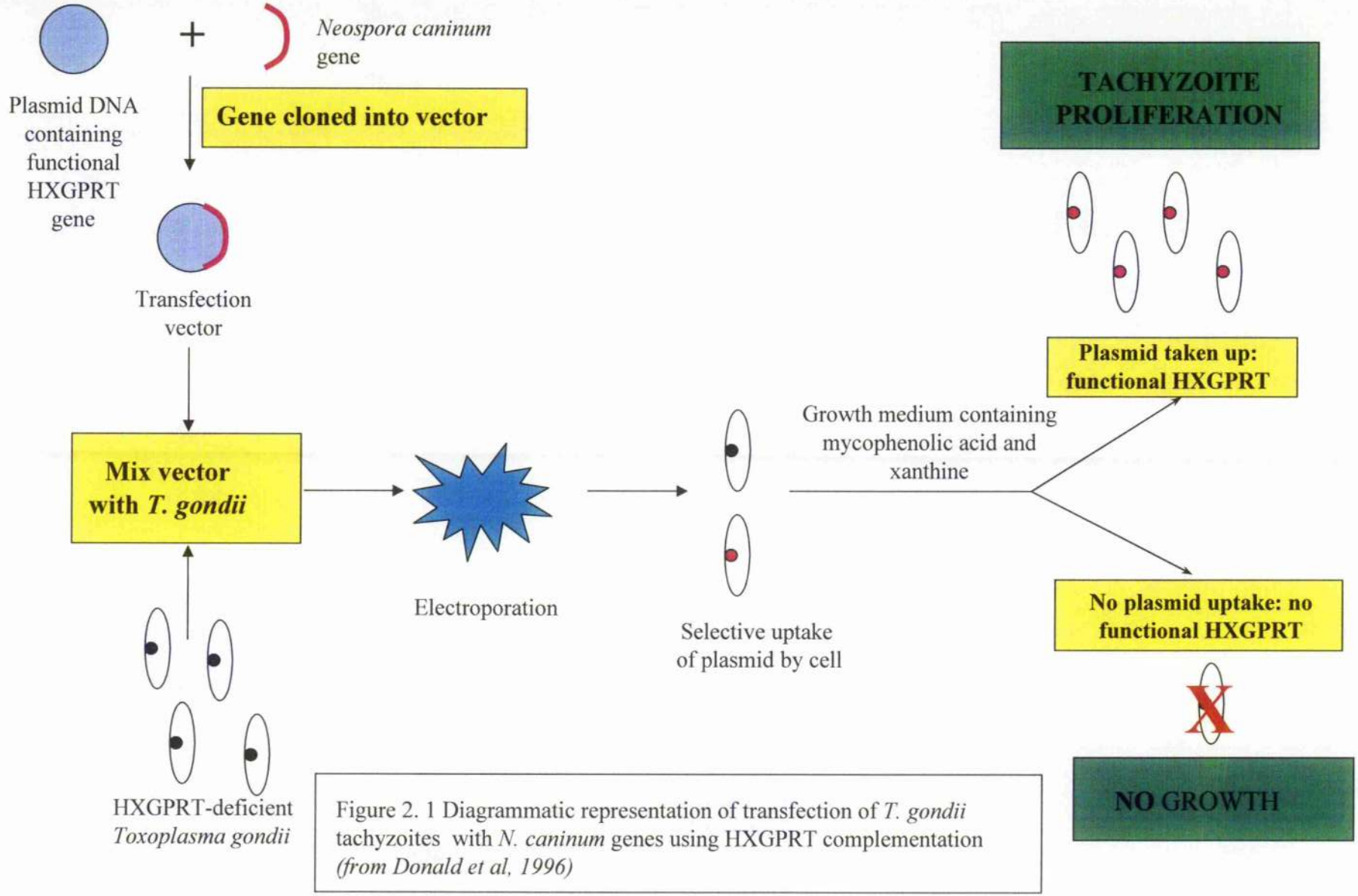


Figure 2. 1 Diagrammatic representation of transfection of *T. gondii* tachyzoites with *N. caninum* genes using HXGPRT complementation *(from Donald et al, 1996)*

2.1.1.1 Complementation of HXGPRT knockout T. gondii tachyzoites

Selection using complementation of HXGPRT-deficient tachyzoites with a gene encoding for an active HXGPRT enzyme can be either positive (that is, selecting for the *presence* of the active HXGPRT) (Pfefferkorn & Borotz 1994) or negative (selecting for the *absence* of the gene) (Ullman & Carter 1995).

Since *T. gondii* is auxotrophic for purines, the parasite requires the uptake of these compounds for survival and so the parasite has evolved a degenerate system to ensure that it will obtain sufficient purines, irrespective of the growth environment (Chaudhari *et al*, 2004). The parasite has two mechanisms by which it can obtain guanosine monophosphate (GMP), a necessary component of nucleotide biosynthesis. These are shown in Figure 2.2.

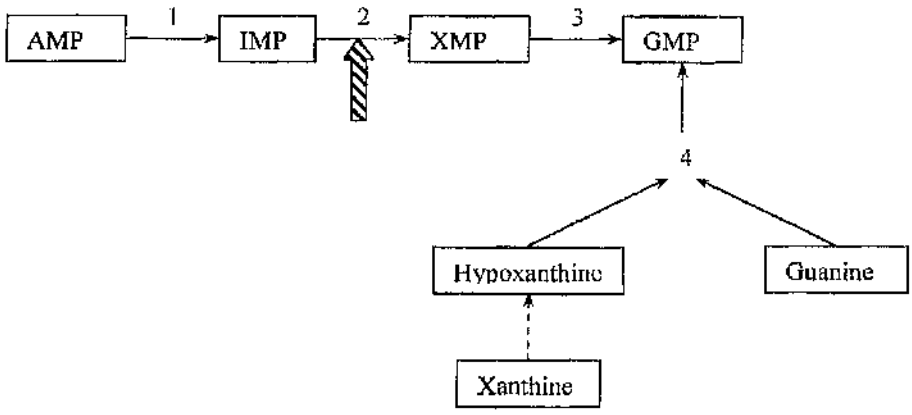


Figure 2.2 Schematic diagram of the two metabolic pathways used by *Toxoplasma gondii* to ensure a supply of guanosine monophosphate (GMP). Adenosine monophosphate (AMP) is metabolised to inosine monophosphate (IMP) by AMP deaminase (1), which in turn undergoes metabolism to xanthine monophosphate (XMP) by IMP dehydrogenase (2). XMP is finally metabolised to GMP by XMP-glutamine aminotransferase (3). GMP is also produced by the combination of hypoxanthine and guanine in the presence of HXGPRT (4). Xanthine is the initial precursor of hypoxanthine. Mycophenolic acid inhibits IMP dehydrogenase (at hatched arrow) *Adapted from Chaudhari* et al, *2004*

In *T. gondii* tachyzoites, both the AMP→IMP→XMP→GMP and (hypoxanthine + guanine)→GMP pathways are operational. In HXGPRT knockout parasites

(ΔHXGPRT), however, only the former is active. This is the basis for the selection. When ΔHXGPRT tachyzoites are transfected with the HXGPRT and then allowed to grow in medium supplemented with mycophenolic acid (MPA) and xanthine only the parasites that contain the active HXGPRT gene will survive (Pfefferkorn and Borowitz, 1994; Donald and Carter, 1996). MPA is an inhibitor of IMP dehydrogenase, which in turn stops the metabolism of IMP to XMP and hence the production of GMP by this route. Xanthine is a precursor of hypoxanthine so by adding xanthine in the cultures increases the levels of hypoxanthine available to the parasite. If the active HXGPRT gene is co-transfected with another gene this gene may also be expressed by the selected parasites.

2.1.1.2 Restriction Enzyme-Mediated Transfection

Transfection of *T. gondii* and other organisms is aided by Restriction Enzyme-Mediated Integration (REMI), originally used in transfection of *Saccharomyces cerevisiae* (Manivasakam & Schiestl, 1998). REMI-based transfection, as the name suggests, has a particular restriction enzyme added to the transfection mix. In *T. gondii*, BamHI (the enzyme used by Manivasakam and Schiestl (1998)) was shown to increase integration of the transfection vector by 2-5 fold, while addition of NotI increased integration of the vector by a further 29-46 times over the control in which no enzyme was added (Black *et al,* 1995).

The mechanism by which REMI works is not fully understood. Most reports suggest that the integration of the linearised vector is a simple ligation reaction, when the vector inserts into the appropriate sites caused by the restriction enzyme (Kuspa &

Loomis 1992; Riggle & Kumamoto, 1998). Other studies report that the integration is a non-homologous end-joining event (Manivasakam & Schiestl 1998).

2.1.2 Transfection of *T. gondii* with heterologous genes

2.1.2.1 Green Fluorescent Protein (GFP)

Green fluorescent protein (GFP) is a commonly used protein in gene expression studies. It originated from *Aequoria victoria* (Tanahashi *et al* 1990) and has been used in studies of gene expression in *T. gondii* previously (Striepen *et al.* 1998). The use of GFP has been optimised in *T. gondii* to allow for fluorescent visualisation without the need for multiple-copy insertions, and also to enable GFP to be used as a marker for transient transfection (Kim *et al*, 2001).

2.1.2.2 Escherichia coli β-Galactosidase

β-galactosidase from *E. coli* is commonly used as a marker molecule for transformations (MacGregor *et al*, 1989). Seeber and Boothroyd (1996) adapted the bacterial gene *lacZ* (which expresses β-galactosidase) to be driven by several *T. gondii* promoters of different strengths to enable the use of the β-galactosidase as a marker for transient and stable transformations of *T. gondii* (Seeber & Boothroyd 1996).

2.1.2.3 Transgenic Expression of Leishmania sp. Genes in T. gondii

T. gondii has been used to express genes from *Leishmania sp.*, for example the kinetoplastid membrane protein-11 (KMP-11) (Ramirez *et al.* 2001). In this study, a temperature sensitive *T. gondii* mutant was transfected using the CAT selection system (Soldati & Boothroyd, 1993; Kim *et al* 1993). The transfected organisms

were then used in immunisation studies in mice, to see if an immune response could be generated against cutaneous leishmaniasis in BALB/c mice. T-cell proliferation responses to both the *T. gondii* vector and the heterologous antigen (KMP-11) were observed. When immunised mice were challenged with *L. major*, the mice that had been immunised with the transfected *T. gondii* expressing KMP-11 showed significant immunity against the challenge. The foot lesions in two of this group of mice healed. This study indicated that KMP-11 expressed by *T. gondii* mediated partial protection against *L. major* and that mutant *T. gondii,* transfected with heterologous genes, showed potential as a vaccine delivery system (Ramirez *et al* 2001).

2.1.2.4 Transgenic Expression of Plasmodium sp. Genes in T. gondii

Until recently, *Plasmodium sp.* were notoriously difficult to study by transgenic approaches. This was though to be primarily due to the high A/T levels in the genome (Musto *et al*, 1995). Nevertheless, tools to transfect malaria parasites have now been developed (Waterkeyn *et al* 1999). However, compared to *Plasmodium,* transfection of *T. gondii* is still easier to perform, better characterised and more importantly, transfected tachyzoites are easier to maintain in culture.

In one study, *T. gondii* was engineered to express the circumsporozoite protein (CSP) from the primate malaria *P. knowlesi* (Cristina *et al* 1999). This was done to determine the efficiency of *T. gondii* as a delivery system for vaccination with specific genes from another pathogen, a similar approach to that described in Section 2.1.2.1 for *Leishmania* KMP-11. *T. gondii* was transfected with CSP using the CAT system. The development of a specific humoral response against the CSP protein used in the transfection was observed but without a noticeable antibody response

against *T. gondii* (below the World Health Organisation standard positive level of 15 International Enzyme Units (IU) per ml). This suggested that the CSP transgene was the major immunodominant protein expressed by *T. gondii* tachyzoites, when tested in mice.

2.1.2.5 Transgenic Expression of N. caninum Genes in T. gondii

N. caninum genes have not previously been transfected into *T. gondii*, though the reverse has been achieved (Beckers *et al*, 1997). There should, however, be no theoretical barriers to the transfection of *T. gondii* with *N. caninum* genes. Since *T. gondii* has been transfected with non-apicomplexan genes successfully, transfection with genes from *N. caninum* which is phylogenetically very close to *T. gondii* (Ellis *et al* 1994) should be relatively straightforward. The main issue is the choice of genes for transfection.

Several antigens of *N. caninum* have been studied in detail (for a review of them, see Hemphill *et al* 1999). For the studies described below, two immunologically important antigens were selected for transfection.

2.1.2.5.1 Dense granule protein 7 (NcGRA7)

Dense granule protein 7 (*NcGRA7*), also known as *N. caninum* Dense Granule 1 (NcDG1) – submitted to Genbank under Accession Number U82229 – was first identified by Lally and colleagues at the United States Department of Agriculture (Lally *et al*, 1997). They identified it from a cDNA clone that, when expressed as a recombinant protein, could be used as a capture molecule for an ELISA to identify *N. caninum* specific antibodies in infected cattle. It is homologous to *T. gondii* GRA7, a

29kDa protein shown to be localised to the dense granules by immunofluorescence (Bonhomme *et al* 1998).

Dense granule proteins in both *T. gondii* and *N. caninum* are highly immunogenic, since they are involved in the initial invasion of the host cell by the parasite (Mevelec and Chardres, 1992; Fischer and Stachelhaus, 1998). *NcGRA7* has been studied as a potential vaccine candidate in mouse models. When BALB/c mice were inoculated with plasmid DNA expressing *NcGRA7*, there was limited protection (54%) against vertical transmission following challenge with *N. caninum* (Liddell *et al*, 2003). This was assessed by comparing the amount of parasite DNA in the mothers and pups by PCR (Liddell, *et al*, 2003)

*2.1.2.5.2 Surface Antigen 1 related Antigen 2 (*NcSRS2*)*

N. caninum surface protein p43, also known as NcSRS2 by its homology to *T. gondii* SRS2, was first identified by Hemphill and colleagues (Hemphill *et al*, 1997) (Genbank Accession Number U93870). It has been localised to the surface of tachyzoites as well as to dense granules and rhoptries (Hemphill *et al.* 1996; Howe *et al*, 1998). NcSRS2 is a GPI-anchored protein that contains hydrophobic motifs within its structure that indicates putative transmembrane domains (Howe *et al* 1998; Schares *et al* 2000).

NcSRS2 has been identified as having a role in cell adhesion and invasion (Nishikawa *et al*, 2000). As such it has been identified as a potential vaccine candidate (Nishikawa *et al*, 2001).

2.1.3 Aims and Objectives

The aims of this chapter are to:

- Clone two *N. caninum* genes (*NcGRA7* and NcSRS2) into a transfection vector that will enable the transcription of the *N. caninum* protein in tachyzoites of *T. gondii*.
- Transfect *T. gondii* with the transfection construct and generate stable transfectants.
- Analyse and characterise these transfectants for stability and presence of the transgene by molecular and immunological techniques.

2.2 MATERIALS AND METHODS

2.2.1 Parasite Maintainance

T. gondii and *N. caninum* were maintained by *in vitro* culture in mammalian cell lines. Both parasite species were routinely passaged through African Green Monkey Kidney Fibroblasts (Vero cells), a gift from Dr. E.A. Innes, Moredun Research Institute, Edinburgh, UK. When creating a cloned line of *T. gondii* by limited dilution, Human Foetal Foreskin Fibroblasts (HFFF) (European Collection of Cell Cultures, Porton Down, UK) were used, since they grew slower than Vero cells, and hence were more suitable for cloning by limited dilution. HFFF cells also had the advantage that they stopped growing due to contact inhibition such that once they grew to a confluent layer on the base of the flask proliferation ceased. Vero cells, on the other hand continued to grow even after reaching confluency, eventually detaching from the flask. Cell lines were passaged as described in sections 2.2.1.2 and 2.2.1.3.

2.2.1.1 Preparation of Media

2.2.1.1.1 Growth media

Host cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Paisley, UK) supplemented, in the case of Veros, with 5% (v/v) heat inactivated Foetal Bovine Serum (FBS) (Labtech, Glasgow, UK). IMDM used for the passage of HFFF cells was supplemented with 10% (v/v) FBS. Both media were also supplemented with 100 U/ml penicillin and 100 µl/ml streptomycin sulphate (Invitrogen, Paisley, UK). Medium used for the passage of host cell lines was also used for the growth of parasites. Prior to use in cell culture, the supplemented IMDM

was filtered through a Sartolab V500 sterile 0.2 μm filter attached to a diaphragm pump. The medium was stored at 4 °C for a maximum of one month.

2.2.1.1.2 Cryopreservation Medium

Parasite strains were stored in liquid nitrogen until required. They were suspended in IMDM containing 10% v/v FBS and 12% DMSO. No antibiotics were added to this medium. The cryopreservation medium was filtered through a sterile 0.2 μm membrane and stored at 4°C for a maximum of one month.

2.2.1.2 Passage of Vero Cells

Vero cells were maintained in 25 cm^2 or 75 cm^2 vent-capped tissue culture flasks (Greiner, UK). When the cells had grown to a confluent monolayer, the growth medium was removed and replaced with a 4:1 solution of versine/trypsin (in Hanks' Balanced Salt Solution without calcium or magnesium but containing EDTA). The monolayer was incubated in this solution for 10 min at 37 °C. The flask was tapped gently to aid the detachment of the cells from the base. The cells in versine/trypsin were transferred to a sterile 50 ml tube. A 20 μl aliquot was removed into an Improved Neubauer hamocytometer (Weber Scientific, UK) for later counting. The rest of the cells were centrifuged at 1500 x *g* for 10 min at room temperature. The versine/trypsin solution was removed and the cells resuspended at $1x10^6$ cells/ml. Cell suspension (100 μl) was added to 5 ml or 15 ml of growth medium in a 25 cm^2 or 75 cm^2 flask, respectively. The cells were grown overnight at 37 °C in a 5% CO_2 humid incubator before being infected with parasites.

Vero cells were passaged twice a week, with confluent flasks from the previous week used as the source of the cells.

2.2.1.3 Passage of HFFF Cells

Passage and growth conditions of HFFF cells were similar to those of Vero cells. HFFF cells grew to a monolayer at a slower rate than Vero cells, so were less suitable for long-term parasite maintenance. The cells were detached from the bottom of the culture flask in the same way as described for Vero cells. The cells from one 25 cm^2 flask containing a cell monolayer were resuspended in 10 ml of IMDM supplemented with penicillin, streptomycin and 10% FBS. One ml of this resuspension was added to 4 ml (25 cm^2 flask) while 3 ml was added to 12 ml (75 cm^2 flask) of growth medium. The cells were then incubated at 37°C in a humidified 5% CO_2 incubator.

2.2.2 Parasite Strains

2.2.2.1 Toxoplasma gondii

The Type II *T. gondii* Prugniaud strain (PRU) (Martrou *et al*, 1965) was used as the parental strain for transfections with *N. caninum* genes and was a kind gift from Dr. J. Mattsson, National Veterinary Laboratory, Uppsala, Sweden. The strain used was a knockout strain (PRUΔHX), deficient in a functional hypoxanthine-xanthine guanine phosphoribosyltransferase (HXGPRT) gene, the coding region having been truncated by homologous recombination (Mattsson, pers. commun.). The PRU strain was chosen as Type II strains of *T. gondii* generally cause less pathology in mice that Type I strains (Robben *et al*, 2004). This may be due to the early production of IL-12 by macrophages that infection with Type II *T. gondii* appears to stimulate (Robben *et*

al, 2004). Secondly, an HXGPRT-knockout strain was readily available from the lab of Dr Mattsson.

2.2.2.2 Neospora caninum

One strain of *N. caninum* was used throughout. Experiments were performed using the NC1 strain (Dubey *et al* 1988), a kind gift from Dr. E. A. Innes, Moredun Research Institute, Edinburgh, UK.

2.2.3 Parasite Culture

To ensure maximum infectivity, parasites that were still intracellular were used to infect a flask of host cells, either a confluent monolayer in the case of HFFF cells or, in the case of Vero cells, a flask containing $1x10^5$ cells/ml seeded 24h earlier. The host cells were mechanically disrupted by scraping them off the flask surface, using sterile cell scrapers (Greiner, UK). The medium and cell debris, including the parasites, were centrifuged three times at 1500 x *g* for 10 minutes, with the supernatant being removed and replaced with 10 ml of fresh medium after every centrifugation. After the final centrifugation, the parasites were resuspended in 5 ml of growth medium, and an aliquot was removed for counting using an Improved Neubauer haemocytometer. The host cells were infected at a host:parasite ratio of 1:4 or 1:5 and incubated at 37°C, 5% CO_2. The parasites were sub-passaged into new cells every 72-84 h.

2.2.4 Cryopreservation of Parasites

For long-term storage, parasites were preserved under liquid nitrogen in cryopreservation medium. A minimum of $1x10^8$ parasites was removed by mechanical rupture of the host cell by scraping. They were centrifuged at 400 x *g* for

5min which pelleted much of the larger host cell debris, whilst leaving most of the freed tachyzoites still in the supernatant. This supernatant was removed to a clean tube before being centrifuged at 1500 x *g* for 10min. The pellet of parasites was washed twice in PBS before being resuspended in 1ml cryopreservation medium. This was then placed in an insulated box to ensure a slow freeze and stored at -70°C for 16 h, before being transferred to liquid nitrogen.

Once in liquid nitrogen, the parasites could be stored indefinitely. When required for tissue culture, they were removed from liquid nitrogen and rapidly warmed to 37°C in a water bath before being inoculated into a tissue culture flask with approximately $4x10^5$/ml Vero cells. Any parasites that did not invade after 16 h were removed with a change to fresh growth medium. This had the added benefit of removing traces of DMSO from the cryopreservation medium.

2.2.5 Filtration Harvesting of Tachyzoites

For all procedures requiring parasite material, whether for parasite nucleic acid or protein, one of the major considerations was the removal of host cell debris. Differential centrifugation was used as described (Section 2.2.4) to remove large particles of debris. This procedure was not adequate if the parasites were being harvested for DNA or protein preparation. In these instances, the parasites were filtered free of host cell debris as described below.

Parasites were passed through a 45 mm diameter filter membrane (Whatman) with a 3 μm pore size. This allowed tachyzoites to pass through while stopping cellular debris. Filtered parasites were centrifuged at 2000 x *g* for 10 min, before being

washed three times in sterile PBS. An aliquot of parasites was removed and diluted 1:10 in PBS before being counted in a haemocytometer. The parasites were resuspended at an appropriate concentration, normally 1-2x10^8 parasites per ml, before being transferred to a sterile 1.5 ml tube. Parasite suspensions were then given a final centrifugation at 13,000 x *g*, the supernatant was removed and the parasite pellet either stored at -70°C or resuspended in cell lysis buffer as described below.

2.2.6 Extraction of Genomic DNA from Tachyzoites

DNA was extracted from 1x10^8 tachyzoites using GenomicPrep™ Cells and Tissue DNA Isolation Kit (Amersham Biosciences, Amersham, UK) following the manufacturer's instructions. Briefly, the pellet of tachyzoites was washed by centrifugation (13,000 x *g* for 10 min) in PBS before being resuspended in 600 µl cell lysis buffer (an anionic detergent), in which the tachyzoites were stable at room temperature for up to 18 months (according to the manufacturer). When DNA was required, 3 µl of RNAse A was added, and the lysed cells were incubated at 37°C for 15 to 60 min. Protein Precipitation Solution (300 µl) was added, mixed by vortexing and centrifuged at 13,000 x *g* for 3 min. The supernatant containing the extracted DNA was poured into a fresh tube that contained 600 µl isopropanol. This was mixed by inverting the tube followed by centrifugation at 13000 x *g* for 3 min to pellet any DNA that was precipitated by the isopropanol. Ethanol (70% v/v) (600 µl) was added to the pellet, and again centrifuged at 13,000 x *g* before being air-dried for 15 min. Another 600 µl of 70% v/v ethanol was added, the tube contents centrifuged at 13,000 x *g*. The pellet was fully air-dried to remove any remnants of ethanol before 100 µl of DNA Hydration Solution was added. The pellet was rehydrated overnight at room temperature before being stored long term at -20°C. The quantity

of DNA extracted was assessed by removing a 5 µl aliquot and running it against a known quantity on a 1% agarose gel at 120 V for 1 h. This was stained with ethidium bromide and visualised under ultraviolet light on a transilluminator.

2.2.7 Production of Complementary DNA (cDNA)

2.2.7.1 Extraction of Whole Cell RNA

Total cell RNA was isolated using Tri Reagent® (Sigma-Aldrich,UK) (containing acid guanidinium thiocyanate) according to manufacturer's protocol. Briefly, $1x10^8$ tachyzoites were resuspended in 1 ml Tri Reagent® and 200 µl of chloroform was added and the sample shaken vigorously and allowed to stand at room temperature for up to 15 min. The mix was then centrifuged at 12,000 x *g* for 15 min at 4°C resulting in three distinct layers – the organic phase, an interface layer and an upper aqueous layer. The aqueous layer contained the RNA and this layer was carefully aspirated into a fresh tube and 500 µl isopropanol was added, before being allowed to stand at room temperature for 10 min. The sample was then centrifuged at 12,000 x *g* for 10 min at 4°C, to pellet the RNA. The pellet was then washed in 1 ml 75% (v/v) ethanol in distilled water. The sample was vortexed and then centrifuged at 7500 x *g* for 5 min at 4°C and the resulting pellet was air-dried in a laminar flow hood for 5-10min before being resuspended in 100 µl DEPC-treated water.

To ensure that no contaminating DNA was present, the resuspended pellet was treated with 50 U/ml DNAse I (Stratagene). DNAse I is an endonuclease that cleaves DNA and removes it from the RNA preparation. The mix was incubated at 37°C for 30 minutes. The RNA was then stored at -70°C until required.

2.2.7.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

A mix of 1-2 µg RNA (isolated as described in Section 2.2.7.1 above), 2 µl DEPC-treated ddH_2O and 3 µl oligo-dT (Stratagene) were incubated at 70°C for 10 min, before being allowed to cool to room temperature. This heating inactivated the DNAse I as well as encouraging annealing of the primers to the RNA. Once the mixture had cooled, 4 µl Reverse Transcriptase Buffer, 2 µl 100 mM DTT, 2 µl dNTPs (Stratagene) were added, along with 0.5 µl RNAse inhibitors (Promega) and 1 µl Reverse Transcriptase (Stratagene). This was incubated at 30°C for 10 min and then heated to 42°C for 45 min. The mixture was then heated to 95°C for 5 min to denature the enzyme before being cooled and stored at -20°C. The resulting cDNA was quantified by running a small (5 µl) aliquot on a 1% agarose gel against a known concentration of DNA and visualised as described above

2.2.8 Primer Design and Polymerase Chain Reaction (PCR)

2.2.8.1 Design of Specific Primers

Specific primers (Invitrogen, UK) were designed around the 5′ and 3′ ends of the parasite cDNA sequences to be amplified. These primer sequences ($NcGRA7^F$ $NcGRA7^R$, $NcSRS2^F$ and $NcSRS2^R$) are shown in Table 2.1 and contain no degeneracy. These primers were used to amplify sequences for subcloning, and also for the identification of the presence of transgenes in *T. gondii*.

PRIMER NAME	SEQUENCE (5′-3′)
NcGRA7^{F}	ATGGCCCGACAAGCAACCTTC
NcGRA7^{R}	TTCGGTGTCTACTTCTGCTC
NcSRS2^{F}	AACATGGCGACGCATGCTTGTGTG
NcSRS2^{R}	TGATCAGTACGCAAAGATTGCCGT
NcGRA7_NsiI^{F}	TTGatgcatCGACAAGCAACCTTCATC
NcGRA7_PacI^{R}	CCGttaattaaCTATTCGGTGTCTAGTTC
TOPOSeq^{F}	GATCCACTAGTAACGGCC
TOPOSeq^{R}	GTGTGATGGATATCTGCA
PlasmidSeq1^{F}	TCGAGGTCGACGGTATCGATA
PlasmidSeq1^{R}	TCACCGTTGTGCTCACT
SAG1-UTR_BglII^{F}	CGATagatctGTGATCACCGTTGTGCTCAC
SAG1-UTR_NotI^{R}	CGATgcggccgcTCGGGGGGGCAAGAATTGTG
NcGRA7_BglII^{F}	CGATagatctAAAATGGCCCGACAAGCAACCTTC
NcGRA7-**myc**_BglII^{R}	CGATagatctCTA**GAGGTCCTCCTCCGAGATGAGCTTC TGCTC**GCCGCCTTCGGTGTCTACTTCTGCTC
PlasmidSeq2^{F}	CGCGCAAAAGACATCCAACAA
PlasmidSeq2^{R}	GCACGAAGTGTGTTTTCCTTT
NcSRS2_BglII^{F}	CGATagatctAAAATGGCGACGCATGCTTGTGTG
NcSRS2_BglII^{R}	CGATagatctTCAGTACGCAAAGATTGCCGTTGCA
myc_BamHI^{F}	CGATggatcc**GAGCAGAAGCTCATCTCGGAGGAGGAC CTC**
myc_BamHI^{R}	CGATggatcc**GAGGTCCTCCTCCGAGATGAGCTTCTGC CTC**

(a)

PRIMER SET	ANNEALING TEMPERATURE (°C)
NcGRA7^{F} /NcGRA7^{R}	50-65
NcGRA7_NsiI^{F} / NcGRA7_PacI^{R}	55-60
NcSRS2^{F}/ NcSRS2^{R}	55-60
NcGRA7BglII^{F}/ NcGRA7myc-BglII^{R}	55
NcSRS2-BglII^{F}/ NcSRS2-BglII^{R}	55
MYC^{F}/ MYC^{R}	*

(b)

Table 2.1Primer sequences (capitals) including relevant restriction sites (lower case) (a) and annealing temperatures (b) of primer sets. Range of temperatures indicates that the annealing of the primers was obtained at several temperatures in the range.

* This primer set was not used in PCR. The primers were mixed, heated to 95°C and then cooled to room temperature to anneal into primer dimer.

2.2.8.2 Design of Primers Containing Restriction Enzyme Sites

Primers that were required to contain restriction enzyme recognition sites were designed with some degeneracy engineered into both the 5′ and 3′ ends to allow for incorporation of these sites. This was important when the PCR-amplified sequence was to be inserted into a cloning vector digested with the same enzymes. The two ends of the plasmid and the PCR amplicon containing the restriction enzyme recognition sites could then ligate together to form a construct that contained the PCR product. Along with the restriction enzyme recognition sequence, an additional 3-5nt were designed into both forward and reverse primers. These additional nucleotides were required to ensure that the restriction enzyme annealed to the DNA efficiently, since enzyme annealing (and digestion capability) may be hampered if the recognition site was engineered at the end of the DNA molecule.

2.2.8.3 Polymerase Chain Reaction (PCR)

PCR is a technique whereby large quantities of specific sequences of DNA can be amplified from a very small number of original copies. A thermostable DNA polymerase (*Taq)* obtained from the extremophile *Thermus aquaticus* is commonly used to amplify copies of DNA from an original single-stranded template in the presence of oligonucleotide primers designed to anneal to the 5' ends of the template. The primers anneal to the template to create a double strand of DNA. This step is done at an optimal annealing temperature worked out for each primer set. The *Taq* polymerase catalyses the attachment of free nucleotide triphosphates, complementary to the template strand, to the 3' end of each primer. The complex of template DNA annealed to the complementary new strand was then denatured (95°C) and both are

then free to be used as templates. In this way, an exponential number of copies can be obtained rapidly.

2.2.9 Subcloning of PCR Products

2.2.9.1 Cloning Reaction

Before being inserted into transfection vectors, PCR-derived sequences were subcloned by TOPO TA® cloning (Invitrogen, UK). This allowed for the quick generation of large amounts of DNA for ligation into transfection vectors, as well as enabling restriction digest analysis and sequencing of the insert.

TOPO TA® uses the pCR®2.1-TOPO® vector. This was linearised with topoisomerase I bound to the overhanging "sticky ends" of the plasmid. The linearised plasmid was mixed with approximately 50 ng DNA (in 1-4 µl ddH_2O). This mix was incubated for 5 min at room temperature and 1 µl of 6 x TOPO® Cloning Stop Solution was added immediately to the reaction mix, which was then placed on ice.

2.2.9.2 Chemical Transformation of Escherichia coli

Chemically competent One Shot® TOP10F *E. coli* (Invitrogen) were transformed according to the manufacturer's instructions. Briefly, 2 µl of the cloning reaction described above was added to the vial of *E. coli* and the mix was incubated for 30 min on ice. The cells were heat-shocked for 30 s at 42°C and then transferred back to ice where 250 µl of SOC medium was added. The cells were shaken for 30 min at 37°C before aliquots of between 10-50 µl were transferred to LB agar selection plates containing 50µg/ml ampicillin and 40 mg/ml X-Gal in DMF. These were

incubated overnight at 37°C. Between 10 and 20 positive (white) colonies were picked for overnight culturing in selection broth prior to preparation of plasmid for restriction digest and sequence analysis. Sequencing of the insert was performed using the TOPOSeq^{F} and TOPOSeq^{R} primers (Table 2.1)

2.2.9.3 Plasmid Preparation from Overnight Cultures

Positive white colonies from the transformation were picked into 2-5ml LB Broth containing 50μg/ml ampicillin. These were then incubated on a shaker overnight at 37°C. The plasmids were extracted from the bacterial DNA using the QIAprep® Miniprep Kit (Qiagen) according to the manufacturer's protocol. Briefly, 1ml of overnight culture was pelleted at 13,000 x *g* for 5min. The pellet was resuspended in 250μl of supplied resuspension buffer P1 (containing 50mM Tris pH 8.0, 10mM EDTA and 100μg/ml RNAse A). An equal volume of Cell Lysis Buffer P2 (containing 200mM NaOH and 1% SDS) was added to this mix that was then inverted 4-6 times to evenly mix the buffers. 350μl Protein Precipitation Solution N3 (a proprietory buffer from Qiagen) was added, and the tube was inverted 4-6 times to prevent localised precipitation. The mix was centrifuged at 13000 x *g* for 10min to pellet to precipitated proteins. The supernatant containing the plasmid was applied to a QIAprep column. This contained a silica-gel membrane that selectively adsorbs plasmid DNA in high salt buffer. The plasmids, when passed through the membrane by centrifugation at 13,000 x *g* for 1min, adsorb to the membrane, with the rest of the supernatant components passing through and being discarded. The column was washed by centrifugation (13,000 x *g* for 1min) with 500μl Wash Buffer (PBS) to remove traces of nuclease activity. The final wash of the column was with ethanol-based buffer PE , before the column was transferred to a clean tube and the plasmid

DNA was eluted using elution buffer EB (10 mM Tris.Cl, pH 8.5) by centrifugation (13,000 x g for 1min).

An aliquot of the plasmids (2-10µl) diluted 1:10 and 1:100 in ddH_2O was run on 1% agarose at 100V, and visualised with ethidium bromide under UV. When run against known standards, this allowed the quantification of plasmid concentration.

2.2.10 Restriction Enzyme Digest Analysis

To confirm the presence of an insert in the pCR®2.1-TOPO® vector, plasmid DNA was digested using *Eco RI*, which has recognition sites at the 5'- and 3' ends of the multiple cloning site of pCR®2.1-TOPO®. The digestion of the plasmid with this enzyme excised any insert present. The plasmids that showed an excised fragment of the appropriate size were subsequently sequenced for further confirmation of the insert identity.

2.2.11 Sequencing of Plasmid DNA Inserts

Big Dye® Terminator Sequencing Kit (Applied Biosystems) was used to sequence the insert of plasmids. A mix of 8µl Dye Terminator Buffer, 2µl template plasmid, 1µl of each of the forward and reverse primers and 8µl ddH_2O was made, and underwent the following thermocycling reaction: 10sec at 94°C then 5s at 50°C then 2min at 60°C, for 25 cycles. Following the sequencing reaction, any unincorporated dyes were removed using a Dye-Ex Spin Kit (Qiagen). Briefly, this involved resuspending the resin in the spin column by vortexing for 10s, snapping the end off the column and placing in a 1.5ml tube. The column was then centrifuged at 750x g for 3min. The column was transferred to a clean 1.5ml tube, and the sequencing

reaction was applied to the resin bed. The column was again centrifuged at 750x *g* for 3min. The column was discarded and the cleaned-up reaction was sequenced using an ABI Prism Sequencer (Applied Biosystems) (Sequencing of reactions was kindly carried out by the Parasitology staff, Intervet International, Boxmeer, The Netherlands).

2.2.12 Engineering of *pP30/11GFP Transfection Vector*

The plasmid *pP30/11GFP* was used as a backbone for the initial transfection experiments (Seeber & Boothroyd, 1996) and was a gift from Dr. J. Mattsson, National Veterinary Institute, Uppsala, Sweden. This plasmid contained a *T. gondii* SAG-1 promoter and a gene encoding for green fluorescent protein (GFP). A map of this plasmid is at Figure 2.3.

2.2.12.1 Preparation of Plasmid for Ligation

The GFP gene was inserted between *Nsi I* and *Pac I* restriction sites on the *pP30/11GFP*. This gene was cut out of the backbone by a sequential restriction enzyme digest using these two enzymes, according to the manufacturer's instructions (Promega). Briefly, 50µg of plasmid was mixed with 10% *Nsi I* buffer and 10 U *Nsi I* and incubated at 37°C for 4 hours. This mix was run on a 1% agarose gel (100V, 1h), and the linearised plasmid was excised and extracted from the gel using a Gel Extraction Kit (Qiagen). The purified linearised plasmid was resuspended in a total of 30µl including 5% *Pac I* buffer, 5% bovine serum albumin (BSA) and 10 U *Pac I*. This was further incubated for 4 hours, before being run on another 1% agarose gel. A fragment of ~700 bp, equating to the GFP gene, was excised, leaving the

remainder of the backbone linearised and ready for ligation with a new gene sequence, in this case *NcGRA7*.

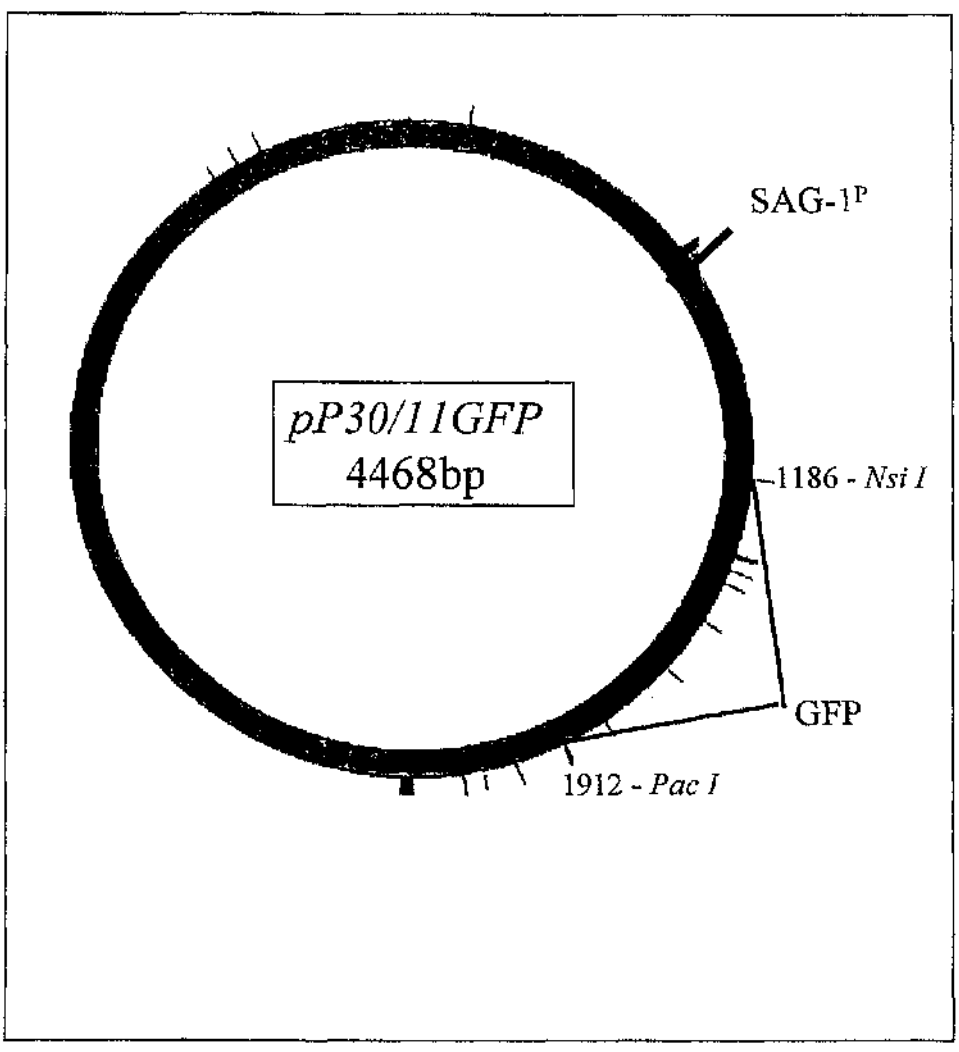


Figure 2.3 *pP30/11GFP* plasmid backbone, showing location of GFP insertion between unique *Nsi I* and *Pac I* sites, and also the location of the *T. gondii* SAG-1 promoter (SAG-1^{P})

2.2.12.2 Preparation of NcGRA7

To be compatible with ligation into the linearised plasmid described previously, the gene *NcGRA7* required the addition of an *Nsi I* site at the 5' end and a *Pac I* site at the 3' end of the sequence. This was achieved by engineering these sites into oligonucleotides specific for *NcGRA7* as described in Section 2.2.8.2. Table 2.1 shows the sequences of both NcGRA7_NsiI^{F} and NcGRA7_PacI^{R}. *NcGRA7* was then amplified by PCR.

The amplified *NcGRA7* was then inserted into a TOPO TA vector as described in Section 2.2.9 and sequenced as described in Section 2.2.11. M13 primers were used to sequence across the ligation sites to ensure that the *Nsi I* and *Pac I* sites were intact.

2.2.12.3 Ligation Reaction

The *NcGRA7* sequence containing the *Nsi I* and *Pac I* sites was inserted into the linearised vector backbone using T4 DNA Ligase. Several vector:insert ratios were used to ensure optimal ligation events occurred. A 1:1, 1:2 and 1:5 vector:insert ratio was used in mixes that also contained T4 DNA Ligase and buffer (Promega). The mixes were incubated at 4°C overnight. The ligation reactions (3µl) were used to transform One Shot®TOP10F Chemically Competent *E.coli* as described in section 2.2.9.2.

Plasmids from transformed bacteria, grown overnight in LB broth, were extracted using QIAprep Miniprep Kit as described previously. The presence of the insert in the plasmid was confirmed by restriction digest analysis. The direction of the insert within the plasmid was confirmed by sequence analysis.

2.2.12.4 PCR Confirmation of Presence of NcGRA7

Specific primers for *NcGRA7* were used to check if the ligation described above had successfully integrated *NcGRA7* into the plasmid. PCR was performed as described in Section 2.2.8.3 using the primers NcGRA7^{F} and NcGRA7^{R} and the annealing temperatures shown in Table 2.1.

2.2.12.5 Sequence Confirmation

To ensure that the *NcGRA7* had been inserted into the plasmid in the correct frame relative to the promoter, sequencing was performed as described in Section 2.2.12. Primers were designed based on the backbone of the plasmid (PlasmidSeq1^{F} and PlasmidSeq1^{R}). These can be seen in Table 2.1. The sequencing reaction covered the promoter, junctions and 5' and 3' ends of the *NcGRA7* sequence.

2.2.13 *pIntervet*

The *pIntervet* vector (Figure 2.4) was based on a *pBluescript* backbone with a *T. gondii* tubulin promoter inserted and was a kind gift from Ms N. van Poppel, Intervet International, Boxmeer, The Netherlands.

2.2.13.1 The c-myc *Epitope Tag*

A major difference in this transfection strategy was the introduction of a tagging sequence *c-myc*. The *c-myc* epitope was derived from the products of oncogenes in human cancerous tissue (Constant *et al.* 2000) and is commonly used as an antibody-recognised epitope in recombinant proteins where no monoclonal antibody is available for the original protein. The amino acid sequence and possible codons are shown in Table 2.2.

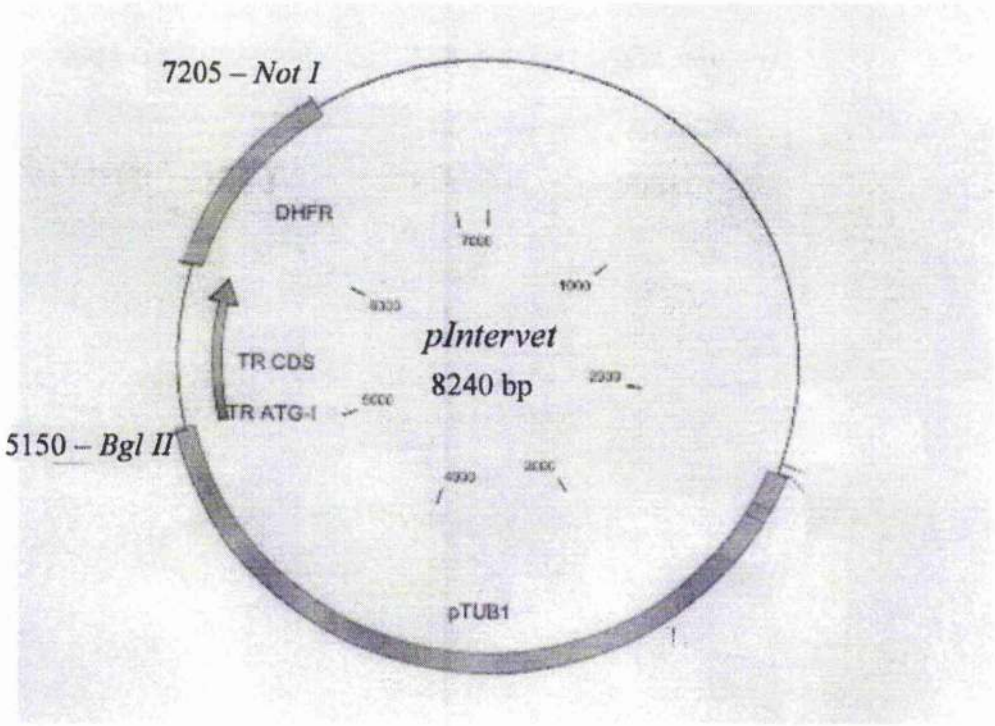


Figure 2.4 *pIntervet* backbone containing Tubulin promoter (pTUB1). The T_R insert, along with the DHFR 3'-untranslated region (UTR), were removed at the *Bgl II* and *Not I* sites prior to insertion of the *T. gondii* SAG-1 3'-UTR and *NcGRA7-myc* or *NcSRS2-myc* sequences.

Abbreviation	GLU	GLN	LYS	LEU	ILE	SER	GLU	GLU	ASP	LEU
Codon(s)	gaa (31.0) gag (38.0)	caa (12.4) cag (24.5)	aaa (19.8) aag (36.9)	uua (3.0) uug (16.0) cua (4.2) cuc (23.8) cug (23.5) cuu (14.7)	aua (3.5) auc (23.0) auu (16.8)	agc (14.2) agu (9.2) uca (7.4) ucc (12.8) ucg (14.6) ucu (13.8)	gaa (31.0) gag (38.0)	gaa (31.0) gag (38.0)	gac (34.1) gau (19.2)	uua (3.0) uug (16.0) cua (4.2) cuc (23.8) cug (23.5) cuu (14.7)
Codon Bias for *T. gondii*	***gag***	***cag***	***aag***	***cuc***	***auc***	***ucg***	***gag***	***gag***	***gac***	***cuc***

Table 2.2 Optimisation of the *c-myc* epitope sequence for expression in *T. gondii*.
Numbers in brackets indicate the prevalence of use of each codon in *T. gondii* sequences on Genbank (www.ncbi.nlm.nih.gov)

2.2.13.1.1 c-myc *Optimised for Expression in* T. gondii

Like all eukaryotic organisms, *T. gondii* DNA exhibits codon bias coding. Table 2.2 shows the amino acid sequence of the *c-myc* tag, along with all possible codons that code for that amino acid. Beside each codon is the percentage usage of that particular codon by *T. gondii*, obtained from the European Bioinformatics Institute (EBI) website (www.ebi.ac.uk/parasites/cutg.html). The *c-myc* tag designed for optimal use in *T. gondii*, taking into account the codon bias, is shown.

2.2.13.1.2 Insertion of c-myc T*ag Using Oligonucleotide Primers*

The optimised gene sequence for the *c-myc* tag was designed into the reverse primer to ensure that when the amplified gene was expressed, the *c-myc* tag was also expressed (see Figure 2.5). It was also necessary to ensure when engineering the *c-myc* epitope into the gene sequence not to insert the tag into sequences important for the function of the protein. For example, any membrane targeting sequences or cleavage sites were avoided as potential sites of integration of the *c-myc* tag.

2.2.13.1.3 Insertion of c-myc T*ag into a Gene Sequence by Ligation*

The presence of targeting sequences at the 3′ end of the amplified sequence of NcSRS2 meant that the use of engineered primers was not possible. In this case, a *c-myc* dimer was created with a restriction enzyme recognition site engineered into the 5′ and 3′ ends. This restriction enzyme would also have a recognition site once within the coding sequence of the gene of interest. The gene was cut with *Bam HI* and the *c-myc* primer dimer was ligated into this site using T4 DNA Ligase (Promega). This allowed for the insertion of a *c-myc* tag within the coding region of the gene (Figure 2.6).

