Characterisation of potentially host-protective material from the abomasal parasite, *Teladorsagia circumcincta*

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Research carried out at Moredun Research Institute, Edinburgh
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The work carried out in this thesis is my own original work, except where otherwise stated, and it has not been submitted for any other degree or professional qualification.
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This work is dedicated to my grandmother, Margaret Craig.
ABSTRACT

One of the major causes of parasitic gastroenteritis in sheep and goats worldwide is the roundworm, *Teladorsagia circumcincta* (Nematoda: Trichostrongylidae). Infection results in clinical symptoms including inappetance, diarrhoea and impaired weight gain and is responsible for considerable financial losses to the farming industry. At present, control of *T. circumcincta* involves a combination of grazing management and anthelmintic drugs. However, the emergence of drug-resistant nematode strains has motivated the search for alternative methods of control. The main aims of this study were to identify and characterise proteins from *T. circumcincta* that may induce a protective immune response in the host and to learn more about the biology of the worm.

In order to identify possible protective antigens, a complementary DNA (cDNA) library prepared from adult worms was screened with serum from an animal that was protected against a single challenge infection after vaccination with a *T. circumcincta* protein fraction (S3 TSBP). Forty five immunopositive cDNA clones were identified, of which sixteen had similarity to galectin. Of the remaining clones, the majority shared similarity with two metabolic enzymes, methylmalonate semialdehyde dehydrogenase and 10-formyltetrahydrofolate dehydrogenase, which have not been characterised in nematodes. A single clone with similarity to the antioxidant enzyme, catalase, was also identified. These three enzymes were selected for further investigation on the basis of their roles in nematode metabolism and therefore, their potential as vaccine candidates.

Characterisation of *T. circumcincta* excretory/secretory material (ES) was also performed. L4 and adult worms were cultured *in vitro* and the proteins released were separated by 1D electrophoresis and analysed by Tandem Liquid Chromatography Mass Spectrometry and N-terminal sequencing. This identified proteins showing similarity to, amongst others, metabolic enzymes, structural components, antioxidants, globin-like proteins and cysteine proteases, present in online databases but not previously characterised in *T. circumcincta*.
This study has identified several novel *T. circumcincta* proteins that may have potential as future vaccine or drug targets. It has also provided further information regarding the biology of the worm.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<td>1D</td>
<td>one dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>ASP</td>
<td><em>Ancylostoma</em> secreted protein</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celcius</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>CRD</td>
<td>carbohydrate recognition domain</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
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<td>DAB</td>
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</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
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<td>digoxygenin</td>
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<td>DNA</td>
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</tr>
<tr>
<td>dNTPs</td>
<td>deoxyribonucleotide triphosphates</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded ribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E64</td>
<td>L -trans-epoxysuccinyl- leucylamido(4-guanidino)butane</td>
</tr>
<tr>
<td>EBI</td>
<td>European Bioinformatics Institute</td>
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EDTA ethylenediamine tetra-acetic acid
ELISA enzyme-linked immunosorbent assay
ES excretory/secretory
EST expressed sequence tag
FEC faecal egg count
FTDH 10-formyltetrahydrofolate dehydrogenase
g grams
GI gastrointestinal
h hour
HCl hydrochloric acid
HRP horse radish peroxidase
HSP heat shock protein
IEF isoelectric focussing
Ig immunoglobulin
IL interleukin
IFN-γ interferon-γ
IPTG isopropyl-β-thiogalactopyranoside
kb kilobases
kDa kilo Dalton
l litre
LMI larval migratory inhibitory
LB Luria-bertani
LC Liquid chromatography
M molar
MgSO₄ magnesium sulphate
min  minute
ml  millilitre
MMSD  methylmalonate semialdehyde dehydrogenase
mRNA  messenger ribonucleic acid
MS  mass spectrometry
N₂  liquid nitrogen
NaCl  sodium chloride
NaOH  sodium hydroxide
NCBI  National Centre for Biotechnology Information
OD  optical density
ORF  open reading frame
PBS  phosphate buffered saline
PCR  polymerase chain reaction
pfu  plaque-forming units
pH  \(-\log_{10}\) (hydrogen ion concentration)
PMSF  phenylmethylsulfonyl fluoride
PO  immunopositive clone
PPR  periparturient rise
Prx  peroxiredoxin
RNA  ribonucleic acid
RNAi  ribonucleic acid interference
ROS  reactive oxygen species
rpm  revolutions per minute
RPMI-1640  Roswell Park Memorial Institute Media 1640
RT-PCR  reverse transcription polymerase chain reaction
<table>
<thead>
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<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>S1</td>
<td>soluble protein fraction (PBS fraction)</td>
</tr>
<tr>
<td>S2</td>
<td>membrane-associated protein fraction (Tween 20 fraction)</td>
</tr>
<tr>
<td>S3</td>
<td>membrane-bound protein fraction (Triton X-100 fraction)</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SSC</td>
<td>standard sodium citrate</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded ribonucleic acid</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate EDTA buffer</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>tris buffered saline with 0.5% (v/v) Tween 20</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tpx</td>
<td>thioredoxin peroxidase</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)amino methane</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>octylphenoxylpolyethoxethanol</td>
</tr>
<tr>
<td>TSBP</td>
<td>thiol sepharose-binding proteins</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polyoxyethylene (20) sorbitan monolaurate</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indoyl-β-D galactopyranoside</td>
</tr>
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CHAPTER 1: GENERAL INTRODUCTION

1.1 MORPHOLOGY AND CLASSIFICATION

_Teladorsagia circumcincta_ is a nematode worm of the family Trichostrongylidae. The trichostrongyles are small roundworms less than 1 cm in length and are recognisable by their reduced buccal capsule and, in the adult male, a prominent copulatory bursa with two spicules (Urquhart, Armour, Duncan et al., 1987). _T. circumcincta_ is a member of the Ostertagiinae, a group of trichostrongyles that causes disease known as ostertagiosis in ruminants and includes the closely related bovine parasite _Ostertagia ostertagi_.

There has been some disagreement as to how many discrete Ostertagiinae genera and species exist. Three _Teladorsagia_ 'forms' have been identified, known as _T. c. circumcincta_, _T. c. trifurcata_ and _T. c. davitani_. Whether these are three separate species or three morphs of one species is still unresolved, but the latter is the more accepted theory due to several bodies of work. _T. c. circumcincta_ and _T. c. trifurcata_ have been shown to interbreed successfully, resulting in infective and fertile progeny with the characteristics of one or the other parent rather than a range of intermediate forms (Suarez & Cabaret, 1992). Morphological studies have shown that while the spicules of male _T. c. circumcincta_ are slender, in _T. c. trifurcata_ and _T. c. davitani_ they are stouter and accompanied by an enlarged sclerotized dorsal portion of the genital cone not found in _T. c. circumcincta_ (Lichtenfels, Puillitt, & Lancaster, 1998). However, certain characteristics such as a single ventral cuticular ridge and a 2-2-1 bursal ray pattern are common to _T. c. circumcincta_, _T. c. trifurcata_ and _T. c. davitani_ but not to other members of the Ostertagiinae (Lichtenfels, Puillitt, & Lancaster, 1998). Allozyme electrophoresis has been used to demonstrate only very small genetic distances between the three 'forms' (Andrews & Beveridge, 1990; Gasnier, Cabaret, & Suarez, 1993) and other research has shown that they exhibit similar patterns of resistance/susceptibility to anthelmintics (Leignel & Cabaret, 2001). On the basis of this evidence, the most current theory is that _T. circumcincta_ is a single polymorphic species that can be placed in the genus _Teladorsagia_ within the Ostertagiinae.