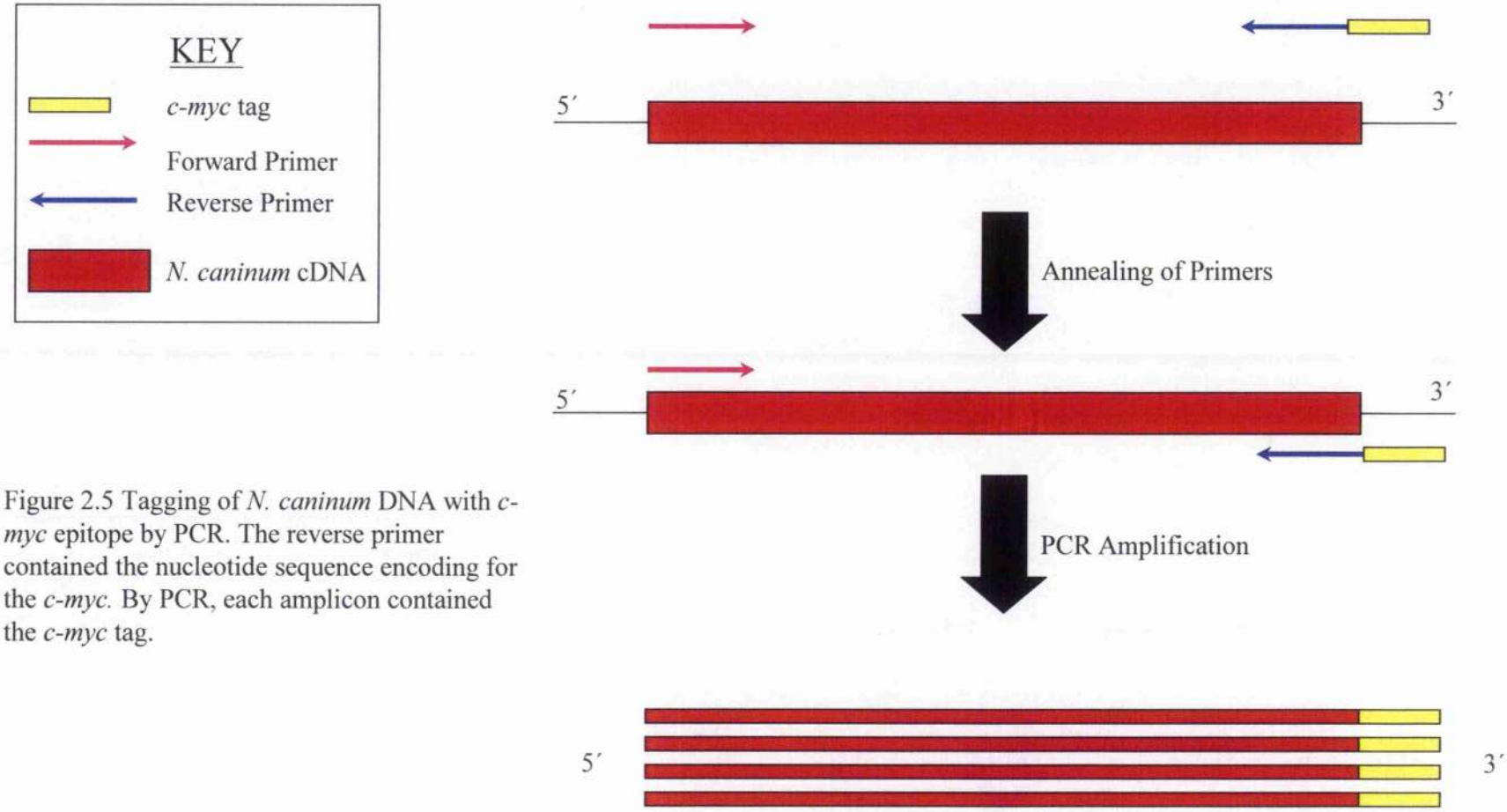


Figure 2.5 Tagging of *N. caninum* DNA with *c-myc* epitope by PCR. The reverse primer contained the nucleotide sequence encoding for the *c-myc*. By PCR, each amplicon contained the *c-myc* tag.

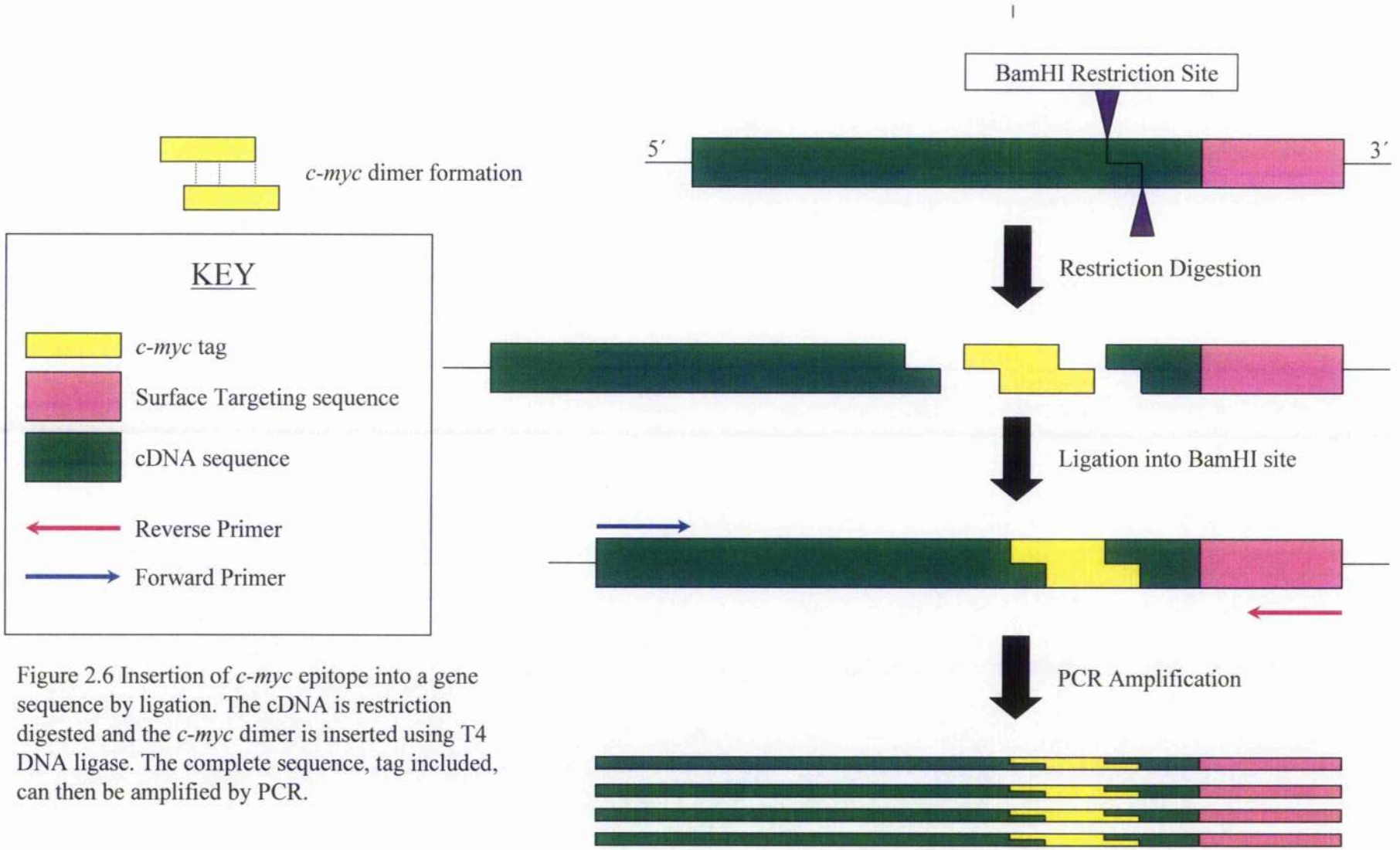


Figure 2.6 Insertion of *c-myc* epitope into a gene sequence by ligation. The cDNA is restriction digested and the *c-myc* dimer is inserted using T4 DNA ligase. The complete sequence, tag included, can then be amplified by PCR.

2.2.13.2 Engineering Transfection Vector with NcGRA7

2.2.13.2.1 Preparation of Vector Backbone

Figure 2.7 shows the strategy employed to create the transfection vectors that encoded for *NcGRA7* and *NcSRS2*. The *pIntervet* plasmid was cut at the *Bgl II* and *Not I* restriction sites indicated, using 10U of each enzyme in buffers according to the manufacturer's instructions (Promega). This removed the T_R gene and 3' untranslated region (UTR) of DHFR. The 3'-UTR of *T. gondii* SAG-1 was amplified using primers with *Bgl II* and *Not I* sites engineered into the forward and reverse primers respectively. These primers are shown in Table 2.1 (SAG1-UTR_BglII^F and SAG1-UTR_NotI^R). This was ligated as previously described in Section 2.2.12.3 into the cut linearised *pIntervet* backbone to form *pIntervet2*.

2.2.13.2.2 Preparation of NcGRA7

The *NcGRA7* coding sequence was amplified using primers containing a *Bgl II* site at both the 5' and 3' ends, as well as the coding sequence for a *c-myc* tag at the 3' end (see Section 2.2.13.1.2 above). The primers used are shown in Table 2.1 (NcGRA7_BglII^F and NcGRA7-**myc**_BglII^R). This sequence was ligated into the *pIntervet2* as described in Section 2.2.12.3. The plasmid was then used to transform *E. coli* and purified as described in Sections 2.2.9.2 and 2.2.9.3.

2.2.13.2.3 PCR Confirmation of Presence of NcGRA7

Specific primers for *NcGRA7* were used to check if the ligations described above had successfully integrated *NcGRA7* into the plasmid. PCR was performed as described in Section 2.2.8.3 using the primers NcGRA7^F and NcGRA7^R and the annealing temperatures shown in Table 2.1.

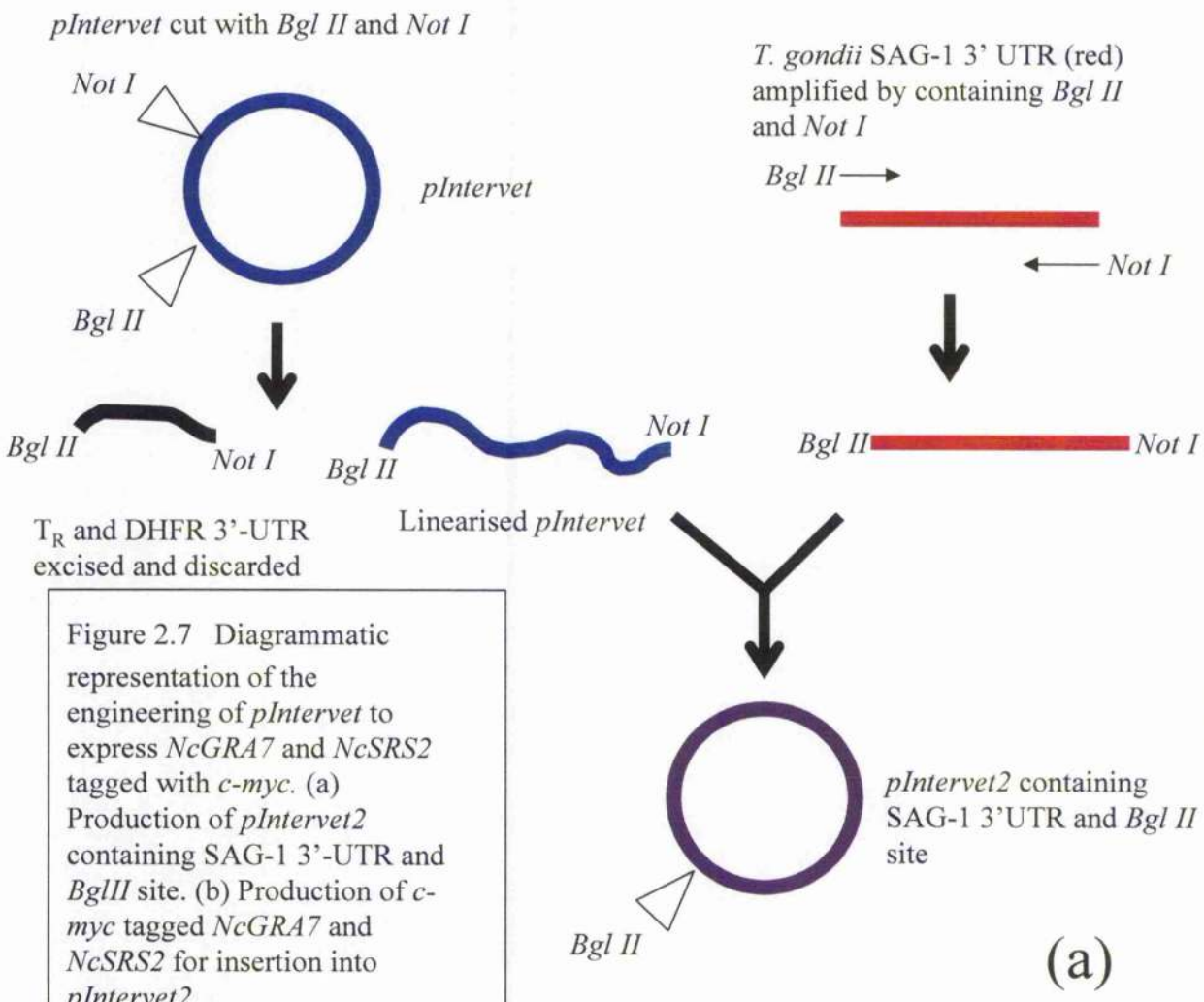
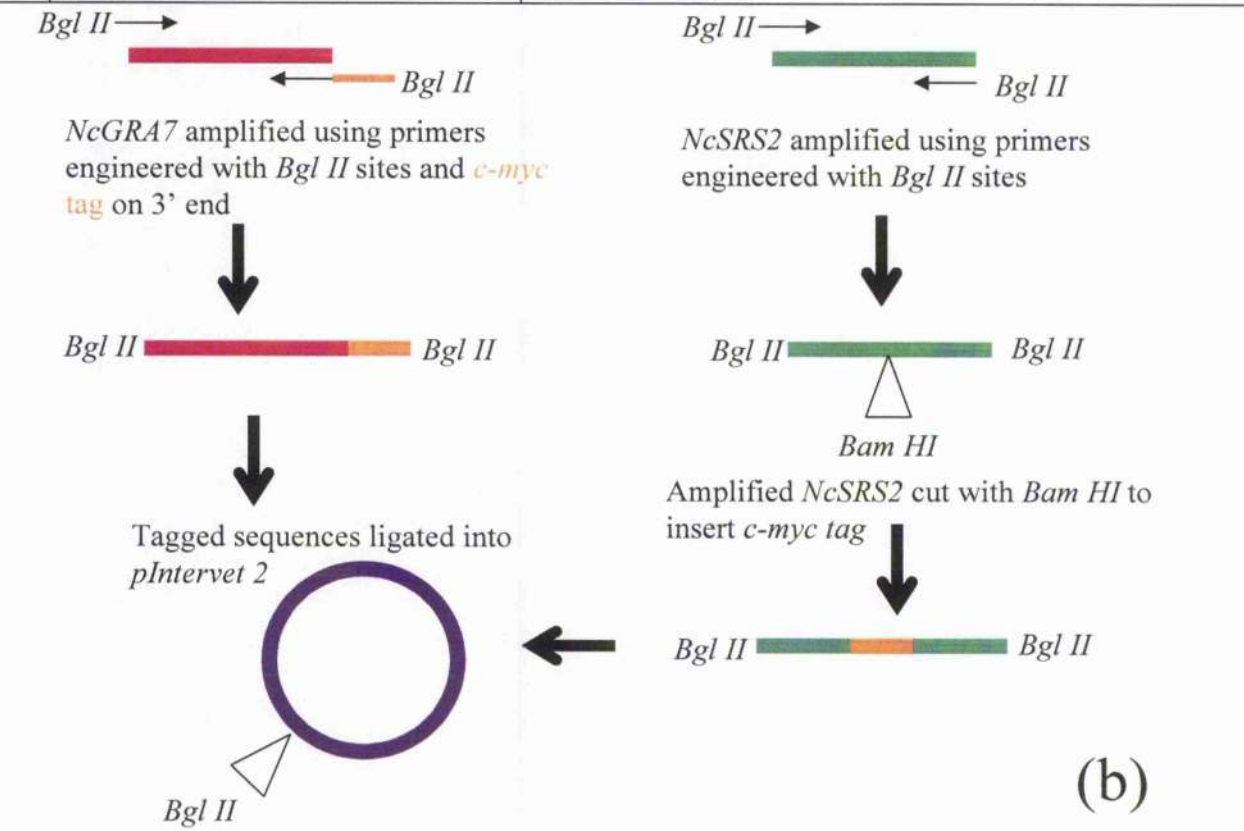


Figure 2.7 Diagrammatic representation of the engineering of *pIntervet* to express *NcGRA7* and *NcSRS2* tagged with *c-myc*. (a) Production of *pIntervet2* containing SAG-1 3'-UTR and *BglII* site. (b) Production of *c-myc* tagged *NcGRA7* and *NcSRS2* for insertion into *pIntervet2*



2.2.13.2.4 Sequence Confirmation

To ensure that the *NcGRA7* had been inserted into the plasmid in the correct orientation relative to the promoter, sequencing was performed as described in Section 2.2.11. Primers were designed based on the backbone of the plasmid (PlasmidSeq2^{F} and PlasmidSeq2^{R}). These can be seen in Table 2.1. The sequencing reaction covered the promoter, junctions and 5' and 3' ends of the *NcGRA7* sequence including the *c-myc* tag.

2.2.13.3 Engineering Transfection Vector with NcSRS2

A similar strategy was used to engineer *pIntervet* to express *NcSRS2.* The *pIntervet2* backbone was prepared as described in Section 2.2.13.2.1.

2.2.13.3.1 Preparation of NcSRS2

NcSRS2 was amplified using primers designed with *Bgl II* sites engineered at both the 5' and 3' ends, to allow ligation with *pIntervet2*. This was done using primers NcSRS2_BglII^{F} and NcSRS2_BglII^{R} (Table 2.1). The amplicon was then cut using *Bam HI*, which cut once in the sequence.

2.2.13.3.2 Insertion of c-myc *tag*

Insertion of a *c-myc* tag was performed as described in Section 2.2.13.1.3. The *c-myc* primer dimer was prepared by mixing 1 µl of each of the **myc**_BamHI^{F} and **myc**_BamHI^{R} primers (100mM solutions). The primer sequences are shown in Table 2.1. The primer dimer was ligated into the *Bam HI* site within the *NcSRS2* as described in Section 2.2.12.3.

2.2.13.3.3 Ligation of NcSRS2 *into* pIntervet2

Ligation of the *NcSRS2* containing the *c-myc* tag into the *pIntervet2* backbone was carried out as described in Section 2.2.12.3.

2.2.13.3.4 PCR Confirmation of Presence of NcSRS2

Specific primers for *NcSRS2* were used to check if the ligations described above had successfully integrated *NcSRS2* into the plasmid. PCR was performed as described in Section 2.2.8.3 using the primers $NcSRS2^F$ and $NcSRS2^R$ and the annealing temperatures shown in Table 2.1.

2.2.13.3.5 Sequence Confirmation

To ensure that the *NcSRS2* had been inserted into the plasmid in the correct frame and orientation relative to the promoter, sequencing of the *NcSRS2* within the plasmid construct was performed as described in Section 2.2.11. Primers were designed based on the backbone of the plasmid ($PlasmidSeq2^F$ and $PlasmidSeq2^R$). These can be seen in Table 2.1.

Sequencing was also carried out using the specific *NcSRS2* primers $NcSRS2^F$ and $NcSRS2^R$ to indicate whether the *c-myc* tag had been inserted within the *NcSRS2* sequence in the correct alignment.

2.2.14 Selection Vector

Transfected parasites contained both the vector containing the gene of interest as well as the positive selection vector *pMINI*. *pMINI* contained a functional copy of the *T. gondii* HXGPRT gene which was knocked out of the parasites used in

transfection (J. Mattsson, pers. commun.). The gene was under the control of the *T. gondii* dihydrofolate reductase (DHFR) promoter (see Figure 2.8)

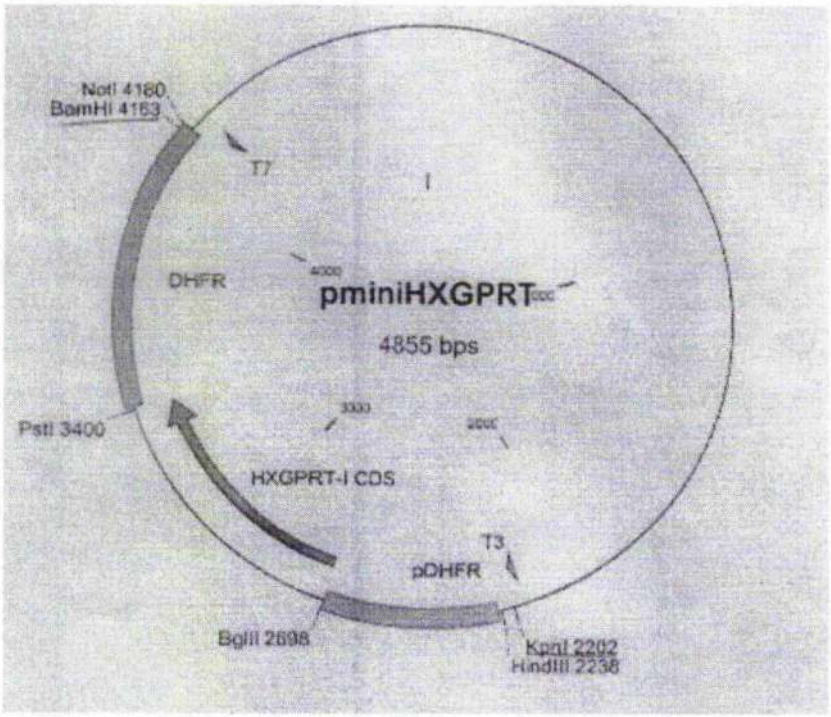


Figure 2.8 *pMINI* plasmid containing a functional copy of *T. gondii* HXGPRT under the control of *T. gondii* DHFR promoter

2.2.15 Positive Control Vector

To check the efficiency of co-transfection, transfection was performed using a plasmid encoding for *E. coli* lacZ, under the control of *T. gondii* tubulin promoter (*pTUBlacZ*) (Figure 2.9). In the presence of *pTUBlacZ*, X-Gal is metabolised into a blue precipitate (see below).

2.2.16 Transfection Strategy

2.2.16.1 Co-Transfection with pMINI

A co-transfection strategy was performed whereby transfection vectors were engineered to contain heterologous *N. caninum* genes (*NcGRA7* and *NcSRS2*). *T. gondii* was transfected with two plasmids – the transfection vector and the selection vector (*pMINI*). The selection vector *pMINI* contained a functional *T. gondii* hypoxanthine-xanthine guanine phosphoribosyltransferase (HXGPRT) gene. This was used to select parasites that had been successfully transfected, since those that did not possess this plasmid were not able to survive the selection process (section 2.2.16.4).

2.2.16.2 Preparation of Plasmids Prior to Transfection

Prior to transfection of *T. gondii*, the 2 plasmids – transfection vector (or control vector) and selection plasmid (*pMINI*) were digested by *Bam III* or *Not I* to linearise the vector. The plasmids were incubated at 37°C for 2-3h and an aliquot run on an agarose gel to check that digestion had occurred completely.

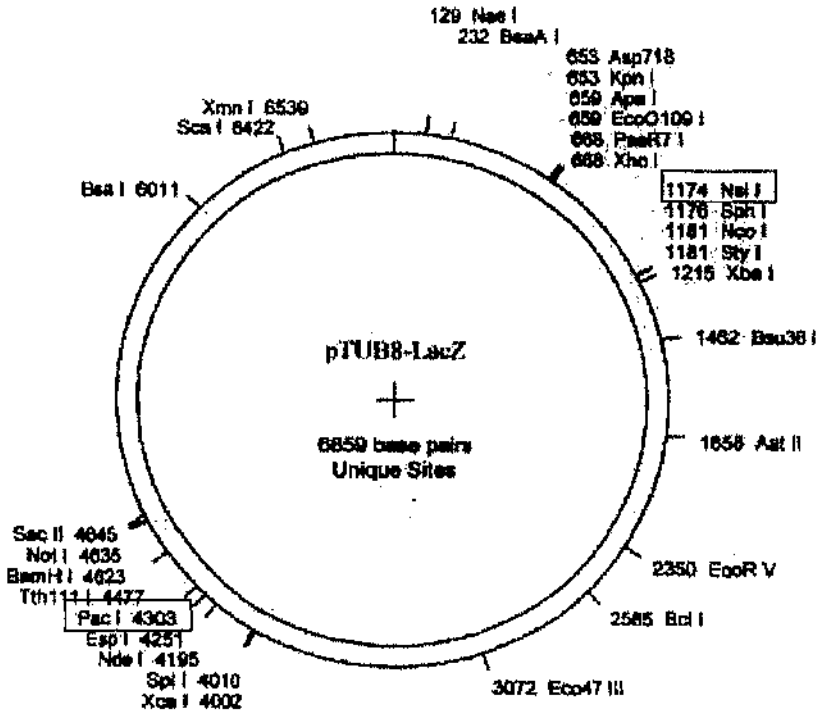


Figure 2.9 *pTUBlacZ* plasmid. *E. coli lac Z* under the control of *T. gondii* tubulin promoter. *E. coli lacZ* was inserted between the *Nsi I* and *Pac I* sites indicated

The linearised plasmids were then precipitated using ice-cold ethanol. This has the advantage of reducing the volumes of each preparation needed to acquire the appropriate ratio of plasmids. A ratio of 5:1 transfection vector : *pMINI* was used to ensure co-transfection occurred. The plasmids were mixed in the following ratio:

1x plasmid mix : 0.1x 2M sodium acetate : 2.5x 90% ethanol (ice-cold)

The mix was centrifuged at 13000x *g* for 10 min at 4°C. The supernatant was removed and 500µl ice-cold 80% ethanol was added. This was centrifuged again at 13000x *g* for 2 min. The supernatant was removed and the pellet of plasmids allowed to air dry in a laminar flow hood. The pellet was resuspended in 50µl of cytomix (120mM KCl, 0.15mM $CaCl_2$, 10mM K_2HPO_4/KH_2PO_4, 25mM Hepes pH7.6, 2mM EDTA pH 7.6, 5mM $MgCl_2$, adjusted to pH 7.6 with 1M KOH) supplemented with 1mM ATP and 3mM glutathione prior to transfection.

2.2.16.3 Transfection

PRUΔHX *T. gondii* tachyzoites (1×10^8), newly lysed out of host cells in culture, were harvested by centrifugation at 5000x *g* for 8 min. The pellet was resuspended in 4ml cytomix (see above) and centrifuged at 4000x *g* for 5 min at 4°C. The pellet of parasites was resuspended in 3ml cytomix supplemented with 1mM ATP and 3mM reduced glutathione.

For each transformation, 700µl of resuspended parasites was used. *Bam III* (100U) were added to the suspension of parasites. The parasite suspension was added to the ethanol-precipitated transfection vector or the control transfection vector containing

E. coli lacZ and selection vector *pMINI*. This mix was electroporated with 2.0kV, 50Ω, 25μF using a Gene Pulser II (Biorad). The electroporated parasites were used to infect confluent HFF cells. The infected cells were incubated at 37°C for 9h prior to selection.

2.2.16.4 Selection Medium

Positive selection of parasites that contained the selection vector was carried out in the presence of 25μg/ml mycophenolic acid (MPA) and 25mg/ml xanthine (Xan) (both Sigma-Aldrich, Poole, UK). The presence of these chemicals killed any parasite that did not contain the HXGPRT gene, found on the selection vector. Parasites that remained viable were cloned using limited dilution.

2.2.16.5 Cloning by Limited Dilution

Briefly, extracellular tachyzoites were serially diluted in a 96-well plate, on which a confluent HFF monolayer was growing. The plates were left untouched for 5 days to ensure that each parasite created only one plaque of growth. Wells were visually scored for the presence or absence of growth plaques. The contents of wells that contained only one plaque of growth were transferred to a well of a 24-well plate containing confluent HFF cells, and from there to a 50cm^2 tissue culture flask. Culturing of the cells and parasites was then carried out as previously described.

The cloned transfected parasites were passaged as normal using MPA- and Xan-supplemented IMDM with 10% FCS for up to ten rounds of passage. This was to ensure stable transformation of the parasites through several generations.

2.2.17 X-Gal Staining

Parasites that were transfected successfully with *pMINI/pTUBLacZ* remained alive in the stringent selection of MPA/Xan-supplemented growth medium. Tachyzoites containing expressed *lacZ* metabolise X-Gal, generating a blue precipitate. Those parasites that only took up the *pMINI* survived in selection while not changing colour.

The monolayer of HFFs containing intracellular tachyzoites was washed once with PBS, before being fixed for 10 min at room temperature in fixative solution (2% formaldehyde, 0.2% EM-grade glutaraldehyde, 2mM $MgCl_2$, 0.02% Triton X-100, 0.04% deoxycholate (DOC) in PBS). The monolayer was then washed with staining solution (100mM Na_2PO_4, 1.3mM $MgCl_2$, 3mM $K_4(FeCN)_6$, 3mM $K_3(FeCN)_6$, 0.02% Triton X-100, 0.04% DOC) without X-Gal, which was added at a 1:75 dilution of a 20mg/ml stock solution. The cells were then visualised under an inverted microscope using 400x magnification (Axiovert).

2.2.18 PCR Confirmation of Transfection

Specific PCR was carried out as described previously to confirm the presence of the heterologous sequence using primers $NcGRA7^F$, $NcGRA7^R$, $NcSRS2^F$ and $NcSRS2^R$ (Table 2.1). The amplification of NcGRA7 using $NcGRA7^F$ and $NcGRA7^R$ was performed using 30 rounds of PCR, with an annealing temperature of 50°C, while the specific amplification of NcSRS2 used an annealing temperature of 55-60°C.

2.2.19 Southern Blot Hybridisation

2.2.19.1 Southern Blotting

Southern Blot was used to transfer DNA from an agarose gel to a nylon membrane (Sambrook *et al*, 1989). Before blotting, the agarose gel was submerged in Denaturation Solution (0.5N NaOH, 1.5M NaCl) for 45 min. The gel was transferred to Neutralisation Solution (1M Tris-HCl pH 7.4, 1.5M NaCl) for 30 min at room temperature. The gel was then placed in the blotting system as described. Briefly, the gel was placed on top of a 3M filter paper wick (Whatman) that was soaked in 20x SSC transfer buffer (3M sodium chloride, 300mM sodium citrate). A nylon membrane (Hybond) was placed covering the gel with a sheet of 3M filter paper on top. A pile of paper was added to the stack, this was to help the buffer pass through the gel, membrane and filter paper by capillary action. A glass plate with a weight on top completed the Southern Blot apparatus. This was left overnight to ensure complete transfer of the DNA from the gel to the nylon membrane. The agarose gel was examined under UV to confirm the transfer. The DNA was cross-linked onto the nylon membrane using a Spectrolinker XL-1000 UV Cross-linker (Spectronics Corp., Nebraska, USA).

2.2.19.2 Labelling of DNA Probe

To identify specific DNA sequences blotted onto nylon, probes were made that were homologous to the sequence. These were derived from the transfection plasmids, or were amplified from cDNA by PCR using primers NcGRA7^{F}, NcGRA7^{R}, NcSRS2^{F} and NcSRS2^{R} (Table 2.1) situated at the 5' and 3' ends of the respective sequences. They were labelled with [α-^{32}P]-dATP using Prime-It II Random Primer Labelling Kit (Stratagene) which uses Klenow Enzyme with no exonuclease activity to synthesise probes which contain the radionucleotide. A mixture of 25ng of DNA

template, 10µl random oligonucleotide primer and ddH_2O was heated in a boiling water bath for 5min. After a pulse centrifuge, 10µl of primer buffer (containing dCTP, dGTP and dTTP), 5µl of [α-^{32}P]-dATP and 1µl Exo(-) Klenow enzyme were added, the reagents mixed and then incubated at 37°C for 2-10 min. Finally, Stop Reagent (2µl) was added.

To remove any unincorporated radionucleotides, and therefore reduce potential background, the radiolabelling reaction was passed through a Microspin G-25 Column (Amersham Biosciences). The resin in the column was resuspended by vortexing, before the base was removed and the whole unit was placed in a clean 1.5ml tube. The column was pre-spun with no sample at 735 x *g* for 1min. The column was removed to a new 1.5ml tube, and the sample was added slowly to the middle of the resin bed. The column was centrifuged at 735 x *g* for 2min. The purified probe was collected in the bottom tube.

2.2.19.3 Hybridisation of Radiolabelled Probe to Membrane

Prior to the addition of radiolabelled probe, the membrane was incubated for a minimum of 2h at 55°C in Prehybridisation Solution (4x SSC, 0.05% SDS, 5x Denhart's Reagent (from a 50x stock) and 40µg/ml salmon sperm DNA (denatured by heating at 100°C for 10 min). Denhart's Reagent (50x) was made by dissolving 5g Ficoll 400, 5g polyvinylpyrrolidone and 5g BSA in 500ml ddH_2O.

The radio-labelled probe was added to the Prehybridisation Solution and incubated with the membrane overnight at 55°C. After this, the membranes were removed from the probe and incubated with Wash Buffer A (4x SSC, 0.1% SDS) at 50°C for 30

min, with one buffer change, then for 30 min at room temperature with Wash Buffer B (0.1x SSC, 0.1% SDS), again with one change of buffer. These washes removed any probe that had not been hybridised to the DNA on the membrane. The membranes were exposed to X-Ray film.

Membranes were stripped of any hybridised radio labelled probe by soaking them in a boiling solution of 0.5% SDS in ddH_2O until the solution cold. This was repeated a further 3 times. To ensure that the radio labelled probe had been removed, the stripped membrane was exposed to X-Ray film. Any hybridisation observed by a discolouring of the X-Ray film after exposure meant that the membrane was not fully stripped and was washed a further 2-3 times in boiling SDS solution, and exposure was repeated until the film remained clear on exposure.

2.2.20 Western Blotting

For the Western Blotting of parasites, tachyzoites were harvested and washed as described in section 2.2.5. Pellets of parasites ($1x10^8$) were resuspended in 60µl of 2x SDS Sample Buffer (100mM Tris.Cl (pH to 6.8), 200mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol). The samples, along with Broad Range Molecular Weight Marker (Promega), were boiled for 10min and then run on a 15% discontinuous polyacrylamide gel as described (Sambrook *et. al.*, 1989). This comprised 30% acrylamide/bis-acrylamide, 1.5M Tris-HCl (pH 8.8), 1% SDS, 1% ammonium persulfate and 0.05% TEMED. The samples were run on Protean II SDS-PAGE equipment (Biorad) at 120V for 1½h. Prior to blotting, the gels were equilibrated in 1x transfer buffer (10% stock transfer buffer (30.36g Tris/144g glycine in 1l ddH_2O)/20% methanol/70% ddH_2O) for 20min at room temperature on a rocking table. Blotting was carried out in a Protean II Western Blot system (Biorad)

according to manufacturer's instructions. The blotting cassette was set up according to manufacturer's instructions. The cassette was soaked in transfer buffer, before being placed in the electrophoresis tank along with a block of ice. The apparatus was placed on a stirring table and electrophoresed at 120V for 1h.

To check the transfer of the proteins, the membranes were stained with Ponceau S (0.5% solution of Ponceau S powder in 1% acetic acid), a non-permanent stain of proteins. The membranes were incubated overnight at 4°C on a rocking table in Blocking Solution. The Blocking Solution used was 5% dry milk dissolved in TBS-T (25mM Tris.Cl, pH 7.4 in 8g/l NaCl, 0.2g/l KCl, 0.1% Tween-20). The membranes were washed in 2 x 5min washes of TBS-T

2.2.20.1 Polyclonal Antisera

Polyclonal antiserum was obtained against *NcGRA7*. This was a kind gift from Dr S. Liddell, USDA, USA. The polyclonal antiserum was diluted 1:250 and used as a primary antibody to probe the proteins blotted onto nitrocellulose. The membrane was incubated in the primary antibody for 3 h at room temperature. The membrane was washed 3 times in TBS-T for 5 minutes. A secondary antibody (swine anti-rabbit IgG) labelled with horseradish peroxidase and diluted 1:1000 was incubated with the membrane for a further 2 h at room temperature.

Antibody binding was detected using an ECL™ Detection Kit (Amersham Life Sciences). An equal volume of Detection Reagent 1 (0.5-1% boric acid, 0.1-0.5% NaOH, <0.1% sodium perborate trihydrate, <0.1% kathon CG) and Detection Reagent 2 (0.5-1% boric acid, 0.1-0.5% NaOH, <0.1% 5-amino-2,3-dihydro-1,4-phthalazinedione free acid, <0.1% dimethyl sulphoxide, <0.1% (E)-3(4-hydroxy

phenyl)-2-propenoic acid, <0.1% kathon CG) were mixed in the dark. The membrane was covered with this mix and incubated at room temperature in the dark for a maximum of 2 min before the mix was removed and excess blotted off. The membrane was wrapped in Saran wrap and exposed to X-Ray film for 30 seconds, 1 min, 5 min and 10 min.

2.2.20.2 Antibodies Against c-myc *Tag*

The membranes were probed with a primary monoclonal antibody raised in mice against the c-myc epitope, which was engineered into the coding sequence of *NcGRA7* and *NcSRS2* in the *pIntervet* plasmids. The anti-*c-myc* monoclonal antibody clone 9E10 (Sigma-Aldrich) was diluted 1 in 1000 in TBS-T. The membranes were incubated in the primary antibody solution for 2-3h at room temperature before being washed 3 times in TBS-T for 5 min each wash. They were then incubated in a 1:2000 dilution of HRP-labelled anti-mouse IgG1 (Diagnostic Scotland, Carluke, UK) for 2 at room temperature before being washed a further three times in TBS-T as above.

Antibody binding was detected using ECL as described in Section 2.2.20.1.

2.2.21 Immuno-Fluorescent Antibody Test (IFAT)

2.2.21.1 Preparation of Slides

Tachyzoites ($1x10^6$) were pelleted by centrifugation at 1500x *g* for 10 min. The pellets were washed twice in PBS before being resuspended in 50µl of PBS. The parasites suspension (10µl) were loaded onto a microscope slide and allowed to air-dry in a laminar flow hood, before being fixed for 15 min in acetone. The slides were then stored at -20°C until required.

2.2.21.2 Incubation of Slides

Tachyzoite slides were immersed in 2% formaldehyde in PBS overnight at 4°C, before being washed by immersion in PBS to remove the formaldehyde and to rehydrate samples. The slides were placed in blocking buffer (0.01% Tween-20, 0.05% sodium azide, 1% BSA in PBS) for 20 min at room temperature. The blocking buffer was removed after this time and replaced with a 1:1000 dilution of anti-c-myc monoclonal antibody in Immunobuffer (0.1M Tris.Cl pH7.4, 2.3M NaCl, 0.01% Tween-20, 1% BSA, 0.05% sodium azide). The slides were incubated in the primary antibody dilution for 30-60 min at 37°C in a moisture chamber to ensure the slides did not dry out. The primary antibody was removed by pipetting and the slide was washed twice by dipping in PBS. A 1:400 dilution of FITC-labelled anti-mouse IgG1 was made in Immunobuffer and the slides were incubated in a dark moisture chamber at 37°C for 30 min. The slides were then washed as above. A drop of Vectashield (Vector Laboratories Inc., California, USA) was placed on the slide as a fluorescence enhancer and the slides were visualised using a fluorescent microscope with a FITC filter.

2.3 RESULTS

2.3.1 Transfection 1: *pP30/11GFP* Expressing *NcGRA7*

2.3.1.1 Preparation of Plasmid Backbone

The plasmid *pP30/11GFP* contained a GFP gene sequence under the control of the *T. gondii P30* (SAG-1) promoter. The GFP gene was inserted between an *Nsi I* site and a *Pac I* site as shown in Figure 2.3. It was necessary to remove this GFP sequence, approximately 700bp in length, from the plasmid backbone so that the *N. caninum NcGRA7* gene could be inserted. The plasmid was cut using *Nsi I* and *Pac I* restriction enzymes as described in Section 2.2.12.1.

Figure 2.10 shows the results of the digest described above. A fragment of ~700bp was excised from the plasmid backbone. This was the expected size of the GFP gene and suggests that the restriction digest excised the correct fragment from the plasmid backbone. The linearised plasmid backbone without the GFP gene and with *Nsi I* and *Pac I* sites at the 5' and 3' ends respectively was gel purified (Qiagen) and used in subsequent ligation experiments to create a plasmid expressing *NcGRA7*.

2.3.1.2 Preparation of NcGRA7

NcGRA7 was amplified from cDNA of *N. caninum* using primers NcGRA7_NsiI^{F} and NcGRA7_PacI^{R} (as shown in Table 2.1). These primers had an *Nsi I* site engineered into the forward primer and a *Pac I* site engineered into the reverse primer. When the sequence was amplified (as shown in Figure 2.11), the *NcGRA7* sequence thus contained these sites at the 5' and 3' ends. To ensure that the PCR reaction had left these restriction enzyme sites intact, the amplicon was sub-cloned into a TOPO TA cloning vector as described in Section 2.2.9, prior to being sequenced as described in Section 2.2.11.

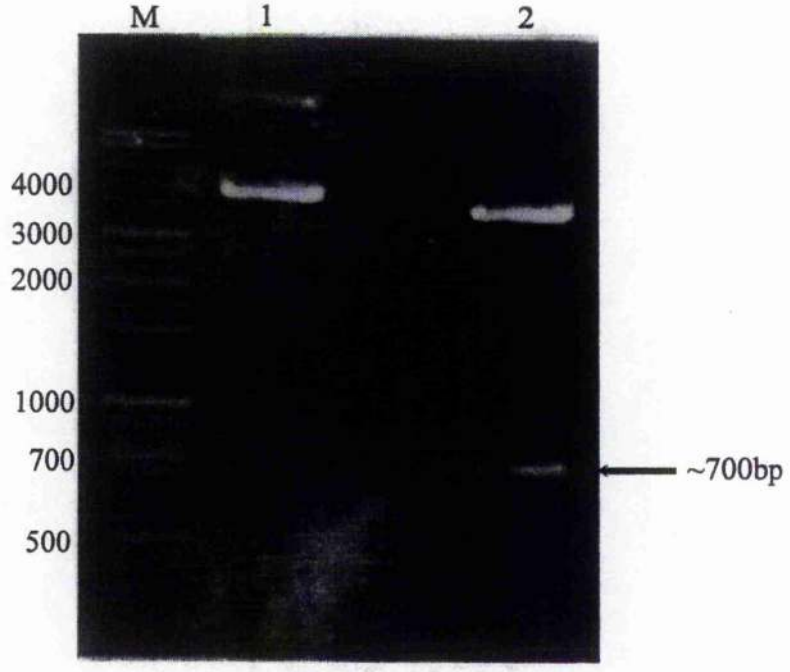


Figure 2.10 Sequential restriction digest of *pP30/11GFP* using *Nsi I* and *Pac I* to remove GFP (~700 bp). Lane M is a molecular weight marker, lane 1 shows linearised *pP30/11GFP* (cut only with *Nsi I*), lane 2 show *pP30/11GFP* cut with *Nsi I* and *Pac I*. A fragment approximately 700 bp has been excised from the plasmid (as indicated by the arrow) and the reduced size of the plasmid backbone.

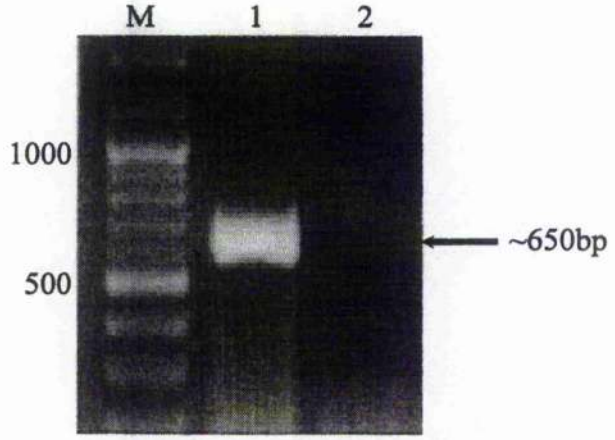


Figure 2.11 PCR amplification of *NcGRA7* from cDNA of *N. caninum* using NcGRA7_Nsi^{F} and NcGRA7_PacI^{R} primers. Lane M is a molecular weight marker, lane 1 contained the *N. caninum* cDNA as template, lane 2 replaced the DNA template with distilled water, as negative control. A band of ~650 bp has been amplified, which is theexpected size for *NcGRA7*.

Figure 2.12 shows the results of a restriction digest of the TOPO TA cloning vector that contained the *NcGRA7* insert. The plasmid was cut using *Eco RI* to cleave a fragment of ~700bp, which was the expected size of the *NcGRA7* with the restriction sites and fragments of the cloning vector between the insert and the *Eco RI* sites. Figure 2.13 shows an alignment of the sequencing results using the sequencing primers TOPOSeq^{F} and TOPOSeq^{R}, compared to the sequence obtained from Genbank for *NcGRA7*. The *EcoRI* sites initially used to cleave the ~700bp fragment, the intact *Nsi I* and *Pac I* sites should be noted. The sequencing confirmed that the gene sequence was that of *NcGRA7*. The polymorphisms observed upstream of the *NcGRA7* may be the result of poor sequencing reaction, rather than actual polymorphisms.

2.3.1.3 Production of Transfection Vector

The *NcGRA7* sequence containing *Nsi I* and *Pac I* sites was cut out of the TOPO TA cloning vector and ligated into the linearised plasmid backbone as described in Section 2.2.12.3. The plasmids were then used to transform *E. coli* as described in Section 2.2.9.2 and purified. PCR was carried out using primers NcGRA7^{F} and NcGRA7^{R} (Table 2.1) to confirm the presence of the *NcGRA7* gene. Figure 2.14 shows a 1% agarose gel of the PCR product, stained with ethidium bromide and visualised under UV. A fragment of ~650bp can be seen, which is the expected size of the *NcGRA7* gene as described in Genbank.

The transfection vector was sequenced to ensure that the inserted *NcGRA7* gene was in the correct frame relative to the P30 promoter. Primers PlasmidSeq1^{F} and PlasmidSeq1^{R} (Table 2.1) were used. Figure 2.15 shows an alignment between the sequenced plasmid and the expected boundary between promoter and gene sequence.

Figure 2.15 shows that *NcGRA7* was in the correct orientation and frame with the promoter.

This transfection vector *(pP30/NcGRA7)* was subsequently used for transfection PRUΔHX *T. gondii* tachyzoites, as described below.

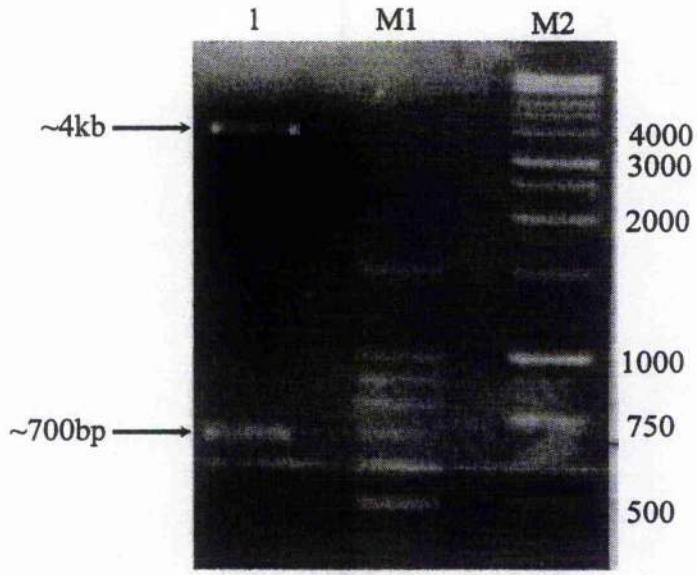


Figure 2.12 *Eco RI* digestion of TOPO TA cloning vector containg *NcGRA7*. *Eco RI* sites were located up- and downstream of the insertion site, and when used to digest the plasmid released the insert plus fragments of the TOPO vector. *NcGRA7* is ~650bp which, when added to the vector fragments, should produce an excised fragment of approximately 700bp, as seen in lane 1. The plasmid backbone of ~4kb is also observed. M1 and M2 are molecular weight markers.

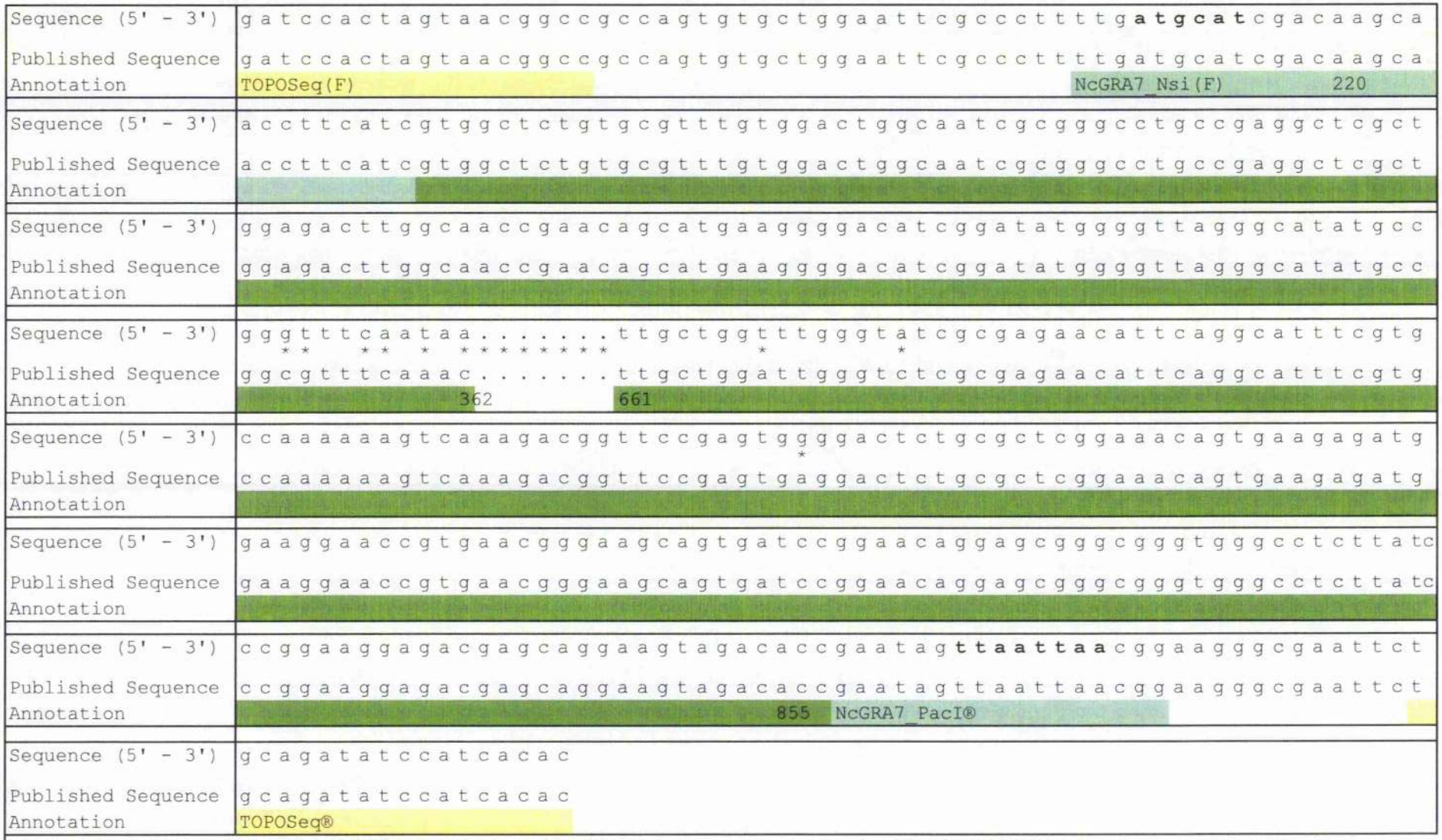


Figure 2.13 Sequence Alignment of TOPO TA cloning of *NcGRA7* gene sequence (Accession Number U82229)
The sequencing primer sequences (cream), the restriction sites (bold) and NcGRA7 sequence (green) are highlighted.
engineered with Nsi I and Pac I restriction sites at the 5' and 3' ends. Single nucleotide polymorphisms are marked *.

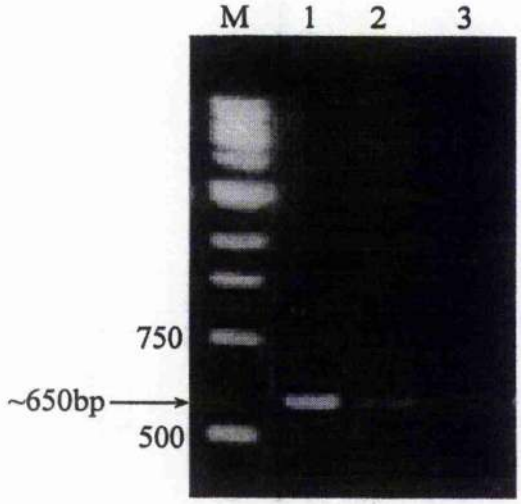


Figure 2.14 PCR amplification of *NcGRA7* from *pP30/NcGRA7* using primers $NcGRA7^{F}$ and $NcGRA7^{R}$. Lane M is a molecular weight marker, lane 1 contains the plasmid *pP30/NcGRA7* as template DNA, lane 2 contains *N. caninum* cDNA as template, and lane 3 contains no template. A PRC product of ~650bp is amplified from both *pP30/NcGRA7* and *N. caninum* cDNA.

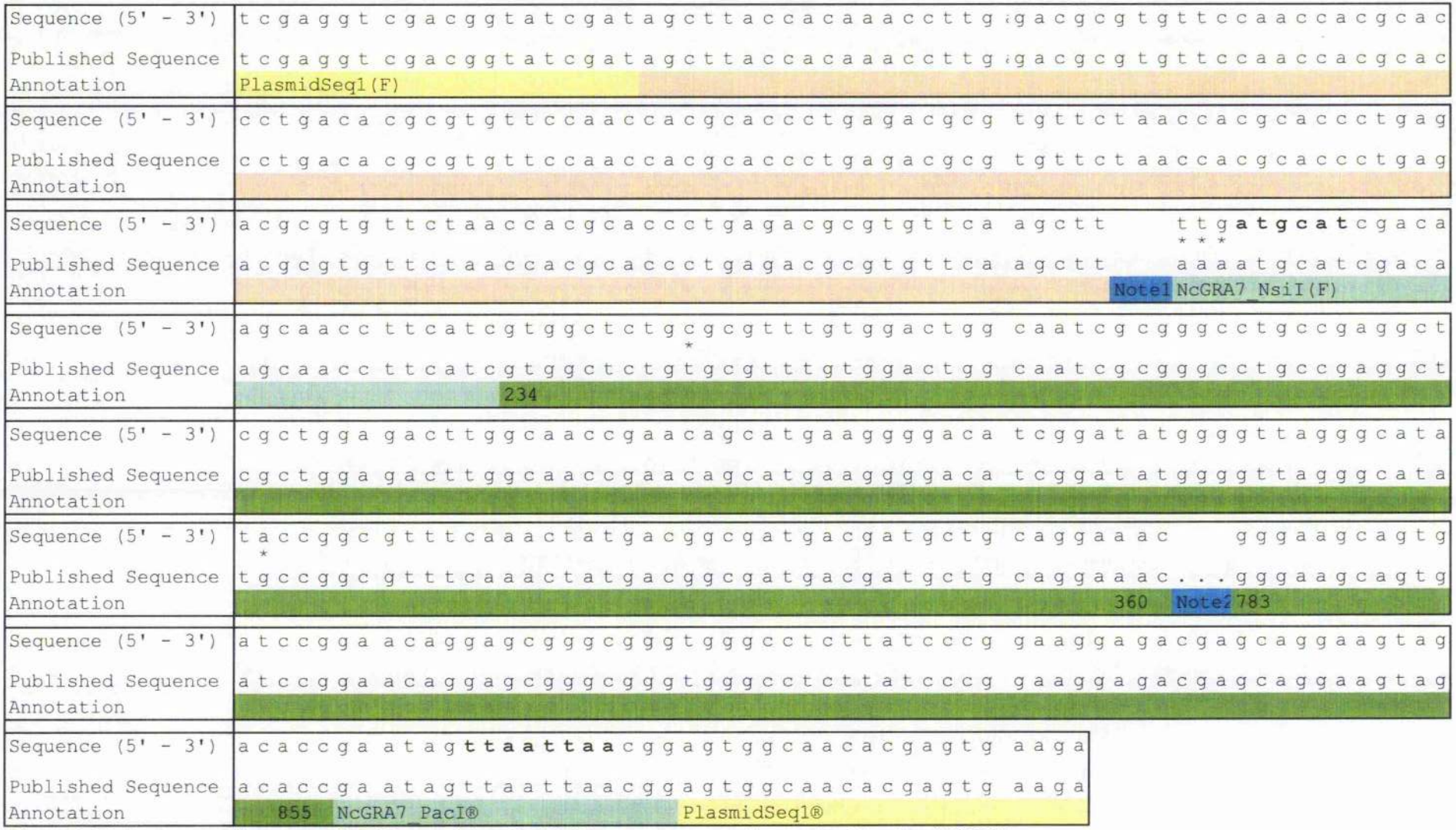


Figure 2.15 Sequence Alignment of pP30 containing *NcGRA7* gene sequence (Accession Number U82229) engineered with Nsi I and Pac I restriction sites (bold) at the 5' and 3' ends.
Primers are shown in cream (sequencing) and light green (specific). Promoter is brown; gene sequence green
Note 1: Plasmid backbone sequence not shown. Note 2: middle of NcGRA7 also sequenced (not shown)
Single nucleotide polymorphisms indicated by *

2.3.2 Transfection of *T. gondii* Tachyzoites

2.3.2.1 Transfection Experiment 1

Transfection of *T. gondii* tachyzoites was carried out as described in Section 2.2.16. This was a co-transfection with *pP30/NcGRA7* and the *pMINI* plasmid containing the active HXGPRT gene. The transfected *T. gondii* were passaged a minimum of 10 times in selection medium for positive selection for the presence of a functional HXGPRT gene. Parasites were then cloned by limited dilution.

2.3.2.1.1 PCR

Initial PCR experiments were carried out on genomic DNA from populations of the transfected *T. gondii* as described in Section 2.2.18. NcGRA7^{F} and NcGRA7^{R} primers were used to detect specifically for the presence of *NcGRA7*. Figure 2.16 shows a 1% agarose gel of the results of this initial PCR. A fragment of ~650bp can be seen, and can be assumed to be *NcGRA7*, since a fragment of the same size was not amplified from gDNA of untransfected tachyzoites of *T. gondii*, but can be seen amplified from the gDNA of *N. caninum* tachyzoites.

However, following cloning by limited dilution, subsequent PCR analysis indicated that the clones no longer contained the *NcGRA7* gene. Transfection Experiment 1 was thus successful in producing transiently transfected parasites, but unsuccessful in producing stable transfectants. A second transfection experiment was therefore attempted using a different approach described below.

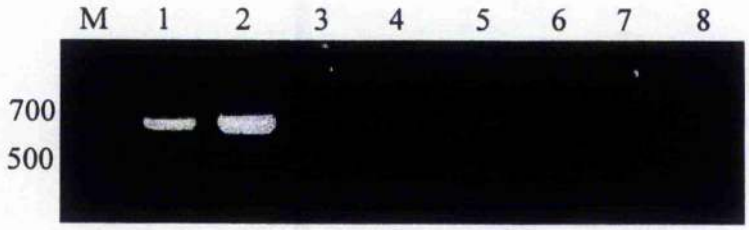


Figure 2.16 PCR amplification using $NcGRA7^F$ and $NcGRA7^R$ primers. Lanes 1-5 contain five different preparations of DNA from populations of transfected *T. gondii*. Lane 6 contains gDNA from untransfected *T. gondii* and lane 7 contains gDNA from *N. caninum*. Lane 8 contains no DNA as template. A fragment of ~650bp can be seen in lanes 1 and 2, and correlates with that fragment observed in lane 7. No fragment has been amplified from untransfected *T. gondii* gDNA.

2.3.2.2 Transfection Experiment 2: pIntervet Expressing NcGRA7 and NcSRS2

2.3.2.2.1 Preparation of Plasmid Backbone

The *pIntevet* plasmid was the basis for a new transfection vector containing a *T. gondii* TUB-1 promoter. The T_R gene and 3'-UTR of DHFR were excised at the flanking *Bgl II* and *Not I* restriction enzyme cutting sites (see Figure 2.4). Figure 2.17 shows a 1% agarose gel separation of the linearised *pIntervet* backbone and an excised fragment of approximately 2kb which equated to the T_R gene sequence and the DHFR 3'-UTR.

The 3'-UTR of *T. gondii* SAG-1 was amplified using primers containing *Bgl II* and *Not I* sites (SAG1-UTR_BglII^F and SAG1-UTR_NotI^R as shown in Table 2.1). The expected size of this is 327bp, and this can be seen in Figure 2.18. This is a picture of the PCR product using these primers, run on a 1% agarose gel and visualised under UV. This fragment was ligated into the *pIntervet* backbone. This backbone (*pIntervet2*) was used in subsequent ligation reactions as described below.

2.3.2.2.2 Preparation and Characterisation of NcGRA7-myc *Transfection Vector*

2.3.2.2.2.1 Preparation of *NcGRA7* tagged with c-myc

NcGRA7 containing the *c-myc* epitope was obtained using the primers NcGRA7_BglII^F and NcGRA7-**myc**_BglII^R (Table 2.1). When amplified, the *NcGRA7* sequence would have the *c-myc* tag at the 3' end. Figure 2.19 shows the PCR product using these primers and *N. caninum* cDNA as template DNA. A fragment of ~650bp has been amplified, and this correlated with the expected size of *NcGRA7*.

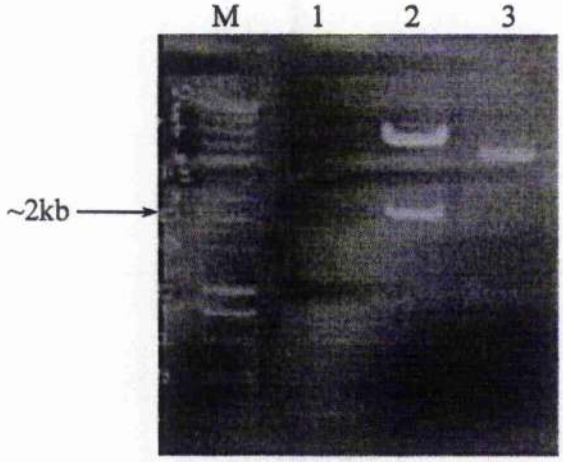


Figure 2.17 Digestion of *pIntervet* using *Bgl II* and *Not I* to release a ~2kb fragment containing a T_R gene and DHFR 3'-untranslated region. Lane 1 contains no digest, lane 2 the cut *pIntervet* plasmid and lane 3 the uncut *pIntervet* plasmid.

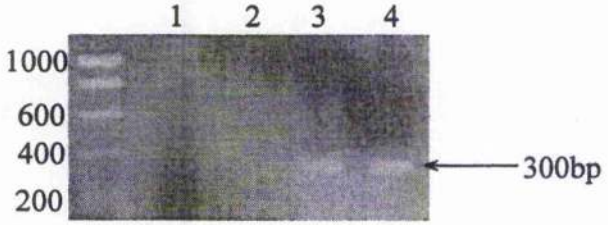


Figure 2.18 PCR amplification of *T. gondii* SAG-1 3'-untranslated region (UTR), using primers SAG1-UTR_BglII^F and SAG1-UTR_NotI^R. Lane 1 is blank, lanes 2-4 contain different *T. gondii* gDNA preparations. The SAG-1 3'-UTR is 327bp, and a product corresponding to this size can be seen in lanes 3 and 4.

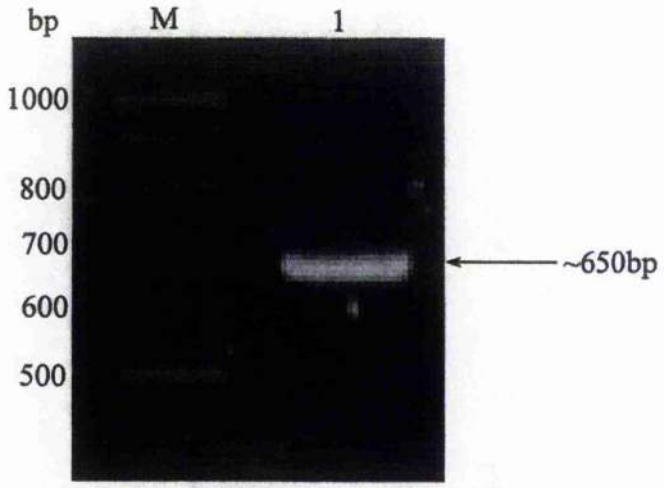


Figure 2.19 PCR amplification of *NcGRA7-myc* from *N. caninum* cDNA using NcGRA7_BglII^{F} and NcGRA7-**myc**_BglII^{R} primers. Lane M is a molecular size marker and lane 1 contains the *N. caninum* cDNA A negative control containing no template DNA was also run, with no amplification product present (data not shown). *NcGRA7* is approximately 650bp, this correlates with the amplification product in lane 1.

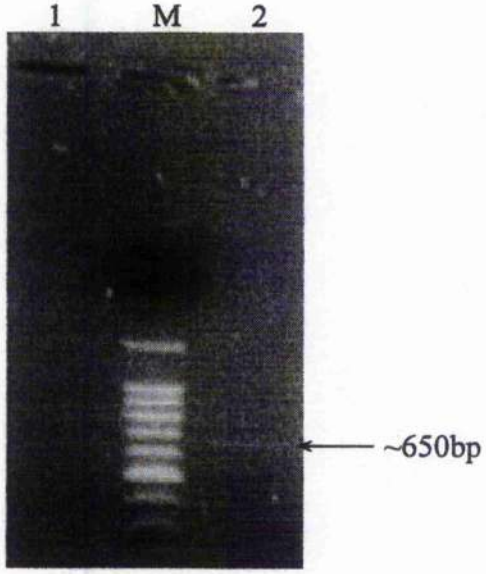


Figure 2.20 PCR amplification of *pIntervet2* plasmid expressing *NcGRA7-myc*. Primers NcGRA7^{F} and NcGRA7^{R} were used. Lane 1 contained *pIntervet2* without *NcGRA7-myc*. A PCR product of the expected (~650bp) size was seen in lane 2 indicating the presence of *NcGRA7* in the plasmid.

This sequence was ligated into the *pIntervet2* as described in Section 2.2.12.3. This ligated plasmid was used to transform *E. coli* as described in Section 2.2.9.2 and purified. PCR was carried out to check the presence of the *NcGRA7* sequence in the plasmid. Primers NcGRA7^{F} and NcGRA7^{R} were used, and the resulting PCR product can be seen in Figure 2.20.

The alignment of the *NcGRA7-myc* in relation to the TUB-1 promoter and 3'UTR was carried out by sequencing across the junctions between the plasmid backbone and the *NcGRA7* sequence. Primers PlasmidSeq2^{F} and PlasmidSeq2^{R} were used. Figure 2.21 shows the alignment between the promoter, the *NcGRA7-myc* sequence and the 3'UTR. The *NcGRA7-myc* sequence was inserted in the correct alignment and in the correct frame relative to the promoter and 3'-UTR. The *c-myc* tag is also present.

This plasmid was subsequently used in transfection experiments as described below.

2.3.2.2.2.2 Preparation of *NcSRS2* tagged with *c-myc*

To allow ligation into the *pIntervet2* backbone, *NcSRS2* was amplified from *N. caninum* cDNA using primers containing the *Bgl II* site engineered into both the forward and reverse primers (NcSRS2_BglII^{F} and NcSRS2_BglII^{R} as shown in Table 2.1). Unlike in *NcGRA7* the *c-myc* tag was inserted by ligation into a *Bam HI* site within the coding sequence of *NcSRS2*, as described in Section 2.2.13.1.3. The *c-myc* epitope primer-dimer containing *Bam HI* sites at the 5' and 3' ends was ligated into the *Bam HI* site within *NcSRS2*. This ligation was then amplified by PCR to obtain sufficient copies for further integration with the *pIntervet2* backbone. Figure 2.22

shows a picture of this PCR amplification. The PCR product of approximately 1.2kb was obtained and this was the expected size for *NcSRS2*.

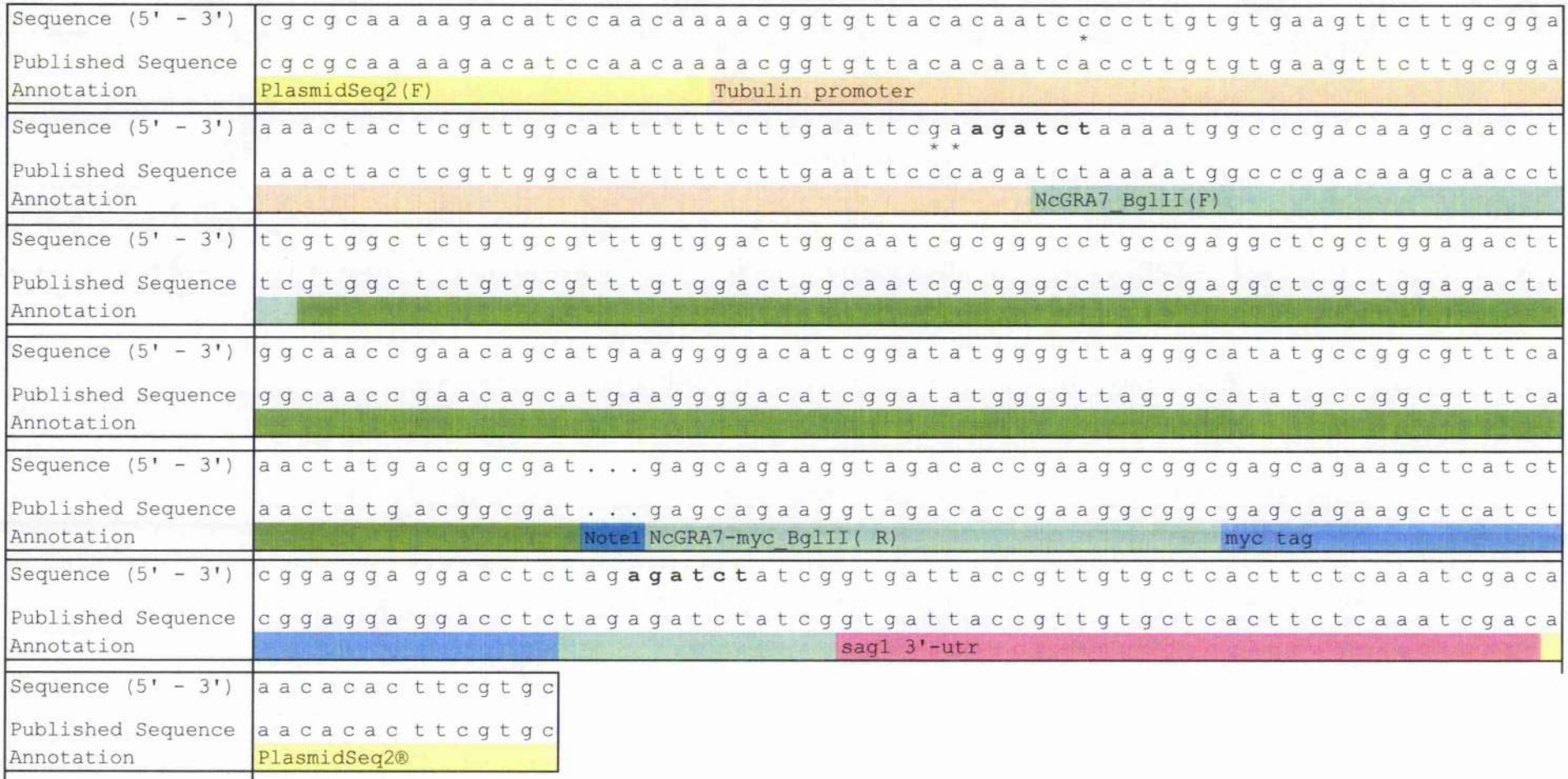


Figure 2.21 Sequence Alignment of pIntervet2 containing *NcGRA7* gene sequence (U82229) engineered with *Bgl II* sites at 5' and 3' ends (in **bold**) and a 3' c-myc tag. Primers are shown in cream (sequencing) and light green (specific). Promoter is brown; gene sequence green; myc tag blue and UTR pink
Note 1: middle of NcGRA7 also sequenced (not shown)
Single nucleotide polymorphisms indicated by *

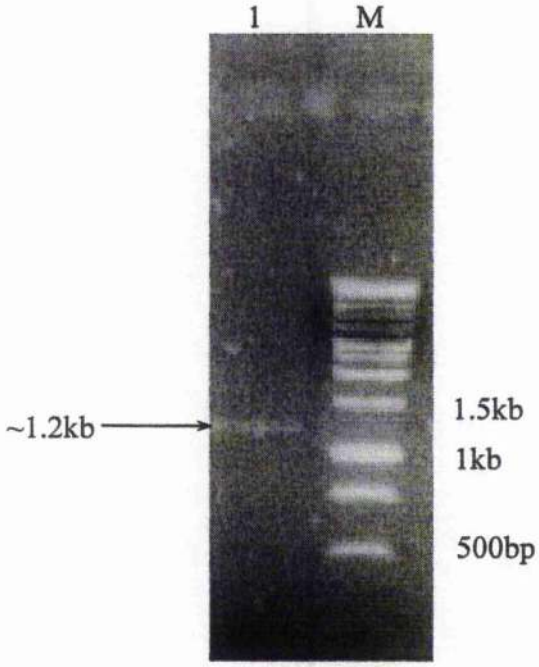


Figure 2.22 PCR amplification of *NcSRS2* containing a ligated *c-myc* tag. This was amplified using primers $NcSRS2^{F}$ and $NcSRS2^{R}$. Lane 1 contains ligation reaction as template, a negative control containing no template was also performed but no product was observed (data not shown).

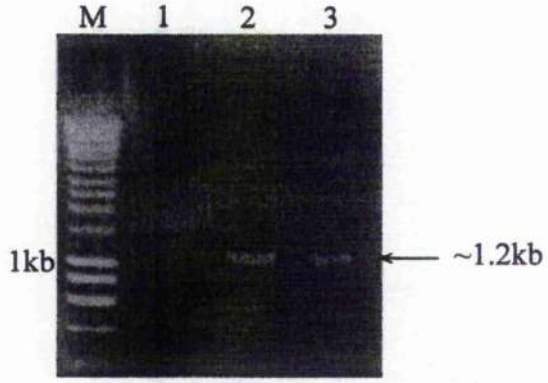


Figure 2.23 PCR amplification of *NcSRS2* from *pIntervet2* containing *NcSRS2* (lane 2) and *N. caninum* cDNA (lane 3). Lane 1 contains no template. Primers $NcSRS2^{F}$ and $NcSRS2^{R}$ were added to each reaction.

This fragment was ligated into the *pIntervet2* backbone as described in Section 2.2.12.3. PCR was performed using NcSRS2^{F} and NcSRS2^{R} specific primers to determine the presence of the *NcSRS2* sequence. Figure 2.23 shows a PCR product of ~1.2kb that corresponds to that obtained from *N. caninum* cDNA.

The alignment of the inserted *NcSRS2-myc* sequence was determined by sequencing across the junctions between the promoter, *NcSRS2-myc* and the 3'UTR. Primers PlasmidSeq2^{F} and PlasmidSeq2^{R} were used. Figure 2.24 shows the alignment of the various plasmid components. The *NcSRS2-myc* sequence is in the correct frame relative to the promoter and the 3'-UTR. Additionally, the *c-myc* sequence has been inserted into the *NcSRS2* gene in the correct alignment.

This plasmid was then used for subsequent transfection experiments as described below.

2.3.2.3 Transfection Experiments

Transfections were performed as described in Section 2.2.16. *T. gondii* transfected with *pMIN1* and *pIntervet2* containing either *NcGRA7* or *NcSRS2* were passaged a minimum of 10 times in selection medium for positive selection for the presence of a functional HXGPRT gene. Parasites were then cloned by limited dilution.

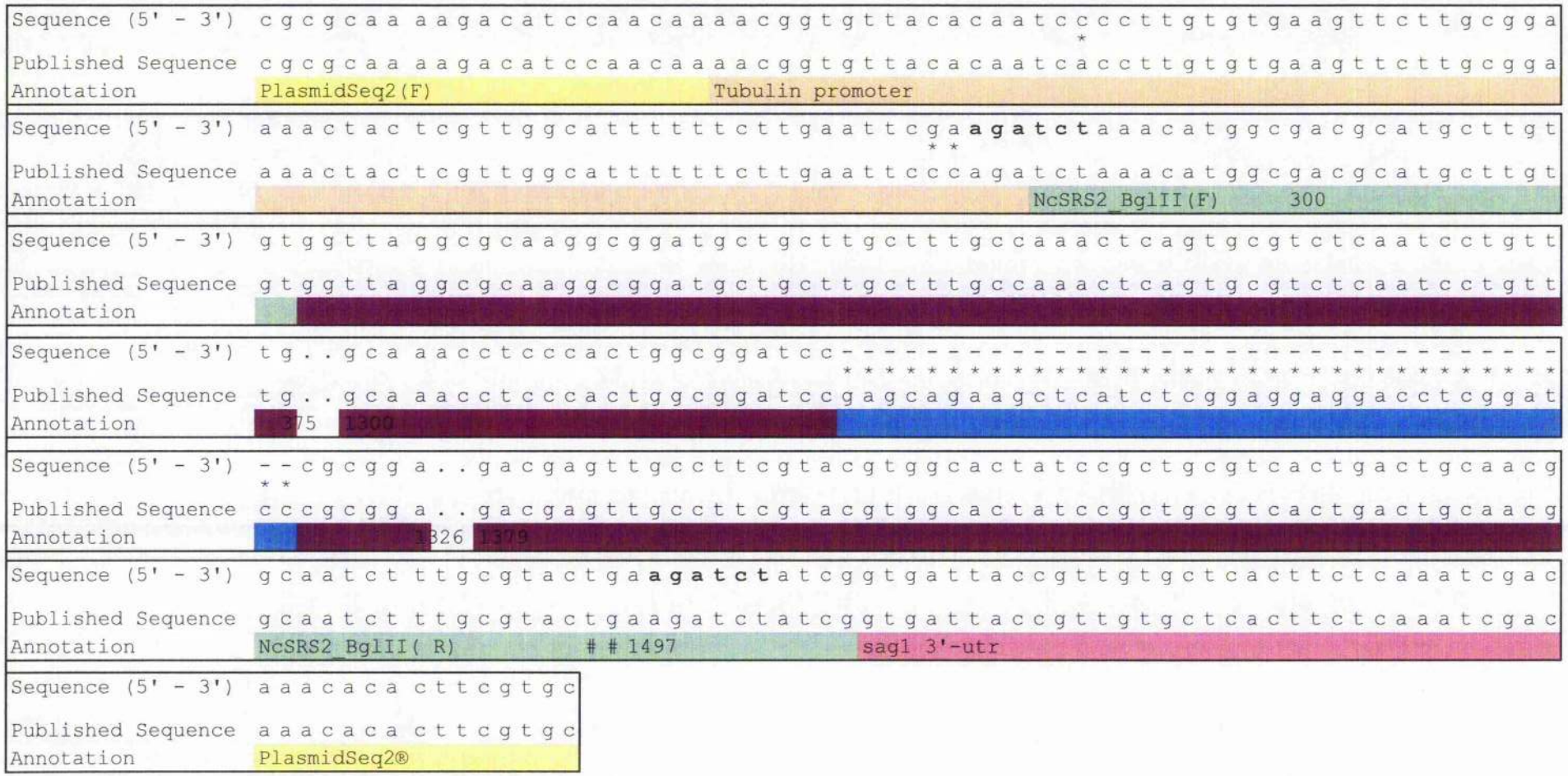


Figure 2.24 Sequence Alignment of pIntervet2 containing *NcSRS2* gene sequence (Accession Number U93870) engineered with *Bgl II* sites at 5' and 3' ends (in **bold**) and an internal c-myc tag. Primers are shown in cream (sequencing) and light green (specific). Promoter is brown; gene sequence purple; myc tag blue and UTR pink. Single nucleotide polymorphisms indicated by *

2.3.3 X-Gal Staining of Parasites: Transfection Control

T. gondii tachyzoites were transfected with *pMINI* and *pTUBlacZ* using the same conditions as the parasites transfected with vectors containing *N. caninum* genes. They were selected with MPA and Xan for 10 passages to ensure a stable transfection had occurred and then fixed and stained for the presence of *pTUBlacZ* with X-Gal. Parasites were fixed whilst still intracellular, since this allowed for the staining of the parasitophorous vacuole contents. This meant that any blue precipitate remained within and around the transfected parasites.

The monolayer of parasite-infected host cells was fixed onto the flask surface as described in Section 2.2.17. Blue precipitate spots were observed without the use of a microscope. Under 400x magnification, however, individual intracellular tachyzoites were identified (Figures 2.25 and 2.26) within the parasitopherous vacuoles. Characteristic rosette formations of tachyzoites (Figure 2.25) as well as tachyzoites in a vacuole in the final stages prior to lysis of the host cell (Figure 2.26) were observed. Both the vacuoles containing the small and large numbers of tachyzoites were stained blue with the X-Gal precipitate, which was indicative of the presence of *lacZ* under the control of a *T. gondii* promoter, in this case the tubulin promoter. The presence of the blue precipitate, in addition to the survival of the parasites in selection, indicated that co-transfection did occur.

Untransfected *T. gondii* tachyzoites were also fixed and stained in the manner described above. No blue precipitate was observed (data not shown).

Figure 2.25 Recently invaded *T. gondii* tachyzoites in the characteristic rosette pattern (arrow). Note the blue halo surrounding the rosetted tachyzoites, indicating the presence of a functional lacZ transgene.

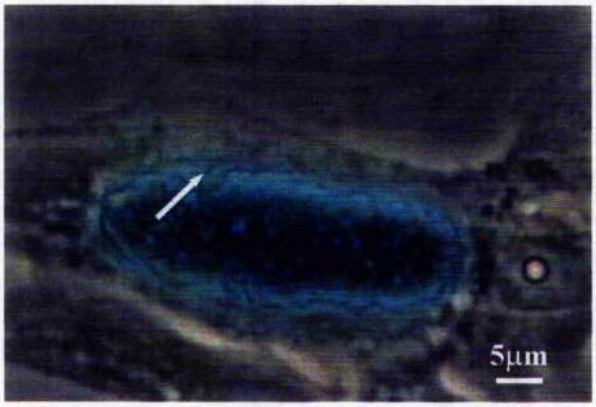


Figure 2.26 Heavily infected HFFF cell, close to the point of lysis. The bright blue of the vacuole indicates the presence of a functional lacZ transgene.

2.3.4 Characterisation of *T. gondii* Transfected with *pIntervet2* Constructs

2.3.4.1 PCR

2.3.4.1.1 NcGRA7

The presence of *NcGRA7* was observed by PCR using the specific primers NcGRA7^{F} and NcGRA7^{R}. PCR was carried out on cloned lines of transfected *T. gondii* tachyzoite and the amplication by PCR of a ~650bp fragment in 3 of the 5 cloned lines can be seen in Figure 2.27. Amplification was seen from clones GC4, GB2 and GE2, as well as from *N. caninum* cDNA, but not in GC5 or GG3, nor from the *T. gondii* parental PRUΔHX strain. The clones identified as being positive for the transgene were used in subsequent characterisation experiments.

2.3.4.1.2 NcSRS2

The presence of *NcSRS2* was observed by PCR using primers NcSRS2^{F} and NcSRS2^{R}. PCR was carried out on gDNA from four cloned lines of transfected *T. gondii* tachyzoites (identified as SC8, SC10, SD9 and SG8). Amplification occurred in 3 of the 4 clones (Figure 2.28) as well as from *N. caninum* gDNA. Only SC10 did not have a fragment of approximately 1200bp amplified from it, indicating the lack of *NcSRS2*. This suggested that clones SC8, SD9 and SG8 contained the transgene, while SC10 did not. No amplification was seen from *T. gondii* DNA.

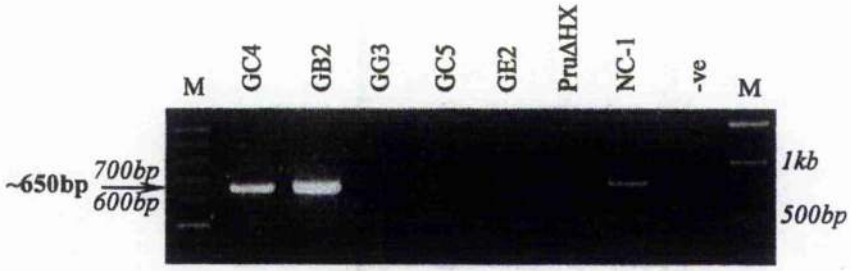


Figure 2.27 PCR amplification of *NcGRA7* using specific primers NcGRA7^{F} and NcGRA7^{R}. Lanes M contain 100bp or 1kb marker. A fragment of approximately 650bp was amplified in lanes containing DNA from clones GC4, GB2, GE2 and NC-1. Negative control contained primers but no template DNA. No amplification was seen in the other lanes.

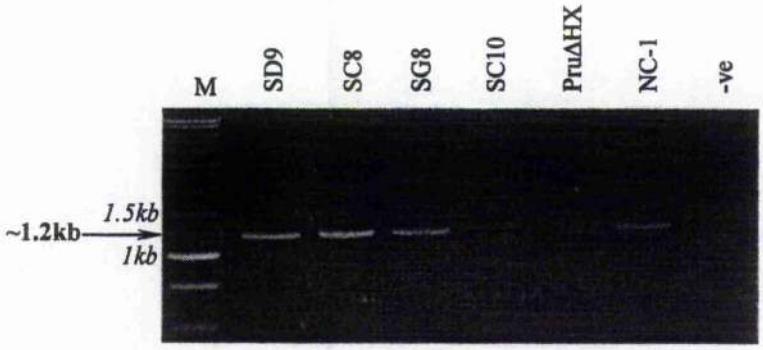


Figure 2.28 PCR amplification of *NcSRS2* using specific primers NcSRS2^{F} and NcSRS2^{R}. Lane M contains 1kb marker. A fragment of approximately 1.2kb was amplified in lanes containing DNA from clones SD9, SC8, SG8 and NC-1. No amplification was seen in the other lanes. (Negative control contained primers but no template DNA)

2.3.4.1.3 Cloned Lines in Selection Without Heterologous Genes

The presence of cloned lines that did not contain either *NcGRA7* or *NcSRS2* yet still grew under mycophenolic acid/xanthine selection for a number of passages indicated one of two scenarios. First, the parasite was transfected with *pMINI* only, or second, parasites were stably transfected by *pMINI* but only transiently transfected with *pTUBGRA7-myc/pTUBSRS2-myc*. The growth of the parasites in selection medium indicated that they must still have contained a functional HXGPRT that could only have come from stable transfection with *pMINI*. However, it appeared in these lines that either the transgenes from *N. caninum* had been lost by the clones or the transfected parasites developed resistance to MPA and so could survive without the functioning HXGPRT gene.

2.3.4.2 Hybridisation of Specific Probes to Southern Blot of Tachyzoite DNA

To test for the presence of *NcSRS2* and *NcGRA7* in *T. gondii* Southern Blotting was performed. The sequence of *NcGRA7* contains two *Acc I* sites that should generate a restriction fragment of 450bp. *NcSRS2* contains two *Nsp I* sites that should generate a fragment of 1kb. Genomic DNA from cloned lines transfected with *NcGRA7* or *NcSRS2*, along with DNA from the untransfected knockout *T. gondii* PRUΔHX and wild-type *N. caninum* NC-1 was digested with either *Acc I* or *Nsp I*. The digested DNA was separated by electrophoresis on 1% agarose gels before being blotted as described in Section 2.2.19. It was then probed with radiolabelled *NcGRA7* or *NcSRS2* gene sequence, obtained from cDNA by PCR to look for the presence of these genes in the transgenic parasites.

2.3.4.2.1 Hybridisation with Radiolabelled NcGRA7 *as Probe*

Hybridisation to a 450bp fragment was observed in lanes containing DNA from clones GB2, GE2 and GC4 and from NC-1 (Figure 2.29). No hybridisation was seen in lanes containing DNA from PRUΔHX or clones GG3 or GC5. There was also an extra band of hybridisation observed in lanes containing clones GE2 and GC4, which was approximately 600bp in size.

2.3.4.2.2 Hybridisation with NcSRS2 *as Probe*

Hybridisation to a fragment of approximately 1kb was observed in the lanes containing DNA from NC-1 and transfected clones SD9, SC8 and SG8 (Figure 2.30). Although there was a level of hybridisation in the other lanes, notably SC10, this was attributed to non-specific hybridisation, since the band that was hybridised in SC10 was not present in the other lanes. Additional annealing to different sized fragments was also observed in the Southern Blot though this was thought to be mis-annealing or the generation of extra *Nsp I* sites due to the integration of the transfection vectors.

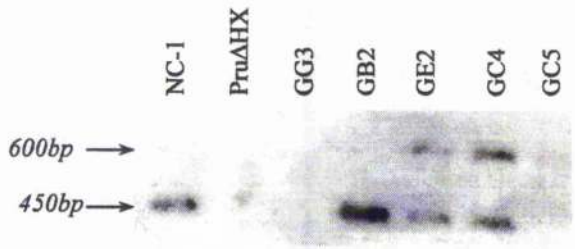


Figure 2.29 Southern Blot hybridisation of *Acc I*-digested genomic DNA of *pTUBNcGRA7-myc/pMINI* transfected *T. gondii*. Probe was a 450bp PCR-amplified *NcGRA7* from cDNA of *N. caninum*. Hybridisation to a 450bp fragment occurred in lanes containing DNA from NC-1, GB2, GE2 and GC4. No hybridisation was seen in the other lanes (PruΔHX, GG3 or GC5). There was also hybridisation to a fragment of ~600bp in lanes with DNA from GE2 and GC4.

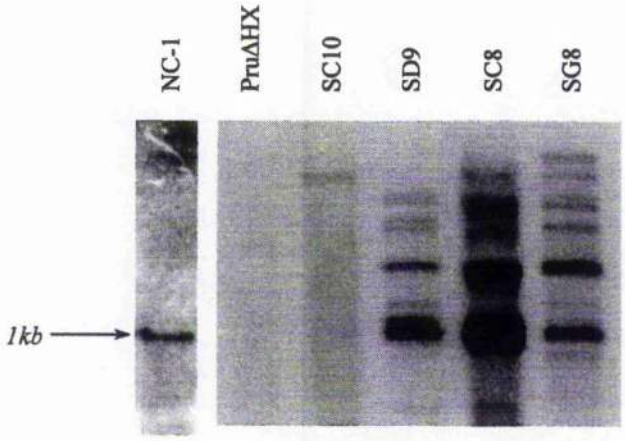


Figure 2.30 Southern Blot hybridisation of *Nsp I*-digested genomic DNA of *pTUBNcSRS2-myc/pMINI* transfected T. gondii. Probe was a 1kb PCR-amplified *NcSRS2* from cDNA of *N. caninum*. Hybridisation to a 1kb fragment occurred in lanes containing DNA from NC-1 and clones SD9, SC8 and SG8. No hybridisation was observed in the lanes containing digested DNA from parental *T. gondii* PruΔHX nor from clone SC10.

2.3.4.3 Western Blotting

Parasites transfected with *pIntervet2* containing *NcGRA7-myc* or *NcSRS2-myc* were lysed and the SDS-soluble fraction run on a 15% acrylamide gel. The lysates were then blotted onto nitrocellulose optimised for ECL detection, and a strip was stained with Ponceau S to determine the efficiency of transfer. The rest of the membrane was probed first with an anti-*c-myc* monoclonal antibody then with a secondary anti-murine IgG1 antibody that was linked to an HRP molecule. ECL was used to detect any antibody binding. However, specific anti-*c-myc* binding was not observed in any blots (data not shown). There were bands of proteins highlighted but these were present not only in those cloned lines that were positive for the transgene by PCR and Southern Hybridisation, but also in lines that were negative by these methods and also in untransfected *T. gondii*. Repeated attempts to minimise non-specific antibody binding were unfortunately unsuccessful. Thus it was decided to use immunofluorescent labelling to determine expression of the transgenes in tachyzoites.

2.3.4.4 Immunofluorescent Labelling of c-myc epitope in transfected parasites

In the absence of monoclonal antibodies raised against either *NcGRA7* or *NcSRS2*, localisation of these molecules within the tachyzoites was carried out by tagging them with an epitope that can be identified easily using commercially available monoclonal antibodies, in this case against *c-myc*.

In both transfected lines, specific sites of localisation could be seen. Figure 2.31 shows cloned line GB2. Similar results were seen in the other two cloned lines (GE2 and GC4) that were PCR- and Southern Blot positive. No localisation was observed either in the negative control, (non-transfected *T. gondii*) or in the cloned lines that

were negative by PCR and Southern Blot (GG3 and GC5). Similarly, Figure 2.32 shows localisation in cloned line SC8 that was also seen in SD9 and SG8, but not in SC10 or in non-transfected *T. gondii*. As can be seen in Figures 2.31 and 2.32, localisation appeared to be to discrete structures throughout the cell. This would be expected in the parasites transfected with *pIntervet2* expressing *NcGRA7-myc*, since dense granules are distributed throughout the cytosol. This was not, however, expected in parasites transfected with *pIntervet2* expressing *NcSRS2-myc* since the *NcSRS2* was expected to be transported to the surface of the parasite.

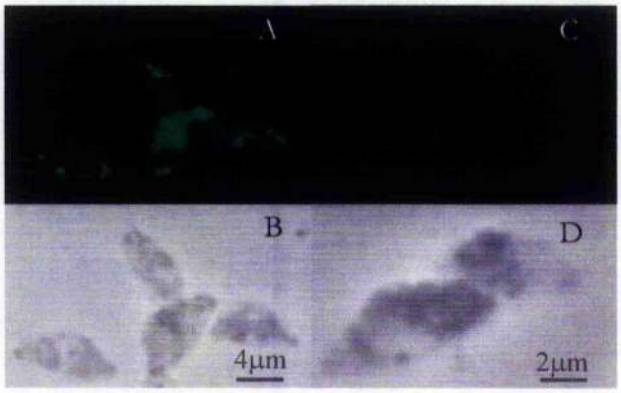


Figure 2.31 Immunolocalisation of *c-myc* tagged *NcGRA7* transfected into *T. gondii*. Monoclonal antibodies against *c-myc* were used to probe fixed *T. gondii* tachyzoites, with anti-mouse IgG, conjugated to FITC used as secondary antibodies. Picture A is *T. gondii* transfected with *pTUBNcGRA7-myc* (cloned line GB2), B showing the same picture under phase-contrast. Pictures C and D are of non-transfected *T. gondii* probed with the same antibodies

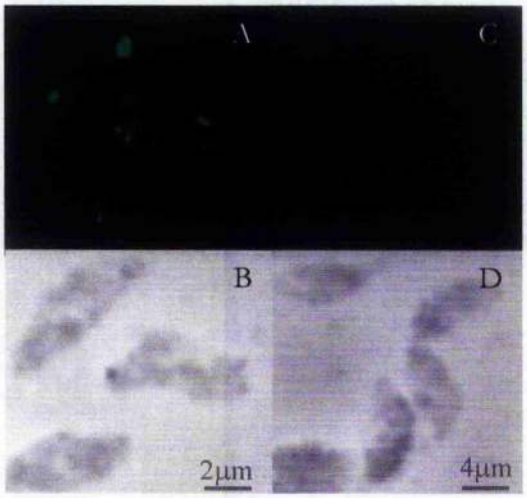


Figure 2.32. Immunofluorescent localisation of *c-myc* tagged *NcSRS2*. Anti *c-myc* antibodies raised in mice were used to probe fixed and permeabilised *T. gondii*. FITC-conjugated anti-mouse IgG was used as secondary antibody. Picture A shows the localisation of *c-myc* in *T. gondii* transfected with *pTUBNcSRS2-myc* (clone SC8). Picture B shows clone SC8 under phase contrast. Pictures C and D show the non-transfected *T. gondii*, both under fluorescence and phase contrast.

2.4 DISCUSSION

In this chapter two *N. caninum* genes, *NcGRA7* and *NcSRS2*, were successfully expressed as transgenes in *T. gondii* tachyzoites by stable integration using a co-transfection technique. PCR and Southern Blotting did not conclusively confirm the integrity of the transformation, merely the presence of the trangenes. Since suitable antibodies against *NcGRA7* and *NcSRS2* were not available at the time, both genes were tagged with a *c-myc* epitope that was co-expressed. Although expression of the recombinant protein in *T. gondii* could not be confirmed due to non-specific antibody binding in Western Blot experiments, analysis by IFAT demonstrated that both transgenes were expressed in *T. gondii* tachyzoites. However, the successful generation of the recombinant parasites was not without some difficulties, which are discussed below.

2.4.1 Generation of Stably Transfected *T. gondii*

The generation of stably transfected *T. gondii* using a co-transfection method has previously been thought to be a relatively straightforward technique in which simultaneous transfection of both the transgene and the selectable marker constructs occurred reliably. Stable co-transfections of *T. gondii* have been carried out previously (Black and Seeber, 1995) with no mention of an apparently stable transfection resulting in a transient transfection of the transgene, as was the case in early transfections carried out in the experiments described in this chapter. The most obvious explanation for this was that whilst the selectable marker became stably integrated, the construct containing the *N. caninum* transgene was only transiently transfected and was lost to subsequent generations. The transient nature of the initial transfections was also unexpected since the parasites were kept in the selection medium containing xanthine and mycophenolic acid for many more cycles of

passage than was normally required. The concept of a stably transfected parasite retaining one of the transfected constructs but not the one containing the gene of interest has not been reported in *T. gondii*. However, since this occurred twice in this study, this event may be more common than previously reported. It is likely that a transfection technique that utilises only one construct – one that contains both the gene of interest and the selection cassette – raises fewer technical difficulties than a technique that relies on the integration of two constructs. Unfortunately, attempts to perform transfection based on this type of construct were unsuccessful in this study.