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1.2 LIFE CYCLE

*T. circumcincta* has a direct life cycle, as shown in Figure 1.2. Eggs are passed in the faeces of the host (sheep or goat). Hatching of the first larval (L1) stage occurs due to enzymic degradation of the inner shell membrane and water uptake by the larva from its surroundings, causing it to swell and burst the outer layers of the egg (Urquhart, Armour, Duncan et al., 1987). The L1 feed on microbes present in the faeces and moult to the second (L2) then the infective third stage larva (L3) within a couple of weeks. Under unfavourable conditions such as drought or a harsh winter climate, the L3, within the retained cuticle of the L2, can enter a state of arrested development for some months (Urquhart, Armour, Duncan et al., 1987). When humid conditions prevail, the L3 migrate onto vegetation where they are ingested by the ovine host. They exsheath in the rumen and invade the mucosa of the abomasum where further development to the fourth stage larvae (L4) and immature adult (sometimes called the fifth stage larvae or L5) occurs in the lumen of an abomasal gland. However, if the host environment does not present optimal conditions, development of the parasitic stages may also become arrested for periods of up to 6 months (Gordon, 1953; Sommerville, 1953). This inhibited development has been linked to infection rate (Dunsmore, 1960), with a higher proportion of worms becoming arrested in larger infections, but is usually attributed to seasonal factors. Larvae ingested in late autumn/winter are more likely to become arrested and worm populations in ewes have been shown to consist of mainly immature L4 in winter but adults in the spring (Connan, 1968; Reid & Armour, 1972). The periparturient rise, discussed in the next section, is often attributed to the maturation and fecundity of these arrested larvae. When development is continuous, the L5 usually emerge around 18 days post-infection onto the mucosal surface where they reach sexual maturity and begin egg production (Urquhart, Armour, Duncan et al., 1987).
Adults feeding in abomasum

\[ L_5 \]

Pasture

\[ L_4 \]

Infective L3

\[ L_1 \]

Eggs

\[ L_2 \]

Host

Figure 1.2. Direct life-cycle of *T. circumcincta*.
1.3 EPIDEMIOLOGY

In temperate areas, there is a peak in faecal *T. circumcincta* egg output by ewes between April and June. This is known as the periparturient rise (PPR) because it occurs between 6-7 weeks after the start of lambing, just prior to weaning of lambs, and lasts on average 6-8 weeks (Brunsdon, 1964; Crofton, 1958). It results in high numbers of infective larvae on the pasture during mid-summer when susceptible young lambs begin grazing and is therefore an important factor in determining the rate of infection of such lambs (Gibson & Everett, 1973; Crofton, 1958). This phenomenon is also known as the ‘spring rise’ but does not appear to be caused directly by environmental factors (Crofton, 1958). Much work has been done to examine the mechanism behind the PPR. Earlier studies suggested that the hormones involved in lactation might play a role. The treatment of non-pregnant ewes with diethyl stilboestrol resulted in increased faecal egg counts (FEC), which the authors suggested might be due to oestrogen-induced elevated levels of prolactin (Salisbury & Arundel, 1970). Later work was inconsistent with this theory however, reporting that an induced rise in prolactin levels had little or no effect on FEC and that inhibition of prolactin production in pregnant ewes did not effect the PPR (Coop, Mellor, Jackson et al., 1990; Jeffcoate, Fishwick, Bairden et al., 1990). It is now generally thought that there is a periparturient relaxation of immunity (PPRI) in ewes but the causes of this relaxation are still not clear. Jeffcoate et al (1990) showed no reduction of humoral antibody production in response to horse red blood cell inoculation in *T. circumcincta*-infected ewes during the PPR as compared to barren ewes. Jeffcoate et al (1992), however, demonstrated that pregnant ewes challenged with *T. circumcincta* showed sizeable increases in egg output only during the PPR period and that the commencement of lactation was closely related to elevated levels of circulating anti-*T. circumcincta* IgA. In sheep, IgA in the milk is protective against *T. circumcincta* infection in the lamb and is primarily synthesised in the gut and transported to the mammary gland via the serum. Jeffcoate et al (1992) suggested that, in the ewe, this may temporarily reduce local gastrointestinal antibody levels allowing the establishment of incoming or re-animation of arrested larvae that would otherwise be inhibited. The PPRI has also been linked to
nutritional factors with increased intake of metabolizable protein by pregnant ewes resulting in lower FECs and plasma pepsinogen levels and higher proportions of globule leukocytes (GLs) in the abomasal tissue (Houdijk, Kyriazakis, Jackson et al., 2000; Houdijk, Kyriazakis, Coop et al., 2001). It is likely that a combination of factors related to pregnancy and lactation are involved in the PPR.

Once ingested, most of these larvae develop and mature within a few weeks and cause what is known as Type I disease. If larvae are ingested late in the season i.e. from early October onwards, they are likely to become arrested and their subsequent re-animation and maturation in late winter/early spring causes Type II disease (see below).

1.4 PARASITIC GASTROENTERITIS

The disease caused by *T. circumcincta* can be divided into what are known as Type I and Type II ostertagiasis. In Type I, the symptoms are less severe due to a gradual intake of parasites when grazing. The acute disease associated with Type II is caused by the simultaneous development of a large population of arrested larvae ingested late in the previous year, leading to a much more debilitating infection.

When present in sufficient numbers in the abomasum, *T. circumcincta* can induce significant morphological and biochemical changes. Lesions are created in the form of expanded abomasal glands occupied by L4, vacated glands and areas of localised necrosis or fibrosis of the abomasal mucosa (Urquhart, Armour, Duncan et al., 1987; Coop, Sykes, & Angus, 1977). Serum levels of the hormone gastrin, which regulates the organisation and function of the gastric epithelium, become elevated around 24hr after adults emerge (Anderson, Hansky, & Titchen, 1985). This rise is associated with mucosal cell hyperplasia and a rapid decrease in numbers of HCl-secreting parietal cells in the abomasum (McLeay, Anderson, Bingley et al., 1973; Coop, Sykes, & Angus, 1977; Scott, Hodgkinson, Khalaf et al., 1998). The pH of the abomasal fluid can rise from an uninfected range of 2 to 3.5 to a maximum of up to 7.5 (McLeay, Anderson, Bingley et al., 1973; Scott, Khalaf, Simcock et al., 2000). The activation of pepsinogen to pepsin requires a pH of less than 4 and the
activity of pepsin is optimal at around pH 2. This might explain why plasma pepsinogen levels are raised during infection even though pepsinogen secretion is shown to decrease (Coop, Sykes, & Angus, 1977; Sykes & Coop, 1977). Due to damage caused by the worms, the abomasal epithelium becomes more permeable to molecules such as pepsinogen and plasma proteins during infection (Holmes & MacLean, 1971). Sub-optimal pH in the abomasum may provide even higher levels of inactivated pepsinogen that then leak into the plasma. It has been shown that elevated pH is due to a chemical rather than physical stimulus from the worms as the same effect is seen with adults prevented from contact with the mucosa (Simpson, Simpson, Simcock et al., 1999). It is also possible that a worm secretory product stimulates pepsinogen release in vitro during the early stages of infection (McKellar, Mostofa, & Eckersall, 1990).

Experimental infection with larval stages produces similar results to those observed when adult worms alone are transferred directly into the abomasum but the effects are delayed until 5-6 days post-infection (Lawton, Reynolds, Hodgkinson et al., 1996; Scott, Hodgkinson, Khalaf et al., 1998). These observations suggest that it is the presence of the adults, outside of the abomasal glands, which is responsible for the majority of these changes (Scott, Dick, Irvine et al., 1999; Scott, Khalaf, Simcock et al., 2000). This theory is strengthened by histological studies, showing an influx of inflammatory cells into the abomasum at this time. Resulting symptoms include appetite depression and reduction in food utilisation. The latter can have serious consequences even when the infected sheep show no other outward clinical signs of parasitism (Parkins, Holmes, & Bremner, 1973; Sykes & Coop, 1977). Inappetance may be a result of, amongst other factors, increased gastrin secretion (McLeay, Anderson, Bingley et al., 1973). Sykes and Coop (1977) reported a significant reduction in nitrogen digestibility in young parasite-naive sheep given a trickle infection of 4,000 L3 daily. This was attributed mainly to a reduction in protein digestion. This study also showed a reduction in appetite, efficiency of metabolizable energy utilisation and deposition of minerals in the skeleton (Sykes & Coop, 1977). Gastrointestinal motility and rate of digesta flow are also affected and in heavier infections, 50,000 worms for example, increased flow of material through the gut results in diarrhoea (Holmes, 1987). These symptoms are dose-related but even light
infections, for instance 2,000 worms, can result in impaired weight gain and reduction in fleece and carcass quality in lambs (Coop, Sykes, & Angus, 1977; Urquhart, Armour, Duncan et al., 1987).

1.5 CONTROL

1.5.1 Anthelmintics

Anthelmintics are anti-parasitic drugs that react with specific target sites in the parasite and disrupt a critical aspect of their biology (reviewed by Kohler, 2001). The benzimidazoles have been the most commonly used class of anthelmintics. They affect the ultrastructure of nematode intestinal cells by inhibiting microtubule development. It is thought that the drug works by binding to the beta-subunit of the protein tubulin, preventing further polymerisation of the molecule and hence inhibiting microtubule formation (Kohler, 2001). Microtubules are involved in a variety of vital cellular processes including the secretory vesicle transport system, the disruption of which may lead to the untimely release of digestive enzymes into the cell cytoplasm (Shompole, Yao, Cheng et al., 2002). The worm intestine appears to be the major site of action. Treatment of *Haemonchus contortus* with fenbendazole results in gross pathology and DNA fragmentation in the anterior intestine. This is associated with inhibition of vesicle transport and the subsequent distribution of their contents in the cytoplasm and throughout the worm body (Jasmer, Yao, Rehman et al., 2000; Shompole, Yao, Cheng et al., 2002).

The macrocyclic lactones are also effective nematocides in both animal and human infections and include the drugs known as avermectins (Hotson, 1982). These affect the somatic muscles of the worm causing a flaccid paralysis. The mode of action is still unclear but they do bind to an invertebrate-specific family of glutamate-gated chloride (GluCl) channels, containing α-type subunits. This may create irreversible chloride ion currents leading to polarisation of the cell membrane and subsequent muscle paralysis (Hotson, 1982; Kohler, 2001). They also inhibit pharyngeal pumping and it is thought that this may cause worm death due to starvation (Kohler, 2001).
Another widely used group, of which levamisole is a member, are the nicotinic agonists. Like the above group, these also affect the parasite's nervous system. Unnatural excitation is caused when the drug binds to nicotinic acetylcholine receptors on the surface of the somatic muscles (Martin, 1997; Moreno-Guzmán, Coles, Jiménez-González et al., 1998; Kohler, 2001). This results in rapid spastic paralysis of the muscles allowing expulsion of the parasites from the host (Kohler, 2001).

However, nematode resistance to anthelmintics, first reported to the benzimidazoles over 30 years ago, is a growing problem worldwide (reviewed by Waller, 1997). Isolates of *T. circumcincta* resistant to one or more classes of anthelmintic have been identified in Scotland (Jackson, Jackson, & Coop, 1992; Bartley, Jackson, Johnston et al., 2003), England and Wales (Hong, Hunt, & Coles, 1996), throughout Europe (Bjorn, Monrad, & Nansen, 1991; Bauer, 2001; Chartier, Soubirac, Pors et al., 2001; Papadopoulos, Himonas, & Coles, 2001), and in New Zealand (Leathwick, Moen, Miller et al., 2000a; Leathwick, Moen, Miller et al., 2000b), Australia (Rifikin, Callinan, Freemantle et al., 1984; Overend, Phillips, Poulton et al., 1994) and South America (Torres-Acosta, Dzul-Canche, Aguilar-Caballero et al., 2003). Until recently, levamisole was still regarded as widely efficacious. However, a paper published in 2002 estimated that up to 3.5% of pig herds in an area of Germany were infected with levamisole-resistant strongylid parasites (Niven, Anderson, & Vizard, 2002). Since then, there have been reports from Tanzania of persistent levamisole resistance in *H. contortus* and, from Malaysia, of rising levels of resistance to all three classes of anthelmintic in ovine gastrointestinal nematodes (Keyyu, Mahingika, Magwisha et al., 2002; Chandrawathani, Waller, Adnan et al., 2003). In Australia, an estimated 80% of sheep farms in areas with high annual rainfall have resistance to both benzimidazoles and levamisole (Waller, 1997). Recently, two separate cases of *T. circumcincta* strains resistant to all three major classes of anthelmintic have been reported from Scottish farms (Bartley, Jackson, Johnston et al., 2003). It therefore appears that incidences of multiple anthelmintic resistance in many species of parasitic nematodes of sheep are likely to become commonplace. But how and why does this resistance develop and can its increase in prevalence be halted or at least slowed?
A conserved point mutation responsible for benzimidazole-resistance has been identified at amino acid (aa) position 200 in the isotype 1 beta-tubulin gene. Phenylalanine has been replaced by tyrosine in resistant *T. circumcincta* and *H. contortus*, preventing high-affinity binding at the drug's site of action (Kwa, Veenstra, & Roos, 1994; Elard, Comes, & Humbert, 1996; Elard & Humbert, 1999). Resistance to the macrocyclic lactones appears more complex, involving more than one gene. As mentioned, the site of action has been identified as GluCl channels. Simultaneous mutations of three separate genes encoding GluCl sub-units are necessary to confer high-level resistance to ivermectin in *Caenorhabditis elegans* (Dent, Smith, Vassilatis et al., 2000). Several studies with ivermectin-resistant *H. contortus* have been conducted. One showed that feeding in resistant worms is less sensitive to treatment with ivermectin, indicating that inhibition of pharyngeal pumping is somehow blocked (Sangster & Gill, 1999). In another, *T. circumcincta* resistance was also linked to prevention of starvation (Gill & Lacey, 1998). However, in the same study, it appeared that ivermectin-induced expulsion of *H. contortus* and *Trichostrongylus colubriformis* was related to motility due to paralysis of the somatic musculature rather than the pharynx and that a single gene was responsible for conferring resistance (Gill & Lacey, 1998). It is apparent that there may be multiple species-specific factors involved in ivermectin action and its complexity may explain why resistance to this drug has been slower to emerge (Kohler, 2001). Little is known of the mechanism behind the newly emerging resistance to levamisole but early work indicates that it is similar to that of the benzimidazoles i.e. mutations causing alteration of the drug binding sites (Kohler, 2001).

Anthelmintic resistance is defined as 'the genetically transmitted loss of sensitivity in worm populations that were previously sensitive to the same drug' (Kohler, 2001). In other words, worms containing a genetic mutation, which blocks the action of a particular drug, such as those described above, persist within the host as susceptible genotypes are cleared by the treatment. This advantage is passed on to future generations and if the same form of treatment is continued, the entire
population can become resistant. Under-dosing of flocks magnifies this problem. For example, treatment with the dosage of fenbendazole recommended by the manufacturer can completely eliminate *T. circumcincta* worms heterozygous for the benzimidazole-resistant gene from the host. However, if this dose is quartered, only worms homozygous for the susceptible gene are cleared (Silvestre, Cabaret, & Humbert, 2001). This means that the next generation of worms from that host could be made up almost entirely of those either heterozygous or homozygous for the resistant gene. Therefore, if a farmer does not give a high enough dose of anthelmintic to his sheep he is actively selecting for resistance. Seeding such a pasture with a susceptible strain of the parasite in order to replace the resistant strain has been shown to be effective in some cases (van Wyk & van Schalkwyk, 1990), but is not really practical for a working farm. Even more worrying is evidence that the establishment of a resistant population of worms can be irreversible. Studies with benzimidazole-resistant *T. circumcincta* have shown that there appears to be no ‘trade-off’ for resistance in this case i.e. here are no differences in fitness between resistant and non-resistant worms when anthelmintic treatment is not a factor (Elard, Sauve, & Humbert, 1998). This means that resistant worms will always have the selective advantage and can therefore not be replaced by non-resistant strains.

In areas where resistance to multiple anthelmintics is yet to emerge, it may still be possible to control *T. circumcincta* and preserve anthelmintic efficacy using a cocktail of drugs from different groups (Waller, 1997). However, such areas may soon be few and far between and the need for non-chemotherapeutic methods of control is growing more urgent.

### 1.5.2 Grazing Management

The ideal method of helminth control would be one that would ‘prevent or limit contact between the parasite and host’ (Brunsdon, 1980), so that infection does not have an opportunity to occur. Grazing management can help towards this goal. For example, areas of land can be left ungrazed or ‘rested’ for specified periods or grazed with sheep and cattle alternately, i.e. host species susceptible to different species of nematode. The aims of such practices are to attempt to create ‘safe pastures’ i.e.
those with little or no contamination by parasites (Brunsdon, 1980). Susceptible young animals can then be moved to such pastures at times when numbers of infective larvae are likely to be high in other areas. However, this method has several problems. Firstly, in the case of trichostrongylid parasites, a pasture may need to be ‘rested’ for over three months in order to be classified ‘safe’ (Brunsdon, 1980). Many farms may not have enough acreage to leave areas of pasture unused for such periods and alternate grazing is not an option for those exclusively farming sheep or cattle. Secondly, drenching of livestock that have previously grazed elsewhere with anthelmintic before moving them to a safe pasture is necessary to prevent re-infection of the pasture. This method could select for anthelmintic-resistant strains so that instead of creating a safe pasture, it could create pasture infected exclusively with resistant worms, as described above (Leathwick, Vlassoff, & Barlow, 1995). Thirdly, it has been suggested that even an anthelmintic-free system of grazing management may select for other traits beneficial to the parasite such as reduced host specificity or increased longevity of infective larvae (Barger, 1997).