When a different backbone (i.e. the *pP30/11GFP* or *pIntervet* plasmids) was used in the transfection vectors containing *NcGRA7* and *NcSRS2*, the transfection appeared to be stable and indeed the genes of interest were retained by the *T. gondii* tachyzoites until the end of the experiments, as assessed by PCR and Southern Blot. More than one band of hybridisation was observed in both hybridisations. In *NcGRA7* integration, it may have been the case that there was more than one *pIntervet2* expressing *NcGRA7-myc* integrated into the parasite, though this would still be cut to leave a 450bp fragment.

2.4.2 Characterisation of Parasite Clones

In Southern blots of DNA from parasites transfected with *NcSRS2*, the multiple fragments highlighted in the lanes containing SD9, SC8 and SG8 may indicate the presence of multiple integrations by more than one plasmid. The lack of bands in the parental *T. gondii* PRUΔHX (control) would suggest that each of the fragments highlighted in the transgenic parasites did indeed contain *NcSRS2*. The different sizes of bands highlighted contain the heterologous fragment along with some parental parasite DNA as was between the restriction site at the beginning of the heterologous

fragment and the next *Nsp I* site in the genome. Nevertheless, the overriding conclusion was that in both transfected lines, the heterologous sequences were present.

In addition to confirming the expression of the transgenes, immunofluorescence was performed on transfected tachyzoites to see if heterologously expressed proteins were localised to the same area in *T. gondii* as they were in *N. caninum*. The tagging of the proteins with *c-myc* was designed in such a way that the *c-myc* tag sequence would not affect targeting signals present in the gene sequence. This in turn should have enabled the cell to transport the protein to the correct location. It was expected that *NcSRS2* would be trafficked to the cell membrane (Howe *et al*, 1998), while *NcGRA7* would be localised in dense granules (Hemphill *et al*, 1998). However, both labelled proteins appeared to be localised in vacuolar structures, generally near the periphery of the tachyzoites, though some were observed towards the middle of the cell. This may indicate that both proteins had been localised to dense granules and the targeting sequence of the *NcGRA7* was unaffected. This does not, however, explain the localisation of *NcSRS2-myc*. It was expected that the heterologous *NcSRS2-myc* would localise to the surface of the cell, particularly since the targeting sequence identified in the gene was not disturbed by the importing of the *c-myc* sequence. No localisation was seen at the surface of the parasite, all localisation in parasites transfected with *NcSRS2* being in vacuolar structures, similar to that seen in parasites transfected with *NcGRA7*. It is likely therefore that neither *NcGRA7* nor *NcSRS2* were localised correctly and both ended up in vesicles, either for expulsion by the cell or for degradation.

In conclusion, stably transfected *T. gondii* tachyzoites expressing transgenic *N. caninum* antigens were successfully produced. In the following chapter, the nature of the immune response elicited by these transgenic parasites was studied.

CHAPTER 3:

IMMUNISATION OF RODENT MODELS WITH TRANSGENIC *TOXOPLASMA GONDII* EXPRESSING *NEOSPORA CANINUM* GENES

- ◊ Select an appropriate mouse strain for immunisation with transgenic *T. gondii* expressing genes from *N. caninum*.
- ◊ Conduct preliminary analysis of the immune response in mice inoculated with transgenic parasites.

3.1 INTRODUCTION

3.1.1 Background

Transgenic *T. gondii* has been previously shown to be effective at generating a specific immune response to the heterologous proteins that were expressed (Charest *et al*, 2000; Ramirez *et al*, 2001). These studies utilised attenuated *T. gondii* transfected with genes from *Leishmania major* and *Plasmodium yoelii* as a live delivery system to examine ways to deliver antigens and to generate an appropriate cell-mediated immune response. However, there is nothing in the literature about using transgenic *T. gondii* to stimulate a specific immune response against *N. caninum* parasites. The work in this chapter describes some preliminary studies examining the effect of inoculating mice with transgenic *T. gondii* parasites. The eventual aim of these studies would be to characterise the immune responses raised against the transgene and determine the protective effect (if any) of these responses.

3.1.2 Immune Responses to *T. gondii* During Acute Infection

During an acute *T. gondii* infection, a Type I T-cell response is characteristically induced; that is one where there is a high level of IFNγ, TNFα and IL-12 as well as a prevalence of $CD4^+$ T-cells (Mordue *et al*, 2001). Elevated levels of these three cytokines have been shown to exacerbate the pathology associated with acute toxoplasmosis, the symptoms being extensive liver and lymphoid tissue damage (Mordue *et al*, 2001). It is primarily $CD4^+$ T-cells that are important in protection in the acute disease, since $CD4^+$ T-cells are the major source of IFNγ (Gazzinelli *et al*, 1996).

$CD8^+$ T-cells are also important in the development of protective immunity against *T. gondii* (Parker *et al*, 1991). Mice that were depleted of $CD8^+$ T-cells were unable to prevent the development of cysts of *T. gondii*, and protect against the disease (Parker *et al* 1991).

3.1.3 Acute *N. caninum* Infection

3.1.3.1 Models of Acute Neosporosis

Mice have primarily been used to model acute neosporosis (Lindsay *et al*, 1990), although it is comparatively more difficult to infect mice with *N. caninum* than with *T. gondii*. Other species have also been examined, including gerbils (Dubey *et al*, 2000). In this study by Dubey *et al* (2000), tachyzoites of *N. caninum* were found in intestinal lesions and also in the brains of infected gerbils. The acute pathology observed in gerbils is similar to the pathology in mice, though more severe involving acute pneumonia, ulcerative lesions in the brain and pancreatitis (Lindsay *et al*, 1990). It should be noted that the response to *N. caninum* in mice is dependent on several factors, including dose of parasite and genetic background of both the mouse (discussed in Section 3.1.3.2) and the parasite (Dubey and Lindsay, 1996).

3.1.3.2 Immune Response to N. caninum *During Acute Infection*

The situation in acute neosporosis is similar to that in acute toxoplasmosis, where a Th1 response is essential for survival, and in particular IFNγ and IL-12 has been shown to be critical for survival of mice infected with *N. caninum* (Khan *et al*, 1997; Baszler *et al*, 1999; Nishikawa *et al*, 2001; Ritter *et al*, 2002). Khan and colleagues demonstrated that if IFNγ and IL-12 were depleted, mice became more susceptible to developing clinical signs of infection (Khan *et al*, 1997). In IFNγ knockout mice, the

disease quickly becomes lethal, primarily due to the lack of activation of macrophages by IFNγ (Nishikawa *et al*, 2000).

T-cells are also important in the protection against acute neosporosis (Tanaka *et al*, 2000). In mice depleted of $CD4^+$ T-cells, there was a reduced level of *N. caninum* specific antibodies and IFNγ, and the mice succumbed quicker to infection than the control mice with normal levels of $CD4^+$ T-cells (Tanaka *et al*, 2000).

A specific humoral response has also been demonstrated to be protective against infection with *N. caninum*. In B-cell deficient μMT mice that could not produce antibodies, infection with *N. caninum* was fatal, whereas C57/BL6 mice with functioning B-cells showed no clinical symptoms when infected with the parasite (Eperon *et al*, 1999). This study suggests that a humoral response had a role in protection.

3.1.4 Immunological Cross-Reactivity Between *N. caninum* and *T. gondii*

3.1.4.1 Serological Cross Recognition

Until the discovery of the parasite in 1988 (Dubey *et al*, 1988), *N. caninum* infections were often mis-diagnosed as being *T. gondii* infections, despite the fact that the serum from the infected dogs did not recognise *T. gondii* antigens (Dubey *et al*, 1988). The mis-diagnosis was primarily due to the morphological similarities between the two species. These similarities were confirmed by phylogenetics (Mugridge *et al*, 1999). There is, however, evidence of the serological cross-recognition of some *N. caninum* and *T. gondii* antigens, as demonstrated by Western blotting of one- and two-dimensional SDS-PAGE (Bjerkas *et al*, 1994; Sundermann *et al*, 1997; Harkins *et al*,

1998; Hemphill *et al*, 2000). When *N. caninum* lysate is probed with serum from a *T. gondii*-infected animal, or *vice versa*, there were several commonly recognised bands that indicated immunological cross-recognition (Harkins *et al*, 1998). The use of two-dimensional SDS-PAGE technology has enabled the identification of individual *N. caninum* proteins recognised by *N. caninum* specific and *T. gondii* specific sera, as well as several proteins that are only recognised by homologous sera, including two spots in the 11-18kD pI 5-6 range (Heckeroth *et al*, 2000) (cited in Hemphill (2000)).

3.1.4.2 T-Cell Cross-Recognition

Cellular cross-recognition between *N. caninum* and *T. gondii* is less well characterised than the humoral immune responses discussed above. A study has shown that T-cells taken from *N. caninum* infected cattle would also proliferate when stimulated with a crude lysate antigen of *T.gondii* (Lunden *et al*, 1998). However, this study also suggests that, while there was a proliferative effect, there was a reduced production of IFNγ in the heterologous stimulation. This may suggest that, while there are common T-cell epitopes in *N. caninum* and *T. gondii*, the functionality is different (Lunden *et al*, 1998). This is supported by further work where sheep infected with *T. gondii* were not protected against abortion when challenged at mid-gestation with *N. caninum*, despite demonstrating a cell proliferation response to both *T. gondii* and *N. caninum* antigen *in vitro* and an antibody response that was cross-reactive between *T. gondii* and *N. caninum* (Innes *et al*, 2001a)

3.1.4.3 Cross-Protective Immunity

In initial challenge experiments, mice infected with *N. caninum* were not protected against subsequent infection with *T. gondii* tachyzoites (Lindsay *et al*, 1990).

However, in later studies by Kasper and Khan (1998), evidence of cross-protective immunity between *T. gondii* and *N. caninum* was observed in a murine model (Kasper and Khan, 1998). In this study, *N. caninum* infected mice did not succumb to a secondary infection of *T. gondii*. It was shown that $CD8^+$ T-cells from the *N. caninum* infected mouse proliferated in the presence of antigen from *T. gondii* as well as *N. caninum* and also produced significant levels of IFNγ, suggesting that the cross-reactivity between antigens of *T. gondii* and *N. caninum* have different modes of action in $CD4^+$ and $CD8^+$ T-cells. However, when mice infected with *N. caninum* tachyzoites are challenged with an oral dose of *T. gondii* oocysts from a Type II (i.e. less virulent) strain (TS-4), there is some protection against parasite-induced mortality (Lindsay *et al,* 1998). In the same study by Lindsay and colleagues (1998), when *N. caninum* infected mice are challenged with tachyzoites of a Type I strain of *T. gondii* (RH), the results mirror those of previous work by Lindsay and co-workers (1990), in that there is no protection (Lindsay *et al*, 1998). This may suggest that the challenge dose and genetic background of the parasite is critical in determining the immune response generated. Furthermore, in studies carried out in large animals, it was shown that sheep immunised with *T. gondii* were not protected against challenge by *N. caninum* (Innes *et al*, 2001a). In this study, there was no cross-protection to challenge by *N. caninum* and foetal loss was evident (Innes *et al*, 2001a).

3.1.5 Immunisation Studies Using Transgenic *T. gondii*

Transgenic pathogens have been used in immunisation studies, though these have been primarily viral (McMahon-Pratt *et al*, 1993) or bacterial (Saklani-Jusforgues *et al*, 2003). Recombinant vaccinia virus has been used to immunise against *L. major* using the parasite molecule gp46/M2 (McMahon-Pratt *et al*, 1993). A protective

immune response, both cellular and humoral, was demonstrated (McMahon-Pratt *et al*, 1993). *Listeria monocytogenes* have been engineered to express *Leishmania major* LACK protein to stimulate a $CD4^+$ T-cell response (Saklani-Jusforgues *et al*. 2003).

Recombinant *T. gondii* has been used previously in a study to examine the immune response to a protein from *Plasmodium yoelii,* circumsporozoite protein (CSP) (Charest *et* al, 2000). Attenuated, temperature sensitive *T. gondii* (strain ts-4) were engineered to express CSP. In this study the focus was the priming of $CD8^+$ T-cells to develop protective immunity against *P. yoelii.* A specific protective response involving CD8+ T-cells after challenge with *P. yoelii* was observed. When the $CD8^+$ T-cells were depleted, the protection against challenge was compromised (Charest *et al*, 2000).

The same attenuated strain of *T. gondii* (ts-4) that was used by Charest *et al* (2000) was engineered to express the *Leishmania major* kinetoplastid membrane protein-11 (KMP-11) (Ramirez *et al*, 2001). KMP-11 was expressed as either cytoplasmic or membrane-bound proteins in *T. gondii.* These recombinant *T.gondii* were inoculated into BALB/c mice, which were then challenged with *L. major*. There was a specific proliferative response by T-cells *in vitro* to the transfected gene product (KMP-11), as well as significant protection when the animals were challenged with live *L.major* (Ramirez *et al*, 2002).

These studies suggest that the use of attenuated *T. gondii* as a delivery vehicle for parasite vaccines is potentially useful. Not only is *T. gondii* a eukaryotic organism, with the appropriate cellular mechanisms for expressing eukaryotic proteins, but also

it is shown to generate a Th1 response, protective against intracellular pathogens (Wakelin, 1996)

3.1.6 Aims and Objectives

The aims and objectives of the work in this chapter are to:

- Select an appropriate mouse strain for immunisation with transgenic *T. gondii* expressing genes from *N. caninum*.
- Conduct preliminary analysis of the immune response in mice inoculated with transgenic parasites.

3.2 MATERIALS AND METHODS

Note: All animal work was carried out according to the regulations as stated in the Animals (Scientific Procedures) Act, 1986. This Act ensures that animals used in scientific experiments do not endure unnecessary pain or suffering. Animals that show signs of discomfort, for example ruffling of coats, loss of weight, lethargy and a hunched or tottering gait, as scored against set criteria (Table 3.1), are euthanised when these symptoms exceed a statutory level as judged by a professional handler, Mr Steve Wright (Moredun Research Institute, the project licence holder). This is judged, under the Act, to be when an animal scores 4 for two days running, or scores 5 on one day, according to the scoring systems shown in Table 3.1.

A. Presumed Febrile Response. Determined by Appearance of Coat	
Symptom	***Score***
sleek glossy coat	*0*
ruffled coat	*1*
stary stiff coat	*2*

B. Dehydration. Determined by Weight Loss	
Symptom	***Score***
weight maintained at pre-infection level	*0*
10% weight loss	*1*
20% weight loss	*2*

C. General Demeanour	
Symptom	***Score***
bright / active	*0*
hunched	*1*
tottering gait	*1*
reluctance to move	*1*

Table 3.1 Symptoms and scores for assessing symptoms of experimental animals, as defined by the Animals (Scientific Procedures) Act, 1986. Animals were scored using these criteria. Animals scoring 4 on 2 consecutive days or ≥ 5 on 1 day were euthanised.

3.2.1 Selection of Rodent Model Strain

The response to the transgenic *T. gondii* was analysed in a small animal model. Initial experiments were conducted to determine a suitable strain of mouse and selection of an appropriate dose of parasite for the study. Titration of the dose of parasites was done to determine a dose that would prime the immune system without causing severe disease. This experiment was also an opportunity to determine which strain of mouse would be most appropriate to use, since it has been shown previously that different genetic types of mice have varying susceptibility to *T. gondii* infection (Luo *et al*, 1997; Schluter *et al*, 1999).

3.2.1.1 Mouse Strains

Initially, two inbred strains (BALB/c and C57/Black 6) were used in parasite titration experiments. These mice were given various doses of *T. gondii* PRUΔHX parasite inoculum, ranging from $1x10^5$ down to $1x10^2$, the experimental design is detailed in Table 3.2.

In addition, infections were also conducted using outbred mice of the Porton strain (Moredun Research Institute). Work done recently in Quackenbush outbred mice suggested that outbred mice were more resistant to *N. caninum* infections (Quinn *et al*, 2002). Type II *T. gondii*, of which the Prugniaud strain was one example, were also thought to be avirulent in mice (Dubey *et al* 2002). It was interesting to see if this would also be the case with recombinant *T. gondii* infection of an outbred strain of mouse, and also whether the fact that the strain of *T. gondii* used in the experiments were genetic knockouts not possessing a functional HXGPRT gene.

3.2.1.2 Parasites and Experimental Design

Tachyzoites of *T. gondii* were grown and prepared as described in Chapter 2. Mice were inoculated intraperitoneally with various doses of *T. gondii* PRUΔHX tachyzoites ranging from $1x10^5$ down to $1x10^2$ (see Table 3.2). Trials 1 and 2 were designed to try the widest range of doses, with drug treatment being administered when the clinical symptoms in the mice were noticeable. (Drug treatment in these trials was in the form of "intradine" therapy administered in the drinking water (0.5ml Intradine/500ml water). Intradine is a 33% solution of sulphadimidine sodium, and is administered at a final concentration of 0.3mg/ml drinking water.) In Trials 3(a), 3(b) and 4, the drug treatment was given to all mice whether they showed clinical symptoms or not at 3 days post infection. This Intradine was given as "wet mash" (dried feed soaked in the medicated water as described above. This is more palatable to the mice and therefore a more effective way of administering treatment.

PRUΔHX Dose	Trial 1: BALB/c (n=6)	Trial 2: BALB/c (n=7)	Trial 3(a): C57BL/6 (n=5)	Trial 3(b): Porton (n=5)	Trial 4: BALB/c (n=6)
1×10^5	✓	-	-	-	-
5×10^4	✓	-	-	-	-
1×10^4	✓	-	-	-	✓
5×10^3	✓	-	✓	✓	-
1×10^3	✓	-	✓	✓	✓
5×10^2	✓	-	✓	✓	-
2.5×10^2	-	✓	-	-	-
1×10^2	✓	-	-	-	✓

Table 3.2 Dose of tachyzoites given to mouse strains BALB/c, C57/Black 6 and Porton. The experiment was repeated on two occasions in BALB/c mice. The tick (✓) indicates that the dose was used in the titrations. Other doses were not used (-).

3.2.2 Immunisation of Rodent Model with Transgenic *T. gondii*

From the initial titration experiments, the outbred Porton mice were selected for the study because they were least likely to succumb to infection. The inbred mouse strains

were found to be highly susceptible to infection with the PRUΔHX *T. gondii*. Groups of Porton mice were used to characterise the humoral immune response following inoculation of transgenic *T. gondii* expressing *N. caninum* GRA7 or SRS2. The experimental design is detailed in Table 3.3. The mice were inoculated intra-peritoneally with $1x10^2$ tachyzoites (prepared as described in Chapter 2). Control mice were inoculated with the same volume of PBS (100µl) as that used to resuspend the parasites in the experimental groups. Two mice were removed from each group and sacrificed at days 0, 14, 28 and 48 respectively post infection. The remainder of the mice were factored in to ensure that there were sufficient mice throughout the course of the experiment in case of natural death or euthanisation of animals. 0.1-1.0ml of blood was taken from a direct heart puncture immediately after death as a source of serum, which was prepared from blood as described in Chapter 3.2.5.

Experimental Design

Group n=12	Parasite Strain	Inoculum Dose (per animal)	Number of Mice Sampled			
			day 0	day 14	day 28	day 48
A	PRUΔHX x NcGRA7	100 tachyzoites	2	2	2	2
B	PRUΔHX x NcSRS2	100 tachyzoites	2	2	2	2
C	PRUΔHX (untransfected)	100 tachyzoites	2	2	2	2
D	NC1	100 tachyzoites	2	2	2	2
E	Control - PBS only	0	2	2	2	2

Table 3.3 Groups of 12 Porton mice were infected with $1x10^2$ *T. gondii* PRUΔHX tachyzoites, either transfected with NcGRA7, NcSRS2 or untransfected; or $1x10^2$ *N. caninum* NC-1 strain tachyzoites. Control mice were inoculated with PBS only.

3.2.3 Immunofluorescent Localisation of Transgene Products

To assay the presence of *N. caninum*-specific antibodies in serum from infected mice, the immunofluorescent localisation of transgene products was performed. Slides were prepared and probed with antibodies as described in detail in Chapter 2.2.19. However, instead of using anti-*cmyc* antibodies as the primary antibody, the separated blood serum from the infected mice was used. The blood was separated with the

serum being extracted from the other blood components by centrifugation at 13,000 x g for 10 minutes in a microcentrifuge. The serum was aspirated into a clean 1.5ml tube and stored at -20°C. This serum was used diluted 1:500 in Tris buffered saline (TBS) / Tween-20 solution (1000:1 TBS:Tween-20). This serum was used as primary antibody to probe *N. caninum* tachyzoites to detect specific binding to *N. caninum* molecules. The secondary antibody was the same as previously described in Section 2.2.19, being a 1:1000 anti-mouse IgG (labelled with FITC) in TBS-T.

3.2.4 *In Vitro* Growth Rate of Parasites

To assess whether any differences in pathogenicity were due to variation in growth rates, a uracil uptake assay was carried out to quantify the *in vitro* multiplication rate of the different parasite strains (an adaptation to the protocol described in Pfefferkorn and Pfefferkorn (1981)). Twenty-four well culture plates were seeded with 2 x 10^4 Vero cells/well in a total of 1ml of IMDM (supplemented with 10% foetal calf serum (Labtech, UK)). The plates were incubated at 37°C, 5% CO_2 overnight. *T. gondii* tachyzoites, either PRUΔHX or PRUΔHX expressing NcGRA7 or NcSRS2, or *N. caninum* NC-1, were added to 4 wells each at a concentration of 6 x 10^4 in 60µl of IMDM. The cells plus parasites were then incubated for 0, 2, 12, 24 and 48h at 37°C.

After the allotted time, 5µCi of (5,6-[^{3}H]) uracil (Perkin-Elmer) was added to each well. The plate was incubated a further 4h at 37°C, before being chilled at -20°C for 3min. 1ml of ice-cold TCA (0.6M) was added to each well to precipitate any non-integrated [^{3}H] uracil, and the plate was incubated on ice for 1h to fix the monolayer onto the base of the well. The TCA was then removed from the wells and the plate was immersed in a bowl of water overnight.

The plates were air-dried for 5-10min before 0.1M NaOH was added. This was incubated at 30°C to dissolve any TCA precipitate. 0.25ml from each well was removed and placed in a scintillation vial containing 3ml of acidified scintillation fluid (2.5µl of acetic acid per 2.5ml of scintillate). These were then read in a scintillation counter (Perkin Elmer).

3.3 RESULTS

3.3.1 Inoculation of Different Mouse Strains with PRUΔHX *T. gondii*

Four experiments were carried out to evaluate the effect of inoculation of different parasite doses into different strains of mouse, with a view to selecting the optimal mouse strain for inoculation with transgenic parasites. Three strains of mice were tested with different inoculation doses and with different drug regimes. All experimental animals were examined daily for clinical signs according to the criteria laid out in the Animals (Scientific Procedures) Act (1986) (see Section 3.2) and any animals scoring 4 for two days, or over 5 for one day were removed from the experiment and euthanised.

3.3.1.1 Trial 1: BALB/c

Adult female BALB/c mice were inoculated with different doses of PRUΔHX *T. gondii* ranging from $1x10^5$ to $1x10^2$ tachyzoites per mouse (Table 3.2). By day 1 p.i., the mice were all showing some degree of coat ruffling, an indication that they were not healthy. However, this settled down in the mice in the lower dosage groups ($5x10^3$ tachyzoites and less) by day 2 p.i. The drinking water was supplemented with intradine (day 5 p.i.) to help the mice cope with the infection better, however by day 6 p.i., all the mice infected with higher doses ($1x10^5$, $5x10^4$ and $1x10^4$ tachyzoites) had either died or been euthanised. By day 10 p.i., all remaining mice in the other dose groups had been euthanised according the Home Office Guidelines or had died.

3.3.1.2 Trial 2: BALB/c

One further group of BALB/c mice were inoculated with $2.5x10^2$ tachyzoites (Table 3.2). As in Trial 1, all the mice showed a degree of coat ruffling by day 1 p.i., though

this had settled down by 48h p.i. Intradine therapy commenced on day 4 p.i., a day earlier than in Trial 1, to try and regulate the infection at an earlier stage than in Trial 1. However, by day 7 p.i. all mice were suffering from the infection such that they were all dead or euthanised by day 10 p.i.

Other mouse strains were then tried to compare and contrast their responses to the lower doses of PRUΔHX *T. gondii*. In addition the drug regime was re-evaluated to start treatment earlier and to administer in feed as opposed to in water.

3.3.1.3 Trial 3(a): C56BL/6

Another-inbred strain, C57BL/6, was used. These mice were infected with $5x10^2$, $1x10^3$ or $5x10^3$ tachyzoites of PRUΔHX *T. gondii* (Table 3.2). Again, mice showed ruffled coats on day 1 p.i. followed by a smoothing of the coats on day 2 p.i. The intradine was given in wet mash from day 3 p.i.. By day 7 p.i., the mice infected with $5x10^3$ tachyzoites showed severe clinical symptoms, and all were dead or euthanised by day 11 p.i. The mice in the other groups fared slightly better. By the end of the experiment (day 21 p.i.) 3 of the 5 mice inoculated with $1x10^3$ tachyzoites, and 2 inoculated with $5x10^2$ tachyzoites had succumbed to the infection.

3.3.1.4 Trial 3(b): Porton

Porton mice were treated in the same manner as the C57BL/6 mice described in Section 3.3.1.3, including the drug treatment in wet mash from day 3 p.i. Again, by day 1 p.i. the mice had ruffled coats, though this had cleared by day 2 p.i. In contrast to the result with the C57BL/6 mice, by the end of the experiment on day 21 p.i., only

two of the mice (from the group inoculated with $5x10^3$ tachyzoites) had succumbed to infection and had been euthanised.

The conclusion of Trial 3 was that Porton mice were less likely than C57/Bl6 to succumb to a low dose of *T. gondii* Prugniaud knockout strain PRUΔHX.

BALB/c mice were further examined to test the effect of administration of the drug starting on day 3 p.i. in wet mash as was used in Trials 3(a) and 3(b).

3.3.1.5 Trial 4: BALB/c

Three groups of BALB/c mice received doses of tachyzoites as shown in Table 3.2. Similar to the other experiments, the mice showed ruffling of their coats that cleared by day 2 p.i. The mice in the group inoculated with $1x10^4$ tachyzoites began dying on day 4 p.i., and by the end of the experiment on day 28 p.i., only two mice inoculated with $1x10^2$ tachyzoites had survived.

This final trial confirms the result that the outbred Porton strain of mouse was hardier than the two inbred mouse strains tested and the Porton strain was selected for further experimentation. The genetic background of the host animal was an important factor determining survival with an infection of PRUΔHX *T. gondii*.

3.3.2 Immunisation regimen of Porton Mice

Mice were inoculated with *T. gondii*, transfected with NcSRS2 or NcGRA7, untransfected *T. gondii*, wild-type *N. caninum* NC-1 or PBS (control). PBS was used as a control since the parasite inocula ($1x10^2$ tachzyoites in 100µl) were suspended in

sterile PBS prior to inoculation. Serum samples from each group were taken at day 14, 28 and 48 days p.i (Table 3.3).

Table 3.4 summarises the clinical response of the mice following inoculation. It highlights when mice were found dead, were euthanised because they were too sick to continue in the experiment, and also when samples were removed for analysis. In the group infected with PRUΔHX x NcGRA7 (Group A), one mouse died on day 9 pi and one was culled on day 18 pi, while in Group B (inoculated with PRUΔHX x NcSRS2) one mouse was culled on day 12 pi and another on day 45 pi due to severe clinical symptoms. In the group inoculated with NC1 (group D), only one mouse was euthanised on day 39 pi. However, in Group C (inoculated with untransfected PRUΔHX *T. gondii*) 50% of the mice were removed from the experimental group due to severe clinical symptoms by day 28 p.i.. Four mice died naturally, and two were euthanised. Only in the negative control (group E) were there no cullings / deaths due to severe clinical responses to the inoculum.

It was also noticeable that all the groups had mice surviving to day 48 p.i., the end of the experiment, except Group C, where all the mice had died or been euthanised by 28 days p.i.. This suggested that the transfected strains of *T. gondii* showed less pathogenicity in Porton mice than their untransfected counterparts.

GROUP A PRUHX- x NcGRA7		
Day p.i.	Event	No. of Mice Remaining
0	Inoculation of Mice	12
0	2 samples taken	10
9	1 died	9
14	2 samples taken	7
18	1 culled	6
28	2 samples taken	4
48	2 samples taken	2
48	End of Experiment	2*

GROUP B PRUHX- x NcSRS2		
Day p.i.	Event	No. of Mice Remaining
0	Inoculation of Mice	12
0	2 samples taken	10
12	1 culled	9
14	2 samples taken	7
28	2 samples taken	5
45	1 culled	4
48	2 samples taken	2
48	End of Experiment	2*

GROUP C PRUHX-		
Day p.i.	Event	No. of Mice Remaining
0	Inoculation of Mice	12
0	2 samples taken	10
8	3 died, 1 culled	6
11	1 culled	5
14	2 samples taken	3
18	1 died	2
28	2 samples taken	0
28	End of Experiment	0 mice remaining

GROUP D NC1		
Day p.i.	Event	No. of Mice Remaining
0	Inoculation of Mice	12
0	2 samples taken	10
14	2 samples taken	8
28	2 samples taken	6
39	1 culled	5
48	2 samples taken	3
48	End of Experiment	3*

GROUP E CONTROL: PBS		
Day p.i.	Event	No. of Mice Remaining
0	Inoculation of Mice	12
0	2 samples taken	10
14	2 samples taken	8
28	2 samples taken	6
48	2 samples taken	4
48	End of Experiment	4*

Table 3.4 Porton mice inoculated with PRUΔHX *T. gondii* transfected with NcGRA7 and NcSRS2, untransfected PRUΔHX *T. gondii*, NC1 *N. caninum* and PBS as negative control. (p.i. – post inoculation). "Culled" mice were euthanised because they had significant clinical symptoms as defined by law to warrant their removal from the experiment. * Extra mice in each group were to account for natural death / euthanisation of animals, to ensure that in each sample group n=2. These mice were euthanised at the end of the experiment.

3.3.3 Immunofluorescent Localisation of Transgene Products

Slides of *N. caninum* were made and probed with sera from infected mice (primary antibody). Since the mice were infected with *T. gondii* tachyzoites expressing *N. caninum* GRA7 and SRS2, it was anticipated that there would be some specific antibodies against these two *N. caninum* molecules. A secondary antibody that was conjugated with FITC and raised against mouse IgG was added. This secondary antibody bound to any mouse IgG that had attached to the tachyzoites during the primary reaction. The sera used were from mice of each group, at days 0 and 48 p.i. Figure 3.1 shows that there was little or no specific binding of antibody in any of the groups of mice at day 0 p.i.

After 48 days of infection, there was little evidence of specific binding of serum proteins to *N. caninum* tachyzoites, as is shown in Figure 3.2. There was evidence of nuclear staining of *N. caninum* when probed with sera from mice in Groups A and B (inoculated with *T. gondii* expressing NcGRA7 and NcSRS2, respectively). Stronger fluorescence was observed in tachyzoites probed with sera from Groups C and D (sera from mice inoculated with PRUΔHX and NC1, respectively).

However, some antibody binding was also observed in Group E (PBS control). This may suggest that the fluorescence seen in Groups A-D was not as specific as first thought. In Groups A and B, the fluorescence was focussed around the nucleus, suggesting that there was some level of specific binding, whereas the fluorescence in Groups C and D was focused into granular structures throughout the cytoplasm. More studies would be required to make any relevant conclusions.

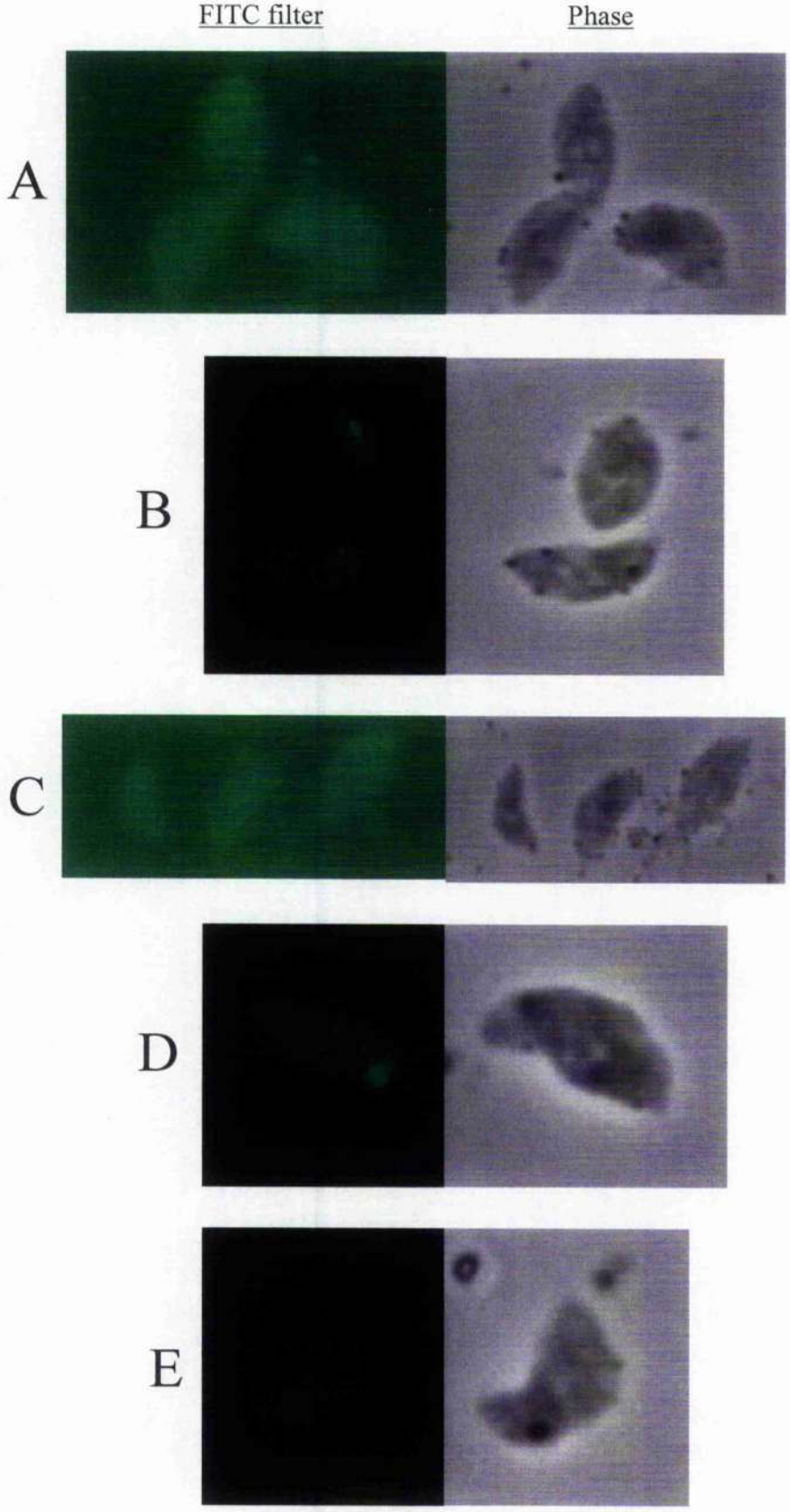


Figure 3.1 IFAT slides of *N. caninum* tachyzoites. Parasites were fixed onto slides and probed with sera from infected mice (A: PRUΔHX x NcGRA7 B: PRUΔHX x NcSRS2 C: PRUΔHX D: NC1 E: PBS) at day 0 p.i. Secondary antibody was rabbit anti-mouse IgG labelled with FITC. Visualised under FITC filter and phase contrast.

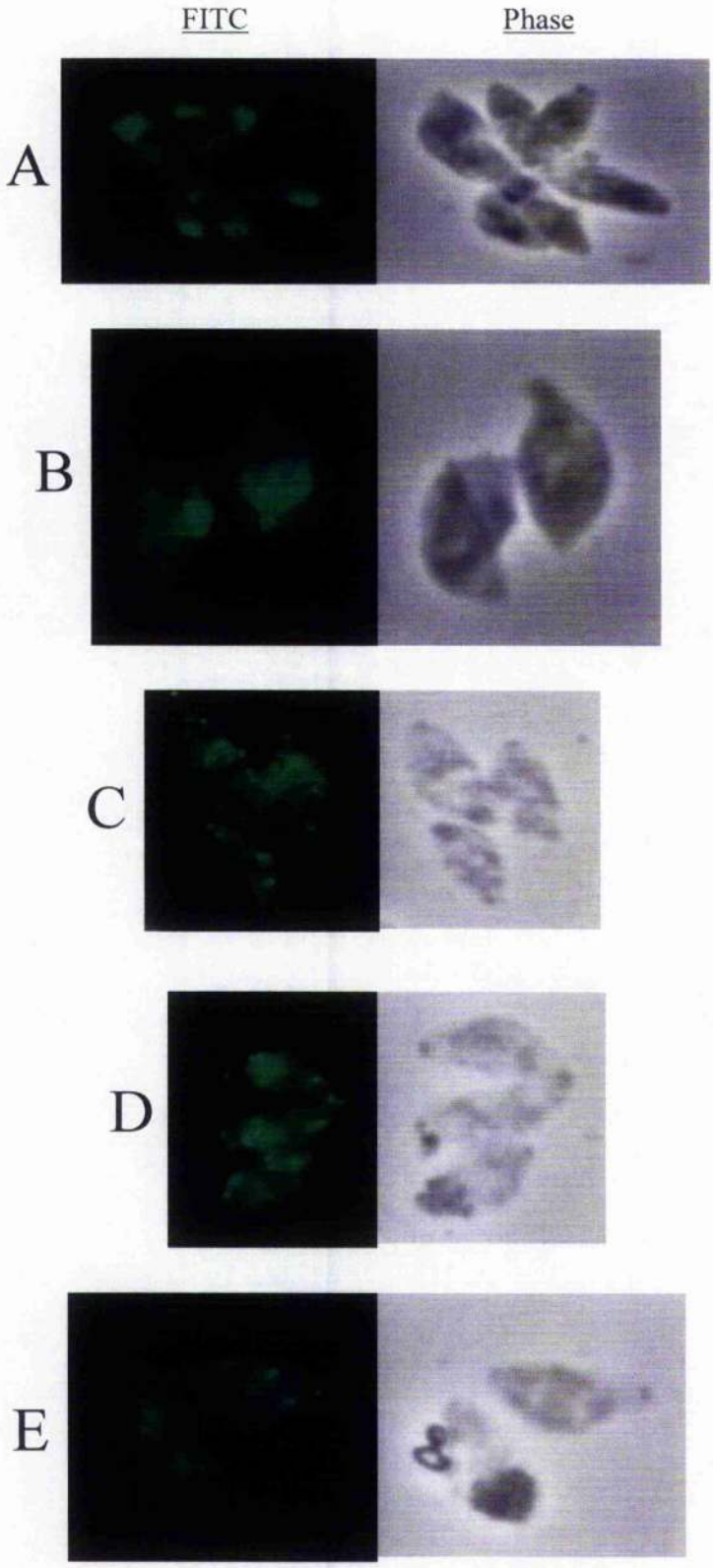


Figure 3.2 IFAT slides of *N. caninum* tachyzoites. Parasites were fixed onto slides and probed with sera from infected mice (A: PRUΔHX x NcGRA7 B: PRUΔHX x NcSRS2 C: PRUΔHX D: NC1 E: PBS) at day 48 p.i (day 28 for Group C). Secondary antibody was rabbit anti-mouse IgG labelled with FITC. Visualised under FITC filter and phase contrast.

3.3.4 Rate of Parasite Multiplication *in vitro*

In vitro growth rates of tachyzoites of *T. gondii* (PRUΔHX) and *N. caninum* (NC1), as well as transformed *T. gondii* (PRUΔHX x NcGRA7; PRUΔHX x NcSRS2), were assayed to determine whether any differences in the observed pathogenicity in the infected mice might be correlated with differential *in vitro* growth rates. Growth was assayed *in vitro* by measuring the differential incorporation of (^{3}H) uracil into parasite RNA.

Figure 3.3 shows the multiplication rate of *T. gondii* PRUΔHX x NcGRA7, PRUΔHX x NcSRS2, PRUΔHX and *N. caninum* NC1. Parasite multiplication was assayed at five timepoints - 0h, 2h, 12h, 24h and 48h. There is no noticeable difference between any of the *T. gondii* strains or *N. caninum*. The fact that there is no difference between transfected and non-transfected *T. gondii in vitro* does not necessarily correlate directly to the *in vivo* results. However, if indeed this were the case, it would suggest that any pathology caused in the mouse was not due to a quicker parasite proliferation in one group than another, but to some other difference between the parasite strains.

3.3.5 Additional Data

Further experiments were conducted following the original submission of this thesis to provide additional data for this chapter. Acknowledgement is made to staff at the Moredun Research Institute, Edinburgh (Paul Bartley and Steve Wright), the National Veterinary Research Institute, Uppsala (Jens Mattson) and the University of Liverpool (Sophia Latham) for their contribution.

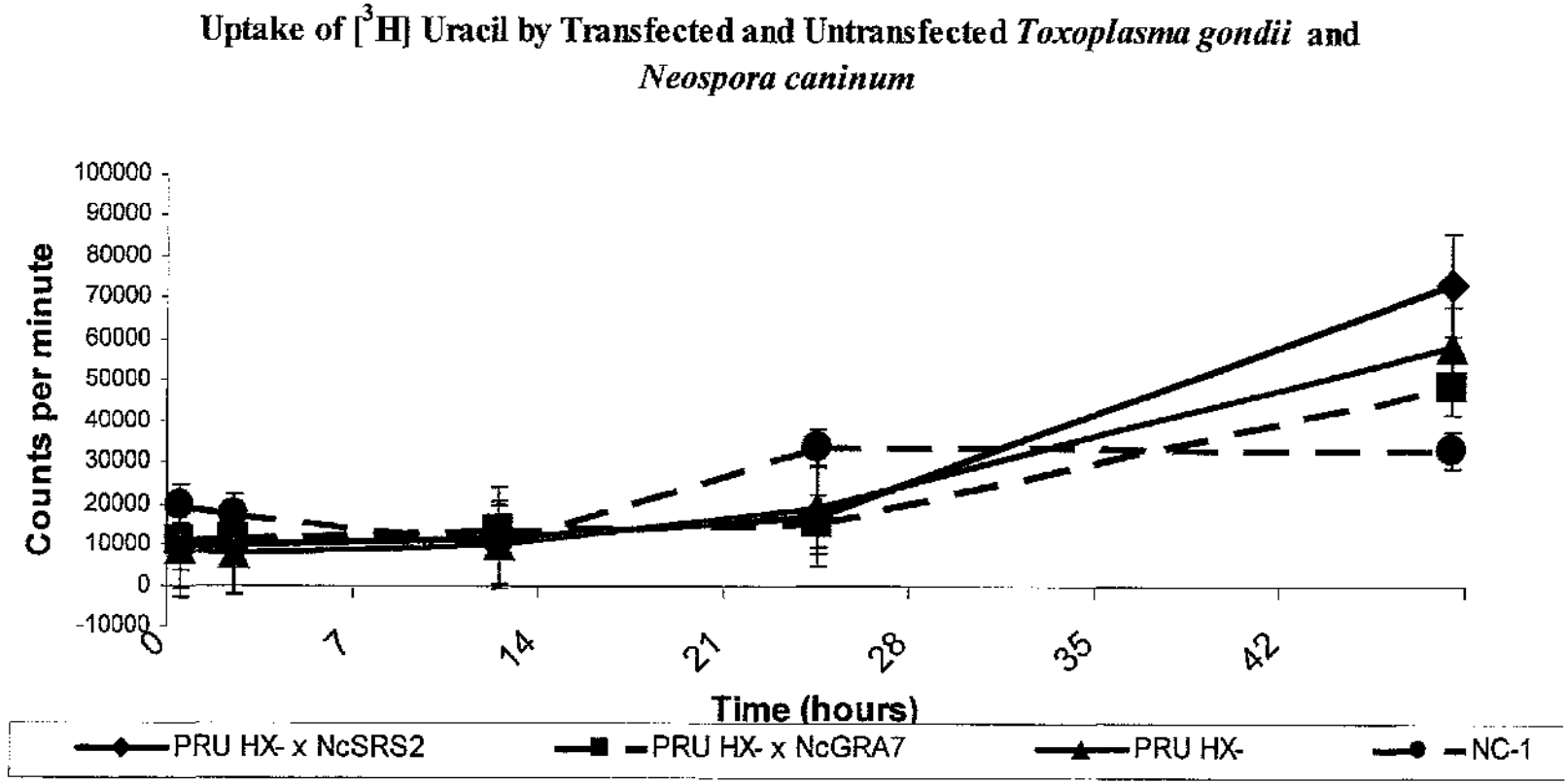


Figure 3.3 Graph of uptake of [^{3}H] uracil by transfected and untransfected *T. gondii* PRUΔHX and *N. caninum* NC1 using a procedure adapted from Pfefferkorn and Pfefferkorn (1981).

Experiments were performed using the *T. gondii* PRU transfected with *N. caninum* GRA7 (PRUΔHX x NcGRA7) DNA was extracted from the tachyzoites and the presence of the transgene confirmed by PCR (S. Latham, pers. commun.). Following this, a series of experiments were carried out to determine the cell-mediated and humoral immune responses in mice following inoculation of the transgenic parasites.. The immunisation studies were performed as described in Section 3.2.2 with the splenic T-cell proliferation assays being carried out as described in Section 4.2.5. The Western Blot analysis was carried out using the technique described in Section 2.2.20 and the IFAT studies were performed using the technique described previously (Buxton and Finlayson, 1996) with the modification of using a goat anti-mouse antiserum conjugated to fluorescein isothiocyanate (FITC) (Sigma, Poole, UK) as a secondary antibody.

3.3.5.1 Rodent Immunisation Studies

Five Porton strain mice were inoculated with 1 x 10^4 tachyzoites of *T. gondii* containing the *N. caninum* GRA7 transgene. One mouse was culled and the spleen removed, along with a sample of blood taken from a direct heart puncture immediately after death as a source of serum, at day 28p.i.; the other four mice (including the control mouse that was inoculated with 100µl of PBS) were culled and samples were collected at day 34 p.i. The spleen cells were co-cultured with a variety of antigens (see Table 3.5) and the proliferation measured as described in Section 4.2.5.

Antigen ID	Antigen	FinalConcentration (μg/ml)
Medium	Medium – IMDM + 10% FCS + Penicillin/Streptomycin	N/A
Con A	Concanavalin A	5
TG	S48 *T. gondii* (sonicated)	5
NC(R)	NC1 (ribolysed)	5
NC(S)	NC1 (sonicated)	5
GRA7(10)	PRUΔHX x Nc GRA7 (sonicated)	10
GRA7(5)	PRUΔHX x NcGRA7 (sonicated)	5
GRA7(2.5)	PRUΔHX x NcGRA7 (sonicated)	2.5
GRA7(1)	PRUΔHX x NcGRA7 (sonicated)	1
GRA7(0.5)	PRUΔHX x NcGRA7 (sonicated)	0.5

Table 3.5 Antigens used to stimulate spleen cells from mice inoculated with *T. gondii* PRU strain transfected with *N. caninum* GRA7 gene. The GRA7 antigens were prepared from the transfected tachyzoites PRUΔHX x NcGRA7.

The mean counts per minute (indicating proliferation) of the splenic cells, as well as the standard deviation and Stimulation Index (SI) for each mouse is shown in Table 3.6. Cell viability was confirmed for all spleen cells by the levels of mitogenic responses seen to ConA. Antigen specific proliferative responses were observed following co-culturing with water soluble fractions of sonicated PRU HX x GRA7at 10μg.ml . A dose response to the decreasing levels of PRU HX x GRA 7 antigen was observed in all the animals. Two animals (Animals 0 and 1) gave an SI value of greater than 3 following culturing with sonicated S48 *T. gondii* tachyzoite antigen. One animal (Animal 0) gave an SI value of greater than 3 following culture with the NC1 *N. caninum* lysate antigens.