1.5.3 Genetic Selection

There exists considerable variation, both between and within host breeds, in disease resistance; the ability to produce a suitable and effective immune response, and resilience; the ability to ‘maintain a relatively un-depressed production level under parasite challenge’ (Woolaston & Baker, 1996). Exotic breeds of sheep are known to be more resistant to gastrointestinal nematodes than European breeds but may have other characteristics such as wool or meat type that are not suitable for particular climates or markets (Windon, 1996). There can also be significant variation between flocks or bloodlines of the same breed (Windon, 1996). There is considerable evidence that genetic factors are involved in this variation between individuals (Smith, 1988), and that these factors may exhibit a significant degree of heritability (Windon, 1996). For example, certain genes associated with the structure of the host major histocompatibility complex (MHC) in naturally T. circumcincta-infected Soay sheep have been linked to lower faecal egg counts (FECs) and higher rates of survival in young animals (Paterson, Wilson, & Pemberton, 1998). It is
therefore possible to selectively breed host species with increased resistance to gastrointestinal nematodes (reviewed by Woolaston & Baker, 1996; Bisset & Morris, 1996; Windon, 1990). The relative resistance of an individual can be measured by FECs, or blood haematocrit (relative proportions of cells and plasma in blood, obtained by centrifuging a blood sample) in the case of H. contortus, as this parasite is a blood feeder (Woolaston & Baker, 1996). Those with high FEC and low haematocrit values can then be eliminated from the gene pool. Such breeding programs have been carried out with promising results for H. contortus and T. circumcincta (Bouix, Krupinski, Rzepecki et al., 1998; Kahn, Knox, Gray et al., 2003).

1.5.4 Nematophagous Fungi

Another promising method of biological control is through the introduction of nematophagous fungi to the diet of the livestock. Predacious fungi such as Duddingtonia spp. have specialised structures on the mycelium that trap nematodes and destroy them (Waller & Larsen, 1993). Several studies have also shown that Duddingtonia flagrans can survive passage through the ruminant digestive tract to prey on developing larvae in the faeces (Chandrawathani, Jamnah, Waller et al., 2002; Gronvold, Wolstrup, Nansen et al., 1993; Paraud & Chartier, 2003). When D. flagrans spores are fed to sheep, goats or cows infected with gastrointestinal nematodes, they can reduce the numbers of infective larvae present in the faeces by up to 90%. This method has proved successful for animals infected with T. circumcincta (Waghorn, Leathwick, Chen et al., 2003; Paraud & Chartier, 2003), T. colubriformis (Waghorn, Leathwick, Chen et al., 2003), H. contortus (Chandrawathani, Jamnah, Waller et al., 2002; Waghorn, Leathwick, Chen et al., 2003) and Ostertagia ostertagi (Gronvold, Wolstrup, Larsen et al., 1993).

Endoparasitic fungi, such as Harposporium anguillulae, also live in ruminant faeces and release small infective units called conidia that are ingested while the developing worms are feeding. Once inside, they germinate and grow causing death by lysing the worm’s internal organs and releasing more conidia. This species has
been shown to kill almost all *H. contortus* L3 present when added to faecal cultures (Charles, Roque, & Santos, 1996). However, literature on their suitability as an addition to host feed is currently unavailable. Nematophagous fungi could be used to reduce pasture contamination in the long term. However, they will not affect infective larvae that have already moved out of the faeces onto the vegetation or kill worms already infecting the animals they are fed to and so may need to be used in conjunction with anthelmintics initially.

### 1.5.5 Dietary Supplementation

The nutritional status of the host affects its ability to produce an effective response to infection with gastrointestinal nematodes (reviewed by Coop & Kyriazakis, 2001). Calves fed a low protein diet have significantly higher egg counts after trickle infection with *O. ostertagi* than those given high protein feed (Mansour, Dixon, Rowan et al., 1992). Increasing the amount of metabolizable protein in the diet content can also result in decreased female worm size and fecundity and increased numbers of circulating eosinophils in *T. circumcincta*-infected lambs (Valderrábano, Delfa, & Uriarte, 2002). It can also greatly reduce the effects of the periparturient relaxation in immunity in pregnant and lactating ewes (Houdijk, Kyriazakis, Jackson et al., 2000; Houdijk, Kyriazakis, Coop et al., 2001; Houdijk, Kyriazakis, Jackson et al., 2003). The expense of giving flocks a high protein diet, however, may outweigh the benefits of this approach.

Another approach may be the supplementation of ruminant diet with vegetation from plants rich in tannins. These are phenolic secondary metabolites with anti-bacterial and anti-parasitic properties produced by some plants. Condensed tannins (CTs) are stable within the ruminant digestive tract and appear to cause little or no harm to the host (Coop & Kyriazakis, 2001). Several studies have investigated the use of tannin-rich feed as an anthelmintic. Lambs fed on CT-rich lotus and infected with *T. circumcincta* or fed on *Quebracho* extract and infected with *T. colubriformis* showed lower FECs and worm burdens than controls fed a CT-free diet (Niezen, Waghorn, & Charleston, 1998; Athanasiadou, Kyriazakis, Jackson et al., 2000). However lambs infected with both *T. circumcincta* and *T. colubriformis*
and fed on CT-rich sulla only showed a reduction in *T. circumcincta* FECs and worm burdens (Niezen, Charleston, Robertson et al., 2002). CTs may enhance nutrient uptake in ruminants by forming complexes with dietary protein, which dissociate lower in the digestive tract, allowing greater uptake of amino acids (Coop & Kyriazakis, 2001). It has been suggested that it is by this method rather a direct anthelmintic effect that CTs reduce infection levels but emerging evidence that even short-term exposure to tannins can increase host resistance to infection may prove otherwise (Coop & Kyriazakis, 2001).

Increasing the intake of a trace metallic element, molybdenum, by dietary supplementation reduced egg and worm counts by up to 90% in lambs infected with *H. contortus* or *T. colubriformis* (Suttle, Knox, Angus et al., 1992; McClure, McClure, & Emery, 1999). Molybdenum may harm the worms by enhancing the host inflammatory response either directly or indirectly due to a reduction in the effectiveness of local host-derived, Cu-dependent, anti-inflammatory enzymes (Suttle, Knox, Angus et al., 1992). However, high levels of molybdenum intake can also be toxic to the host due to impairment of copper metabolism (McClure, McClure, & Emery, 1999), so calculation of optimal dosages would have to take existing dietary intake into account and be carefully administered.

Finally, supplementation of the host diet with copper oxide wire particles (COWP) can reduce levels of gastrointestinal (GI) nematode infection. Orally introduced COWP remain in the abomasum and are corroded by the acidic environment, releasing soluble copper which can have an anthelmintic effect (Knox, 2002). By giving 5 g of COWP to sheep five days before infection, *H. contortus* burdens were reduced by 96% compared to controls (Bang, Hamilton, & Sykes, 1990). However, the reduction was less for *T. circumcincta* (56%), possibly due to changes in abomasal pH during infection, and there was no effect on *T. colubriformis*. Also, in a subsequent trial, although egg and worm counts were reduced, such a high efficacy against *H. contortus* could not be reproduced (Knox, 2002), and a similar study in cattle failed to give any anthelmintic effects (Dimander, Höglund, Uggla et al., 2003).
A combination of these alternatives to anthelmintics could be useful in controlling levels of infective larvae on pasture or enhancing the immune response of livestock to certain helminths. However, some may be expensive or troublesome to implement. It would, therefore, be even more useful if animals could be vaccinated against helminth infection.

1.6 FACTORS AFFECTING HOST IMMUNITY

It is difficult to design an effective vaccine without first understanding something about the mechanisms behind the development of immunity to the target organism. Resistance to *T. circumcincta* results from the host immune system targeting one or more of three stages of the infection: the infective L3, the emerging L4 or the established adults (Miller, 1996). Infective larvae can be rejected before establishing in the gut or be prevented from reaching adulthood when arrested development or expulsion of the L4 is induced. Reduction of adult worm size, reduction of female fecundity and expulsion of the adult nematode population can also occur. Stear, Strain & Bishop (1999) stated that worm length is a heritable characteristic in sheep i.e. genetic factors are responsible for determining the degree of inhibition of worm growth when an animal capable of a protective immune response is infected with *T. circumcincta*. As worm fecundity is closely related to worm length in *T. circumcincta* (Stear, Strain, & Bishop, 1999; Stear, Bairden, McKellar et al., 1999), this growth inhibition should reduce egg output by females.

The magnitude of the immune response is dependent on several variables. As well as the genotype and nutritional status of the host, pregnancy and lactation in females are implicated. The size and frequency of the challenge dose and the age of the host are also important parameters. Expulsion of gastrointestinal nematode infections by parasite-naïve animals, common in murine models, is rare in ruminants (Balic, Bowles, & Meeusen, 2000a).
1.6.1 Level of infection and protective immune responses

When single primary doses of 50,000 infective larvae were given to naïve 10-month old sheep, a local immune response, manifested as a marked increase in lymphoblast concentration in the gastric lymph, could be detected. However, only a small reduction in worm numbers occurred and the responses varied greatly between individuals (Smith, Jackson, Jackson et al., 1983a). In another study, small but frequent doses with 2,000 larvae daily for 8-10 weeks were given to sheep 10 months of age, an infection regime similar to what they might experience when grazing on a naturally infected pasture. The sheep were then treated with anthelmintic to clear the infection and given a 50,000 L3 challenge infection (Smith, Jackson, Jackson et al., 1983b). Again, this resulted in increased local lymphoblast production but also in elevated IgA concentrations in the gastric lymph that peaked 7 days after challenge. Worm numbers were much lower and a high proportion of worms were arrested at L4. Subsequently, IgA levels have been correlated with reduction of worm size and fecundity in *T. circumcincta* infections (Stear, Strain, & Bishop, 1999).

1.6.2 Age-related immunity

The age of the host when it first encounters the infective parasite also influences its susceptibility to infection, with younger lambs tending to be most vulnerable. In a comparison of immunity in 4½ and 10-month-old lambs, both groups were given small daily immunizing infections of *T. circumcincta* larvae for 8 to 9 weeks, treated with anthelmintic then given a large challenge infection of larvae (Smith, Jackson, Jackson et al., 1985). The local immune responses were measured through cannulation of the gastric lymph duct and daily analysis of the collected fluid. The 10-month-old lambs showed significant reductions of worm numbers, stunting of adult worm growth and developmental arrest in almost all developing larvae, but this was not the case for the younger lambs. Both age groups showed a similar response to primary infection but the secondary response differed. In younger lambs, the latter had similar timing but a very different magnitude from that of the 10-month-olds, with total IgA levels and rates of lymphoblast production being significantly reduced. The authors concluded that the susceptibility of young lambs
to heavy infections is due to their inability to generate an adequate secondary mucosal response (Smith, Jackson, Jackson et al., 1985).

1.7 MECHANISMS OF HOST RESISTANCE

Resistance to gastrointestinal nematodes varies greatly with host and parasite species and, as mentioned above, between host individuals. Rodent models are used extensively to investigate the immune mechanisms behind resistance to nematodes as they are much cheaper and easier to use than ruminants. Specific rodent gene knock-out strains can be used to look at effectors of the immune system. However, there is often disagreement as to whether rodent data can be extrapolated to the ruminant immune response. There are some characteristic immune responses that appear consistently in such infections and these will be reviewed in this section.

1.7.1 Mast Cell Hyperplasia and Globule Leukocyte Generation

Increased local concentrations of mucosal mast cells (MMC) and globule leukocytes (GL - intraepithelial mast cells) are a common feature of gastrointestinal nematode infections, particularly when the host has been subjected to previous infections with that parasite (Balic, Bowles, & Meeusen, 2000a). Degranulation of mast cells releases a variety of mediators, such as histamine, proteases, leukotrienes and cytokines, that may aid worm expulsion (McKay & Bienenstock, 1994). Histamine-mediated excitation of the enteric nerves may lead to expulsion by implementing a 'washer/sweeper' mechanism involving increased water secretion and alteration of gut movements to flush out the antigen (McKay & Bienenstock, 1994).

A sheep mast cell-derived proteinase (SMCP) has been isolated from MMCs in the abomasal tissue of sheep (Huntley, Gibson, Brown et al., 1987). The exact role of mast cell proteinases in the sheep immune response is unclear. They are thought to be involved in a variety of processes including the proliferation of muscle cells and fibroblasts, upregulation of interleukin(IL)-8 synthesis, and recruitment of eosinophils to the site of infection (Pemberton, McAleese, Huntley et al., 2000).
gastric mucosa of sheep immune to *H. contortus* contained significantly higher concentrations of SMCP than naïve controls and larval challenge stimulated release of SMCP into the sera and gastric lymph of immune but not naïve sheep (Huntley, Gibson, Brown et al., 1987). A temporary increase in the pepsinogen content of the gastric lymph was found to be concomitant with the release of SMCP after challenge of previously infected sheep with *T. circumcincta* larvae (Huntley, Gibson, Brown et al., 1987). This information, plus the demonstration of a negative correlation between worm counts and SMCP levels (Stevenson, Huntley, Smith et al., 1994), indicates a role for this enzyme and other mast cell mediators in resistance to nematode infection in sheep.

A negative correlation between adult worm burden and abomasal GL number, but not MMC number, has been reported in sheep infected with *T. circumcincta* or *T. colubriformis* (Stear, Bairden, Duncan et al., 1995; Douch, Harrison, Elliott et al., 1986). Moreover, mast cell-deficient mice could not expel the nematode *Strongyloides ratti*. When these mice were injected with either bone marrow cells from normal mice or with bone marrow-derived cultured mast cells, only mice receiving the bone marrow cells were able to expel *S. ratti* infection (Abe & Nawa, 1987a; Abe & Nawa, 1987b). These mice showed a high proportion (>90%) of mast cells located intra-epithelially, close to the level seen in mast cell-sufficient mice, whereas the bone marrow-derived mast cell-treated mice had few donor MMCs present and a much lower proportion were within the epithelium (Abe & Nawa, 1987a). However, mice of the same mast cell-deficient strain have been shown to expel *Nippostrongylus brasiliensis* without mast cell supplementation (Uber, Roth, & Levy, 1980). This observation may indicate that different parasites are susceptible to different effector responses. It has been established that goats are less able to expel *T. circumcincta* infections than sheep (Macaldowie, Jackson, Huntley et al., 2003). However, a recent study comparing *T. circumcincta* larval development in young goats and sheep with the same trickle infection regime showed that goats had a higher concentration of total mast cells and that a significantly higher proportion of these were GLs than in the sheep (Macaldowie, Jackson, Huntley et al., 2003). This implies that the sheep were better able to expel or arrest incoming worms for reasons other than high GL numbers. It appears therefore, that the role of mast cells may be
similar in *T. circumcincta* and *N. brasiliensis* infections. This might be expected, as *T. circumcincta* is evolutionarily more closely related to *N. brasiliensis* than *S. ratti* (Blaxter, De Ley, Garey et al., 1998). The role of mast cells in expulsion is therefore still open to argument but may depend on the infecting nematode or host species.

### 1.7.2 Mucus Production and Content

Mucus is a viscous sticky substance that coats the GI tissue in animals mounting a protective immune response to GI nematodes (Miller, 1987). It may physically trap or inhibit the movement in a number of ways. Mucus appears to exhibit a chemical larval migratory inhibitory (LMI) activity, particularly in previously immunised animals (Douch, Harrison, Buchanan et al., 1983; Claerebout, Agneessens, Shaw et al., 1999). Other factors could be antibody, leukotrienes, histamine, prostaglandins and amines or a combination of these. Experiments have been conducted to test the ability of mucus from sheep resistant to *T. colubriformis* to prevent the migration of worms (Douch, Harrison, Buchanan et al., 1983; Douch, Harrison, Buchanan et al., 1984; Harrison, Pulford, Hein et al., 2003a). Larval migration was inhibited by up to 93% on agar plates and L3 clumped together when exposed to gastrointestinal mucus from resistant sheep or those with very low egg counts after infection (Douch, Harrison, Buchanan et al., 1983; Harrison, Pulford, Hein et al., 2003a). Also, when a mixture of larvae and mucus from immune sheep were implanted into the intestine of naïve sheep, the larval establishment was reduced and the larvae were displaced distally compared to controls (Harrison, Pulford, Gatehouse et al., 1999). LMI activity was apparently non-specific, with mucus from *T. colubriformis*-resistant sheep also inhibiting the migration of *H. contortus*, *Nematodirus spathiger* and *T. circumcincta* (Douch, Harrison, Buchanan et al., 1983). These authors showed that substances other than primary amines, prostaglandin or histamine must be responsible, as these did not inhibit larval migration at the concentrations present in the mucus. It was suggested that leukotrienes may be involved but other work did not find any correlation between leukotriene concentration and LMI activity (Balic, Bowles, & Meeusen, 2000a), so their role is still not clear. It has now been shown that mucus anti-larval activity to
T. colubriformis may in part be due to antibody (IgG1 and IgA) present in the mucus that binding specifically to an L3 surface-carbohydrate antigen (Harrison, Pulford, Hein et al., 2003a). In calves infected with the abomasal nematode O. ostertagia, a close relation of T. circumcincta, previously immunised animals show higher levels of LMI activity than naïve controls (Claerebout, Agneessens, Shaw et al., 1999).