Animal 0			
Antigen	cpm (mean)	St. Dev.	SI
Media	17.67	8.50	1.00
Con A	4926.67	189.04	**278.87**
TG	55.33	19.09	**3.13**
NC(R)	70.33	12.90	**3.98**
NC(S)	64.67	20.65	**3.66**
GRA7(10)	403.33	76.07	**22.83**
GRA7(5)	283.33	28.22	**16.04**
GRA7(2.5)	200.00	62.39	**11.32**
GRA7(1)	93.67	8.08	**5.30**
GRA7(0.5)	100.67	13.58	**5.70**

Animal 1			
Antigen	cpm (mean)	St. Dev.	SI
Media	32.00	5.57	1.00
Con A	6320.00	413.17	**197.50**
TG	118.67	41.06	**3.71**
NC1(R)	54.33	10.02	1.70
NC1(S)	76.33	24.91	**2.39**
GRA7(10)	408.67	35.84	**12.77**
GRA7(5)	272.67	82.52	**8.52**
GRA7(2.5)	233.67	48.44	**7.30**
GRA7(1)	154.67	85.89	**4.83**
GRA7(0.5)	111.00	37.24	**3.47**

Animal 2			
Antigen	cpm (mean)	St. Dev.	SI
Media	15.33	6.03	1.00
Con A	8614.00	1059.17	**561.78**
TG	nd	nd	nd
NC(R)	27.33	18.58	1.78
NC(S)	28.33	10.60	1.85
GRA7(10)	162.67	30.44	**10.61**
GRA7(5)	98.33	22.03	**6.41**
GRA7(2.5)	59.67	25.77	**3.89**
GRA7(1)	47.00	18.08	**3.07**
GRA7(0.5)	20.67	6.66	1.35

Animal 3			
Antigen	cpm (mean)	St. Dev.	SI
Media	42.33	14.74	1.00
Con A	6396.33	316.89	**151.09**
TG	86.00	2.65	2.03
NC1(R)	55.00	9.64	1.30
NC1(S)	68.00	35.55	1.61
GRA7(10)	296.33	58.77	**7.00**
GRA7(5)	363.33	81.37	**8.58**
GRA7(2.5)	246.67	82.56	**5.83**
GRA7(1)	144.67	45.96	**3.42**
GRA7(0.5)	265.33	42.19	**6.27**

Animal 4			
Antigen	cpm (mean)	St. Dev.	SI
Media	20.00	6.93	1.00
Con A	5709.33	599.15	**285.47**
TG	42.33	10.69	2.12
NC(R)	25.00	5.29	1.25
NC(S)	29.33	3.79	1.47
GRA7(10)	159.33	11.85	**7.97**
GRA7(5)	103.33	21.03	**5.17**
GRA7(2.5)	65.33	10.07	**3.27**
GRA7(1)	37.33	17.56	1.87
GRA7(0.5)	27.00	15.10	1.35

Table 3.6 Proliferation of murine splenic cells in the presence of heterologous antigens. The mean counts per minute (cpm) are derived from triplicate assays. Standard Deviation is shown (St. Dev.), along with the Stimulation Index (SI). SI > 3 are shown in bold. (nd = not done) The GRA7 antigens were prepared from the transfected tachyzoites PRUΔHX x NcGRA7.

3.3.5.2 Western Blot

Nitrocellulose membranes containing 5µg of *N. caninum* GRA7 fused with Maltose Binding Protein were probed with serum from: 1. Mouse infected with Vero cells (*N. caninum* specific antibody titre <1:16); 2. Mouse infected with *N. caninum* (*N. caninum* specific antibody titre 1: 4096); 3. Mouse infected with PRUΔHX x NcGRA7 *T. gondii* (*N. caninum* specific antibody titre <1:16). A secondary anti-mouse IgG antibody labelled with horseradish peroxidase was used subsequently to detect any specific binding.

Figure 3.4 shows the results of the Western Blot. A large number of strong bands were observed in Lane 1 (*N. caninum* infected mouse serum). Very faint bands (at approximately 47.5 and 60 kDa) were observed in Lanes 2 and 3 (serum from Vero cell infected mice and serum from mice infected with the transfected *T. gondii*, respectively).

3.3.5.3 IFAT

An indirect immunofluorescence test was set up to quantify titres of both *N. caninum* and *T. gondii* specific antibodies in serum from the mice immunised with PRUΔHX x NcGRA7. Tachyzoites of RH strain *T. gondii* and NC1 strain *N. caninum* were used as antigens in the test.

Sera from the infected mice were serially diluted in PBS from 1:16 to 1:1024 for use in the test and the secondary reagents e.g. goat anti-mouse IgG and goat anti-mouse IgM) labelled with FITC were used diluted in glycerol at 1:100 (IgG) and 1:50 (IgM)

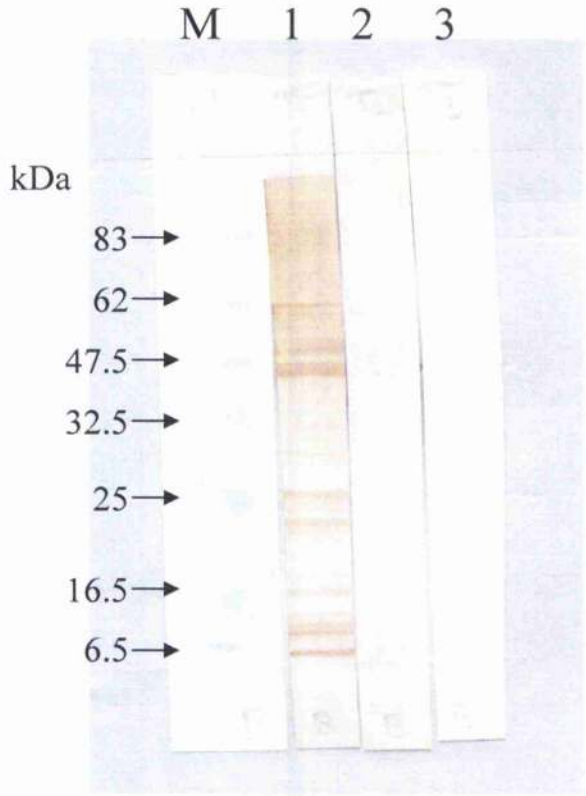


Figure 3.4 Western Blot of nitrocellulose membranes containing recombinant *N. caninum* GRA7. Lane 1 was probed with serum from a *N. caninum* infected mouse. Lane 2 was probed with serum from mice infected with Vero cells. Lane 3 was probed with serum from a mouse infected with *T. gondii* transfected with *N. caninum* GRA7. M is the molecular weight marker.

Table 3.7 shows the results of the IFAT experiments. None of the mice produced IgG or IgM specific for *N. caninum*. However, anti-*T. gondii* IgG titres of 1/1024 were observed in all animals.

Mouse	anti-NC1 IgG	anti-*T. gondii* IgG	anti-NC1 IgM	anti-*T. gondii* IgM
0	<1/16	1/1024	<1/16	<1/16
1	<1/16	1/1024	<1/16	1/64
2	<1/16	1/1024	<1/16	<1/16
3	<1/16	1/1024	<1/16	<1/16
4	<1/16	1/1024	<1/16	<1/16

Table 3.7 IFAT detection of anti-*N. caninum* and anti-*T. gondii* IgG and IgM. The figures indicate the lowest dilution of serum antibodies from experimental animals that generated specific fluorescence using tachyzoites of *T. gondii* or *N.caninum* as antigen..

3.3.5.4 General Conclusions

This additional work was done in an effort to help determine whether the transfected *T. gondii* containing *N. caninum* GRA7 produced specific immune responses to *N. caninum* in experimentally infected mice. Only five mice were used, so the conclusions are only preliminary. There did appear to be some specific proliferative response in cells taken from the spleens of infected mice. Proliferation in cells co-cultured with a crude lysate antigen prepared from PRUΔHX x NcGRA7 antigen was slightly higher than the proliferation caused when the cells were cultured with other antigens. A dose response was noticeable in the infected mice. However, only one out of the five mice showed positive proliferative responses to a crude lysate of *N. caninum* antigen.

The Western Blot and IFAT experiments showed that the mice immunised with PRUΔHX x NcGRA7 did not produce a measurable antibody response against *N. caninum* or more specifically the GRA7 antigen. Whereas, control serum from mice

infected with *N. caninum* recognised several bands in the GRA7 western blot. This may suggest that, although the *T. gondii* is stably transfected with *N. caninum* GRA7 the expressed transgene is either not available or is not being adequately presented to the immune system of the mouse. The addition of a the *c-myc* tag to the sequence of the NcGRA7 gene may have altered the structure of the gene product sufficiently such that the protein was not trafficked within the cell correctly. Some proteins are under post-translational control in *T.gondii* and therefore may not be efficiently expressed. In the case of a GRA-protein that control could also mean that the protein is not correctly targeted.

Alternatively, the results presented here may suggest that the transgenes are not being expressed by *T. gondii*, despite being stably transfected into the parasite. Further studies would be required before a firm conclusion can be reached.

3.4 DISCUSSION

The aim of the experiments described in this chapter was to establish a suitable mouse model to allow us to study the effects of inoculating transgenic parasites into mice. The longer-term aim of this work would be to determine if the immune response generated following infection with the transgenic parasites would be protective against challenge with *Neospora caninum* parasites.

3.4.1 Mouse Strains

In the studies described in this chapter, three different strains of mice were inoculated with untransfected *T. gondii*. There has been work published which suggests that the genetic background of the mouse host may directly affect the outcome of infection with *T. gondii* (Lee and Kasper, 2004). Two inbred strains, BALB/c and C56Bl/6 – and one outbred strain, Porton, were used to determine a suitable animal model to allow us to conduct an *in vivo* assessment of the transgenic parasites. Unfortunately the PRUΔHX *T.gondii* strain of parasites proved to be highly virulent in mice and it took longer than anticipated to establish an inoculation and treatment regime that would allow the mice to become infected but not succumb to disease. The wild-type PRU strain of *T. gondii* has previously been characterised as a Type II strain shown to be relatively benign in OF1 mice (Zenner *et al*, 1999). However, when the HXGPRT gene is knocked out, the virulence of the strain increases, the reason for which is unknown (Dubremetz, pers. commun.). This increased virulence is reduced when the knockout gene is re-introduced.

In previous studies, outbred Swiss-Webster mice have been shown to be more resistant to infection with *N. caninum* than inbred BALB/c mice (Cole *et al*, 1995).

However, Swiss-Webster mice have also been shown to be highly susceptible to *T. gondii* infection, as mice inoculated with one tachyzoite of the RH strain (a Type I strain) rapidly succumbed to infection (Dubey, 1999). The experiments in this chapter would indicate that outbred Porton mice are less susceptible than either BALB/c or C56BL/6 mice to infection with PRUΔHX strain *T. gondii* since there were Porton mice that survived >21d pi, compared to no BALB/c or C57BL/6 mice that survived at this time point following inoculation. The PRUΔHX strain of *T. gondii* used was a knockout of a Type II strain (Howe and Sibley, 1995). Type I strains of *T. gondii* generate higher pathology and are generally more virulent that Type II strains in inbred mice (Howe and Sibley, 1995). Therefore, the Porton strain of mouse was used to conduct some preliminary studies to examine the humoral immune response to the transgenic parasites.

3.4.2 Humoral Immunity

When B-cell deficient mice are challenged with tachyzoites of *N. caninum*, there is an increased susceptibility to infection (Eperon *et al*, 1999). A similar increase in susceptibility is observed when B-cell deficient mice are challenged with *T. gondii* (Kang *et al*, 2000). This suggests that the humoral response is important in protecting against these two organisms.

There was some evidence using localisation studies that mice inoculated with the transgenic parasites produced antibodies that recognised antigens of *N. caninum*. Different staining patterns were observed using antibodies obtained from the mice infected with transgenic parasites compared with the wild type parasites. This suggests that the antibodies raised in the mice infected with transgenic parasites may

have been directed against particular antigens on the *Neospora* parasites. Clearly, further work needs to be done to examine the specificity of this immune response in more detail some of which has been discussed in the additional studies section 3.3.5.

3.4.3 Future Work

The preliminary immunology studies using IFAT to identify a specific antibody response to *N. caninum* in the mice inoculated with the transgenic *T. gondii* has shown that there is potentially a specific response, though time constraints meant that further studies were not possible to confirm if this was indeed the case. These studies would include Western Blot and T-cell proliferation assays to further assess the specificity of the humoral immune response and to examine the cellular immune responses, thought to be important in protective immunity to *N. caninum* (Kaspar and Khan, 1997).

The Western Blotting experiments could be carried out two ways. Firstly, whole parasite lysates of untransfected PRUΔHX *T. gondii*, transfected *T. gondii* expressing NcGRA7 and NcSRS2, and NC1 *N. caninum* would be separated on SDS-PAGE gels and blotted onto nitrocellulose, before being probed with sera from the inoculated mice, and differences in response to the different parasites assayed. In addition, recombinant NcGRA7 or NcSRS2 could be run on an SDS-PAGE gel, blotted onto nitrocellulose and probed with sera from infected mice to detect specific antibodies raised against the transgene products. This second method may be more useful, due to the known level of cross-reactivity between *N. caninum* and *T. gondii* (Hemphill, 2000). However some cross-reactivity may still be observed using the recombinant proteins, particularly when using NcSRS2, which has been shown to be relatively

homologous between *T. gondii* and *N. caninum* (Howe *et al*, 1998). A study by Nishikawa *et al* (2002) suggests, however, despite the homology, TgSRS2 and NcSRS2 are sufficiently different that antibodies raised against one will not recognise the other (Nishikawa *et al*, 2002).

One of the difficulties in trying to dissect the specific immune responses to *N. caninum* with a concurrent infection of *T. gondii* is the level of similarity between the two parasites. It would be easier to detect specific immune responses to the transgene if the *N. caninum* genes were transfected into a parasite or a bacteria species that was unrelated. However the down side of doing that would be that the cross-reactivity of *T. gondii* may be helpful to additionally stimulate the immune response.

The specific proliferative and cytokine response of spleen and lymph node cells to NcGRA7 and NcSRS2 would also be interesting to study. This would require culturing immune cells *in vitro* and stimulating them with a number of different antigens including whole *N. caninum* NC1 antigen lysate, whole *T. gondii* PRUΔHX antigen lysate, lysates prepared from recombinant PRUΔHX expressing NcGRA7 and NcSRS2, and the recombinant antigens NcGRA7 and NcSRS2. Hopefully by using these recombinant antigens it would be possible to dissect the cellular response against the transgenes as well as the parasite carrier. In the study by Ramirez *et al* (2002), a specific cell proliferative response to recombinant *L. major* KMP-11 was identified, and mice were protected following challenge with *L. major*. (Ramirez *et al.*,2001). This suggests that *T. gondii* may be useful as a live delivery vehicle for heterologous proteins, and it would be interesting to see if this was also the case for *N. caninum* infections. To do this we would challenge the mice with live *N. caninum*

tachyzoites following immunisation with the transgenic parasites to determine whether the mice had generated protective immunity.

In addition to using techniques to help determine the specificity of the immune response, it is also important to check expression of the protein and how this may be processed and presented to the immune system.

CHAPTER 4:

STIMULATION OF BOVINE T-LYMPHOCYTES BY *N. caninum* PROTEINS SEPARATED USING DIFFERENT SDS-PAGE METHODS

- ◊ Prepare and separate whole *N. caninum* protein lysate by one- and two-dimensional polyacrylamide gel electrophoresis
- ◊ Prepare by electroelution several protein fractions for lymphoproliferation assays and assess feasibility of using this antigen to stimulate bovine T-cells
- ◊ Produce $CD4^+$ T-cell lines from *N. caninum* infected cattle
- ◊ Quantify proliferation of bovine $CD4^+$ T-cells to different fractions
- ◊ Identify T-cell immunodominant fractions for downstream analysis by mass spectrometry

4.1 INTRODUCTION

4.1.1 Separation of Proteins by One-Dimensional Polyacrylamide Gel Electrophoresis (PAGE)

4.1.1.1 Sodium Dodecylsulphate PAGE (SDS-PAGE)

One-dimensional Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a standard technique within all molecular biology and biochemistry laboratories. It is based on a technique developed in the 1970s to analyse the protein conformational changes in the production of viral particles (Laemmli, 1970).

The basis of this method is that polymerised polyacrylamide forms a matrix through which proteins, and other charged molecules, can pass in an electric current. The distance they can penetrate through this matrix is dependent on the size of the molecule i.e. large, branched molecules pass slowly through the matrix whilst smaller molecules can pass more quickly. The addition of the detergent SDS aids the passage of these molecules through the matrix and crucially removes the influence of charge, inherent in many proteins, on their migration in the electric field.

There are many uses for SDS-PAGE separated proteins, including the identification of specific proteins using immune sera in Western Blotting (Wastling *et al*, 1994). This technique enables the transfer of proteins separated by SDS-PAGE onto a nitrocellulose membrane, prior to being probed by specific antibodies. Specific binding between proteins and antibodies, and subsequent visualisation using labelled, secondary antibodies against the initial specific antibody, can help the identification of individual proteins.

4.1.1.2 Two Dimensional PAGE (2DE)

Separation of proteins in two dimensions by isoelectric point as well as molecular weight is now a widely used technique in proteomics. It was first developed in 1975 (O'Farrell *et al*, 1975) but it was not until the development of immobilised pH strips that it became a more practical, reproducible technique (Gorg *et al*, 1988). The immobilised pH strips are strips of polyacrylamide polymerised onto plastic in which the pH of this acrylamide varies across the strip. When a current is passed through this strip in the presence of proteins, the proteins will focus according to their isoelectric point. This strip is then placed on top of a SDS-polyacrylamide gel. Similar to one-dimensional SDS-PAGE, the proteins are then separated according to their molecular weight (Laemmli, 1970).

Proteins separated by 2-DE can be used for mapping the proteome, or partial proteome, of an organism. This technique has been used to map the partial soluble proteome of the apicomplexan parasite *T. gondii* (Cohen *et al*, 2002) as well as identifying proteins that are recognised by antibodies against *T. gondii* (Dlugonska *et al*, 2001). Alternatively, the separation in two dimensions can be used to isolate individual proteins for analysis where individual protein spots are picked, digested by trypsin and identified by mass spectrometry (Cohen *et al*, 2002).

4.1.2 Elution of Proteins from SDS-PAGE gels

4.1.2.1 The Necessity for Protein Elution

Elution of proteins from the polyacrylamide matrix is often not required for further analysis of the proteins, for example mass spectrometric analysis can be performed following in-gel digestion (see Chapter 5). However, if the proteins of interest are required for testing biological activity or reactivity in living systems, for example in

in vitro tissue culture, then the presence of polyacrylamide and other gel contaminants may be detrimental to the system. Both acrylamide and SDS have been shown to be toxic to cells *in vitro* (Grant and Acosta, 1994; DeJongh *et al*, 1999). In such cases, separation of the protein from the gel matrix is essential.

4.1.2.2 Benefits of Protein Elution

Proteins eluted from SDS-PAGE gels have the advantage over proteins blotted onto membranes in that they are eluted into solution, usually a very weak Tris buffer (Gulle *et al*, 1990). This has the advantage over membrane-bound proteins in that eluted protein samples do not contain membrane components influence further experimental designs.

Proteins can also be eluted from native polyacrylamide gels (Schagger *et al*, 1994). This has the advantage of maintaining the secondary structure of the protein, unlike in SDS-PAGE gels where proteins are denatured.

4.1.2.3 Use of Electroeluted Proteins in Immunological Studies

The majority of the work studying the cellular immune response using electroeluted proteins has been carried out using *Mycobacterium sp.* (Gulle *et al*, 1993; Gulle *et al* 1995). No studies on the T-cell response to *N. caninum* infection have been carried out using this technique. This chapter aims to identify individual proteins from *N. caninum* that stimulate T-cells from *N. caninum*-infected cattle to proliferate *in vitro*. It is therefore appropriate to look in more detail at the immunology of *N. caninum* before discussing previous work identifying proteins that produce a cellular response against *N. caninum*.

4.1.3 Immunity to *N. caninum* Infections

4.1.3.1 Humoral Immune Responses

The role of antibodies in *N. caninum* infections is relatively uncharacterised. B-cell deficient mice (uMT) that do not have the ability to make antibodies when infected with *N. caninum* tachyzoites, showed an increased mortality and pathology as compared to the corresponding wild type mice. This suggests a protective role for antibodies (Eperon *et al* (1999)). This role may be in the prevention of the invasion of host cells by tachyzoites. Monoclonal antibodies identifying parasite antigens of various molecular weights (36, 42 and 70kDa) significantly inhibited the invasion of the parasite into host cells *in vitro* (Nishikawa *et al*, 2000). However, once the parasite is inside the cell, antibody is no longer effective and cell-mediated immune mechanisms are required to control the infection.

The humoral immune response to *N. caninum* infection is very useful in the diagnosis of infection. Specific antibodies to *N. caninum* antigens have been shown to be produced in response to natural and experimental infection (Conrad *et al*, 1993). The antigens against which these specific antibodies have been produced have been used extensively in ELISA diagnostics, and the antibodies themselves have been used in antigen capture assays (Dubey *et al*, 1997); these are discussed further in section 4.1.3.3. In addition, immune sera has been used to identify immunodominant *N. caninum* antigens (reviewed in Hemphill *et al*, 1999) and discussed in section 4.1.3.3.

4.1.3.2 Cell-Mediated Immune Responses

4.1.3.2.1 Mice

As *N. caninum* is an obligate intracellular parasite, there has been much interest in trying to understand the cellular response against *N. caninum*. The role of $CD4^+$ and $CD8^+$ cells in the protective immune response against *N. caninum* has been studied in mice (Tanaka *et al*, 2000). In the study by Tanaka and colleagues (2000), mice were treated with monoclonal antibodies against $CD4^+$ and/or $CD8^+$ T-cells to deplete these populations. The mice were then challenged with *N caninum* tachyzoites. Mice with intact $CD8^+$ T-cells (i.e. the $CD4^+$ T-cells were destroyed) survived post-*N. caninum* challenge, whereas all the mice treated with anti-CD8 monoclonal antibody (i.e. only had $CD4^+$ T-cells) died following challenge (Tanaka *et al*, 2000). $CD4^+$ T-cells also have a role in the production of IFNγ, known to be important in protection against acute neosporosis (Khan *et al*, 1997) and providing help for the production of specific antibodies (Tanaka *et al*, 2000). In *in vivo* studies, mice depleted of IFNγ (and IL-12) have shown increased susceptibility to *N. caninum* infection (Khan *et al*, 1997), (Baszler *et al*, 1999).

4.1.3.2.2 Bovine responses

$CD4^+$ T-cells have also been recognised as important in cattle infected by *N. caninum*, both for their production of IFNγ to control the parasite (Lunden *et al*, 1998; Marks *et al*, 1998) and for their direct cytotoxic effects (Staska *et al*, 2003). It appears that the dual role of $CD4^+$ T-cells in being directly cytotoxic and producing IFNγ is important in protecting against *N. caninum* infections. IFNγ has been shown to have a role in significantly inhibiting intracellular multiplication *in vitro* (Innes *et al*, 1995).

4.1.3.2 Identification of N. caninum Antigens using Antibodies

The identification of *N. caninum* antigens is of interest for the development of more diagnostic reagents, vaccine candidates, and to improve our understanding of parasite biology. An antigen, identified as being a potential vaccine candidate, is the *N. caninum* SAG-1 related surface protein 2 (NcSRS2) as discussed in Chapter 2. The NcSRS2 molecule, when delivered as part of a recombinant vaccinia virus vector, has shown potential as a vaccine candidate after demonstrating protection against vertical transmission in mice (Nishikawa *et al*, 2001).

Many other *N. caninum* proteins have been recognised by serum antibodies taken from infected animals (Lally *et al*, 1997; Atkinson *et al* 2001). In the paper by Lally and colleagues (1997), the authors identified a dense granule protein, initially termed NcDG1, though later referred to as NcGRA7 due to its homology to GRA7 in *T.gondii*. The gene product was recognised by screening a cDNA library of *N. caninum* using sera from several infected cattle. Atkinson *et al* (2001) used a similar screening technique, using mouse sera, to identify two gene products. In other studies, further antigens of *N. caninum* are recognised by immune sera (reviewed in Hemphill, 1999). Polyclonal antisera, raised against *N. caninum* tachyzoites in rabbits, have been used to identify approximately twenty antigens of varying sizes (Barta and Dubey, 1992). Murine monoclonal antibodies raised against *N. caninum* tachyzoites (Cole *et al*, 1993) identified eight major and several minor antigens, ranging in size from 97.4kDa (the largest) to 31kDa (smallest) and were localised in the apical complex, dense granules and parasitophorous vacuole (Cole *et al*, 1994). The production of monoclonal antibodies against cell lysates identified several antigens (molecular weights of 25kDa, 65kDa and 116kDa), which were

subsequently used to set up diagnostic assays for bovine neosporosis (Bjerkas *et al*, 1994; Cole *et al*, 1994; Baszler *et al*, 1996).

4.1.3.3 Identification of Pathogen Antigens Using T-Cell Assays

The identification of appropriate antigens from pathogenic organisms is important when developing effective vaccines. Antigens recognised by T-cells are particularly important in the case of intracellular pathogens, as T-cells are known to have an important role in protective immunity.

4.1.3.3.1 Mycobacterium sp.

Proteins that stimulate an immune response to pathogens, particularly a cellular response (since humoral responses can be detected using Western Blotting) have been identified by stimulating T-cells with eluted proteins (Gulle *et* al, 1993; Pinto *et al*, 2000). Much immunological work with eluted proteins has centred on the identification of the cellular responses to proteins of *Mycobacterium bovis* or *M. tuberculosis* (Gulle *et al*, 1990; Gulle *et al*, 1993; Andersen and Heron, 1993; Gulle *et al*, 1995), with a view to using any identified proteins as components of subunit vaccines. One study looked at the difference in bovine immune responses between viable BCG and γ-irradiated BCG (Gulle *et al*, 1995). Different responses were seen at a cellular proliferation level between different groups. The T-cells from cattle immunised with either the viable or irradiated form of BCG responded similarly to *M. bovis* fractionated lysate. However, T-cells from cattle immunised with viable BCG recognised fractions of the *M. bovis* lysate that cells from cattle immunised with irradiated BCG did not (Gulle *et al*, 1995). This indicated that the use of electroeluted proteins is a useful and appropriate method for stimulating T-cells to identify immunologically active proteins.

4.1.3.3.2 Toxoplasma gondii

T-cell assays have also been used to identify antigens from *T. gondii* (Saavedra *et al*, 1991). In this study, a library of *T. gondii* cDNA expressed in a lambda vector was screened with sera from an immune human donor to identify antigens recognised by the humoral immune response. These antigens were further screened using a T-cell clone and an antigen of 54kDa was identified.

This experiment was limited by the initial screening of the expression library by immune sera. To assay a larger number of *T. gondii* proteins without the use of immune sera, and also to harness the separation of proteomic techniques, a lysate of *T. gondii* tachyzoites was separated by 2-DE SDS-PAGE. The resulting separation was electroeluted from the gel into T-cell proliferation assays (Reichmann *et al*, 1997). In this study, a *T. gondii* parasite lysate was electrophoretically separated in two dimensions, with individual proteins being used in proliferation assays using cells from a *T. gondii*-specific T-cell clone, chosen for its induction of toxoplasmocidal activity in co-cultured macrophages (Reichmann *et al*, 1997). A fraction of approximately 40kDa stimulated proliferation of the cells (Reichmann *et al*, 1997).

4.1.3.3.3 Neospora caninum

Little work has been done using T-cell proliferation assays to identify immunodominant antigens of *N. caninum*; indeed, only one study to date has been published with this aim (Marks *et al*, 1998). In this study, whole *N. caninum* lysate (NC1 strain) was separated on a one-dimensional SDS-PAGE gel and transferred onto nitrocellulose membrane. The membrane was cut into sections containing several protein bands of similar molecular weight before being added to polyclonal

bovine T-cells and autologous antigen presenting cells *in vitro*. The majority of the cells showed little proliferation; however, the cultures containing proteins in the <30kDa range proliferated significantly and produced IFNγ (Marks *et al*, 1998).

The work described in this chapter aims to extend the sensitivity of the experiment described in the paper by Marks and colleagues (1998) by testing protein fractions separated by 2-DE for their ability to stimulate immune T-cells from *N. caninum* infected cattle.

4.1.4 Aims and Objectives

The aims and objectives of this chapter are to:

- Prepare and separate whole *N. caninum* protein lysate by one- and two-dimensional polyacrylamide gel electrophoresis
- Prepare by electroelution several protein fractions for lymphoproliferation assays and assess feasibility of using this antigen to stimulate bovine T-cells
- Produce $CD4^+$ T-cell lines from *N. caninum* infected cattle
- Quantify proliferation of bovine $CD4^+$ T-cells to different fractions
- Identify T-cell immunodominant fractions for downstream analysis by mass spectrometry

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Protein Samples

4.2.1.1 Harvesting and Storage of Parasites

Parasites were harvested from culture as described previously (Chapter 2.2.5). They were pelleted by centrifugation and washed three times in PBS. The parasites were resuspended at a concentration of 1-2x10^8 before being pelleted by centrifugation at 13000x *g* and stored at -70°C prior to use.

4.2.1.2 Preparation of Cell Lysate for One-Dimensional SDS-PAGE

Pellets of tachyzoites (1-2 x10^8) were resuspended in 50-100µl of ddH_2O. The suspension underwent three cycles of freezing-thawing in liquid nitrogen, before being sonicated in a sonicating water bath for 5 mins. The samples were diluted 1:1 in SDS-PAGE loading buffer (100mM Tris.Cl (pH 6.8), 200mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) prior to electrophoresis.

4.2.1.3 Preparation of Cell Lysate for Two-Dimensional SDS-PAGE

The preparation of tachyzoite protein for Two-Dimensional SDS-PAGE (2DE) was similar to that for 1D-SDS-PAGE in that the parasite pellet (1-2 x10^8) was freeze-thawed in liquid nitrogen and sonicated. However, for 2DE, the tachyzoites were initially resuspended in 100µl Lysis Buffer/1x10^8 tachyzoites. Lysis Buffer (Amersham Biotech Ltd.) contained 8M urea, 4% CHAPS and 40mM Tris. The parasite suspension was then freeze-thawed five times before 200µl Rehydration Buffer (8M urea, 2% CHAPS, bromophenol blue (sufficient to give a brilliant blue colour to the solution), 4-5mg DTT and 5µl/ml IPG Buffer (Amersham Biotech Ltd.,

Amersham, UK) were added. This mix was then sonicated for 5 min and then incubated at room temperature for 1h prior to 2DE analysis (Cohen *et al*, 2002).

4.2.1.4 Preparation of NC1 Whole Cell Antigen

N. caninum whole cell antigen was prepared using cycles of sonication, freeze-thawing and centrifugation. For the experiments described below, the tachyzoites (stored until required at -80°C in pellets of 1-2 x10^8 tachyzoites) were resuspended in 200 µl ddH_2O. This suspension underwent three cycles of freezing-thawing in liquid nitrogen. This was followed by 7 x 15s cycles of sonication on ice and the suspension was centrifuged at 10,000 x *g* for 30min. The water-soluble fraction was aspirated from the pelleted cellular fragments and stored at -20°C. Protein concentration was determined by a coloromctric assay comparing the binding of Coomassie-based reagents by the NC1 antigen to that by a range of known concentrations of bovine serum albumin (BSA) (Coomassie® Plus Protein Assay Reagent (Pierce, Rockford, USA)) according to the manufacturer's instructions. Triplicate dilutions of a 2.0mg/ml stock solution of BSA were prepared in ddH_2O. 1ml of each standard and the test sample were transferred to individual tubes (ddH_2O was used as the control). To each tube, 300µl of the Coomassie® Plus Reagent was added, and the tube was inverted to mix the samples and reagent. The absorbance of each of the dilutions of BSA was measured at 595nm and an absorbance curve produced. The absorbance of the unknown water soluble fraction (wsf) antigen was also measured and plotted against the curve. The antigen was resuspended in culture medium at a concentration of 10µg/ml for subsequent assays, and stored at -20°C until required.

4.2.2 SDS-PAGE Electrophoresis

4.2.2.1 One-Dimensional SDS-PAGE

4.2.2.1.1 Sample Preparation

Protein lysates produced as described in Section 4.2.1.2 were incubated at 100°C for 10 mins prior to being loaded into the wells in the stacking gel. 10µl of Broad Range Molecular Weight marker (Promega) was also heated at 100°C for 10 minutes and loaded onto the gel.

4.2.2.1.2 SDS-PAGE Separation

Protein lysates were separated on a 12-15% polyacrylamide-Tris buffered gel (Laemmli, 1970). This consisted of a discontinuous polyacrylamide gel, with a 12-15% resolving gel, buffered by 1.5M Tris.Cl (pH 8.8) and a 5% stacking gel buffered by 1M Tris.Cl (pH 6.8). Both gels contained 10% SDS and 10% ammonium persulphate. Samples were separated in Tris-Glycine SDS-PAGE Running Buffer (containing 10mM Tris Base, 50mM glycine, 0.1% SDS (all Sigma-Aldrich) using 100-120V for 1-1½h before being visualised by either Coomassie Blue or Sypro Orange staining (section 4.2.2.3).

4.2.2.2 Two-Dimensional Gel Electrophoresis

4.2.2.2.1 Isoelectric Focussing (IEF) of Proteins

IEF was carried out using immobilised pH gradient (IPGPhor) strips (Amersham Biotech Ltd.). The proteins were separated according to their isoelectric point. The strips had a pH gradient, either a linear or logarithmic gradient. The strips could have a relatively wide range (pH 3-10 or 4-7), or narrow range (pH 4.0-4.5). The strips

could either be 7cm or 24cm long, depending on the amount of protein to be separated and the size of the PAGE gel used for second dimension separation.

For minigel separation, 7cm strips were used and in this case, 125µl of sample prepared as described in section 4.2.1.2 was loaded into the IPGPhor ceramic strip holder. For large gel separation, 24cm strips were used and 250µl of sample was loaded into the strip holder. The strips were placed onto the sample in the holder and covered by Strip Cover Fluid (Amersham Biosciences). The strips were subjected to the conditions described in Table 4.1.

Step	Voltage (V)	Time (h)	Volt hours (Vh)
1	30	12	360
2	500	1	500
3	1000	1	1000
4	10000	4	40000

Table 4.1 Isoelectric Focussing Conditions for IPGPhor Immobilised pH Gradient strips

Strip rehydration was carried out by passing 30V through the strip, since this enabled better entry of proteins than rehydration without voltage. Isoelectric focussing then occurred in the higher voltage steps.

4.2.2.2.2 Second Dimension SDS-PAGE Separation

Rehydrated strips with isoelectrically focussed proteins were equilibrated prior to SDS-PAGE separation. Equilibration Buffer (500mM Tris.Cl, 6M urea, 30% v/v

glycerol, 2% w/v SDS, bromophenol blue) was supplemented with 10mg/ml DTT (Amersham Biosciences) (Equilibration Buffer I) or 25mg/ml α-iodoacetamide (Sigma Aldrich) (Equilibration Buffer II). Strips were incubated at room temperature on a rocking platform, firstly in Equilibration Buffer I for 15min, then Equilibration Buffer II for 15min.

Strips were placed on top of 12-15% polyacrylamide resolving gels and covered by agarose sealing solution (0.5% w/v agarose in Tris-Glycine SDS-PAGE Running Buffer containing bromophenol blue). The gels were immersed in SDS-PAGE Running Buffer and run at 100-120V for 1.5h (minigel) or 20-25h (large gel), or until the dye front ran to the end of the gel. The gel was removed from the apparatus and the proteins were visualised as described below.

4.2.2.3 Visualisation of Proteins

4.2.2.3.1 Coomassie Blue Staining

Coomassie staining of proteins was commonly used to detect protein separated on either SDS-PAGE or 2DE gels. Gels were submerged in stain (10% v/v acetic acid, 40% v/v methanol, 0.1% w/v Coomassie Blue R250 (Biorad)) for at least 2h, but generally overnight. The gels were destained using 10% v/v acetic acid / 40% v/v methanol. The gels were destained for as long as necessary to visualise either the bands or spots without significant background staining.

4.2.2.3.2 Sypro®Orange Fluorescent Staining

Sypro® Orange Protein Stain (Biorad) binds to the SDS attached to electrophoresed proteins. For 2DE-separated proteins, the PAGE gels were immersed in 0.05% w/v SDS solution to sensitise the proteins to the stain by increasing the binding of SDS.

A 1:5000 dilution in 7% v/v acetic acid of Sypro® Orange stain was made and the gels were soaked in the stain for 30min. After 30min, the gels were removed and washed in 7% v/v acetic acid to remove excess stain. The gels were visualised by Typhoon Laser Scanner under 532nm.

4.2.3 Electroelution of Proteins

4.2.3.1 Sample Preparation

Proteins separated by either SDS-PAGE or 2DE were manually excised from the gel using clean scalpel blades. Protein bands or spots were removed from the gel with as little excess polyacrylamide as possible. Bands from SDS-PAGE gels were further cut into smaller pieces to improve elution. This process was only required for elution using the Model 422 Eluter (Biorad). Elutions using the Bioteluter (Biometra) were carried out using non-stained, non-fixed 2DE gels.

4.2.3.2 Electroelution Using Biorad Model 422 Eluter

Elution from the 422 Eluter (Biorad) was carried out according to the manufacturer's instructions (see Figure 4.1). The membrane caps used were for a molecular weight cut-off of 3500Da. Thus, any proteins above this size were retained by the membrane for later manipulation, whilst other proteins passed through into the elution buffer (10mM Tris). It should be noted that the elution buffer initially contained 50mM glycine and 0.1% SDS, as described in the manufacturer's instructions. Subsequent elution buffers used did not contain glycine or SDS, since these were thought to have potentially adverse effects on the cells used in later analyses. In addition, the Tris concentration was also reduced to 5mM, sufficient for a current to pass through, but reduced the concentration of salts in the final eluted sample.

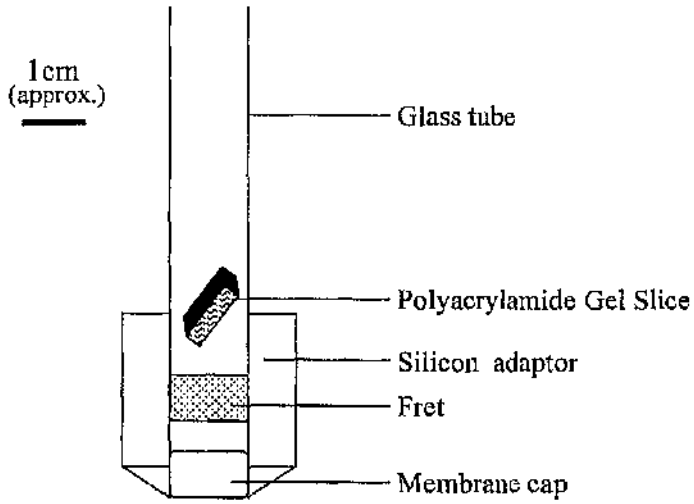


Figure 4.1 Schematic of Model 422 Eluter (Biorad). Polyacrylamide gel slices were placed in the glass tube that was filled with 10mM Tris elution buffer. 10mA per tube / adaptor combination was used to elute proteins from the gel slice through the fret into the membrane cap. The proteins were collected for later analysis. (Adapted from Model 422 Electro-Eluter Instruction Manual)

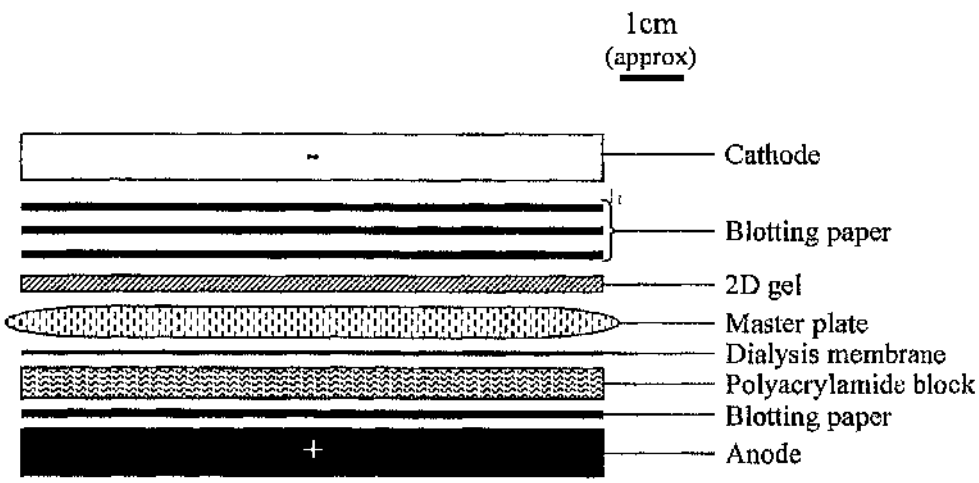


Figure 4.2 Schematic of Bioteluter (Biometra). An unfixed, unstained 2DE gel was laid onto a master plate with 528 holes sealed with dialysis membrane and polyacrylamide gel. 0.8mA/cm^2 gel was used to elute into 5mM Tris elution buffer. (Adapted from Biometra Biotcluter Manual Rev. 02)

Prior to electroelution, the membrane caps were soaked in elution buffer at 60°C for 1h. The membrane cap was attached to a silicon adaptor that was then filled with elution buffer to remove any bubbles of air that may impinge on elution efficiency. The cap-adaptor assembly was attached to the glass sample tube that had a silicon fret to prevent the gel pieces entering the cap-adaptor assembly. Six of these assemblies could be used per elution. The apparatus was fitted into a Mini Protean II tank (Biorad) and the bottom reservoir was filled with elution buffer, as were the glass tube assemblies.

Protein samples, either SDS-PAGE separated bands or 2DE separated spots, excised and prepared as described previously were carefully placed into the glass tubes, and then the upper buffer chamber was completely filled with elution buffer. The system was run at 10mA/tube for 1-3h. After this, the buffer chambers were drained and the buffer in the glass tubes was removed using a plastic Pasteur pipette. The cap-adaptor assembly was carefully removed and the liquid in the cap was decanted to a clean 1.5ml tube. A further 200µl of elution buffer was used to wash the membrane. The samples were concentrated as described in section 4.2.3.4. The caps were washed in 0.05% v/v sodium azide before being stored at 4°C in elution buffer supplemented with 0.05% v/v sodium azide.

4.2.3.3 Electroelution Using Biometra Bioteluter®

Unlike the Model 422 Eluter, the Bioteluter (Biometra) was not compatible for use with proteins that had been fixed or stained and it was used to elute 2DE-separated proteins from unstained gels.

The Bioteluter was set up and run according to the manufacturer's instructions (see Figure 4.2). Briefly, one piece of blotting paper was soaked in 100mM Tris.Cl (pH 8.0) and was placed on the anode, before a 2 cm block of 15% polyacrylamide gel to cover the whole electrode area was laid on top. A dialysis membrane, boiled in 5 mM Tris to remove contaminants, was soaked in ddH_2O and then 100mM Tris.Cl (pH 8.0) and was placed on top of the gel block. The master plate containing 528 holes was laid onto these layers and connected to the base plate by screws. The master plate was then filled with elution buffer (5mM Tris.Cl, pH 8.0).

Each precast 24cm gel (Amersham Biosciences) was cut into quarters, since the gels were too large for the eluter. The gel quarters were then placed onto the master plate, and 4 pieces of blotting paper, soaked in 5mM Tris.Cl (pH 8.0) placed on top. The cathode plate was placed on top of this assembly, and attached using the safety screws. The elution was carried out by passing $0.8mA/cm^2$ of gel surface through the apparatus for 30 min. After elution, the apparatus was disassembled and the elution buffer was removed from the 528 wells using a 12-channel multipipette into 96-well plates. These samples were then concentrated as described below.

4.2.3.4 Concentration and Visualisation of Eluted Proteins

Samples were concentrated using a vacuum centrifuge (Speedivac) at maximum concentration for 1-2h, or until all the liquid had been removed. The samples were resuspended in 50-100µl PBS. A 20µl aliquot of each sample was then analysed for the presence of protein by running the samples on a 15% PAGE. Gels were stained by either silver staining or Sypro® Orange staining, depending on whether they were intended for subsequent mass spectrometry analysis.

4.2.4 Preparation of Bovine Cells for Proliferation Assay

4.2.4.1 Bovine Peripheral Blood Mononuclear Cells

Blood was collected from the jugular vein of cattle into preservative free heparinised evacuated blood collection tubes, Vacutainer ®^{TM} (Becton Dickinson Ltd., Oxford, UK). Each sample was diluted 1:2 with sterile PBS and centrifuged at 450g for 20 mins at 12^{0}C with the brake off in a GS-6R Beckman®^{TM} centrifuge. The buffy coat was removed and diluted 1:2 in Hanks balanced salt solution (HBSS) supplemented with 2% foetal calf serum (FCS), 100U/ml heparin (Sigma, Poole, UK) (wash medium). The cell suspension was layered over lymphoprep (Robbins, Scientific Solihull, UK) and centrifuged at 550g for 30 mins with the brake off. Peripheral blood mononuclear cells (PBMC) were collected from the interface into wash medium and washed three times by repeated resuspension and centrifugation at 300g for 10mins. The cells were counted using an improved Neubauer haemocytometer and viability was determined by exclusion of nigrosin dye. The cells were resuspended at the required concentration in Iscove's Modified Dulbecco's Medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Paisley, UK) (culture medium). The PBMC were used to generate T-cell lines and also as a source of antigen presenting cells in the proliferation assays.

4.2.4.2 Preparation of Antigen Presenting Cells (APC)

Peripheral blood mononuclear cells (prepared as described in section 4.2.4.1) were irradiated with 3000 rads using a [^{37}Cs] source prior to using them as a source of autologous APC in the T-cell proliferation assays.

4.2.4.3 Generation of bovine CD4+ T-cell lines

Peripheral blood mononuclear cells were prepared as described in section 4.2.4.1 using blood taken from two donor cattle 410 and 416. These cattle were Fresian/Holstein breed aged 16-24 months and had been inoculated 12 months previously with 5 x 10^8 live tachyzoites of the NC1 strain (Dubey et al 1988) subcutaneously over the left pre-femoral lymph node (Innes *et al*, 2001). Equal volumes of PBMC at 2 x 10^5 cell/well and NC1 antigen at 5µg/ml final concentration were cultured in 96 well round bottom tissue culture plates (Gibco, Paisley, UK) at 37°C in a humidified 5% CO_2 incubator. After 7d the cells were harvested and diluted approx 1:3 in culture medium supplemented with 10 Units /ml of human recombinant IL-2 (hrIL-2) (Cetus Labs, UK). Cells were cultured for a further 7-10d prior to harvesting and testing in the proliferation assays.

4.2.4.4 Phenotypic analysis of the bovine T-cell lines

An indirect immunofluorescence test was used to test the phenotypic composition of the bovine T-cell lines (Innes *et al*, 1995). In brief, 50µl aliquots of cells at a concentration of 2 x 10^7 cells/ml were resuspended in 50µl of fluorescence activated cell sorter (FACS) medium (HBSS supplemented with 2% FBS and 0.1% sodium azide) and mixed with 50µl of the appropriate monoclonal antibody (Mab) at a pre-determined optimal dilution. Cell and Mabs were incubated at 4°C for 30min and washed three times by repeated re-suspension and centrifugation at 300g for 5min. Cells were then resuspended in 50µl of fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulin (Dako, Glostrup, Denmark) and incubated in the dark for a further 30min at 4°C. Cells were then washed 3 times with FACS medium

and finally fixed prior to analysis in FACS medium containing 1% paraformaldehyde.

The percentage of cells stained with FITC for each Mab was determined using flow cytometry (FACScan, Becton Dickinson, Oxford, UK). Ten thousand cells were analysed per sample. A sample stained with FITC conjugate only was used as a control along with an unstained sample of cells.

A panel of monoclonal antibodies recognising distinct bovine leucocyte populations were used to phenotype the bovine T-cell lines. CC42 recognising (CD2), CC8 recognising (CD4) and CC15 recognising (γ/δ T-cells) were a kind gift from the Institute for Animal Health, Compton, UK. ILA-51 recognising (CD8) and ILA-111 recognising (IL-2 receptor) were a kind gift from the International Laboratory for research on Animal Diseases, Nairobi, Kenya.

4.2.5 Proliferation assays

Proliferation assays with the bovine T-cell lines were performed in the presence of autologous APC at a ratio of 10 APC: 1 T-cell. The cells were set up at 2 x 10^5 T-cells/well in culture medium in 96-well round-bottomed culture plates. Test fractions prepared as described in Section 4.2.3 were added to the cultures in triplicate for each T-cell line along with control antigens, comprising whole NC1 lysate antigen (1μg/ml final concentration), the T-cell mitogen concanavalin A (Con A) (Sigma, Poole, UK) at 5 μg/ml final concentration, Vero cell antigen (1 μg/ml final) and culture medium alone to quantify background proliferation of the cells. One hundred microlitres of each fraction was added to three wells containing cells from Animal

410 and also three wells with cells from Animal 416. The cultures were incubated for 5 days at 37°C in a humidified 5 % $C0_2$ incubator. Cells were pulsed for the final 18h with 18.5kbq [^{3}H]-thymidine (Amersham, Bucks, UK) per well before harvesting onto fibreglass filters (Canberra Packard, Meriden, CT, USA). Cell associated radioactivity was quantified in a gas proportional counter. Results were expressed as mean counts per minute of triplicate cultures. The differential incorporation of [^{3}H]-thymidine between control and test cultures was used as a measure of proliferation expressed as a stimulation index (SI) where SI = cpm of test culture/cpm of negative control culture.

4.3 RESULTS

4.3.1 Elution, Concentration and Visualisation of Proteins Separated by SDS-PAGE

4.3.1.1 One- and Two-Dimensional SDS-PAGE

Proteins from the *N. caninum* whole cell lysate were separated by one- and two-dimensional SDS-PAGE as described in Section 4.2.2. Typical examples of these can be seen in Figure 4.3 and Figure 4.4. Figure 4.3 shows a typical separation of whole tachyzoite lysate when run on a 15% polyacrylamide gel in one dimension and stained with Coomassie blue as described in Section 4.2.2.3.1. Figure 4.4 shows the 2D separation of the whole *N. caninum* tachyzoite lysate, using a 24 cm, pH 4-7 immobilised pH gradient strip and 15% polyacrylamide gel, and stained using Sypro Orange (as described in Section 4.2.2.3.2). 2D SDS-PAGE gels were either stained using Sypro Orange or left unstained for use in the Bioteluter apparatus.