1.7.3 Eosinophilia

Eosinophils are short-lived cells with phagocytic and secretory properties. They can release substances such as leukotrienes and reactive oxygen species and are associated with inflammatory reactions (reviewed by Rothwell, 1989). A build up of eosinophils has been noted in the abomasum and local lymph nodes of naïve lambs given a single primary infection with H. contortus L3 larvae (Charleston, 1965). This is now a recognised response to many helminth infections but the exact role of eosinophilia in host resistance is still unclear. The relatively low level of eosinophilia that occurs during a primary nematode infection appears to be associated with larval rather than adult worms and, without direct contact between eosinophils and larvae, does little to prevent establishment of the parasite (Balic, Bowles, & Meeusen, 2000a). It has been suggested that, if the antigen that stimulates eosinophil-mediated killing is only present in the larvae of a parasite, repeat larval infections may be necessary in order to produce enough of the appropriate specific antibody to stimulate sufficient eosinophil activation (Meeusen & Balic, 2000). After repeat infections with H. contortus L3, considerably higher eosinophil numbers in the abomasum have been recorded (Charleston, 1965). This response has been observed during secondary infections with several other species of GI nematode and suggests a possible role in acquired immunity (Stevenson, Huntley, Smith et al., 1994). Eosinophils collect around the L3 of H. contortus and adhere to the larvae in vitro, causing disruption of the parasite surface, immobilisation and eventually death (Charleston, 1965; Rainbird, Macmillan, & Meeusen, 1998). However, this only occurred in the presence of specific anti-H. contortus antibody and not with the antibody alone indicating that the two work together to affect the parasite (Rainbird, Macmillan, & Meeusen, 1998). The cytokine IL-5 is also essential for eosinophilia as...
it is necessary for the development of immature eosinophils and enhances the capabilities of mature cells (Meeusen & Balic, 2000; Rainbird, Macmillan, & Meeusen, 1998). Thus, this eosinophilia response tends to be T-cell dependent, with concurrent eosinophil and activated T cell recruitment to the site of infection (Meeusen & Balic, 2000).

Accumulation of eosinophils around GI nematodes has also been reported in several species infecting rodent models, such as *N. brasiliensis* and *S. ratti* and, in some cases, worm damage due to eosinophil degranulation has been demonstrated in vitro (reviewed by Rothwell, 1989). Rodent experiments with IL-5-knockouts or treating with anti-IL-5 antibodies gave variable results, depending on the parasite species, and therefore did not point to a definite role for eosinophils (reviewed by Meeusen & Balic, 2000). For example, IL-5 did not appear to be required for mice to expel a primary infection with *Trichuris muris*, *Toxocara canis* or *Trichinella spiralis* or a challenge infection with *Heligmosomoides polygyrus*. However IL-5 knockout mice could not expel a primary or challenge *Strongyloides venezuelensis* infection. However, it was suggested by the authors that this was due to the tendency of mice to reject primary infections and an increase in non-specific inflammatory responses when a nematode infects an animal that is not its natural host. The authors concluded that the available evidence was consistent with a role for eosinophils for killing larval, but not adult, helminths (Meeusen & Balic, 2000). The larvae of *T. circumcincta* develop inside host abomasal glands, which are inaccessible to eosinophils. This strategy prevents direct contact between eosinophils and the worms until they emerge near to maturity and hence may be less susceptible to damage by the products of eosinophil degranulation (Balic, Bowles, & Meeusen, 2000a). However, eosinophil counts are correlated with significant reductions in egg output when sheep over 3 months old are naturally infected with *T. circumcincta* (Stear, Henderson, Kerr et al., 2002). This means that eosinophilia may be a useful marker for genetic resistance but does not confirm that eosinophils are directly responsible for reduced worm fecundity.
1.7.4 Lymphocytes

There is a great deal of conflicting evidence regarding the role of lymphocytes in the development of immunity to gastrointestinal nematodes due to apparent differences between the responses to larvae and adults and different nematode species. This topic has recently been extensively reviewed (Balic, Bowles, & Meeusen, 2000a).

A large increase in lymphocyte output in the gastric lymph has been demonstrated in response to a challenge infection of 50,000 *T. circumcincta* L3 administered to previously infected sheep (Smith, Jackson, Jackson et al, 1983b). This occurred during the first 5 days post-infection and was followed by a peak in IgA-containing cells one day later (Smith, Jackson, Jackson et al, 1983b). It has also been established that transfer of lymphocytes between genetically identical sheep can confer a limited degree of immunity (Smith, Jackson, Jackson et al., 1986). Immunised sheep were given a challenge infection of 50,000 *T. circumcincta* larvae and lymphocytes were harvested from the gastric lymph for several days after. These lymphocytes were washed and administered intravenously to the identical but *T. circumcincta*-naive siblings (i.e. twins, triplets etc) of the donor sheep. The recipients were then challenged with 50,000 *T. circumcincta* L3. They showed a significant reduction of worm biomass compared with controls due to expulsion or stunting of the worms (Smith, Jackson, Jackson et al, 1986). An increase in B cell numbers in the abomasal lymph nodes and tissues has been detected during the first week after primary infection with *O. ostertagi* (Almeria, Canals, Zarlenega et al., 1997a). A similar response was detected in naïve sheep infected with 50,000 *T. circumcincta*, with lymphoblast numbers in the gastric lymph increasing in the first week then being maintained throughout the experiment (Smith, Jackson, Jackson et al, 1983a). As with eosinophilia during primary infection, the elevation of lymphocyte numbers appears to be associated with the larval stages as no further increases occur in response to the adult infection in *H. contortus* or *O. ostertagi* (Balic, Bowles, & Meeusen, 2000b; Gasbarre, 1994).

Previously infected sheep challenged with *H. contortus* larvae also show an increased recruitment of CD4+, γδ+ T-cells and B-cells to the abomasal tissue.
peaking at 3 days (Balic, Bowles, & Meeusen, 2002). Also, increased percentages of γδ+ T-cells and B-cells were found in the abomasal lymph nodes, which was not seen in the primary infection, implying that lymphocyte recruitment is involved in the development of resistance. Immunisation with *H. contortus* gut antigens induces significant protection in sheep, a topic that will be discussed further in the next section. Importantly, if sheep immunised in such a manner are then treated with anti-CD4+ monoclonal antibody to deplete CD4+ cells, immunity against subsequent challenge is partially abrogated (Karanu, McGuire, Davis et al., 1997), confirming the role of CD4+ cells in immunity in this case.

### 1.7.5 Cytokine Expression

In rodent models, T helper (Th) cells can be categorised as Th1 or Th2-type depending on the cytokines they secrete (Miller, 1996). Murine gastrointestinal nematode infections are generally associated with a Th2-type response with secretion of IL-4, -5, -6, -9, -10 and -13 cytokines, resulting in eosinophilia, mast cell proliferation and IgE expression (reviewed by Miller, 1996). Perhaps the most widely studied Th2 cytokine to date is IL-4. For example, studies with both *H. polygyrus* and *T. muris* have shown that blocking of IL-4 action by treatment with anti-IL-4 or anti-IL-4 receptor monoclonal antibody, after a challenge infection, results in significantly lower mast cell and IgE responses compared with controls and prevents worm expulsion (Else, Finkelman, Maliszewski et al., 1994; Finkelman, Shea-Donohue, Goldhill et al., 1997). Treatment of susceptible *T. muris*-infected mice with IL-4 causes expulsion (Koyama & Ito, 1996). Further evidence for the role of IL-4 has been provided by studies with severe combined immunodeficient (SCID) mice. SCID mice with established *H. polygyrus* or *N. brasiliensis* infections were treated with a long acting formulation of IL-4. This resulted in decreased *H. polygyrus* egg production and termination of *N. brasiliensis* infection (Urban, Maliszewski, Madden et al., 1995). However, IL-4 deficient mice can still expel *N. brasiliensis* so it is apparent that while IL-4 can induce expulsion, it is not essential for immunity to this species. Also, treatment of susceptible *T. muris*-infected mice with monoclonal antibody against interferon-γ (IFN-γ), a cytokine
secreted by Th1 cells, also caused expulsion, demonstrating that Th1-type responses may actually play a role in establishing a chronic infection (Else, Finkelman, Maliszewski et al, 1994).

Rodent studies have also outlined the importance of IL-13 in expulsion of gastrointestinal nematodes. IL-13 and IL-4 are related cytokines. They share an overlap in some activities and can both be blocked by anti-IL-4 receptor monoclonal antibody (Onah & Nawa, 2000). However, when IL-4 and IL-13 knockout mice are infected with *N. brasiliensis*, only the IL-4 knockouts can clear the infection (Finkelman, Wynn, Donaldson et al., 1999), suggesting that IL-13 plays a distinct and important role in worm expulsion. Studies with other nematode species in rodents, such as *T. spirals* and *T. muris* have also highlighted putative roles for IL-10, in polarisation of the response towards Th2, and IL-9, in control of intestinal muscle function (Finkelman, Wynn, Donaldson et al., 1999; Khan, Richard, Akiho et al., 2003).

Preliminary studies exploring the cytokine responses of the host to GI nematode infections have been carried out for some ruminant parasite species. This work mainly utilises reverse transcription-polymerase chain reaction (RT-PCR) to detect the expression of specific cytokine messenger ribonucleic acid [mRNA, (Balic, Bowles, & Meeusen, 2000a)]. Such a study has been conducted to analyse cytokine production by mucosal lamina propria lymphocytes (LPL) and abomasal lymph nodes (ABL) in cows given a primary infection of *O. ostertagi* larvae (Almeria, Canals, Zarlenga et al., 1997b). They recorded rapid increases of IL-4 and IFN-γ mRNA transcription occurring before 10 days post-infection and a more delayed IL-10 response between 10 and 60 days (Almeria, Canals, Zarlenga et al, 1997b). The rise in IFN-γ transcription was concurrent with the penetration of the abomasal glands by the infective larvae and indicates stimulation of a Th1-type immune response by the developing larvae (Almeria, Canals, Zarlenga et al, 1997b). IL-4 expression, however, is indicative of a Th2-type response. It has been suggested that other factors must be involved in *O. ostertagi* infections, as a primary dose is not sufficient to stimulate expulsion of the infection even though rises in IL-4 levels are
induced (Almeria, Canals, Zarlenza et al, 1997b). Prolonged exposure to *O. ostertagi* is required before calves develop a reasonable degree of protective immunity. The cytokine profiles of calves immunized with five drug-attenuated infections followed by a challenge dose were compared with those given a single primary infection as described above (Almeria, Canals, Gomez-Munoz et al., 1998). Immunised animals had much lower ALN IL-4 mRNA levels that correlated with worm burden i.e. IL-4 levels are low when worm numbers are low, perhaps due to a lower level of stimulation to the local immune system, or are not related to protection (Almeria, Canals, Gomez-Munoz et al., 1998). IFN-γ mRNA levels did not differ significantly from those in animals with a primary infection but in general, cytokine mRNA levels were lower in the immunised calves (Almeria, Canals, Gomez-Munoz et al, 1998). In other words, this is not a typical Th2-type response.

Within 3 days of a primary infection of lambs with *H. contortus* larvae, a temporary elevation in IFN-γ and IL-10 levels has been recorded in the ALN but this declined to control levels within 5 days post-infection (Balic, Bowles, & Meeusen, 2000a). Contrary to the results for *O. ostertagi*, IL-4 mRNA levels in the ALN of infected sheep were comparable with those of controls and were undetected in the abomasal tissues (Balic, Bowles, & Meeusen, 2000a). A later study, however, demonstrated a strong Th2-type cytokine response in genetically resistant sheep infected with *H. contortus*. ALN IL-5 levels were elevated with a concurrent decrease in IFN-γ secretion. It was suggested by the authors that during *H. contortus* infection in resistant sheep, the Th2-type response is expanded while the Th1-type response is down-regulated (Gill, Altmann, Cross et al., 2000). Another study reported detectable IL-4 levels in the abomasal tissues of sheep, immunized against *H. contortus* then given a challenge infection, which peaked in the ALN after 5 days, compared to unchallenged animals, whereas IFN-γ levels in the tissues peaked 3 days after challenge (Balic, Bowles, & Meeusen, 2000a). Little work has been done on cytokine expression during *T. circumcincta* infection. One study with naturally-infected Soay sheep has suggested that a polymorphism in the IFN-γ gene, leading to its reduced expression or efficacy, may increase resistance (Coltman, Wilson,
Pilkington et al., 2001). This would again suggest that down-regulation of the Th1-type response is required for immunity to this species.

These results demonstrate that the mechanisms involved in protection in ruminant infections may involve a cross-regulatory Th1/Th2 system similar to that postulated in murine models. They also suggest, however, that the effectors responsible for protective immunity may be species-specific for both the host and the parasite.

1.7.6 Specific Antibodies

Studies with rodent models tend to associate elevated IgE and IgG1 levels with resistance to GI nematodes, as would be expected in a typical Th2 type response (Onah & Nawa, 2000). This has been supported by work involving transfer of immune serum to susceptible *N. brasiliensis* or *H. polygyrus*-infected mice, resulting in the development of a species-specific protective immunity (Brindley & Dobson, 1983). Secondary GI nematode infections typically induce a larger and earlier peak in serum antibody titres than primary infections, suggesting an involvement in parasite rejection, but their precise role in ruminant resistance is still far from clear (Balic, Bowles, & Meeusen, 2000a).

**IgA:** As discussed above, IgA has been linked to resistance to *T. circumcincta* in sheep. In experiments by Stear, Strain & Bishop (1999), reduced worm length, and therefore fecundity, was associated with higher levels of L4-specific IgA in the serum post-challenge. A negative correlation has also been recorded between worm length and IgA levels in the gastric lymph (Smith, 1988). It has also been shown that, after infection with *H. contortus*, the majority of Ig-containing cells in the abomasum contain IgA (Gill, Husband, & Watson, 1992). Further evidence for the role of IgA is provided by a study showing that, after repeat infection, IgA levels in the gastric lymph of young lambs were much lower when compared to older animals (Smith, Jackson, Jackson et al, 1985). In addition, it has been demonstrated that, due to its heritability, anti-*T. circumcincta* IgA activity may
be suitable as a marker of resistance (Strain, Bishop, Henderson et al., 2002). Only low levels of anti-*H. contortus* and anti-*T. colubriformis* IgA were found in the serum of animals immune to these species after challenge (Schallig, Van Leeuwen, & Hendrikx, 1995; Douch, Green, & Risdon, 1994). The authors suggested that this may not reflect local IgA responses and this has been demonstrated in other studies where levels of specific IgA tended to be low in the serum but elevated locally in the mucus, tissues and lymph nodes at the site of infection (reviewed by Balic, Bowles, & Meeusen, 2000a). It is unclear whether IgA contributes to resistance by directly inhibiting worm growth or indirectly through induction of other effector mechanisms. IgA is thought to induce the release of cytokines by inflammatory cells (Miller, 1996). When immune sheep are challenged with *T. colubriformis*, IL-5 is expressed by eosinophils and T cells (Bao, McClure, Emery et al., 1996). It has been suggested that binding of local IgA/antigen complexes to eosinophils could stimulate this and that IL-5 release could then induce further rises in IgA production (Miller, 1996).

**IgG**: In rodent models, a role for IgG has been implicated in rapid expulsion. For example, treatment with anti-*T. spiralis* IgG conferred immediate protection against infection with this species (Arasu, Ellis, Iglesias et al., 1994). There is also evidence that suggests involvement of IgG1 in the mechanism by which immunity to *N. brasiliensis* is transferred in serum from immune to naïve rats (Jones, Edwards, & Ogilvie, 1970). In rodents, IgG1 production is stimulated in the Th2 type response. In *T. muris* infections in mice, blocking of IL-4 function results in a large increase in IgG2 levels, leading to a chronic infection. IFN-γ depletion, however, raises IgG1 secretion resulting in expulsion (Else, Finkelman, Maliszewski et al., 1994). A rise in serum IgG1 and, to a lesser extent, IgG2 is generally observed in secondary infection of calves with *O. ostertagi* (Canals & Gasbarre, 1990), and sheep with *H. contortus* (Schallig, Van Leeuwen, & Hendrikx, 1995). During *H. contortus* infection, a six-fold local increase in IgG1-containing cells has been measured by cannulation of the abomasum (Gill, Husband, & Watson, 1992). It has also been shown that lambs bred for genetic resistance to *H. contortus* produce larger quantities of cell-derived IgG1.
after infection than outbred controls (Gill, Altmann, Cross et al, 2000). In natural nematode infections, there is no passive transfer of immunity from ewe to lamb in the colostrum (Miller, 1984).

IgE: For some time, IgE has been strongly associated with immunity to helminth infections, particularly in schistosomiasis in humans (reviewed by Capron & Dessaint, 1992). IgE production is an essential part of the Th2-type response, being up-regulated by IL-4 and down-regulated by IFN-γ (Miller, 1996). Many studies with rodent and human nematode infections have reported a correlation between IgE levels and protection (reviewed by Negrao-Correa, 2001). N. brasiliensis infection in rats is associated with a substantial rise in circulating levels of both parasite-specific and non-specific IgE (Jarrett & Haig, 1976; Jarrett & Miller, 1982). In secondary infections, the total IgE response tends to be rather shortlived, compared to the parasite-specific IgE levels which decline slowly over several weeks (Jarrett & Haig, 1976). In addition, work carried out with T. spiralis in rats has shown that passive transfer of IgE from immune to susceptible adults results in rapid expulsion upon larval challenge (Ahmad, Wang, & Bell, 1991). There is some argument, however, over how beneficial IgE responses, and associated immediate hypersensitivity reactions, can be to the host. IgE is thought to be responsible for increased mucosal permeability, important in the pathogenesis of this type of disease, via activation of mast cells and basophils (Miller, 1996). It has been suggested that high levels of non-parasite specific IgE could actually benefit the parasite by blocking receptors on effector cells (Pritchard, 1993).