4.3.1.2 Efficacy of Model 422 Eluter and Biometra Bioteluter

The two electroeluters were used to elute proteins from the polyacrylamide matrix as described in Section 4.2.3.

4.3.1.2.1 Model 422 Eluter

This eluter was designed for use with 1D SDS-PAGE gels. However, since the aim of this work was to elute from 2D gels, individual protein spots were excised from a 2D SDS-PAGE gel, as indicated by the yellow circles in Figure 4.5. These gel spots were eluted as described in Section 4.2.3.2. Elution was carried out for 1-3 h, and the samples were recovered from the membrane cap of the eluter apparatus. The samples were concentrated and an aliquot from each was run on a 15% SDS-PAGE gel, before being visualised using Coomassie Blue staining. Figure 4.6 is a photograph of

the resulting gel. It demonstrates that the Model 422 Eluter can be used effectively to recover the small concentrations of protein from individual protein spots.

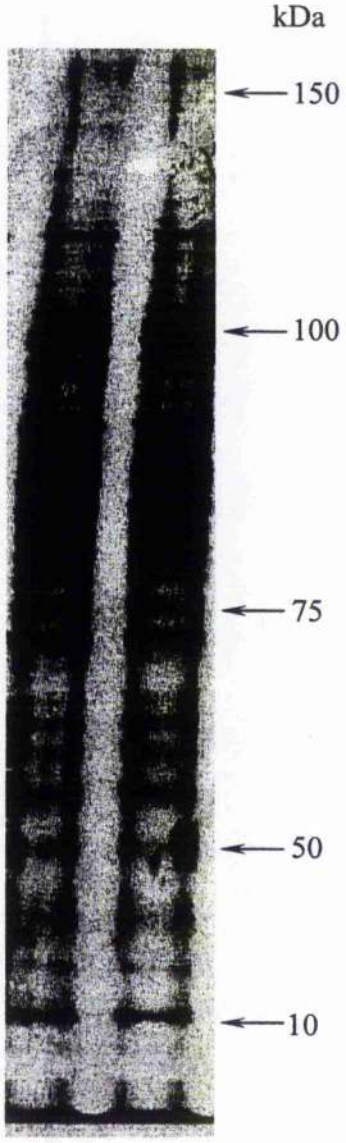


Figure 4.3 One-dimensional SDS-PAGE gel of *N. caninum* whole cell lysate, run on 15% polyacrylamide gel, and visualised using Coomassie Blue staining.

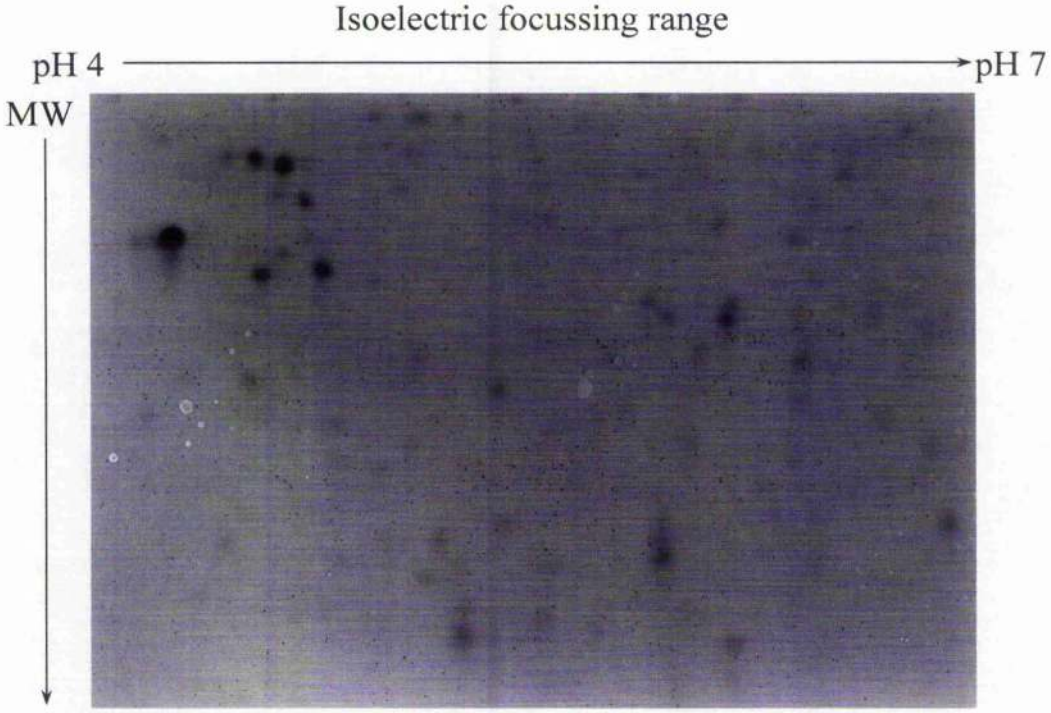


Figure 4.4 Two-dimensional separation of whole cell lysate of *N. caninum*. $2x10^8$ tachyzoites were lysed and focussed on an immobilised 24cm gradient strip, range pH 4 – pH 7. The isoelectrically focussed proteins were then size separated on a 15% polyacrylamide gel and visualised by Sypro® Orange Stain (Biorad).

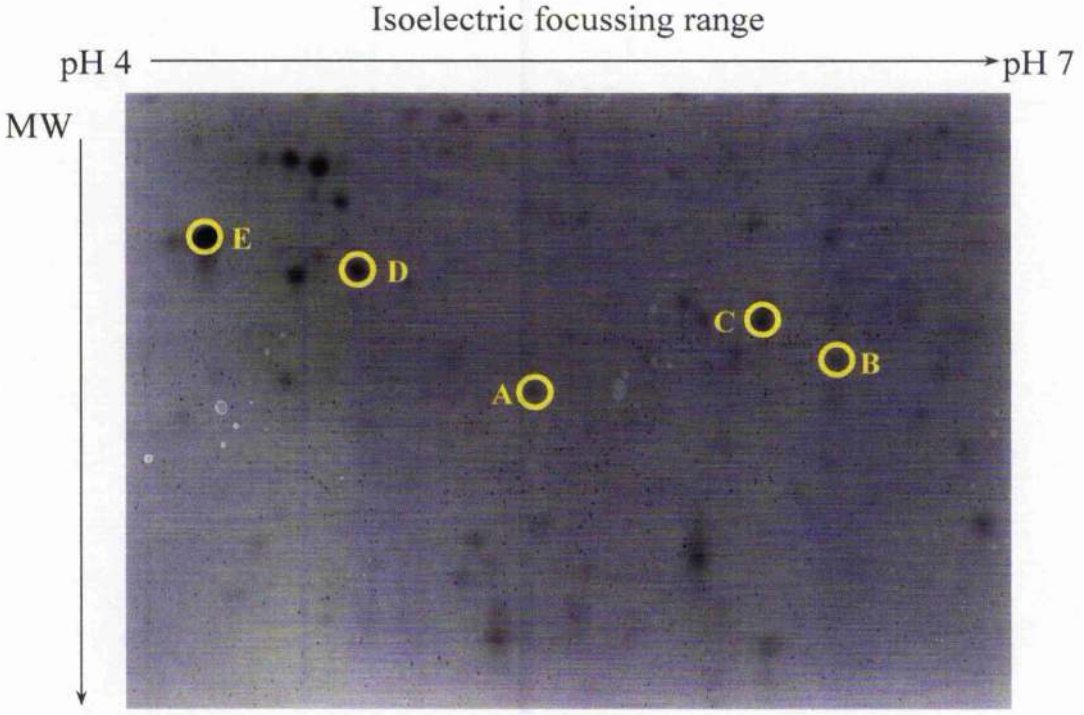


Figure 4.5 Typical 2D gel as described in Figure 4.4. The 5 spots marked in yellow (A-E) were excised and eluted to assess the efficacy of using the Model 422 Eluter with 2D-separated proteins.

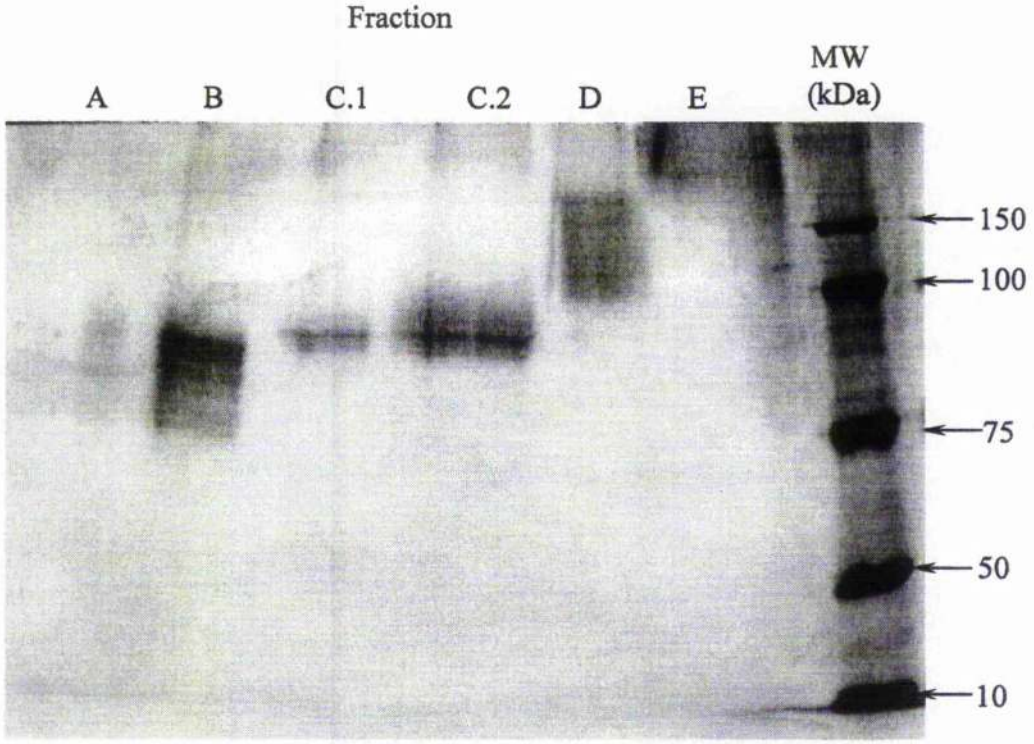


Figure 4.6 Coomassie stained aliquots of eluted proteins from a cell lysate of *N. caninum* run on 15% SDS-PAGE gel.

4.3.1.2.2 Biometra Bloteluter

This eluter was designed to elute directly from unstained 2D SDS-PAGE gels. Unstained gels were placed in the eluter apparatus. Elution was carried out as described in Section 4.2.3.3. Following the elution, samples were removed from the master plate, concentrated and run on a 15% polyacrylamide SDS-PAGE gel. Visualisation was with Coomassie Blue, as described in Section 4.2.2.3.1.

No eluted protein was observed when the Blotcluter was used. Several attempts were made to obtain protein from the Bloteluter, but none were successful. Therefore, the Model 422 Eluter was used to elute proteins from both one- and two-dimensionally separated *N. caninum* whole cell lysate.

4.3.1.3 Preparation of Protein Fractions for Use in T-cell Proliferation Assays

Protein samples were eluted from one- and two-dimensional SDS-PAGE gels of *N. caninum* whole cell fraction. In total, 104 protein fractions were eluted. These derived from several gels, and are identified in Figures 4.7 to 4.13. These 104 protein fractions were used in T-cell proliferation assays.

4.3.2 Bovine CD4+ T-cell Lines from *N. caninum* Infected Cattle

4.3.2.1 Proliferative Responses

T-cell lines generated from two animals, 410 and 416, were assayed for proliferative response to a variety of antigens, as described in Section 4.2.5. Figure 4.14 shows a typical proliferative response to NC1 whole cell lysate (NC1) and Concanavalin A (ConA), as well as medium and Vero cell lysate antigen controls. The cells proliferated in the presence of NC1 lysate and ConA. Table 4.2 shows the Stimulation Index values for this experiment. The proliferation due to NC1 had SI

values of 31.14 and 85.61 (animals 410 and 416) while the proliferation caused by ConA had SI values of 26.04 and 52.50 (Animals 410 and 416 respectively). Very little proliferation was observed in the cultures containing culture medium or Vero cell lysate. Proliferation due to the Vero cell lysate was approximately similar to that in medium alone.

Animal 410			
Antigen	**cpm (mean)**	**St. Dev.**	**SI**
Medium	328.33	39.55	1.00
ConA	8548.33	1084.64	26.04
NC1	10224.67	1958.18	31.14
Vero	511.67	174.02	1.56

Animal 416			
Antigen	**cpm (mean)**	**St. Dev.**	**SI**
Medium	100.10	44.99	1.00
ConA	5255.00	618.93	52.50
NC1	8569.67	788.47	85.61
Vero	130.00	40.73	1.30

Table 4.2 Proliferation (counts per minute – cpm) and Stimulation Index data for bovine T-cell proliferations. Counts per minute are means of triplicate wells.

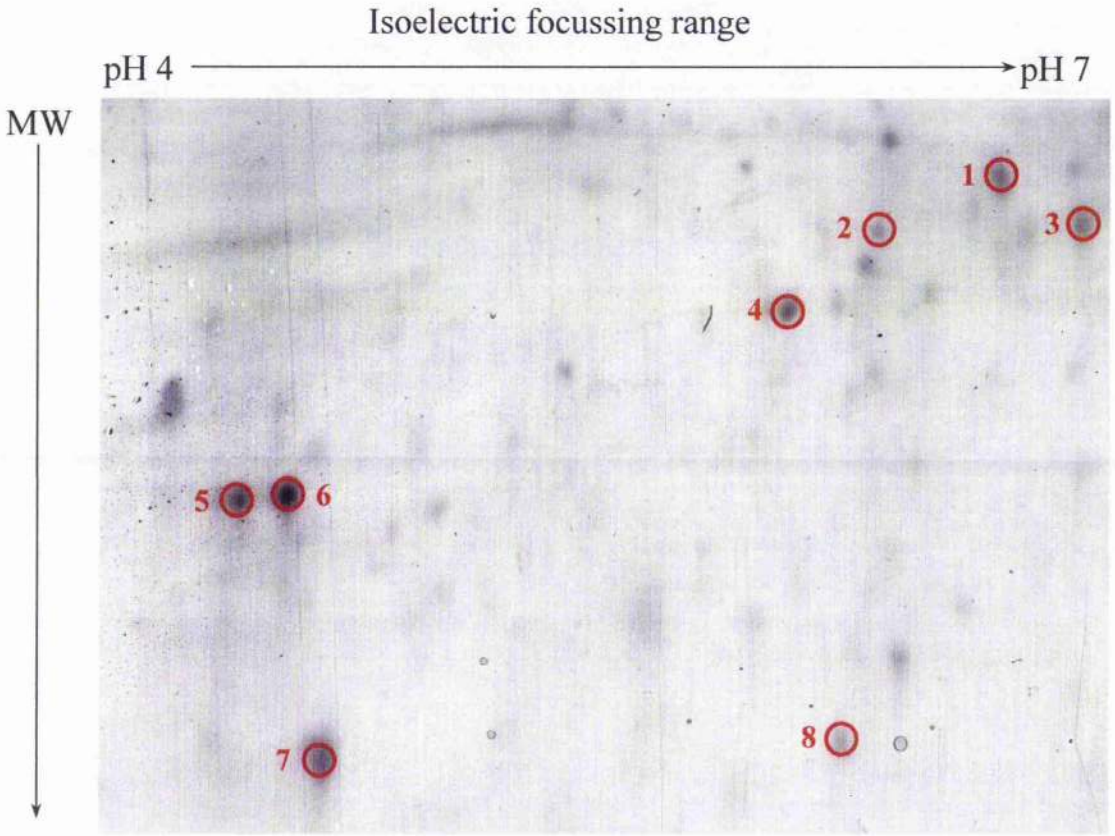


Figure 4.7 Two-dimensional separation of whole cell lysate of *N. caninum.* $2x10^8$ tachyzoites were lysed and focussed on an immobilised 24cm gradient strip, pH 4-7. The isoelectrically focussed proteins were then size separated on a 15% polyacrylamide gel and visualised using Coomassie Blue stain. Numbered circles 1-8 indicate proteins that were excised and eluted.

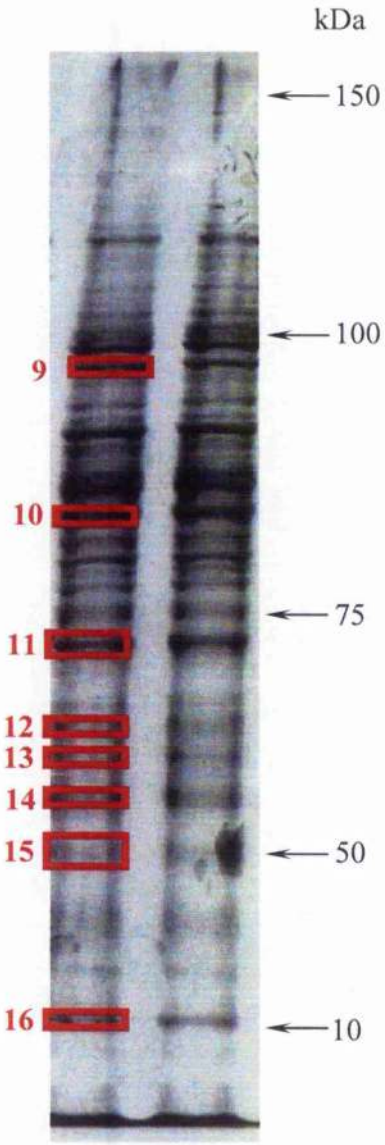


Figure 4.8 One-dimensional SDS-PAGE gel of *N. caninum* whole cell lysate, run on 15% polyacrylamide gel, and visualised using Coomassie Blue staining. The numbered rectangles 9-16 are the bands excised from the gel and the proteins eluted.

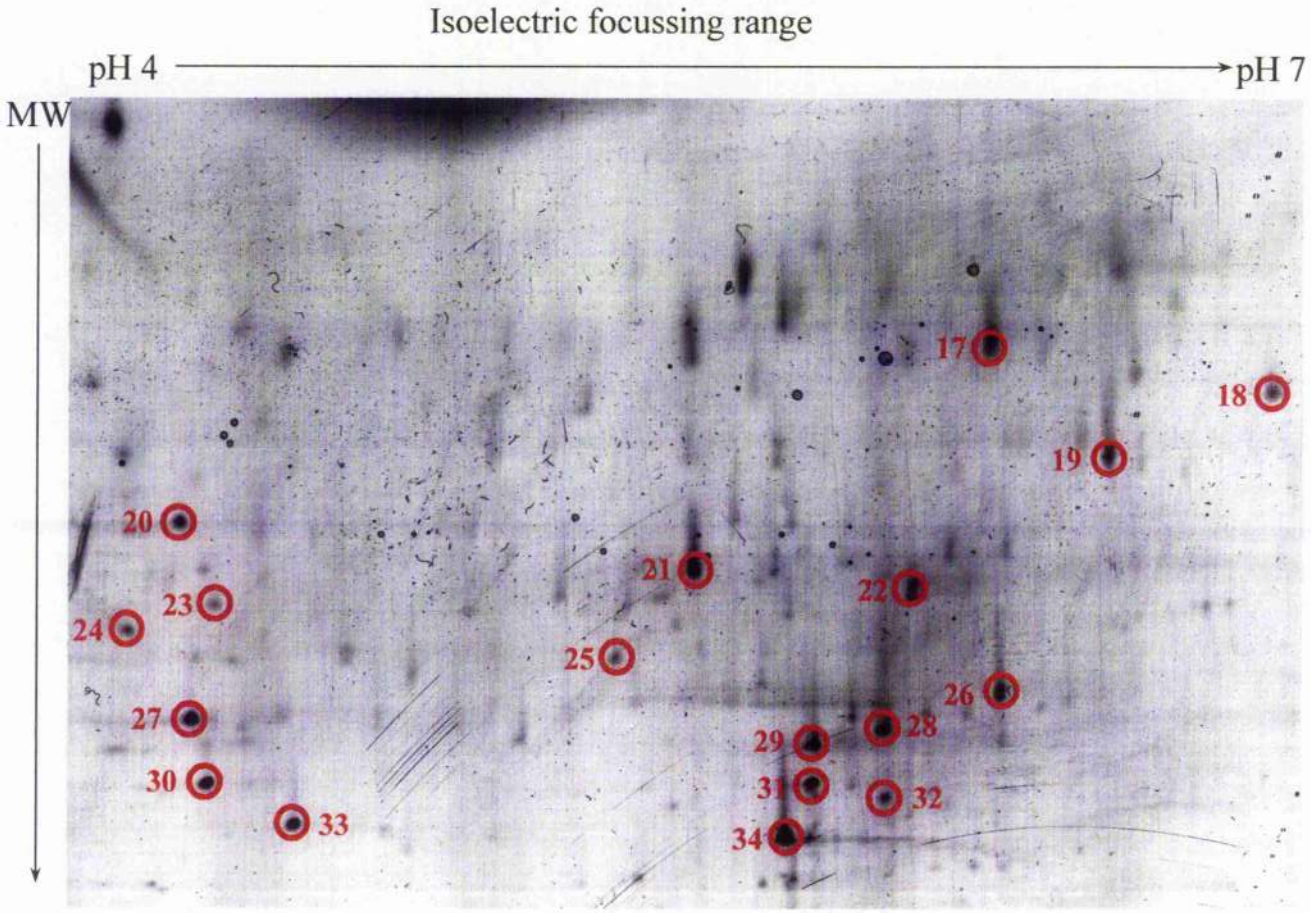


Figure 4.9 Two-dimensional separation of whole cell lysate of *N. caninum.* $2x10^8$ tachyzoites were lysed and focussed on an immobilised 24cm gradient strip, pH 4-7. The isoelectrically focussed proteins were then size separated on a 15% polyacrylamide gel and visualised using Coomassie Blue stain. Numbered circles 17-34 indicate proteins that were excised and eluted.

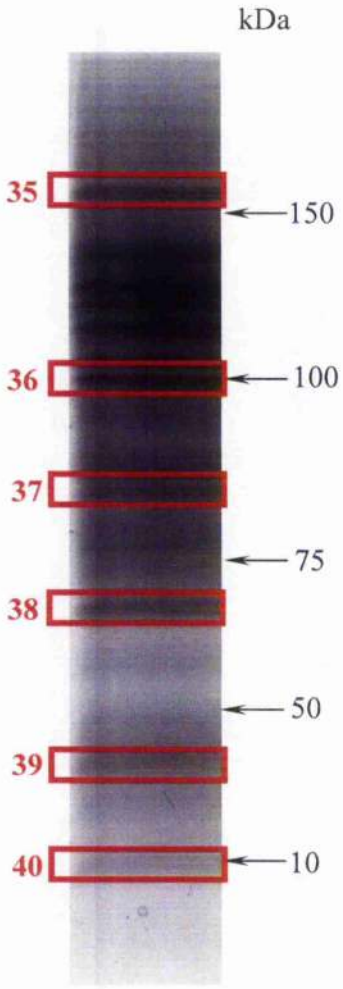


Figure 4.10 One-dimensional SDS-PAGE gel of *N. caninum* whole cell lysate, run on 15% polyacrylamide gel, and visualised using Coomassie Blue staining. The numbered rectangles 35-40 are the bands excised from the gel and the proteins eluted.

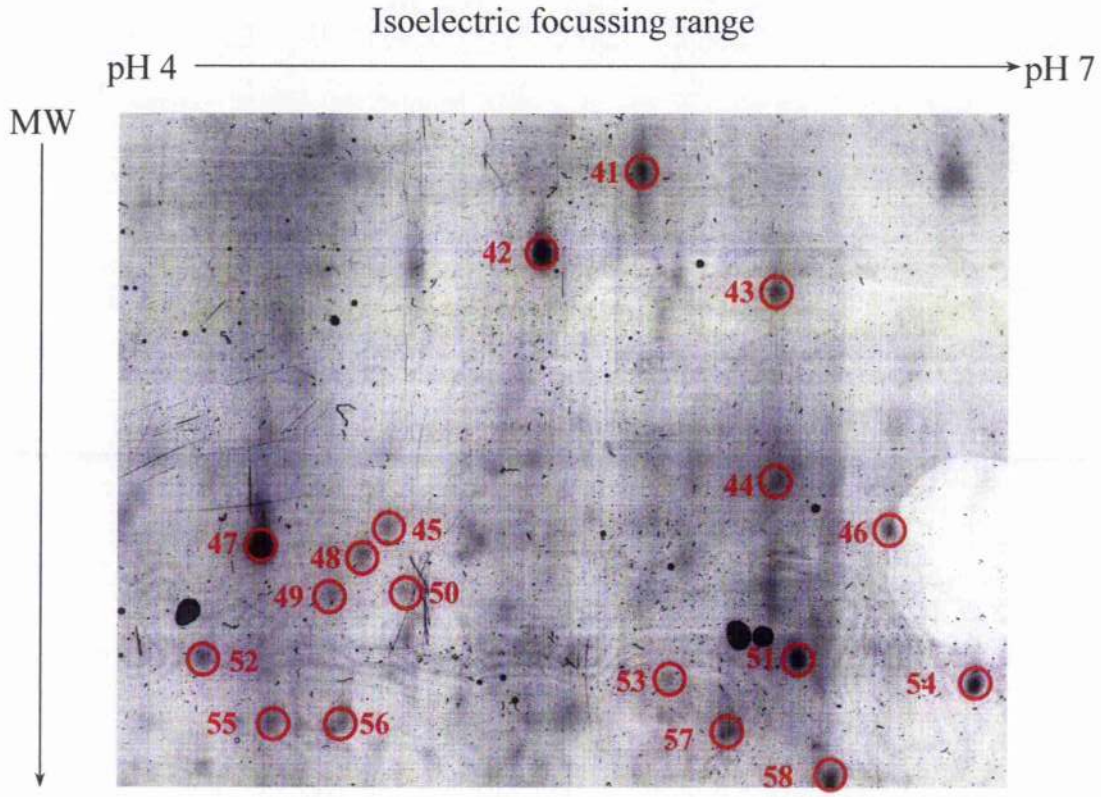


Figure 4.11 Two-dimensional separation of whole cell lysate of *N. caninum*. $2x10^8$ tachyzoites were lysed and focussed on an immobilised 24cm gradient strip, pH 4-7. The isoelectrically focussed proteins were then size separated on a `5% polyacrylamide gel and visualised using Coomassie Blue stain. Numbered circles 41-58 indicate proteins that were excised and eluted.

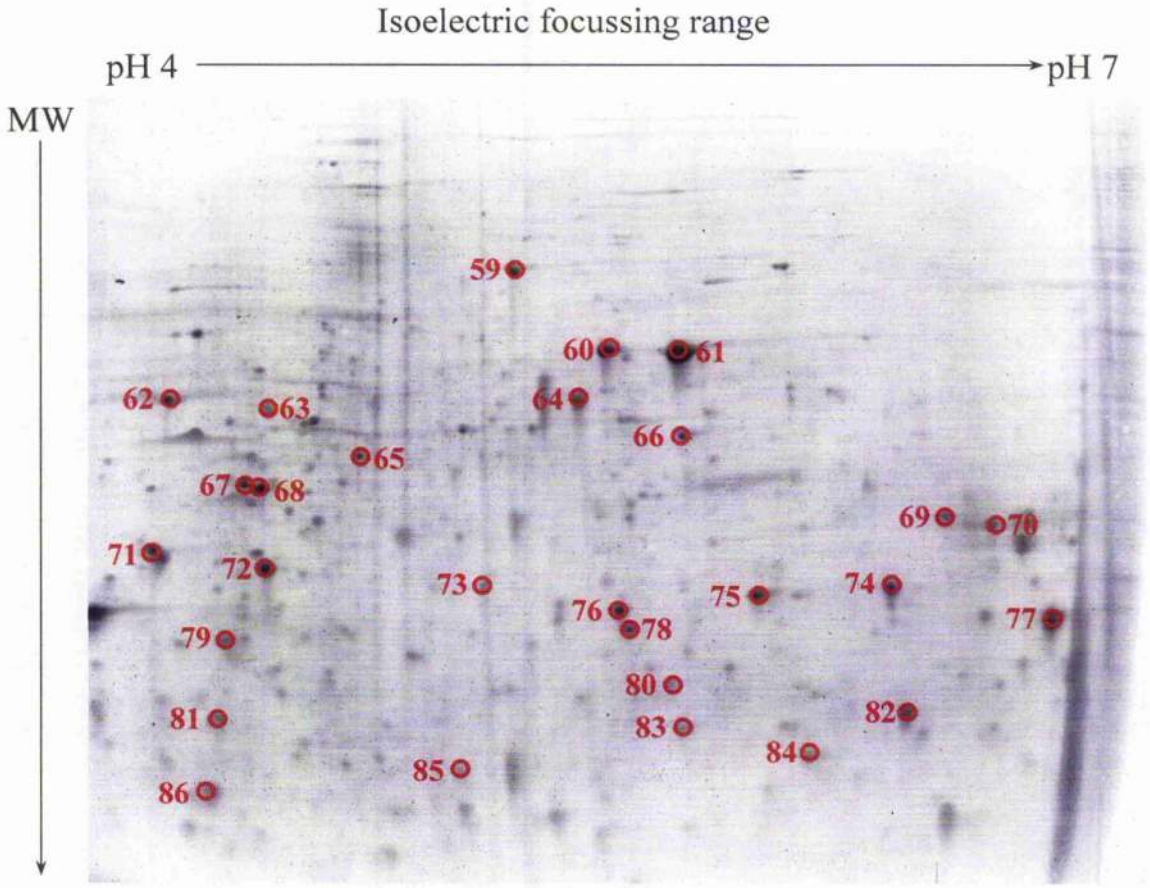


Figure 4.12 Two-dimensional separation of whole cell lysate of *N. caninum*. $2x10^8$ tachyzoites were lysed and focussed on an immobilised 24cm gradient strip, range pH 4 – pH 7. The isoelectrically focussed proteins were then size separated on a 15% polyacrylamide gel and visualised by Coomassie Blue Stain. Numbered circles 59-86 indicate proteins excised and eluted.

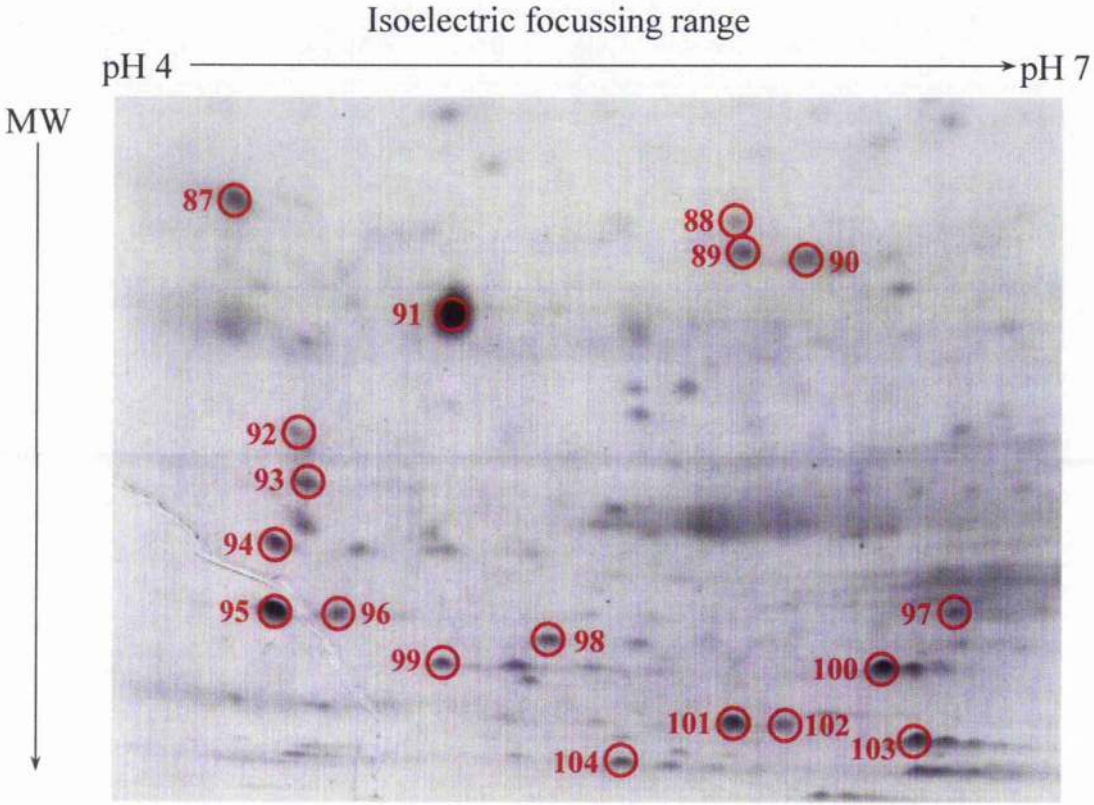


Figure 4.13 Two-dimensional separation of whole cell lysate of *N. caninum.* $2x10^8$ tachyzoites were lysed and focussed on an immobilised 24cm gradient strip, pH 4-7. The isoelectrically focussed proteins were then size separated on a `5% polyacrylamide gel and visualised using Coomassie Blue stain. Numbered circles 87-104 indicate proteins that were excised and eluted.

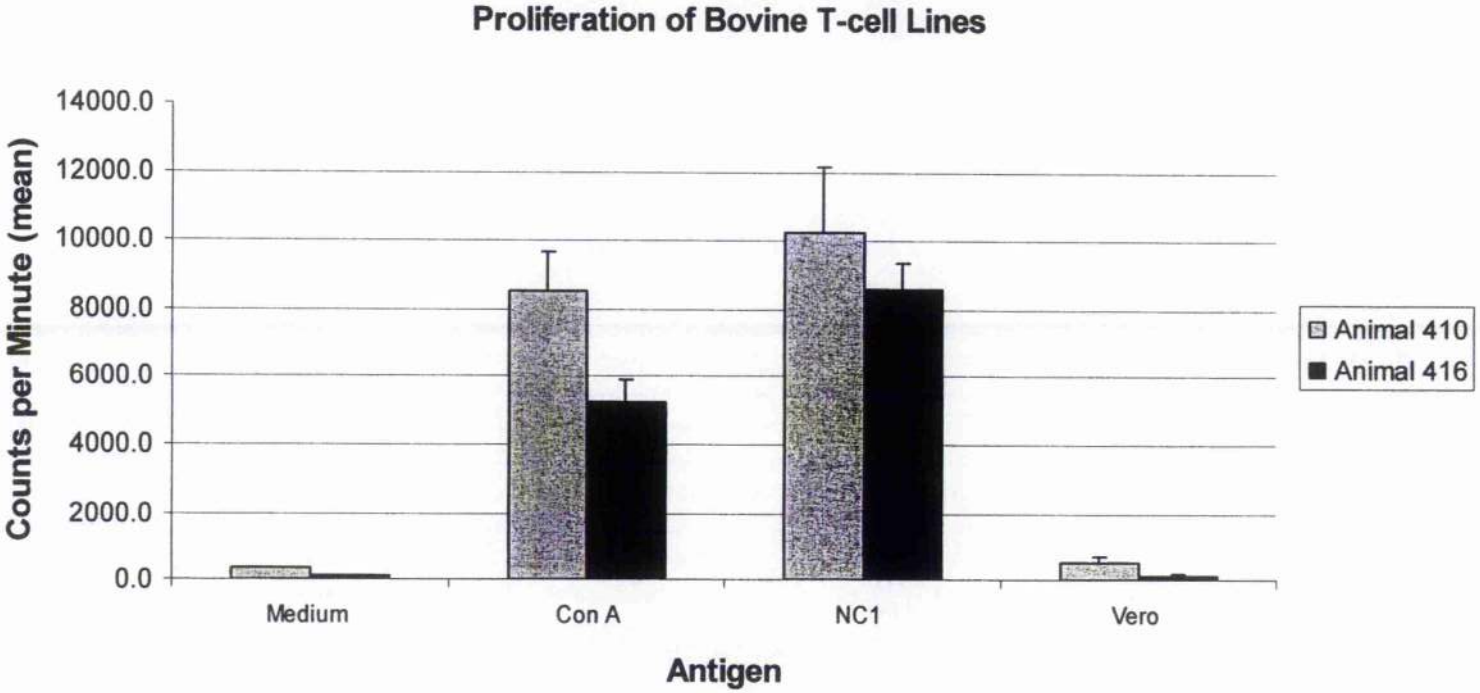


Figure 4.14 *In vitro* proliferation of bovine T-cells in the presence of concanavalin A (ConA) (5μg/ml final concentration); whole *N. caninum* lysate (NC1) (1μg/ml final concentration), Vero cell lysate (1μg/ml final concentration) and culture medium. Error bars indicate Standard Deviation in the means of experiments done in triplicate.

4.3.2.2 Phenotypic analysis

To determine the phenotype of the cells present following *in vitro* enrichment (section 4.2.4.3), indirect fluorescence was used where cells were mixed with monoclonal antibodies against the different bovine leucocyte marker molecules (CD2, CD4, CD8, γδ and IL-2 receptor) before being mixed with FITC-labelled anti-mouse immunoglobulin. When analysed in the FACSCAN (Beckton-Dickenson), the different sub-populations of T-cells within the bovine cell lines were quantified.

Table 4.3 shows the proportions of different T-cell subsets, for the cell lines generated from animals 410 and 416 (also see Figures 4.15 and 4.16 for a graphical illustration, the positively stained cells are shown in the top-right quadrant of the graphs, and the negative stained cells in the bottom right quadrant). In both of the bovine cell lines, the majority of the cells stained positively for the CD2 and CD4 markers, with over 95% of the cells from both animals containing these markers. In addition, approximately two-thirds of the cells expressed the IL-2 receptor. $CD8^+$ T-cells and γδ T-cells were very minor components of the cell lines. These results suggest that the cultured polyclonal cell lines were predominantly of the $CD4^+$ phenotype.

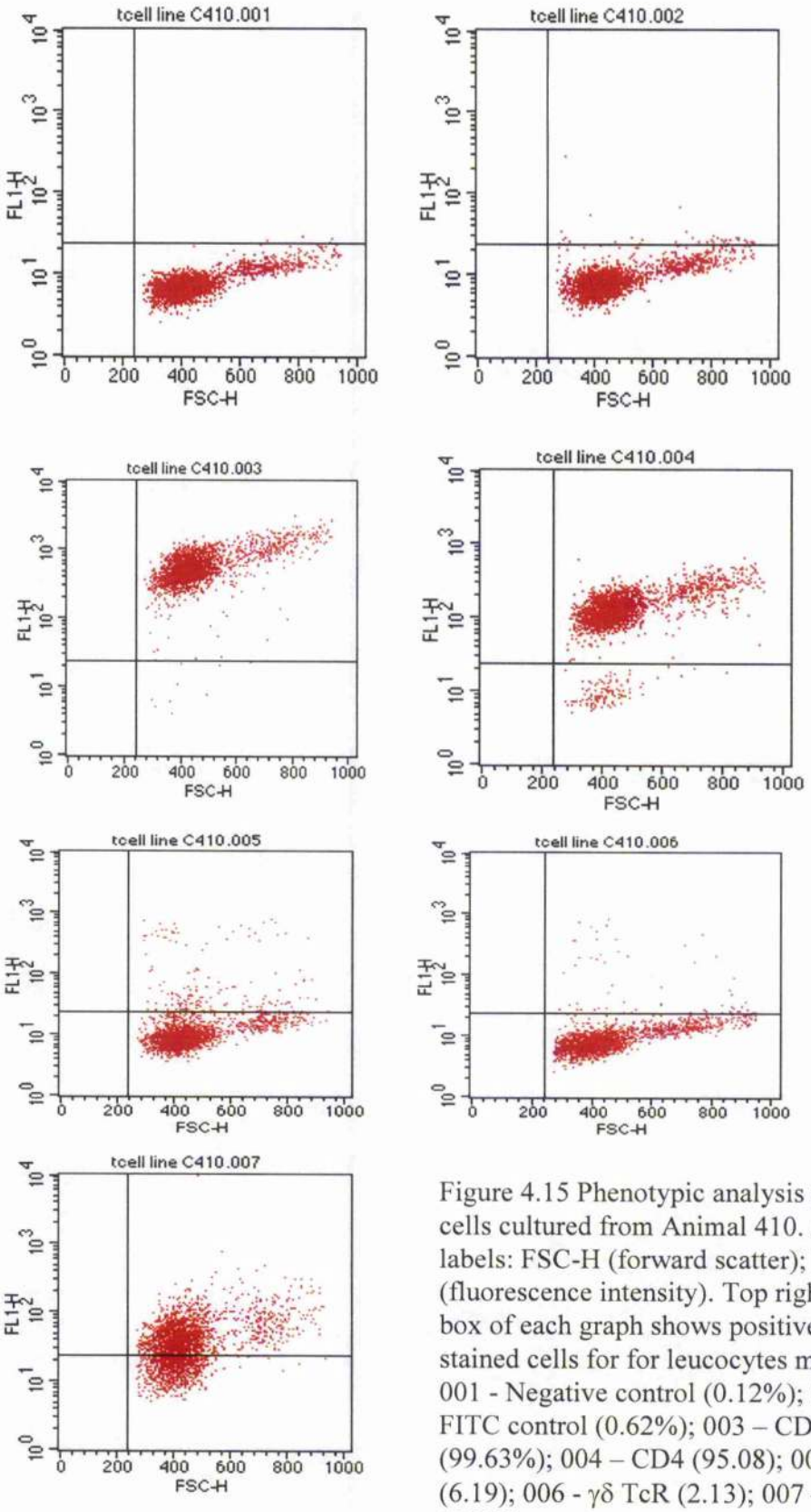


Figure 4.15 Phenotypic analysis of T-cells cultured from Animal 410. Axis labels: FSC-H (forward scatter); FL1 (fluorescence intensity). Top right hand box of each graph shows positively stained cells for for leucocytes markers: 001 - Negative control (0.12%); 002 – FITC control (0.62%); 003 – CD2 (99.63%); 004 – CD4 (95.08); 005 – CD8 (6.19); 006 - γδ TcR (2.13); 007 – IL-2R (66.72)

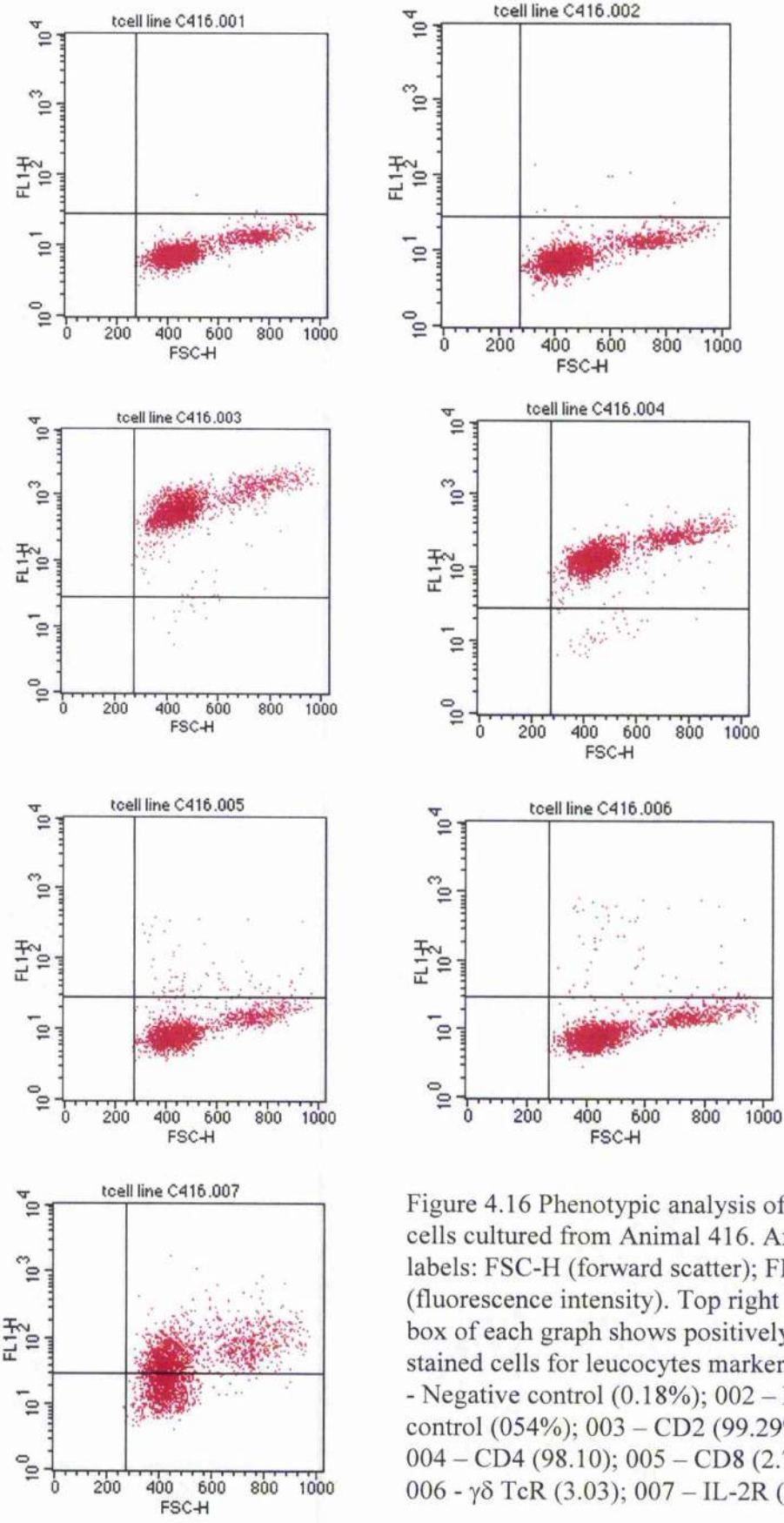


Figure 4.16 Phenotypic analysis of T-cells cultured from Animal 416. Axis labels: FSC-H (forward scatter); FL1 (fluorescence intensity). Top right hand box of each graph shows positively stained cells for leucocytes markers: 001 - Negative control (0.18%); 002 – FITC control (054%); 003 – CD2 (99.29%); 004 – CD4 (98.10); 005 – CD8 (2.72); 006 - γδ TcR (3.03); 007 – IL-2R (64.89)

	Leucocyte Marker	Primary/Secondary Mab	% Positive Cells
Cell Line 410	-ve control	Medium / Medium	0.12
	FITC control	Medium / FITC	0.62
	CD2	CC42 / FITC	99.63
	CD4	CC8 / FITC	95.08
	CD8	ILA-51 / FITC	6.19
	γδ-T cells	CC15 / FITC	2.13
	IL-2 Receptor	ILA-111 / FITC	66.72
Cell Line 416	-ve control	Medium / Medium	0.18
	FITC control	Medium / FITC	0.54
	CD2	CC42 / FITC	99.29
	CD4	CC8 / FITC	98.10
	CD8	ILA-51 / FITC	2.72
	γδ-T cells	CC15 / FITC	3.03
	IL-2 Receptor	ILA-111 / FITC	64.89

Table 4.3 Relative abundancies of different T-cell sub-populations within antigen-reactive cell lines generated from two cattle infected with *N. caninum*.

4.3.3 Proliferation Assays to Identify Immunodominant *N. caninum* Proteins

Several experiments were performed to test the feasibility of stimulating bovine T-cells with electroeluted proteins. In each experiment, different antigen preparation protocols were examined to try and optimise the procedure.

4.3.3.1 Experiment 1: Elution into Tris / Glycine / SDS Buffer

Initial experiments using proteins eluted from one- and two-dimensional gels were performed, using T-cell lines derived from both Animal 410 and Animal 416. The elution protocol is described in Section 4.2.3.2. The proteins were eluted into a buffer containing 10mM Tris, 50mM glycine and 0.1% SDS. For this experiment, proteins were eluted from both 2D and 1D SDS-PAGE gels (samples 1-8 were eluted from 2D gels, samples 9-16 from 1D gels, as seen in Figures 4.7 and 4.8, respectively).

The results obtained for the T-cell line from animal 416, using the eluted fractions are shown in Figure 4.17. There was some evidence of proliferation of cells observed in the wells containing ConA, both NC1 lysates and fractions 2, 5, 12, 13, 14 and 15. It should be noted that two independently prepared NC1 lysates (NC1-Edin. and NC1-Glas.) were used. They were prepared in the same way to the same concentration but using tachyzoites harvested at different times. The mean proliferations expressed as cell counts per minute (with standard deviations) and Stimulation Indexes (SI) for this experiment are shown in Table 4.4. The SI for all the samples was approximately 1, and in the cells co-cultured with ConA, the SI value was only 1.86 due to the high level of proliferation due to medium alone. It was encouraging that the eluted protein fractions did not show signs of being toxic to the majority of the T-cell cultures.

4.3.3.2 Experiment 2: Elution into Tris / Glycine / SDS Buffer

This experiment was not a direct repeat of Experiment 1, since different proteins were eluted. The proteins were eluted from both 2D and 1D gels, and can be seen in Figure 4.9 (samples 17-34) and Figure 4.10 (samples 35-40). The same separation and elution techniques as in Experiment 1 were used, and the T-cell lines were derived again from the same animals as in Experiment 1. The results for this experiment are shown in Figure 4.18, with the mean counts per minute, standard deviation and SI for all samples tested with T-cell lines from both animals, shown in Table 4.5. In this experiment there was proliferation in the cells co-cultured with both whole cell lysate (NC1) and ConA.

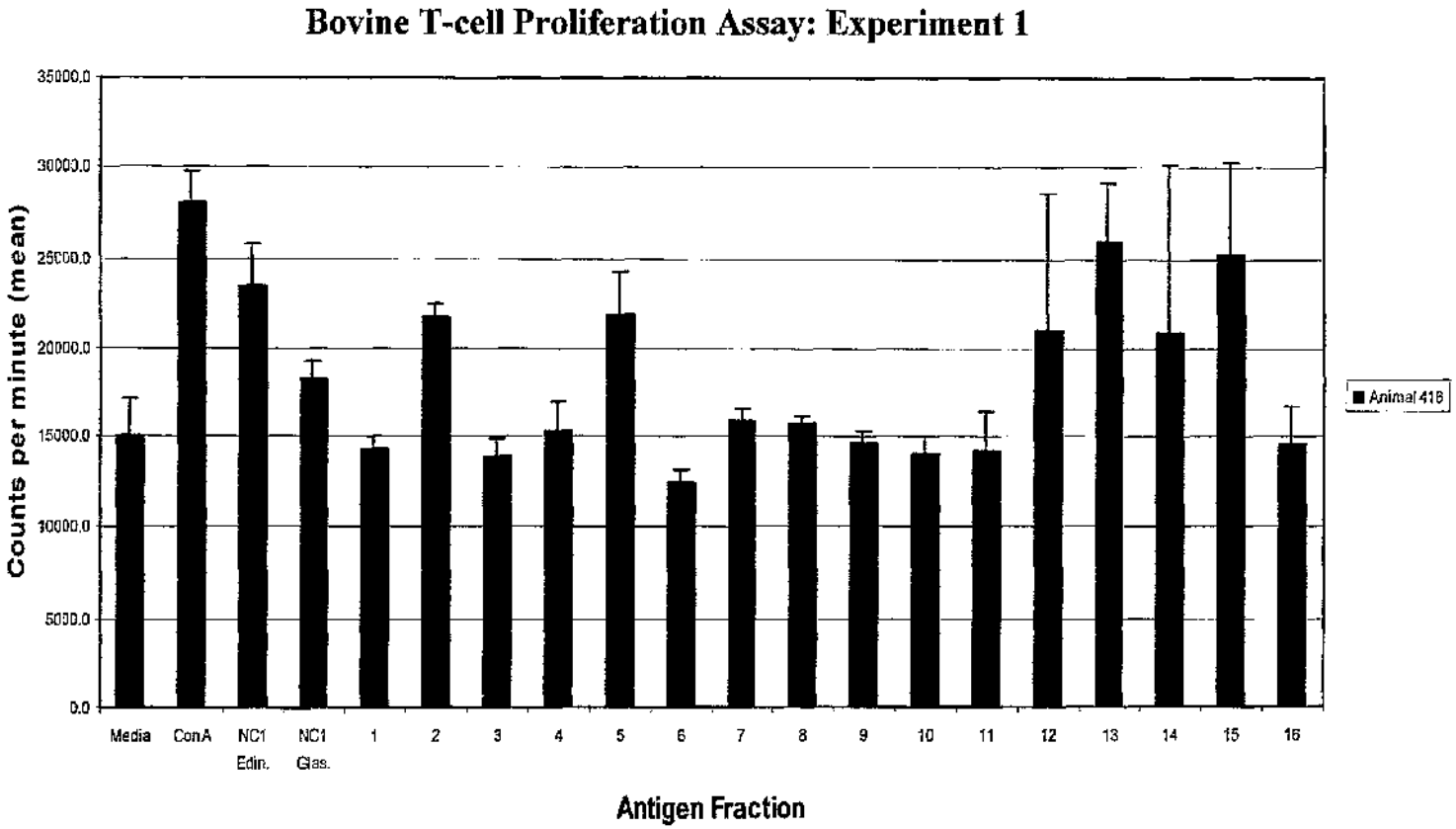


Figure 4.17 *Experiment 1.* The mean lymphoproliferation of bovine T-cells co-cultured with fractions of *N. caninum* antigen eluted from one- or two-dimensional SDS-PAGE gels (samples 1-8 eluted from 2D gels; samples 9-16 from 1D gels) , or with medium alone, two preparations of whole cell lysate (NC1-Edin. and NC1-Glas.) and ConA. Error bars show standard deviation. Cultures were set up in triplicate.

Antigen	Mean cpm	St. Dev.	SI
Media	15040.7	2174.69	1.00
Con A	28012.3	1778.81	1.86
NC1 Edin.	23311.0	2419.93	1.55
NC1 Glas.	18347.3	862.51	1.22
1	14269.7	819.02	0.95
2	21705.0	651.08	1.44
3	13865.3	1000.48	0.92
4	15288.0	1676.10	1.02
5	21826.0	2463.16	1.45
6	12523.7	685.42	0.83
7	15819.7	794.00	1.05
8	15680.3	475.33	1.04
9	14540.3	704.53	0.97
10	13964.3	1096.88	0.93
11	14118.7	2340.11	0.94
12	20934.3	7644.47	1.39
13	25912.3	3238.37	1.72
14	20849.0	9330.78	1.39
15	25265.0	5095.73	1.68
16	14647.7	2086.67	0.97

Table 4.4 *Experiment 1.* Mean lymphoproliferation (with standard deviation) of bovine T-cells (Animal 416) co-cultured with antigens eluted from one- and two-dimensional SDS-PAGE gels Samples 1-8 were eluted from 2D gels; samples 9-16 were eluted from 1D gels. Stimulation Index indicated the stimulation of different fractions and controls in relation to the medium control.

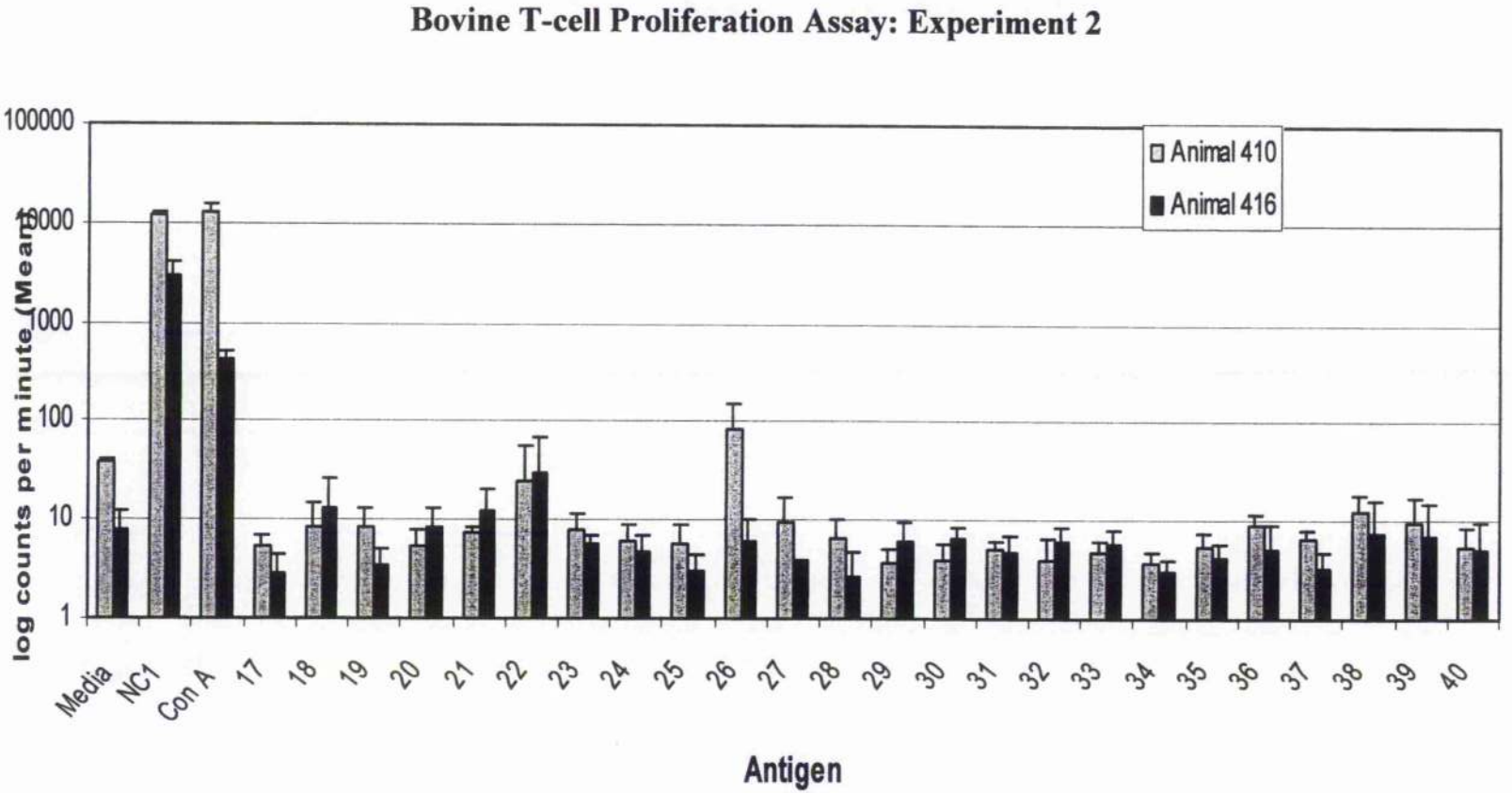


Figure 4.18 *Experiment 2.* The mean lymphoproliferation of bovine T-cells from 2 animals infected with *N. caninum* co-cultured with fractions of *N. caninum* antigen eluted from one- or two-dimensional SDS-PAGE gels (samples 17-34 from 2D gels; samples 35-40 from 1D gels) , or with medium alone, whole cell lysate (NC1) and ConA. Error bars show standard deviation. Experiments were carried out in triplicate. [*Note the logarithmic scale on the y-axis.*]

Animal 410				Animal 416			
Antigen	**Mean cpm**	**St. Dev.**	**SI**	**Antigen**	**Mean cpm**	**St. Dev.**	**SI**
Media	38.00	3.61	1.00	**Media**	8.00	4.36	1.00
NC1	11849.33	890.14	**311.82**	**NC1**	2868.33	1051.95	**358.54**
Con A	13049.33	1968.23	**343.40**	**Con A**	415.67	97.08	**51.96**
17	5.37	1.53	0.14	**17**	2.77	1.57	0.35
18	8.10	6.84	0.21	**18**	12.77	14.07	1.60
19	8.23	4.80	0.22	**19**	3.37	1.53	0.42
20	5.23	2.66	0.14	**20**	8.23	4.50	1.03
21	7.37	1.15	0.19	**21**	12.47	7.61	1.56
22	24.53	32.48	0.65	**22**	29.70	41.58	**3.71**
23	7.77	3.88	0.20	**23**	5.53	1.37	0.69
24	5.90	3.27	0.16	**24**	4.57	2.20	0.57
25	5.77	3.07	0.15	**25**	3.00	1.30	0.38
26	86.80	63.38	2.28	**26**	6.13	3.96	0.77
27	9.77	7.43	0.26	**27**	3.80	0.17	0.48
28	6.57	3.56	0.17	**28**	2.67	2.03	0.33
29	3.67	1.42	0.10	**29**	6.10	3.12	0.76
30	3.90	1.71	0.10	**30**	6.53	2.04	0.82
31	5.00	1.18	0.13	**31**	4.77	1.97	0.60
32	3.77	2.54	0.10	**32**	5.90	2.48	0.74
33	4.67	1.42	0.12	**33**	5.57	2.21	0.70
34	3.57	0.98	0.09	**34**	2.90	1.01	0.36
35	5.43	2.01	0.14	**35**	4.23	1.57	0.53
36	8.67	2.57	0.23	**36**	5.10	3.57	0.64
37	6.33	1.74	0.17	**37**	3.10	1.71	0.39
38	12.33	5.65	0.32	**38**	7.57	8.49	0.95
39	9.57	6.99	0.25	**39**	6.70	7.81	0.84
40	5.43	3.11	0.14	**40**	5.00	4.78	0.63

Table 4.5 *Experiment 2*. Mean lymphoproliferation (with standard deviation) of bovine T-cells (Animals 410 and 416) co-cultured with antigens eluted from one- and two-dimensional SDS-PAGE gels. Fractions 17-34 were eluted from 2D gels; fractions 35-40 from 1D gels. Stimulation Index indicated the stimulation of different fractions and controls in relation to the medium control. SI values > 3 are highlighted in bold.

There appeared to be some level of inhibition of the proliferative response, since the SI values in Table 4.5 indicate that only one fraction (26) in the cultures from Animal 410 and two fractions (18 and 22) in the cultures from Animal 416 are greater than 1. Only one (fraction 22) has an SI greater than 3 (3.71), 3 being the normal cut-off for positive stimulation indexes. The majority of SI values were less than 1, indicating that there maybe something in the sample that may have had some inhibitory effect on the cells.

One explanation regarding the inhibitory effect of the eluted fractions may be that residual SDS, acrylamide or other chemicals from the SDS-PAGE or elution experiments remained in the eluted sample. Since the samples were concentrated from the elution, these residual elements may also be concentrated.

To address the issue of possible contaminants causing problems with the T-cell proliferation assays, SDS was removed from the elution buffer, which (according to the manufacturer's instructions) was necessary for high yield elutions. Nevertheless, this decision was aimed at trying to reduce the potential toxicity to the cells of the eluted proteins.

4.3.3.3 Experiment 3: Elution into Tris /Glycine Buffer

The elution buffer used to elute the proteins (selected from the low molecular weight portion of a 2-DE SDS-PAGE gel) from the SDS-PAGE gels in this experiment contained 10mM Tris and 50mM glycine, the SDS was removed as it was thought that the SDS may account for the problems of toxicity in the cell cultures. Protein samples 41-58 were eluted only from a 2D gel, and can be seen in Figure 4.11

The results from Experiment 3 are shown in Figures 4.19 and 4.20 and Table 4.6. There was proliferation of the cells co-cultured with NC1 and ConA, and limited proliferation in those cultures containing Vero cell lysate or medium alone (Figure 4.19). Four fractions (Fractions 42, 45, 49 and 57) all stimulated the proliferation in cells from Animal 416. Fraction 45 (SI = 6.57) made the cells proliferate most. No eluted fractions stimulated cells from Animal 410 above that seen with medium control, as the low SI values indicate (Figure 4.20).

There were still signs of some inhibition of the cultures containing the eluted fractions, despite there being no SDS in the elution buffer. Therefore, another component of the buffer was removed, this time glycine, to assess if excess glycine in the concentrated samples was toxic to the cells.

4.3.3.4 Experiment 4: Elution into 10mM Tris Buffer

Proteins were eluted into buffer only containing 10mM Tris to assess for toxicity / inhibitory factors in the eluted fractions. Protein samples 59-86 were again eluted from 2D gels as shown in Figure 4.12. This experiment had contamination problems, such that the cell lines from Animal 416 were not available for analysis. The results for Animal 410 are shown in Figure 4.21 and Table 4.7.

The cells co-cultured with NC1 and ConA proliferated strongly. However the responses to all the eluted fractions had SI values of less than 1.

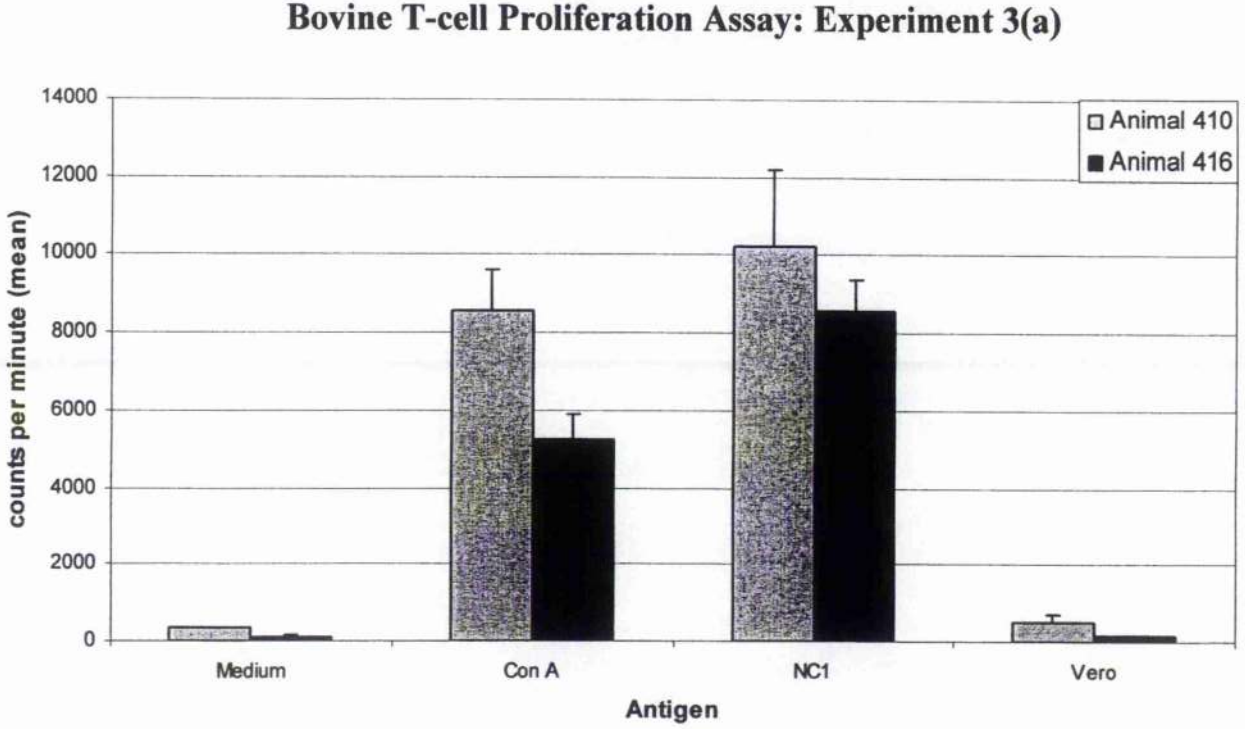


Figure 4.19 *Experiment 3(a).* The mean lymphoproliferation of bovine T-cells from 2 animals infected with *N. caninum* co-cultured with medium alone, whole cell lysate (NC1), ConA and Vero cell lysate. Error bars show standard deviation. Cultures were carried out in triplicate.

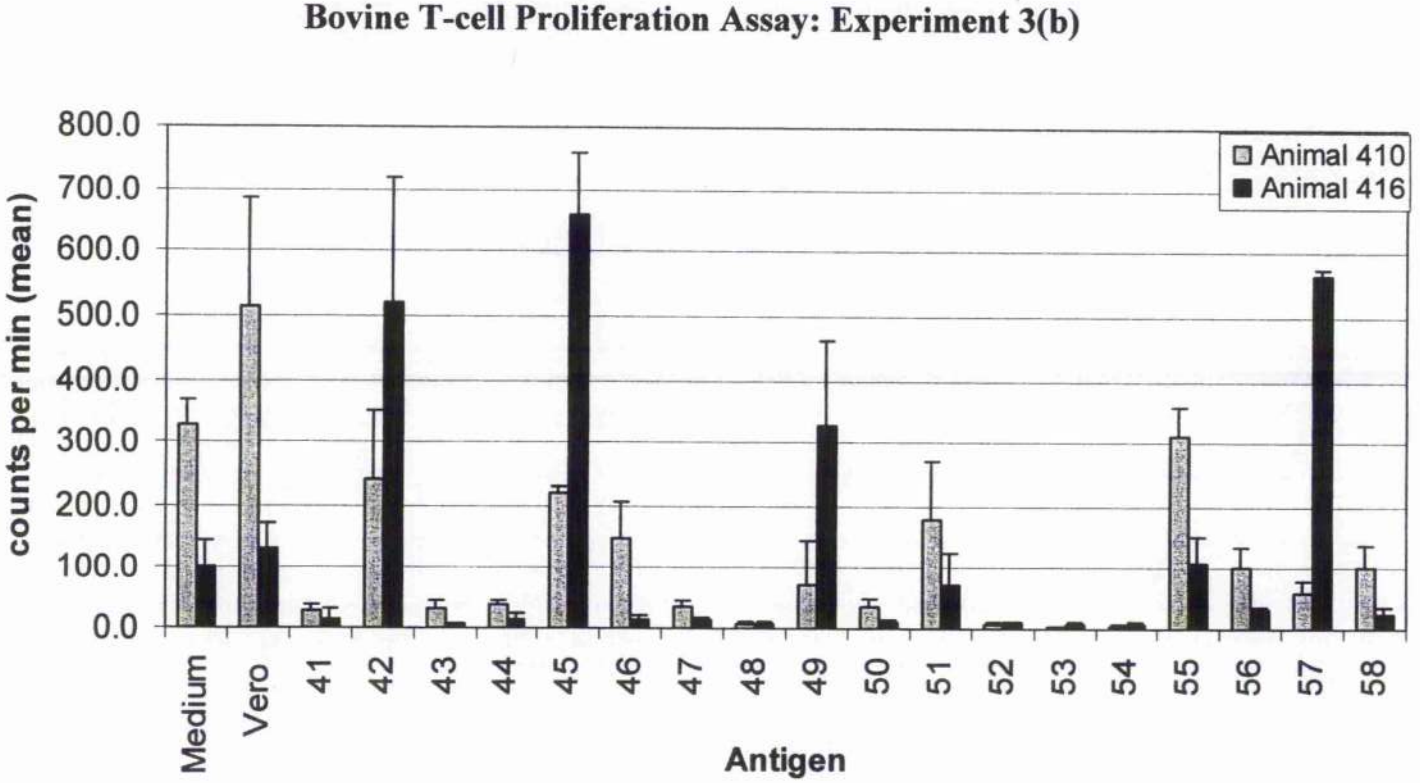


Figure 4.20 *Experiment 3(b).* The mean lymphoproliferation of bovine T-cells from 2 animals infected with *N. caninum* co-cultured with fractions of *N. caninum* antigen eluted from two-dimensional SDS-PAGE gels , or with medium alone and Vero cell lysate. Error bars show standard deviation. Cultures were carried out in triplicate. NC1 and ConA controls shown in Figure 5.6.

Animal 410			
Antigen	**Mean cpm**	**St. Dev.**	**SI**
Medium	328.33	39.55	1.00
Con A	8548.33	1084.64	**26.04**
NC1	10224.67	1958.18	**31.14**
Vero	511.67	174.02	1.56
41	26.33	9.90	0.08
42	239.00	112.60	0.73
43	29.37	15.82	0.09
44	38.33	5.51	0.12
45	219.33	9.29	0.67
46	149.00	58.03	0.45
47	32.13	10.50	0.10
48	8.10	0.72	0.02
49	69.67	74.76	0.21
50	32.10	14.34	0.10
51	177.77	91.38	0.54
52	7.33	3.18	0.02
53	3.57	0.23	0.01
54	4.60	2.97	0.01
55	309.00	49.12	0.94
56	102.23	33.34	0.31
57	57.47	19.86	0.18
58	102.67	36.96	0.31

Animal 416			
Antigen	**Mean cpm**	**St. Dev.**	**SI**
Medium	100.10	44.99	1.00
Con A	5255.00	618.93	**52.50**
NC1	8569.67	788.47	**85.61**
Vero	130.00	40.73	1.30
41	15.13	15.22	0.15
42	520.00	198.40	**5.19**
43	6.53	1.66	0.07
44	14.57	10.03	0.15
45	657.67	102.14	**6.57**
46	13.43	7.36	0.13
47	13.87	2.71	0.14
48	6.43	3.39	0.06
49	327.00	135.24	**3.27**
50	9.70	3.61	0.10
51	71.33	53.14	0.71
52	9.43	1.40	0.09
53	7.57	1.63	0.08
54	5.47	3.36	0.05
55	109.00	41.34	1.09
56	29.53	3.33	0.30
57	563.67	10.50	**5.63**
58	22.00	13.01	0.22

Table 4.6 *Experiment 3*. Mean of triplicate lymphoproliferation assays (with standard deviation) of bovine T-cells (Animals 410 and 416) co-cultured with antigens eluted from two-dimensional SDS-PAGE gels. Stimulation Index indicated the stimulation of different fractions and controls in relation to the medium control. SI > 3 are highlighted in bold.

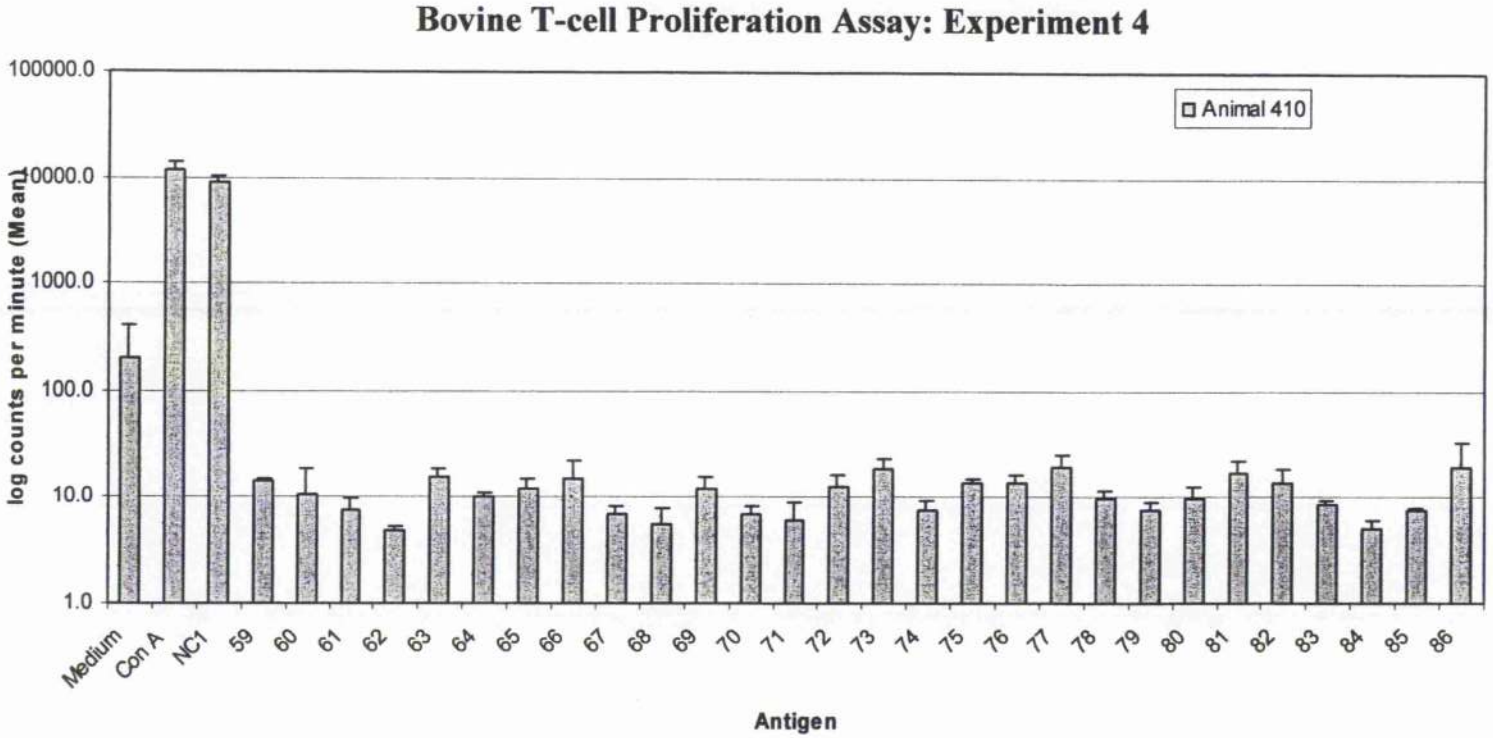


Figure 4.21 *Experiment 4.* The mean lymphoproliferation of bovine T-cells from Animal 410 infected with *N. caninum* co-cultured with fractions of *N. caninum* antigen eluted from two-dimensional SDS-PAGE gels , or with medium alone, whole cell lysate (NC1) and ConA. Error bars show standard deviation. Note the logarithmic scale.

Antigen	Mean cpm	St. Dev.	S.I.
Medium	198.67	219.49	1.00
Con A	12004.67	2139.53	**60.43**
NC1	9129.67	1075.95	**45.95**
59	14.33	0.58	0.07
60	10.67	8.14	0.05
61	7.33	2.52	0.04
62	4.67	0.58	0.02
63	15.67	3.06	0.08
64	10.33	0.58	0.05
65	12.33	3.06	0.06
66	15.33	7.09	0.08
67	6.67	1.53	0.03
68	5.33	2.52	0.03
69	12.33	3.51	0.06
70	6.67	1.53	0.03
71	6.00	3.00	0.03
72	13.00	3.46	0.07
73	19.33	4.73	0.10
74	7.33	2.08	0.04
75	13.67	1.53	0.07
76	14.00	2.65	0.07
77	19.67	6.43	0.10
78	9.67	2.08	0.05
79	7.33	1.53	0.04
80	9.67	3.21	0.05
81	17.33	5.77	0.09
82	13.67	5.69	0.07
83	8.67	0.58	0.04
84	5.00	1.00	0.03
85	7.33	0.58	0.04
86	20.00	13.86	0.10

Table 4.7 *Experiment 4.* Mean lymphoproliferation (with standard deviation) of bovine T-cells (Animal 410) co-cultured with antigens eluted from two-dimensional SDS-PAGE gels. Stimulation Index indicated the stimulation of different fractions and controls in relation to the medium control. SI > 3 highlighted in bold.

A further buffer was used to try and remove as many of the potential toxic components as possible from the eluted proteins. One possibility was that the Tris, when concentrated by vacuum centrifugation, might cause inhibition of the cultures. A weaker Tris buffer was subsequently used.

4.3.3.5 Experiment 5: Elution into 5mM Tris Buffer

On this occasion, a buffer containing only 5mM Tris was used to elute the proteins. 5mM Tris was the weakest buffer that could be used and allow a current (data not shown). Protein samples 87-104 were prepared as described previously from 2-DE SDS-PAGE experiments (and seen in Figure 4.13).

Figure 4.22 and Table 4.8 show the results from Experiment 5. There was some proliferation of cells from both Animals 410 and 416 to NC1 lysate and ConA controls, and little proliferation in the cultures containing cells and medium. One fraction (Number 87) showed signs that it caused cells from Animal 410 to proliferate (SI value of 2.05), although it should be recognised that the actual counts per minute recorded were extremely low.. In cell cultures from Animal 416, there were two fractions (Fractions 87 and 88) that caused some proliferation above that seen in the medium controls (SI values 1.33 and 5.76 in fractions 87 and 88 respectively) . The SI of the cells co-cultured with Fraction 88 was 5.76, higher than the SI=3 cut-off.

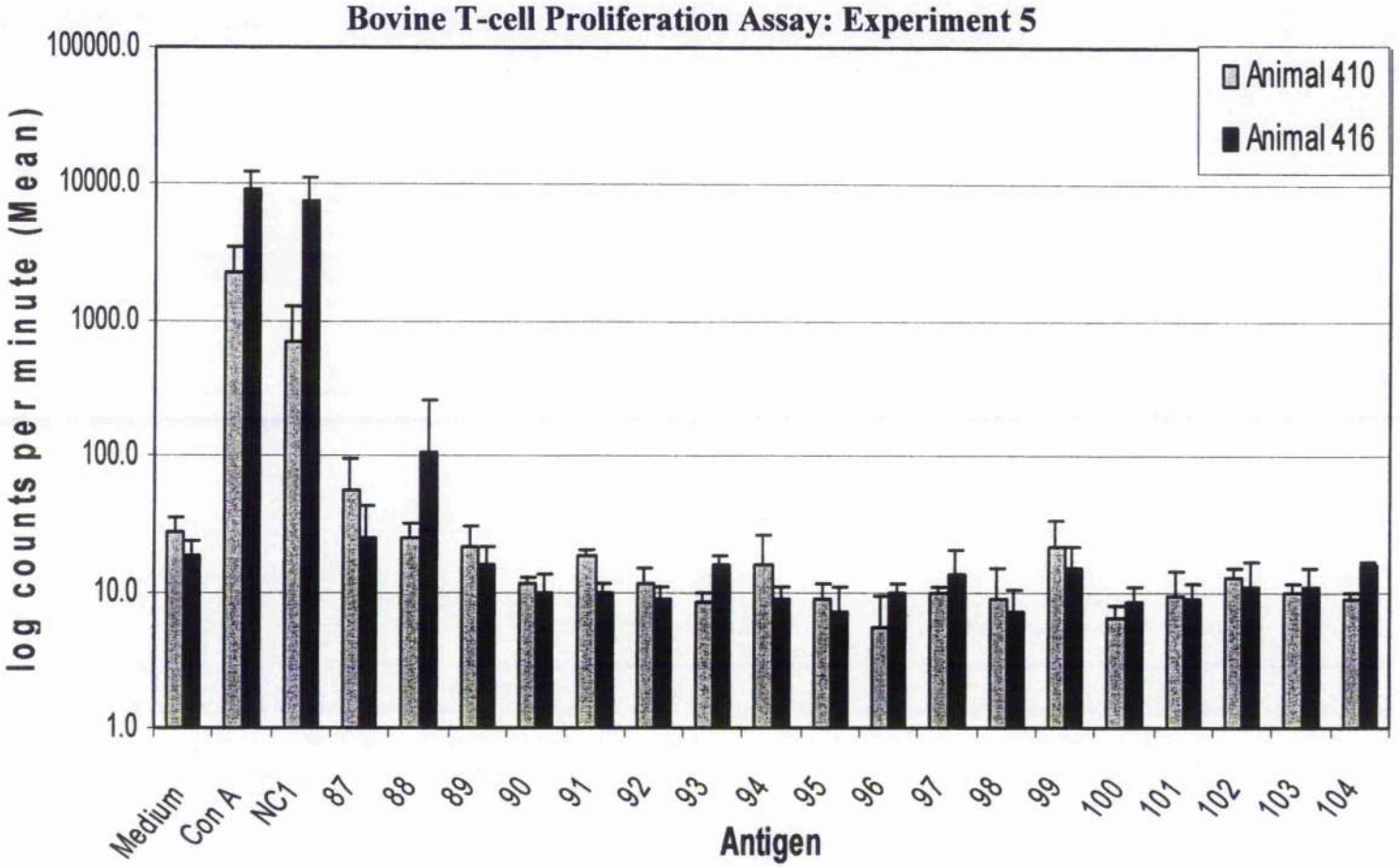


Figure 4.22 *Experiment 5.* The mean lymphoproliferation of bovine T-cells from Animals 410 and 416 infected with *N. caninum* co-cultured with fractions of *N. caninum* antigen eluted from two-dimensional SDS-PAGE gels , or with medium alone, whole cell lysate (NC1) and ConA. Error bars show standard deviation. Note the logarithmic scale.

Animal 410				Animal 416			
Antigen	**Mean cpm**	**St. Dev.**	**SI**	**Antigen**	**Mean cpm**	**St. Dev.**	**SI**
Medium	27.33	9.29	1.00	**Medium**	18.33	4.93	1.00
Con A	2235.00	1156.95	**81.77**	**Con A**	8991.67	3281.15	**490.45**
NC1	688.00	577.19	**25.17**	**NC1**	7656.00	3778.47	**417.60**
87	56.00	37.99	2.05	**87**	24.33	18.77	1.33
88	24.67	7.37	0.90	**88**	105.67	157.05	**5.76**
89	21.00	10.44	0.77	**89**	15.33	5.51	0.84
90	11.67	1.15	0.43	**90**	10.00	3.46	0.55
91	18.00	2.00	0.66	**91**	9.67	1.53	0.53
92	11.33	3.21	0.41	**92**	9.00	1.73	0.49
93	8.33	1.53	0.30	**93**	15.67	2.52	0.85
94	16.00	10.15	0.59	**94**	9.00	2.00	0.49
95	8.67	2.89	0.32	**95**	7.00	3.61	0.38
96	5.67	3.79	0.21	**96**	9.67	1.53	0.53
97	10.00	1.00	0.37	**97**	13.33	6.66	0.73
98	8.67	6.03	0.32	**98**	7.33	3.06	0.40
99	21.00	13.00	0.77	**99**	14.67	6.81	0.80
100	6.33	1.53	0.23	**100**	8.33	2.52	0.45
101	9.33	4.93	0.34	**101**	8.67	3.06	0.47
102	13.00	1.73	0.48	**102**	10.67	5.69	0.58
103	9.67	1.53	0.35	**103**	10.67	4.04	0.58
104	9.00	1.00	0.33	**104**	15.67	0.58	0.85

Table 4.8 *Experiment 5*. Mean lymphoproliferation (with standard deviation) of bovine T-cells (Animal 410 and Animal 416) co-cultured with antigens eluted from two-dimensional SDS-PAGE gels. Stimulation Index indicates the stimulation of different fractions and controls in relation to the medium control. SI > 3 highlighted in bold.

4.3.3.6 General Conclusions

In the five experiments described above, no protein fractions stimulated positive (i.e. SI >3) proliferation in Animal 410 and five fractions (22, 42, 45, 49, 88) stimulated proliferation in Animal 416. The fact that the majority of fractions did not stimulate proliferation was expected, since not every protein of *N. caninum* was expected to cause lymphoproliferation. However, the very low SI values in a number of these experiments would suggest that there maybe something in the fractions that inhibited cell growth and proliferation. To try and identify the inhibitory factor, components of the elution buffer were removed or reduced. The only excess chemicals in the buffer were the 5mM Tris, and small concentrations of chemicals from the SDS-PAGE experiments. More work to identify and remove these potential inhibitors will be required to ascertain which proteins of *N. caninum* were lymphoproliferative. In addition, determining the protein concentration in each of the fractions would help to determine if protein concentration is a limiting factor in these experiments.

4.4 DISCUSSION

4.4.1 Two-Dimensional SDS-PAGE

Two-dimensional electrophoresis (2DE) enables the identification of individual proteins on a polyacrylamide gel, along with the relative ease of obtaining suitable quantities of protein for subsequent mass spectrometric identification. The separation of proteins in two dimensions has the added advantage over traditional one-dimensional SDS-PAGE gels of isolating individual proteins of similar sizes that would normally migrate together in a one-dimensional SDS-PAGE gel. This means that individual proteins can be excised and their primary amino acid sequence, hypothetical structure and antigenicity can be analysed.

4.4.1.1 Problems with 2DE SDS-PAGE

However, there are inherent difficulties associated with 2DE protein separation. The inability to separate membrane (hydrophobic) and very basic proteins on polyacrylamide gels means these two major classes of proteins cannot be investigated as easily as cytoplasmic (hydrophilic) proteins. This becomes a major problem when many of the most immunogenic proteins identified by the humoral immune response are to be found on the surface of cells (Sacks & Sher, 2002; Wastling *et al*, 1994; Carruthers, 1999; Hemphill *et al*, 1999). However, proteins recognised by the cellular immune response are not necessarily found on the surfaces of pathogens like *T. gondii* (for a review, see (Lüder and Seeber, 2001)) and therefore, 2DE separation of *N. caninum* cell lysate is a potentially useful technique to identify those soluble cytosolic proteins. Another limitation of 2DE is the amount of protein that can be separated on a 2DE gel. If too little protein is loaded, few proteins can be observed, even with the most sensitive fluorescent stains. Conversely, if too much protein is in the sample, the gel becomes overloaded and the

proteins do not focus to their isolelectric point. The resulting gel becomes smeared with proteins.

Even with good protein loading, good separation and good visualisation the amount of individual proteins is still very small, in the nanogram range per protein spot (Corthals *et al*, 2000). For many downstream processes except proteolytic digestion for mass spectrometry analysis and Western blotting for antibody probing, the amount of protein is too small. If an extra step of handling (excising of spots from gels and electroelution) and concentration is considered, there is a risk of decreasing the quantity of protein in the sample, such that there is insufficient protein in the sample that cellular proliferation assays are difficult (i.e. low concentrations of protein, diluted into culture assays may also dilute the protein concentration to levels that are undetectable).

4.4.1.2 Alternatives to 2DE-SDS-PAGE

There are alternative methods in the initial stages of development that would mean that 2DE separation would not be required. Direct multi-dimensional liquid chromatography/mass spectrometry (MudPIT – Multi Dimensional Protein Identification Technology (Link *et al*, 2001)) can now be used to bypass completely the polyacrylamide electrophoresis that causes the main difficulties in current proteomic studies and also hampers high throughput protein identification. However, for preparative purposes as described here, polyacrylamide gels are still the most effective method of separating individual proteins for analysis, despite the relatively small quantities of protein separated.

4.4.1.3 Elutions

The presence of small quantities of protein is most probably the case when proteins were eluted from the gels as described in section 4.2.3. Approximately 100μg of protein was loaded onto the gel, and so each spot would likely contain approximately 50-100pg of protein. The extra handling step and the lack of substantial amounts of proteins meant that there was not sufficient protein in the original sample to allow any further loss of any of the proteins during the elution process.

In all the comparisons, using 1D and 2D electrophoresis gels, the Model 422 Eluter was far more effective at eluting significant amounts of proteins compared to the whole 2D elutions carried out on the Bioteluter. The lack of protein in fractions eluted from unfixed 2D gels using the Bioteluter contrasted noticeably with the many fractions containing proteins that were eluted from fixed gels using the Model 422 Eluter. This was despite the fact that the gels were fixed using the Eluter and unfixed using the Bioteluter. The real problem with the Bioteluter was the fact that, though the proteins were unfixed and so more likely to leave the gel matrix when placed in an electric current, the gels were also unstained and so the whole process was carried out without knowing if there were proteins present or not. This meant that the pooling of several gels to increase the overall quantities of each protein was impossible. It also meant that the presence of protein in the eluted sample was unknown right until the end of the experiment. The 422 Eluter, despite being designed for elution of proteins out of a standard SDS-PAGE gel, gave relatively high yields of protein from 2D-separated proteins, compared to the Bioteluter that was specifically designed for 2D gels. The handling of the samples was also much easier using the 422 Eluter, meaning that loss of protein samples through handling was greatly reduced.

The initial aims of this chapter were to separate whole cell lysate of *N. caninum* on both one- and two-dimensional PAGE and prepare protein samples for T-cell proliferation assays. The separation of whole cell lysate was a relatively straightforward procedure; it was the optimisation of the elution technologies that was challenging. Of the two elution apparatuses used, the 422 Electroeluter, not designed for use with 2D gels was the most successful, eluting significant quantities of proteins from both 1D and 2D gels. This was evident when aliquots of the eluted fractions were run on SDS-PAGE gels and stained with Coomassie Blue stain. Unlike other stains, Coomassie Blue is relatively insensitive and cannot be used to detect small quantities of protein. However, proteins were visualised using Coomassie Blue in fractions eluted using the Model 422 Eluter, but not the Bloteluter. The Bloeluter, while designed specifically for 2D gels, did not provide levels of protein that were suitable for assays. However, in other studies, the use of direct blotting of 2-DE separated proteins using the Bloteluter from Biometra (or the equivalent prototype) appeared very successful (Gulle *et al*, 1990; Gulle *et al*, 1993; Andersen and Heron, 1993; Gulle *et al*, 1995).

The method described initially by Gulle *et al* (1990) was the basis for the protocol described in this chapter. Briefly, 100µg of protein was separated by 2-DE and the proteins electroeluted from unfixed gels. T-cells – either directly from patients or previously cultured for 6d and restimulated with 1µg/ml *M. tuberculosis* lysate – were added directly to the eluted fractions. Portions of the cells were irradiated prior to being added to the eluted fraction as antigen presenting cells (APC). Proliferation of the T-cells was measured as described in Section 4.2.5. The protocols for the

experiments described in this chapter only differed from that described by Gulle in that Gulle added the T-cells directly to the fractions. In the experiments described here, the fractions were diluted and added to the T-cells. This was to allow for each fraction to be tested in triplicate, using cells derived from two different animals.

The body of literature from Gulle's laboratory working on identifying T-cell responsive antigens in *Mycobacterium bovis* would indicate that this method of direct elution of proteins into elution buffer before stimulating T-cell cultures from infected animals is feasible (Gulle *et al*, 1993; Gulle *et al*, 1995). In previous work from this lab, proteins eluted from 2-DE gels were directly eluted into T-cells assays, the cells being derived from *M. tuberculosis*-infected patients. This allowed the rapid identification of any potential candidates for sub-unit vaccines (Gulle *et al*, 1990). However, this was not possible for the work described in this chapter since the electrophoresis and elutions were performed at labs in Glasgow and The Netherlands, while the T-cell assays were done in Edinburgh.

In this chapter, it was assessed that the Bloteluter (manufactured by Whatman to the design described in Gulle *et al*, 1990) was not as effective in eluting proteins from gels as the Model 422 Eluter. This was despite the fact that the Bloteluter was designed specifically for 2D gels, while the Model 422 Eluter was designed for 1D gels. The fact that the Bloteluter used unstained gels obviously had practical difficulties, in that the whole process is carried out without knowing if there are proteins present on the gel and it is not until the last step – the SDS-PAGE of concentrated elutions – that the protein levels can be visually assessed. The small sample volume meant that quantification was not possible, since the entire sample

was used in the proliferation assays. In none of the unstained gels was significant protein eluted using the Bloteluter.

One method around using unstained gels has been described for one-dimensional SDS-PAGE (Baskar *et al*, 2000). Immediately after electrophoresis was completed, longitudinal strips were cut from either side of the lane and stained with Coomassie Blue. The rest of the sample was kept unstained. The stained strips could then be used to align with the rest of the sample and individual bands could be excised without staining. This method was inappropriate for use with 2-DE separated proteins; furthermore, even using unstained gels, the elution was only 50-60% efficient (Baskar *et al*, 2000).

4.4.1.4 Protein Quantification

Although not assayed, it is reasonable to assume that the amount of protein within the sample solutions eluted from lysate separated by 2-DE was lower than that present in the whole NC1 lysate (1μg/ml final concentration). The gels used for electroeluting the proteins were loaded with $1x10^8$ tachyzoites, approximately 100μg of protein. Although the amount of protein loaded onto the gels in this chapter was the same as that loaded onto the gels in the studies of Gulle *et al* (1993), when the gels obtained in this chapter were visually compared with those published by Gulle *et al* it appeared that there were higher levels of protein separated on the gels in Gulle's experiment than in the gels in the experiments described in this chapter, as determined by greater numbers of, and more pronounced, spots. Several antigens from *Mycobacterium bovis* crude lysate separated by 2-DE SDS-PAGE were identified in this study (Gulle *et al*, 1993). It should be noted that Gulle and co-

workers did not quantify the protein levels in each sample, because in their studies, proteins were directly eluted into T-cell cultures (Gulle *et al*, 1990; Gulle *et al*, 1993).

Similarly, in the studies described in this chapter, the quantity of protein in any of the eluted fractions was also not assayed. All of each sample was used in the proliferation assays, so the experiments could not be repeated using the same fractions, and quantification assays could not be done, because gel-to-gel variation, even between spots that had migrated to the same points of the gel, meant that an accurate quantification from one gel was not applicable in other gels. Furthermore, the reproducibility of the gels was not sufficiently high to allow gel-to-gel comparisons of quantities of individual protein spots.

4.4.1.5 Use of Eluted Proteins

The ultimate aim of the elution experiments was to obtain protein fractions that could be used in subsequent T-cell proliferation assays. One hundred and four samples were eluted from various gels. Of these 104 samples, only five produced some proliferative response in cells from one or other of the cattle (proliferation being assessed as being shown when the SI value was greater than 3), and none of these produced proliferative response in both Animal 410 and 416. The proliferation caused by these fractions was less than that produced by the positive control (NC-1 lysate) or the T-cell mitogen (ConA). Possible reasons for this are discussed below.

4.4.2 Lymphoproliferation Assays

4.4.2.1 Potential Toxicity of Buffers

Other than the quantity of protein in the fractions (described in Section 4.4.1.4), a further explanation for the lack of stimulation of the cultures may have been some

toxicity of the elution buffers. Inhibition of the growth of the cells *in vitro* when co-cultured with the eluted fractions appeared to have occurred. This can be observed by comparing the stimulation indices (SI) of the various eluted fractions and T-cells compared to the medium control. The SI of the medium control is 1; this is the baseline growth of the cultures with no additional antigens added to the medium. In the majority of cultures, the SI value was <1, suggesting that there were inhibitory components present in the electroeluted antigen fractions. This may have been due to excess salt in the elution sample, which, as described in Chapter 4, was eluted from the polyacrylamide matrix into a weak Tris buffer. This was then concentrated to remove the solvent; however this meant that the solute (containing the protein) had a very high salt concentration, which may have been detrimental to the growth of the cells. The presence of Tris salts in the elution fractions did not appear to be detrimental in other studies using directly eluted proteins in T-cell assays (Gulle *et al*, 1990).

In addition, there may have been traces of SDS within the sample, which is recognised as being inhibitory to the growth of cells in tissue culture (Grant and Acosta, 1994). Gulle *et al* (1993) noted no significant effect on the survival of bovine T-cells in the presence of less than 0.001% SDS, though there was some effect when this concentration increased to 0.025%. However, even when a non-SDS elution buffer was used in Experiments 2-5, there was some inhibition of growth. It is likely that there were maybe other contaminants, other than SDS, that were responsible for the observed inhibition of growth. It is likely that the concentration of the samples increased the likelihood of the cultures being inhibited, whether SDS was present or not.

Because of the likely low concentration of protein within the sample, along with the small volume of the sample, dialysis to remove toxic components was not a viable option. Neither the studies by Gulle and co-workers (Gulle *et al*, 1990; Gulle *et al*, 1993; Gulle *et al*, 1995), or Andersen and Heron (1993) concentrated the eluted samples, prior to T-cell proliferation assay.

4.4.2.2 Concentration of Samples

One difference in the methodology between our experiments and those described of Gulle and colleagues (1990, 1993) is that in our study, the proteins were not eluted directly into the T-cell cultures. Concentration of samples was felt to be required because the amount of protein separated into each spot was low (as can be seen in Figures 4.7, 4.9, 4.11, 4.12 and 4.13). With the low recovery rate expected from the eluters, it was felt that concentrating the samples would be useful.

4.4.3 Future Studies

4.4.3.1 Elution Methods

Electroelution of one-dimensional SDS-PAGE gels for use in lymphoproliferation assays has been done previously (Andersen and Heron, 1993). Whole cell lysates of *M. tuberculosis* were separated on a 1-DE SDS-PAGE before individual protein bands were eluted from the gel for use in T-cell assays. In this study, an immunodominant, secreted protein was identified by causing both proliferation of murine T-cells *in vitro* and also stimulation of IFNγ (Andersen and Heron, 1993).

In previous studies to identify immunodominant T-cell antigens of *N. caninum*, one-dimensionally separated whole cell lysate was blotted onto nitrocellulose, which was then added to the cell cultures (Marks *et al*, 1998). One-dimensional SDS-PAGE

does not have the separation power of 2-DE SDS-PAGE, so several proteins were present in each fraction tested. Nevertheless, several fractions (<30kDa) were identified as containing proteins that caused lymphoproliferation and production of IFNγ in bovine T-cells (Marks *et al*, 1998). This may be a good starting point to test the electroelution technique.

4.4.3.2 Alternatives to Elution

Blotting of 2-DE SDS-PAGE gels onto nitrocellulose before use in T-cell assays is another method that may be considered for future work. T-cells have been shown to proliferate to antigens immobilised on nitrocellulose (Young and Lamb, 1986) without the nitrocellulose affecting the assay. This is technically difficult when dealing with large numbers of proteins and/or small pieces of nitrocellulose.

Coomassie blue and Sypro Orange staining procedures used to identify proteins risks contamination of the membrane with potentially cytotoxic substances. However, there are reports in the literature of the use of colloidal gold stain, to stain nitrocellulose membranes for use in T-cell assays (Horn *et al*, 1999). However, quantification of protein on nitrocellulose is still problematic, but is possible if a comparative molecular weight marker with known concentrations of each weight is also blotted onto the nitrocellulose.

4.4.3.3 Modification of Assay Methodology

Modifications of the methodology to optimise the assay are outlined below. Firstly, and most importantly, quantification of protein in the samples would allow an adequate comparison of T-cell proliferation stimulated by the eluted fractions and the NC1 positive control. This will allow us to determine whether the amount of

Neospora-specific protein in the sample was a limiting factor in this system. A titration of the eluted protein could be carried out, along with titrations of NC-1 lysate to set an upper and lower range of protein concentration that the T-cells would respond to. This would be dependent on (a) the amount of protein that could be adequately separated on a 2-DE gel and (b) a more efficient protein recovery from elution.

4.4.3.4 Inhibition of Cell Growth

The issue of inhibitors of growth of cells in culture is important. Native PAGE, without SDS could be used, however the separation of proteins would not be optimal. Elution could be carried out using a lower concentration of elution buffer, though this would have the effect of reducing the elution:time ration. SDS-PAGE gels and the Tris elution buffer used in this study were also used by Gulle and colleagues (1990), and no toxicity effects were observed in these experiments. It is therefore likely that the concentration of the samples did have an effect in concentrating toxic components.

4.4.4 Conclusion

Five fractions were identified in our study that caused the T-cells to proliferate with an SI of greater than 3. However, further work is required as discussed to assess whether this method of eluting proteins is useful to identify novel immunodominant antigens of *N. caninum* that may be useful as potential vaccine candidates. For example, each fraction would need to be used several times to check reproducibility of the T-cell stimulation, and several T-cell lines from different cattle would need to be produced and used to test the lymphoproliferative capacity of these fractions in a larger cattle group.

CHAPTER 5:

IDENTIFICATION OF *NEOSPORA CANINUM* PROTEINS BY MASS SPECTROMETRY

- ◊ Identify proteins that were identified as immunologically interesting by T-lymphocyte proliferation assays (Chapter 4) by mass spectrometry
- ◊ To compare and contrast mass spectrometry techniques in identifying proteins from an organism with little genomic sequence available

5.1 INTRODUCTION

5.1.1 Use of Mass Spectrometry to Identify Parasite Proteins

Mass spectrometric technologies have been used extensively in disease research, noteably in cancer and heart disease research (reviewed in Jungblut *et al*, 1999). In addition, there is a growing body of work using mass spectrometry techniques to identify parasite proteins, for diagnostics and to study the basic biology. Proteomic tools, for example two-dimensional electrophoresis and mass spectrometry, are useful to identify interesting proteins of parasites. Complete proteome maps of, for example, disease and non-disease states or different life-cycle stages, can identify changes due to different gene expressions that may elucidate further information about the biology of the parasites.

5.1.1.1 Matrix-Assisted Laser Desorption Ionisation Time-of-Flight (MALDI-ToF)

Matrix-Assisted Laser Desoprtion Ionisation Time-of-Flight mass spectrometry (MALDI-ToF) was first described by Hillenkamp *et al* (1991). MALDI-ToF is used to analyse organic molecules such as peptides. Samples are mixed with an absorptive matrix, prior to being ionised using lasers. The matrix transforms the laser light energy into excitation energy, which causes the sample to spontaneously ionise and leave the matrix into the sampling chamber of the mass spectrometer (Hillenkamp *et al*, 2001). Within the chamber, the ionised samples are separated by the Time-of-Flight analyser according to their mass-to-charge ratio. This ratio is calculated by measuring the time the ions take to pass through a charge-free field. The sizes of the ions can then be judged against known, calibrated standards (Hillenkamp *et al*, 2001). This peptide mass fingerprint can then be used to search EST databases.

5.1.1.1.1 The Use of MALDI-ToF to Identify T. gondii Proteins

MALDI-ToF has been used to identify differences in primary amino acid sequence in tachyzoites of *T. gondii* (Zinecker *et al,* 2001) as well as being used to provide an overview of the proteome of the same parasite (Cohen *et al*, 2002). In the work by Zinecker and co-workers (2001), MALDI-ToF was used to study the isoforms of the *T. gondii* molecule TgSAG1. TgSAG1 is the major surface protein of *T. gondii* (Burg *et al,* 1988) and is inserted into the parasite membrane with a glycosyl-phosphatidylinositol (GPI) anchor attachment (Zinecker *et al* 2001). Two different glycoforms of the GPI anchor were analysed by ion exchange chromatography and gel filtration, as well as by MALDI-ToF. Differences in the primary amino acid sequence were observed between the GPI anchor prior to and following attachment to the glycan component of the TgSAG1 molecule (Zinecker *et al*, 2001).

The mapping of the *T. gondii* proteome was demonstrated by Cohen and co-workers (2001) as viable, despite the lack of full genome sequence. There was at the time, however, a moderate coverage of the *T. gondii* genome in the EST databases, for example ToxoDB (http://toxoDB.org). In the study by Cohen *et al*, the use of MALDI-ToF analysis of proteins separated by 2DE SDS-PAGE identified several putative matches to the ToxoDB, and identified post-translationally modified proteins.

However, the global approach to protein identification as performed by Cohen and colleagues (2001) has been recognised as being not only difficult but of limited value as the number of proteins in a eukaryotic cell far outweighs current separation techniques. Pre-fractionation of a cell lysate, to obtain particular subsets of proteins

allows a more detailed analysis, an approach that has been carried out in the apicomplexan parasite *Eimeria tenella* (Bromley *et al*, 2003). In this study, the proteins from the micronemes of the parasite – thought to be involved in host invasion - were purified from *E. tenella* sporozoites, and separated by 2D electrophoresis. Spots were analysed using MALDI-ToF and chemically assisted fragmentation (CAF) MALDI. Several spots were identified from the *Eimeria* sp. specific databases, indicating that the use of MALDI techniques, in combination with bioinformatic database searching is a powerful tool for protein identification in parasites with some genome / EST coverage (Bromley *et al*, 2003). However, for those organisms with limited coverage, a tandem mass spectrometric approach may be more useful.

5.1.1.2 Tandem Mass Spectrometry (MS/MS)

Tandem mass spectrometry (MS-MS) is used to generate primary amino acid sequence data about a peptide by fragmenting specific sample ions inside the mass spectrometer and identifying the resulting fragment ions. This information can then be pieced together to generate sequence information regarding the intact molecule. The use of MS/MS in biochemical analysis has become routine (reviewed in Griffiths *et al*, 2001)

Peptides are firstly proteolytically digested before the daughter ions are sprayed into the quadrupole/time-of-flight analyser of the mass spectrometer. This energy causes the side-chains of the peptides to fragment, and produce a detailed peptide map that can be used to identify the sample by using this map to search the protein databases.

5.1.1.3 Advantage of MS/MS over MALDI-ToF

The advantage of Tandem Mass Spectrometry (MS/MS) over MALDI-ToF when in conjunction with two-dimensional SDS-PAGE is that MS/MS generates peptide fragmentation data that can be used to search through protein sequence databases. MALDI-ToF MS, on the other hand, produces a peptide mass fingerprint for the sample that, though searchable in EST databases, reveals nothing about the primary amino acid sequence of the peptide directly. Therefore, for organisms with limited genome coverage MS/MS is a much more useful tool.

MS/MS has been used to identify potential vaccine candidates from the nematode parasite *Haemonchus contortus* (Yatsuda *et al,* 2003). In this study, 107 proteins were identified from the excretory-secretory proteins. Included in these proteins were ones that had not been identified previously in the excretory-secretory products. This study suggests that the use of MS/MS may be useful to identify proteins that, for some reason such as low concentration, have not been identified previously using other biochemical or molecular techniques (Yatsuda *et al* 2003).

5.1.2 Use of Mass Spectrometry to Identify *N. caninum* Proteins

Mass spectrometry (MS) techniques have been used to identify proteins in disease pathology, particularly in combination with two-dimensional SDS-PAGE (2-DE) (Jungblut *et al,* 1999). In *N. caninum*, which has limited protein, and DNA sequence (approximately 18,000 ESTs), available on public databases, there have been attempts to identify antigenically interesting proteins using a combination of immunoblotting, 2-DE and MALDI-ToF MS (Lee *et al* 2003; Shin *et al,* 2004). In the study by Lee and colleagues (2003), twenty proteins were identified from 31

spots examined. These included excretory proteins from the microneme (NcMIC1) and dense granules (NcDG1, also named NcGRA7; NcGRA1 and NcGRA2). In addition to these proteins identified with *N. caninum* proteins, a further eleven proteins were identified as being significantly homologous to *T. gondii* proteins as to warrant mention. These include heat shock proteins, alpha- and beta-tubulin and metabolic enzymes 9-fructose-1,6-bisphosphatase, lactate dehydrogenase and glyceraldehydes-3-phosphate dehydrogenase (Lee *et al*, 2003). Similar proteins were identified in the later study by Shin *et al*, which used a combination of immunoblotting using anti-*Neospora* IgG, IgE, IgA and IgM to identify immunodominant proteins prior to sequencing (Shin *et al*, 2004).

The results from Lee and co-workers (2003) would suggest that a "shotgun" approach, i.e. selecting proteins at random for sequencing, for identification of proteins from *N. caninum* – either directly or by homology with other closely related organisms would be interesting, since very little is currently known about the proteome of *N. caninum* (Lee *et al*, 2003). The more considered approach by Shin *et al* who used the premise of humoral immunodominance to pre-select those protein spots that were to be sequenced is an interesting one. Since *N. caninum* infections are primarily controlled by a cell-mediated immunity (Lunden *et al*, 1998), the concept of identifying, by MS methods, immunodominant proteins that cause a proliferative response in T-cells is one that has scientific merit, and will be explored further in this chapter.

5.1.3 Aims and Objectives

The aim of the work presented in this chapter is to:

- Identify proteins that were identified as immunologically interesting by T-lymphocyte proliferation assays (Chapter 4) by mass spectrometric techniques.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of Protein Samples for Mass Spectrometry

5.2.1.1 Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-ToF MS)

MALDI-ToF MS was carried out on proteolytically cleaved peptides. Normally the tryptic digestions were performed on proteins within a polyacrylamide matrix. However, since some proteins of interest had been electroeluted (see Chapter 4) into solution, it was desirable to perform the trypsin digest in the solution.

5.2.1.1.1 In-Gel Trypsin Digestion of Proteins Separated by 2-Dimensional Electrophoresis (2-DE)

Tryptic digestion of proteins within a polyacrylamide matrix was performed as described previously (Hillman, 1995). Spots of proteins separated by 2-DE were excised from the gel and cut into smaller pieces to increase the surface area. These pieces were washed in 500µl of 100mM ammonium bicarbonate for 1h, followed by another 1h wash in 500µl of a 100mM ammonium bicarbonate/50% (v/v) acetonitrile. A further 10min wash in 50µl 100% acetonitrile. Any solvent remaining in the tube was removed by vacuum drying the gel pieces in a vacuum centrifuge (DNA Speed Vac, Savant Ltd). The gel pieces containing the protein were incubated for 15min at room temperature in 25mM ammonium bicarbonate (10µl), which contained 0.2µg trypsin (sequence grade) (Promega). The gel pieces were then covered in 20µl of this buffer and incubated overnight at 37°C.

Prior to MALDI-ToF MS, the peptide mixtures from the overnight tryptic digestions were concentrated and excess salts were removed from the digestion using ZipTips™

(10µl, C18) (Millipore) according to the manufacturers instructions. The ZipTip™ resin bed was washed through twice with 50% (v/v) acetonitrile, and twice with 0.1% (v/v) trifluoroacetic acid. The protein sample was mixed with 3µl 3% trifluoroacetic acid. The sample was then loaded onto the ZipTip™ and passed through the resin bed 10 times by aspirating the solution through the tip using an automatic pipette (Gilson). The resin was washed twice in 0.1% (v/v) trifluoroacetic acid. A mixture of 60% (v/v) acetonitrile/0.3% trifluoroacetic acid (10µl) was loaded onto the ZipTip™. The sample was flushed into a clean 1.5ml tube. This whole procedure was repeated to further concentrate the sample. The sample was then ready for MALDI-ToF MS.

Before the samples were analysed in the MALDI-ToF machine, 1µl of MALDI matrix (Promega) was aliquoted into a clean 1.5ml tube. The sample (1µl) was added to this aliquot of matrix and mixed. One microlitre of this mix was placed on the plate, allowed to dry for 20min, before it was placed in the machine for analysis by the MALDI-ToF MS.

5.2.1.1.2 Trypsin Digestion of Proteins Electroeluted into Tris Buffer

This protocol was similar to that described above in section 5.2.1.1.1. However, instead of two ZipTip™ steps, only one ZipTip™ step was performed and after each wash step, the solvents (as described above) were removed by vacuum centrifuge. The samples were mixed with MALDI-ToF matrix as described above.

5.2.1.2 Tandem Mass Spectrometry (MS/MS)

The samples for MS/MS were digested with trypsin as described in Section 5.2.1.1. The digestion mixtures were also washed using ZipTip™ columns. Prior to

electrospray, proteins adsorbed onto the ZipTip™ columns were washed with 1% acetic acid and then eluted from the column using 50% acetonitrile: 1% acetic acid (in water). This was loaded onto a borosilicate capillary prior to mass spectrometry.

5.2.2 Mass Spectrometry of Proteolytic Peptides

5.2.2.1 MALDI-ToF MS

MALDI-ToF MS was carried out using the Voyager DE-STR MALDI-ToF (Applied Biosystems), equipped with a nitrogen laser of 337nm (3ns pulse). After calibration of the machine with trypsin autolytic peaks, obtained by firing approximately 200 laser shots at the trypsin-only control, the same number of laser shots was fired at the sample in the matrix.

The spectrum generated by the MS, was generated using Voyager Data Explorer software (v. 5.01) (Applied Biosystems). This spectrum could be used to search the EST databases using MASCOT software.

5.2.2.2 Tandem MS/MS

Capillaries containing eluted proteins (described previously in Section 5.2.1.2) were placed into the nanospray source of the Q-STAR mass spectrometer (Applied Biosystems). Spray formation was performed in a low-pressure nitrogen atmosphere, and spectra were obtained after 1s scans.

5.2.3 Identification of Peptides/Proteins

The spectra obtained from the different MS experiments were used to search several databases. MALDI-ToF MS data was used to search Genbank

(www.ncbi.nlm.nih.gov/BLAST), while the MS/MS data was used to search Genbank and local version of ToxoDB (http://ToxoDB.org) *T. gondii* databases containing information from the *T. gondii* sequencing project, as well as EST and BAC information (searches were carried out in February 2003). This searching was carried out using MASCOT software.

5.3 RESULTS

5.3.1 MALDI-ToF MS

Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-ToF MS) was initially used to obtain sequence data from eluted protein samples. Proteins eluted from the polyacrylamide gel matrix (Chapter 4) were proteolytically digested using modified trypsin (Promega) and then mixed with MALDI-ToF matrix before being bombarded with a laser to ionise the peptide fragments which passed into the MS machine and analytical spectra obtained.

Figure 5.1 shows a typical trace obtained from the MALDI-ToF analysis of eluted protein sample from NC1 lysate separated in two dimensions by SDS-PAGE. All the high intensity peaks were in the mass/charge ration of between 800 and 1240, and were identified as trypsin fragments. Since trypsin itself is a protein and will also be ionised by the lasers, it was expected that there would be trypsin peaks in the mass spectrometry trace, with a mass/charge ratio of 842.5 (Figure 5.1). However, since there were no other peaks present, this suggested that the proteins were not successfully transferred from the Tris elution buffer into the ZipTip™ and then into the matrix.

This experiment was repeated several times using different eluted proteins; however, the traces obtained were very similar to the one shown in Figure 5.1, that is, the high intensity peaks were identified as trypsin fragments. Any peptide fragments would have been expected to have a higher mass/charge ratio. The poor signal-to-noise ratio, suggested that there was insufficient protein in the sample as no peptides were identified except for trypsin fragments. This was probably related to the fact that the

ZipTip^{TM} protocol was not optimised for proteins in solution. Other MS techniques were therefore tried.

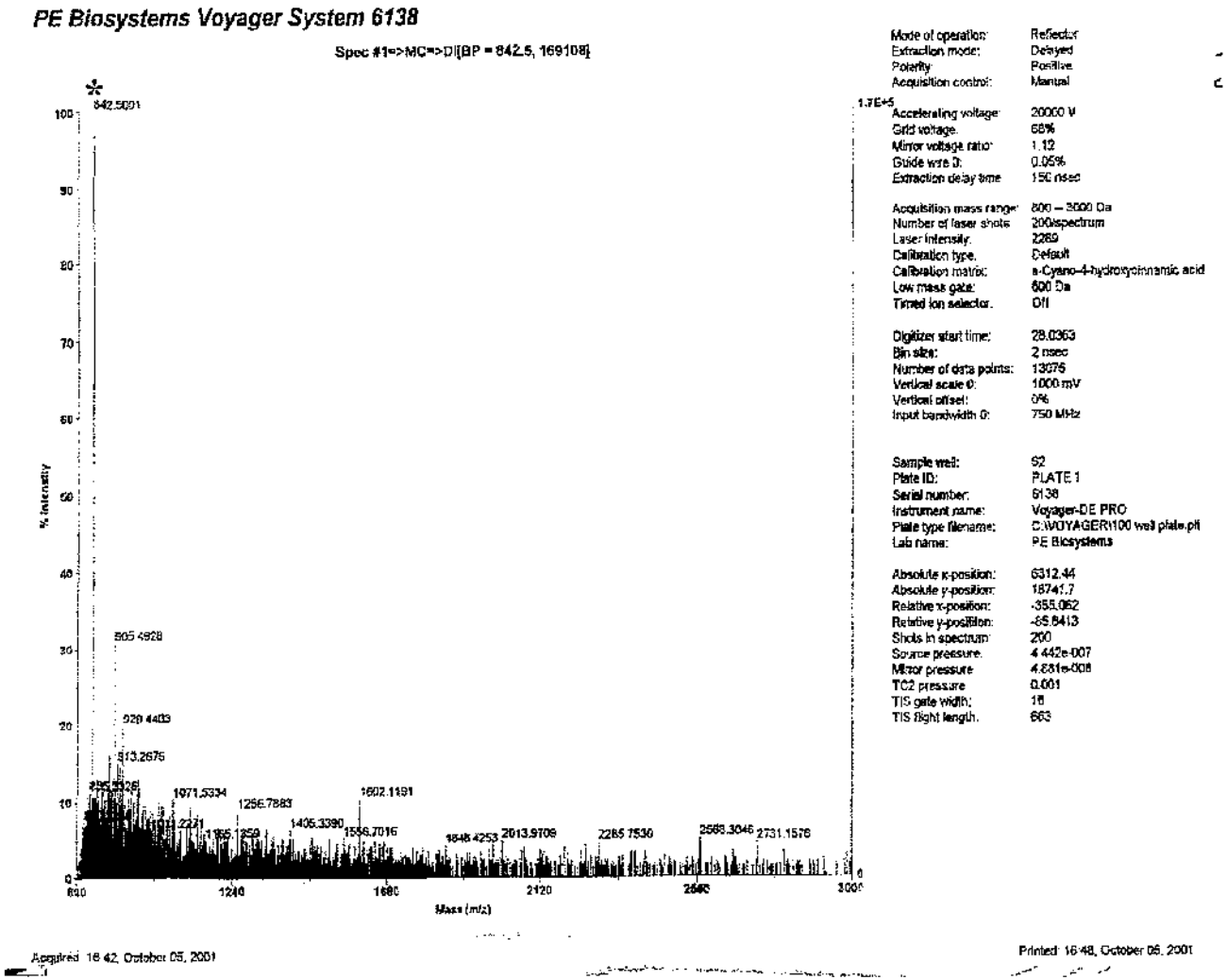


Figure 5.1 MALDI-ToF trace of peptides. Using standard protocols for proteolytic digestion of proteins, this was carried out within the polyacrylamide gel matrix. In this case, because the protein fragments were previously eluted from the gel, the typtic digest was carried out in the liquid phase. The peak with a mass/charge ratio of 842.5 (*) is trypsin

5.3.2 MS/MS

This technique was used to identify proteins that were previously highlighted as potentially immunologically interesting by their stimulation of T-lymphocytes *in vitro* (Chapter 5). Two proteins from these experiments were worthy of further analysis, since they produced a relatively high proliferation in the T-cell cultures, above the medium control. As described in Chapter 4, an aliquot of each protein used in proliferation assays was run on a standard one-dimension SDS-PAGE gel. This allowed the in-gel digestion of the protein for MS/MS analysis. This also removed the risk that the protein would be lost in the handling steps as occurred in MALDI-ToF experiments (section 5.3.1).

Figure 5.2 shows the MASCOT output from one of the proteins identified in Chapter 4 (Fraction number 45 (Animal 416), Experiment 3). The spectra generated from the mass spectrometers were automatically searched using MASCOT; no manual input from the spectra was carried out. The hatched area that spans from 0 to just short of 75 on the Probability based Mowse score, indicates an area where any hits to the database were not regarded as significant. This area also contained several hits against trypsin and human keratin, a common contaminant in MS experiments. There was, however, one significant hit at approximately 90 on the Probability Based Mowse Scale that was obtained. This was identified (Table 5.1) from the database as *N. caninum* superoxide dismutase.

The mass spectrometry data generated by the other protein identified in Chapter 4 (Animal 416, Fraction 42, Experiment 3) did not have any significant hits associated with it (not shown).

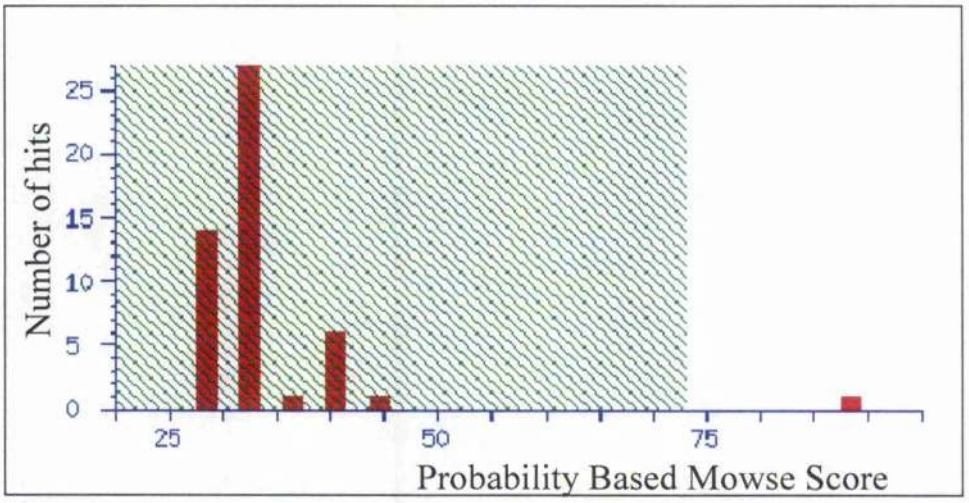


Figure 5.2 Data from Tandem Mass Spectrometry, output through the MASCOT programme. Peaks of peptide identifications within the green shaded area are insignificant hits. Those outside the shaded area are significant. In this case, one significant hit was obtained.

Accession Number	Identifier Number	Description
AF296416_1	AAL62028 G18157554	*Neospora caninum* Superoxide dismutase
LLDEINKEFTSVEK		

Table 5.1 Identification of the one significant hit from protein fraction that had positive T-lymphocyte proliferative activity. Peptide sequence with significant alignment is highlighted.

5.4 DISCUSSION

Modern mass spectrometric techniques provide protein sequence information that would have been very difficult to generate a decade ago.

5.4.1 Lack of Identification Using MALDI ToF MS

The lack of identification of proteins using MALDI-ToF was surprising, since this technique was optimised for identification of proteins from *T. gondii* (Cohen *et al*, 2002). There are a couple of reasons to explain this lack of identification.

Firstly, the protocol normally used for MALDI-ToF was optimised for in gel digestion of proteins (described in Cohen *et al*, 2002). There is currently nothing in the literature to demonstrate that digestion of proteins separated by SDS-PAGE but eluted into an aqueous solution is possible. The reverse phase extraction used in ZipTip^{TM} clean-up of protein samples is also not possible when there is a high salt concentration present. In eluted proteins, the salt concentration in the samples is higher than in normal experimental conditions, since the protein in elution buffer is concentrated so that it can be detected on a preparatory gel.

As described in Chapter 4, the preparatory gels from which the protein spots were eluted were loaded with 100μg of *N. caninum* whole cell lysate. Each spot was estimated to contain approximately 50-100 pg of protein. The likelihood of a 100% efficient elution was negligible, according to the eluter manufacturers, and a more likely elution rate was approximately 50%, reducing the amount of protein within each sample to 25-50 pg. Since the majority of the sample was used in proliferation assay, the amount of protein used in the spectrometry experiments was in the 5-10 pg

range, so there was a possibility that the eluted protein samples were at such a low concentration that, during the digestion process, the majority of the sample was lost. Even the most successful elution, using the Biorad Bioteluter (see Chapter 4), only succeeded in extracting small quantities of protein from the gel. When another handling step was introduced, this already small concentration of protein was reduced substantially.

The limitations of MALDI-ToF were also exacerbated by the lack of sequence data available for *N. caninum* proteins. Identification of the peptide fingerprints generated by MALDI-ToF experiments requires prior data in the databases, since it is the fingerprint of individual peptides rather than direct sequences of peptides that are searched. Without previous fingerprints to compare with, it is difficult to get identification of the peptides generated in an experiment. Unlike *T. gondii* where there have been significant advances in the sequencing of the genome (http://www.sanger.ac.uk/Projects/T_gondii/), there is limited genome sequencing available for *N. caninum* (at the time of searching there was approximately 13,000 ESTs and 81 gene sequences available for *N. caninum*). This lack of genome-, and related protein, sequence limited the use of MALDI-ToF MS. Nevertheless, MALDI-ToF MS has been used to identify several *N. caninum* tachyzoite proteins, either by direct identification or by homology with *T. gondii* proteins (Lee *et al*, 2003).

5.4.2 MS/MS

The use of MS/MS sequencing enabled the identification of a protein that caused lymphoproliferation in T cells obtained from *N. caninum* infected cattle (Chapter 4) as *N. caninum* superoxide dismutase (SOD) (Accession number - Genbank

AAL62028, submitted by Cho *et al*). SOD has been identified as an important molecule in *T. gondii* infections (Hughes *et al*, 1989). SOD is one of several enzymes that pathogens use to counteract the oxidative stress that occurs when macrophages are activated during infection. *T. gondii* is particularly rich in SOD, while *Eimeria bovis* for example is less so. *E. bovis* appears to be more susceptible to oxidative killing than *T. gondii* (Hughes *et al*, 1989).

However, since only one peptide was identified as *N. caninum* superoxide dismutase, further work needs to be done to confirm this result. Since the molecular weight of the protein spot (Fraction 46 in Figure 4.11) can be estimated, this can be compared with the known value for *N. caninum* superoxide dismutase (22kDa).

The other sample that was used in mass spectrometry (Fraction number 42) was not identified as being significantly homologous to any other sequence in the database. This is probably due to the lack of genome sequence available for *N. caninum* though it does suggest that a homologue for this protein was not present in *T. gondii* or other related organisms. The lack of material prevented further information about this unknown protein being elucidated.

5.4.3 Further Studies

The efficiency of protein identification from MS data is dependent on having significant genome sequence with which to compare the spectra obtained. For organisms like *N. caninum,* which currently has limited sequence information, the amount of meaningful data that can be obtained from MALDI-ToF and MS/MS is limited. Other, more powerful techniques could be used to obtain data both from

individual protein spots identified as being immunodominant by T-cell proliferation assay, as well as from mixed samples from, for example, a parasite lysate. One such technique is Multidimensional Protein Identification Technique (MudPIT). MudPIT uses the same technology as MS/MS but in tandem with a liquid chromatographic separation of the proteolytic peptides. This additional step enables mixed protein preparations, for example from a one-dimensional SDS-PAGE, to be run and the different proteins identified.

MudPIT is a powerful tool to dissect the biology, immunology and biochemistry of parasites such as *N. caninum*. The combination of liquid chromatography with tandem MS that is MudPIT, with immunoblotting using immune serum from naturally infected cattle would be a useful starting point in determining the set of proteins that is recognised by the humoral immune response during infection. In addition to this initial screening, by combining liquid chromatography technology with T-cell proliferation assays (such that a whole parasite lysate can by digested before half going for T-cell proliferation studies, the other half going for mass spectrometric identification) should provide strong evidence as to the identification of proteins important for a cellular response. It should be noted at this stage that the samples would contain multiple proteins and that a further chromatographic separation would be required to get individual proteins for analysis. Nevertheless, this technique would also have the potential for a rapid high-throughput screening of a large set of proteins. Fractions that contained protein(s) that caused proliferation of T-cells, as well as being identified by the humoral immune response, could be isolated and fractionated further, until individual proteins could be identified. Alternatively, a "positive" fraction, that is one that causes lymphoproliferation, could

be sequenced and recombinant proteins made from each of the identified proteins, which could then be used as antigen in subsequent immuno-assays. This may be a complex method of identifying immunodominant proteins, but the omission of SDS-PAGE separation would ensure that the limitations of that technology would not limit the proteins available for study to only the soluble ones.

CHAPTER 6:

DISCUSSION

6.1 Background

The aims of the work presented here can be split into two main sections – the use of transgenic *T. gondii* as a live vehicle for delivery of heterologously expressed *N. caninum* genes (NcGRA7 and NcSRS2) (Chapters 2 and 3); and the use of proteomic/mass spectrometric technologies, in combination with cellular immunology assays to identify potential vaccine candidates for neosporosis (Chapters 4 and 5).

T. gondii was selected as an appropriate live delivery system for *N. caninum* genes for use in immunological studies. This was for a number of reasons. Firstly, and perhaps most importantly, *T. gondii* is genetically well characterised, and molecular genetic tools for the transfection of *T. gondii* tachyzoites are well established (Howe and Sibley, 1997). *T. gondii* is also relatively easy to culture *in vitro*. In addition, since *T. gondii* and *N. caninum* are very similar organisms (Marsh *et al,* 1995), the hypothesis was that a specific immune response to heterologously expressed *N. caninum* genes could be "piggy-backed" onto the strong protective Th1-mediated immune response generated against the tachyzoites of *T. gondii* (Mordue *et al* 2001). As proof of concept for this, two already characterised *N. caninum* genes (NcGRA7 and NcSRS2 (Lally *et al*, 1997; Howe *et al,* 1998)) were transfected into tachyzoites of *T. gondii*, and the engineered *T. gondii* characterised by genetic, biochemical and immunological methods (Section 6.2).

The second theme of work in this thesis, i.e. the identification of (novel) immunodominant antigens of *N. caninum* combined the power of two-dimensional SDS-PAGE and modern mass spectrometry techniques with the specificity of T-cell

proliferation assays. This theme also contained an element of technology and technique development, since the elution of proteins from two-dimensional SDS-PAGE gels for use in T-cell proliferation assays is not a common technique (Gulle *et al*, 1990) and required optimisation which took a longer time than first anticipated (See Section 6.3). It was the initial plan that antigens identified by causing proliferation in *N. caninum* specific T-cells, followed by identification by mass spectrometry, could be engineered to be expressed in *T. gondii* tachyzoites for immunological studies. Time constraints meant this was not achieved (see Section 6.4 for future work).

The aims and objectives of the work presented in this thesis as described in Chapter 1 form the basis for the discussion outlined below. These aims and objectives were to:

- Produce and characterise transgenic *T. gondii* that express proteins of *N.caninum.*
- Assess the efficacy of transgenic *T. gondii* as a delivery vehicle for *N.caninum* proteins for stimulation of a specific immune response to *N.caninum* in a small animal model.
- Use a combination of proteomic technologies and immunological assays to identify potentially immunodominant proteins of *N. caninum* that may have potential as vaccine candidates.

6.2 Use of *T. gondii* as a Delivery Vehicle for *N. caninum* Antigens

6.2.1 Engineering T. gondii to Express Proteins of N. caninum

Chapters 2 and 3 explain the production and characterisation of engineered *T. gondii* expressing *N. caninum* GRA7 and SRS2 genes, and the use of these transgenic

parasites in preliminary immunological studies. Several difficulties arose in the production of the transgenic *T. gondii*, not least the fact that co-transfection of *T. gondii* with the transfection vector expressing the transgene and one containing a selection cassette (in this case, an active HXGPRT gene) was not as stable as the literature suggests (Donald and Roos, 1998). Several attempts at generating stably transfected *T. gondii* were made, and while it appeared that the transfection was successful by the fact that the parasites were growing in stringent selection conditions, it was later discovered that the selection cassette had been stably transfected and the transfection vector had been transiently expressed (See Section 2.3.4.1). Co-transfection using HXGPRT as a selectable marker has been shown to be a successful method for transfecting *T. gondii* (Donald *et al*, 1996). Nothing in the literature suggested this preferential expression – where the selection cassette but not the transfection vector was expressed – has occurred.

There were also some difficulties in detecting the transgene when it was stably transfected. PCR and Southern Blotting did indicate the presence of the transgene relatively easily (See Sections 2.3.4.2 and 2.3.4.3). However, the use of immunoblotting to detect the *N. caninum* protein in the cell lysate of the transfected *T. gondii* was not successful, since very high background non-specific binding was such that no specific binding could be identified. Immunofluorescence did suggest the presence of the heterologous protein in tachyzoites of transgenic *T. gondii*. Nevertheless, both immunoblotting and immunofluorescence would have been easier had monoclonal antibodies against the recombinant proteins NcGRA7 and NcSRS2 been available. The monoclonal antibodies would add specificity to detection, without resorting to the addition of a *c-myc* epitope that, despite careful engineering

of the sequences to ensure minimal disruption of the structure of the heterologous proteins (Section 2.2.13.1) may have caused some unforeseen changes in the protein structure that resulted in the protein not being transported to its expected environment within the transgenic *T. gondii*. For example, the heterologous NcSRS2 was expected to be transported to the surface as NcSRS2 in *N. caninum* is located on the surface (Hemphill, 1999).

6.2.2 Mouse Inoculation Experiments

Nonetheless, heterologous protein was observed using monoclonal antibodies against the *c-myc* epitope by immunofluorescence, and the transgenic *T. gondii* were then used to inoculate outbred mice to try and characterise the immune responses, with a view to challenging the mice with *N. caninum* to assay the protection given by the "vaccine" recombinant *T. gondii*. However, the study of the immune response in the mice was limited by time constraints, due in part to the unexpected high levels of virulence of the background strain of *T. gondii* used in the transfection studies (Prugniaud strain (PRU), with an HXGPRT knockout - PRUΔHX). The PRU strain of *T. gondii* was known to be a Type II *T. gondii*, which were less pathogenic than the Type I strain (characterised by the highly virulent RH strain) (Lindsay *et al*, 1998). This did not seem to be the case, since even at low inoculation levels ($1x10^2$ tachyzoites) the inoculated BALB/c mice were still succumbing to infection rapidly (Section 3.3.1). It was later known that the HXGPRT knockout PRU strain demonstrated higher levels of pathogenicity in mice, compared to the wild-type strain, and this may account for the difficulties experienced in the initial titration experiments (Dubremetz, pers. commun.).

BALB/c mice had been used as a model for *N. caninum* infection (Lunden *et al*, 2002), and were the initial choice for these inoculation experiments. Because of the highly virulent nature of the PRUΔHX tachyzoites, different mouse strains were required to be tried (Section 3.3.1), and eventually the outbred Porton strain of mouse was selected.

Unfortunately, there was not time to dissect the immune response fully. A preliminary immunofluorescence study suggested that there may some specific humoral response against the heterologous protein. This is covered in more detail in Section 6.4.

6.2.3 Conclusions

This section of work attempted to combine the use transgenic *T. gondii* expressing heterologous proteins with the study of the immune response against the transgenic parasite. It has been shown in previous studies that this rationale is effective in generating specific immune responses to the heterologous protein (Cristina *et al*, 1999; Ramirez *et al*, 2001). In the work by Cristina, *T. gondii* tachyzoites transformed to express the circumsporozoite protein from *P. knowlesi* were used to inoculate rhesus macaques. When the macaques were challenged with untransformed *T. gondii* tachyzoites, there was a specific humoral response against the immunodominant epitope from the circumsporozoite protein (Cristina *et al*, 1999). The cellular response was not studied in this work. In the experiments of Ramirez *et al* (2001) transgenic *T. gondii* expressing the *Leishmania* kinetoplastid membrane protein-11 (KMP-11) were used to immunise BALB/c mice that were subsequently challenged with *L. major*. A protective proliferative response was observed in those

animals that were immunised with KMP-11 expressing *T. gondii* (Ramirez *et* al, 2001), indicating that *T. gondii* is a useful delivery mechanism for heterologous proteins from other intracellular organisms. More work would be required to confirm if the engineered *T. gondii* in the experiments described in this thesis would generate a specific immune response to *N. caninum*.

There is also a fundamental ethical question underpinning the work described here in this section (i.e. Chapters 2 and 3), not least the justification of the use of animals for these types of inoculation experiments, particularly with such a virulent strain of *T. gondii*, although the experiments were carried out as prescribed under the legal guidelines. The production of transgenic pathogens is an area that may have legal and ethical implications. *T. gondii* is a human pathogen; *N. caninum* is not, as far as is known, though it can infect non-human primates (Barr *et al*, 1994). This suggests that it may be possible for *N. caninum* to infect humans, though there is no evidence currently to support this. Recombination events occur frequently in viral pathogens, often with serious consequences (Enserink, 2003). Is it appropriate to engineer human pathogens, to express heterologous proteins, when so little is known about both organisms? There is currently debate about the safety of genetically modified crops, and although there is no scientific evidence to support the hypothesis that genetically modified food is harmful, it is a valid argument to state that because so little is known about the long-term implications of genetic manipulation of organisms, further studies should be done before genetically modified crops are allowed. This argument is magnified when discussing potential pathogens. Genetically modified pathogens of animals or humans should be heavily regulated and modifications should only be permitted using organisms where significant

knowledge about the genetic makeup of the organism is known. A fully sequenced and annotated genome should be the minimum requirements, but there is an argument that, since the genome is fixed and it is the proteome that really is the complex part of an organism, there should be significant proteomic understanding of the cell before genetic manipulation is permitted. As for pathogens, those that are genetically modified should be considered dangerous until proven otherwise and, as such, be considered Category III organisms, although the "background" parental strains are categorised lower, and indeed *T. gondii* of the S48 strain has been used successfully and safely as a vaccine for many years (Buxton and Innes, 1995). In the studies presented in this thesis, both *T. gondii* PRUΔHX and *N. caninum* NC1 are Category II organisms, as were the transgenic *T. gondii* expressing NcGRA7 and NcSRS2. The transgenic *T. gondii* should have been treated as Category III pathogens.

6.3 The Use of Post-Genomic Technologies and Immunoscreening to Identify Novel Immunodominant Antigens of *N. caninum*

The power of proteomics, bioinformatics and mass spectrometry to identify proteins has been well documented (Malmstrom *et al,* 2002; Wilke *et al* 2003). However, these technologies have not been combined with the specificity of cell-based immunoscreening to detect specific proteins, although they have been used in concert to identify immunodominant antigens of *T. gondii* (Reichmann *et al*, 1997). One of the problems has been that, until recently, two-dimensional SDS-PAGE has been a difficult method to manipulate easily for use in immunological, and in particular cell-based, assays. Removal of the proteins from the polyacrylamide matrix is essential. This can either be by blotting and using the blotted membranes as antigen in T-cell

proliferation assays (Young and Lamb, 1986), or by electroeluting the proteins from the gel for further use in T-cell assays (Gulle *et al*, 1990; *Gulle* et al, 1993).

A further problem, particularly for an organism like *N. caninum* that has little sequence data available, is that biological mass spectrometry (for example MALDI-ToF MS) and bioinformatics required there to be substantial sequence data for peptide fingerprints to be searched against, as is the case for *T. gondii* (Cohen *et al*, 2002). New mass spectrometry techniques, like Multi-Dimensional Protein Identification Technology (MudPIT), may prove useful for identification of immunologically interesting sequences from organisms like *N. caninum*. MudPIT combines the separation power of liquid chromatography with MS/MS peptide analysis. Whole cell lysates could be separated using liquid chromatography before being used in proliferation assays, with a duplicate being used in MS/MS experiments. However, MudPIT is not as sensitive as 2DE approaches, since each fraction will contain many proteins and so it would be more difficult and laborious to identify individual proteins.

6.3.1 Elution of Proteins for Use in Proliferation Assays

Electroelution of proteins from SDS-PAGE gels is not widely a used technique. This is because it is technically challenging, the quantity of protein obtained can be very low (50-60% of the initial total protein concentration of the sample in the gel, according to the manufacturers of the Bioteluter (Section 4.4.1.3)). This technique has, however, been used to identify proteins that cause proliferation in *Mycobaterium bovis*-specific T-cells (Gulle *et al*, 1990).

In the work described in Chapter 4 of this thesis, proteins were electroeluted from two-dimensional SDS-PAGE gels and used in T-cell proliferation assays. Five fractions stimulated a response in the T-cells over that seen in the medium alone control (Section 4.3.3.6). However, there were some concerns that the eluted fractions were in some way inhibiting the T-cell cultures despite the fact that the elution buffers had as many components as possible removed. One possibility was that the concentration of the samples might have also concentrated any toxic components. If this experiment were to be repeated, then this issue of inhibition of the T-cell cultures would need to be addressed (See Section 6.3.3).

The major concern about the electroelution proteins for use in proliferation assays was that it was very difficult to determine the protein concentration in each sample. The volumes of elution were such that the entire sample was required to allow triplicate proliferation assays, and as such traditional 96-well plate based protein assays, comparing against known concentrations of bovine serum albumin, were not possible. Had there been more comparability between the 2D gels used, this lack of protein would not have been an issue and there may have been sufficient protein to allow both the concentration of the protein within the sample and repeat proliferation experiments to be carried out.

6.3.2 Mass Spectrometry (MS)

Two MS methods were tried in the work described in Chapter 5 – MALDI-ToF MS and Tandem MS. MALDI-ToF MS requires a large amount of sequence, or at least EST data to allow a search of the database using the peptide mass fingerprints obtained from the mass spectrometer. As *N. caninum* does not have this large amount

of sequence data (at the time of searching approximately 14,000 ESTs – now 25.000), MALDI-ToF MS was not successful. Tandem MS produced some data that may be used as the basis of further studies.

Two protein fractions that produced high levels of T-cell proliferation in the studies described in Chapter 4 (Section 5.3.2.6) were analysed by Tandem MS. One of these produced a hit to an *N. caninum* sequence, superoxide dismutase, though the other did not generate a significant hit.

6.3.3 Conclusions

The work described in Chapters 4 and 5 has combined to identify one potential protein that stimulates some proliferation of T-cells. However, the main conclusion from this section of work is that electroelution of proteins for use in T-cell proliferation assays is technically difficult. Better methods for achieving the same separation of proteins without the added handling steps of two-dimensional SDS-PAGE separation, electroelution and concentration should be developed. Liquid chromatography may be useful in this, since it is also the basis of separation in MudPIT. The combination of MudPIT and T-cell proliferation assays has the potential to generate some very interesting results.

6.4 Further Work

The work presented in this thesis has largely been the development of techniques and the integration of proteomics, genetic and immunological procedures, and as such there are still several important areas that need to be investigated. Clearly, the use of PRUΔHX *T. gondii* as a delivery vehicle was not fully assessed because of the

unexpected virulence of the parasite in mice, caused by the deletion of the HXGPRT gene (Dubremetz, pers. commun.). The use of a less pathogenic strain (ts-4 for example) has been shown to be effective in other studies (Ramirez *et al,* 2001). From an immunological perspective the preliminary immunofluorescence work should be built on, to study the humoral and cellular responses to the transgenic *T. gondii* in more detail, though this will depend on the availability of recombinant proteins and monoclonal antibodies. In particular, further work would be required to show a specific humoral response against the heterologously-expressed *N. caninum* proteins, against the background *T. gondii* proteins. It would also be interesting to look at the protective response generated in mice inoculated with the transgenic *T. gondii* after challenge with an infection of *N. caninum*, and extending that to studying the protection against vertical transmission in mice and, if successful, cattle. These extension experiments would only be appropriate if there was conclusive proof from the *in vitro* proliferation and serological studies that there was a specific response to the heterologously-expressed *N. caninum* proteins.

Further *in vitro* identification of immunodominant antigens using T-cell proliferation assays and individual proteins will be greatly improved if either (a) the electroelution step is fully optimised or (b) an alternative to electroelution from 2D SDS-PAGE gels, for example liquid chromatographic separation of cell lysate, is used. The latter would have the benefit of reducing handling steps and also being directly compatible with MudPIT technology. The sequencing of the *N. caninum* genome would facilitate the identification of proteins of interest.

Proteins that generate a proliferative response in *in vitro* studies should then be used in extended work studying the humoral and cellular responses to the protein in a larger cohort of cattle. Only proteins that generate a specific humoral and cellular response in the majority of the cattle should be considered for further vaccine candidate studies. In assessing the efficacy of particular immunodominant antigens for use in vaccination of cattle, several factors need to be addressed. Firstly, does the vaccine prevent acute pathology, that is, does the vaccine prevent abortion in cattle. This is an important consideration, since this is the most economically important reason to vaccinate against neosporosis. However, in the long-term, a transmission blocking vaccine, which prevents the transmission of the parasite from the mother to the calf, is important in preventing the disease spreading and eventually it would be removed from the herd.

6.5 Final Conclusions

The work presented in this thesis has presented both scientific and technical challenges, some overcome, some still to be addressed. However, in relation to the aims and objectives laid out in Chapter 1, some progress has been achieved:

- *T. gondii* was engineered to heterologously express NcGRA7 and NcSRS2 from *N. caninum* and preliminary immunological studies suggested that there was some specific humoral response to the heterologous proteins, though this would need to be investigated further.
- Several antigens were identified as causing some proliferation in *N. caninum*-specific T-cell cultures above that seen in the negative controls. One of these was identified as superoxide dismutase from *N. caninum*, and may warrant further investigation.

It was unfortunate that the unforseen technical limitations and optimisations were not overcome sooner, since the work described in this thesis aimed to combine molecular, biochemical and immunological approaches to answer fundamental questions about parasite biology and pathology. This holistic approach is an attractive one, particularly when dealing with a complex pathogen, multiple hosts and interesting immunological situations, such as pregnancy. With the further understanding of the basic biology of both *N. caninum* and hosts, along with increasingly sensitive technology, answers to some of these questions may soon be answered.

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