Elevated total and excretory/secretory (ES) protein-specific IgE levels have been reported in the serum of sheep 2-4 weeks after infection with H. contortus (Kooymen, Van Kooten, Huntley et al., 1997). A negative correlation between worm burden and serum total IgE levels was also recorded, indicating involvement of IgE in protective immunity against H. contortus (Kooymen, Van Kooten, Huntley et al, 1997). However, a similar study with T. circumcincta did not produce the same results, with only low specific IgE levels being detected in a small number of the infected animals (Huntley, Schallig, Kooymen et al., 1998).
It is apparent that, when it comes to the immune response to GI nematodes in ruminants, there is still much that is not understood. In rodent models, protective immunity to such parasites is generally manifested as a Th2-type response with expression of the associated cytokines, such as IL-4 and IL-13, and resulting elevation of IgE and IgG1 levels. However, there does seem to be a host- and parasite species-specific element involved, leading to some disagreement between models. This is perfectly characterised by the work on IL-4, which appears to be essential for the expulsion of some infections but not others. It appears that a similar situation may exist in ruminant infections.

1.8 VACCINE DEVELOPMENT

Using a model, it was determined that a vaccine against GI nematode infections in ruminants, even with less than 100% efficacy in less than 100% of the population, could give as good if not better results than standard programs of control (Barnes, Dobson, & Barger, 1995). When searching for vaccine candidate antigens, there are several possible sources to consider.

1.8.1 Radiation Attenuated Worms

Live worms, attenuated either with X-rays, ultraviolet or γ-radiation, can stimulate a protective immune response but do not reproduce or induce significant pathology in the host. L3 treated with γ-radiation have been used successfully to produce vaccines, against the nematodes *Dictyocaulus viviparus* (cattle) and *D. filaria* (sheep), which have been commercially available for over 30 years (McKeand, 2000). Passive transfer studies in guinea pigs and calves have implicated antibody-mediated mechanisms in protective immunity to *D. viviparus* (Jarrett, Jennings, McIntyre et al., 1955; McKeand, Knox, Duncan et al., 1995a). However, vaccinated animals require continued exposure to the parasite in order to sustain this
immunity (McKeand, 2000). Irradiated larvae have been shown to induce significant protection against the canine intestinal parasite *Ancylostoma caninum* (Boag, Parsons, Presidente et al., 2003). They have also been shown to protect mature sheep against *H. contortus* but did not have the same effect on animals younger than 6 months (Smith & Angus, 1980). There is also concern over the commercial viability of using a whole worm vaccine due to its low stability and consequently short shelf-life, the expense of production using donor host animals in addition to the need for subsequent natural challenge in order to preserve immunity (McKeand, 2000). In fact, there has been a steady decrease in use of the *D. viviparus* vaccine since its introduction in favour of anthelmintics (McKeand, 2000).

### 1.8.2 Excretory/Secretory (ES) Molecules

These are substances released *in vitro*, and presumably *in vivo*, by the parasite that are thought to be essential for parasite maintenance within the host due to functions in host penetration, feeding, evasion of immune response or direct alteration of the gastrointestinal environment (Knox, 2000). They include enzymes such as proteases, acetylcholinesterases (AChE) and antioxidants. They are derived from the body surface or from specialised external excretory-secretory glands, located at the anterior of the worm, and are often released in a stage-specific manner (Knox, 2000). The search for an improved *D. viviparous* vaccine has included investigation of ES material. ES from this species contained several enzymes including proteases (Britton, Knox, Canto et al., 1992), superoxide dismutase [SOD (Britton, Knox, & Kennedy, 1994)] and AChE (McKeand, Knox, Duncan et al., 1995b). ES fractions enriched for the latter protected guinea pigs against infection (McKeand, Knox, Duncan et al, 1995b). However, immunisation with either whole adult ES or with a recombinant form of secreted AChE did not result in significant levels of protection against challenge (Matthews, Davidson, Freeman et al., 2001).

Protection trials with ES material obtained from *in vitro* culture of *O. ostertagi* adults, *H. contortus* L3 and *T. colubriformis* L3 and adults have given promising results, with reductions in egg output ranging from 40 to 70% (Boisvenue, Galloway, & Hendrix, 1987; Emery, 1996). Further purification of *T. colubriformis*
adult ES gave several low molecular weight antigens which conferred protection of up to 50\% in guinea pig models (Emery, 1996). *O. ostertagi* adult ES, enriched for cysteine protease activity, gave reductions in cumulative egg counts of 60\%, increased the proportion of inhibited L4 larvae and reduced overall worm count and size when used to vaccinate 7-month old calves (Geldhof, Claerebout, Knox et al., 2002). Two low molecular weight proteins (15 and 24kDa) were also extracted from the ES of *H. contortus* (Schallig, Van Leeuwen, & Cornelissen, 1997). When the purified native proteins were used to immunise eight month old sheep, they reduced mean FEC and worm burdens by over 70\% and also lessened anaemia compared to controls (Schallig & Van Leeuwen, 1997; Schallig, Van Leeuwen, & Cornelissen, 1997; Schallig, Van Leeuwen, Verstrepen et al., 1997). Vaccination with these antigens stimulated a significant specific serum antibody (IgG1) response and increased mastocytosis in the abomasum after challenge (Schallig, Van Leeuwen, & Cornelissen, 1997). More recent trials showed that the efficacy of this vaccination was dependent on the age of the host, with 9 and 6-month old lambs being protected by up to 82\% but 3-month old lambs not showing any protection (Vervelde, Kooyman, Van Leeuwen et al., 2001). Vaccination with adult *H. contortus* ES has also given promising results. Sheep immunised with a cysteine protease enriched fraction of adult ES showed reductions of 52 and 50\% in egg output and worm burden respectively after a single challenge infection (Bakker, Vervelde, Kanobana et al., 2004). A rise in serum and mucus ES-specific IgG, IgE and IgA was observed post-immunisation and post-challenge (Bakker, Vervelde, Kanobana et al, 2004).

Research into proteins secreted during infection with the hookworms *A. caninum*, *Ancylostoma duodenale* and *Necator americanus* has resulted in the discovery of a family of promising cross-protective vaccine candidates, known as ASPs (*Ancylostoma* secreted proteins), which are currently being further evaluated (Sen, Ghosh, Bin et al., 2000; Zhan, Liu, Badamchian et al., 2003). These ASPs show high similarity to a 24 kDa *H. contortus* ES protein and to the major antigenic component of the protective *O. ostertagi* ES fraction (Geldhof, Vercauteren, Gevaert et al., 2003).
A stage-specific 31 kDa antigen has been identified and isolated from the L3 of *T. circumcincta* using sera from resistant sheep. This protein was located in secretory organelles of the oesophageal glands and was abundant in the *in vitro* ES products released by L3 *T. circumcincta* (McGillivery, Yong, Adler et al., 1992; Morton, Yong, Riffkin et al., 1995). When used to immunise 6-month old lambs, it significantly reduced post-challenge mean egg and worm counts compared with controls and induced a steep rise in serum antibody titres. This antigen also elicited a specific cell-mediated response of peripheral lymphocytes before and after challenge infection with the parasite (McGillivery, Yong, Adler et al., 1992). However, these results were not repeated in subsequent trials (Morton, Yong, Riffkin et al., 1995). As with the first trial, high pre-challenge sera antibody titres were recorded but in this case no protection against the challenge occurred. In all trials with this antigen, including the first, the antibody titres of vaccinated sheep were reduced post-challenge (Morton, Yong, Riffkin et al, 1995). This suggests a lack of humoral response stimulated by *T. circumcincta* challenge and that, where protection was observed, it was due a cell-mediated rather than persistent antibody response (Morton, Yong, Riffkin et al, 1995). It is possible that the protective antigen in the first trial was not present in the 31 kDa complex used in subsequent trials and that the purification process needs to be revised.

### 1.8.3 Hidden or Covert antigens

These are antigens expressed inside the worm e.g. the gut, which are thought not to stimulate an antibody response during a natural infection.

H11 is an integral membrane glycoprotein characterised as a microsomal aminopeptidase and expressed only in the microvilli of the intestine of adult *H. contortus* (Smith, Graham, Munn et al., 1997; Knox, 2000). In trials, H11 has been successfully used to immunise sheep, conferring significant protection against challenge infections including challenge with multiply anthelmintic-resistant and geographically separate strains of the parasite (Smith, 1988; Tavernor, Smith, Langford et al, 1992). Importantly, double vaccination with H11 at 7 and 9 weeks also significantly protected young lambs (<6 months old) which are more susceptible
to infection than older animals (Tavernor, Smith, Langford et al., 1992). Serum IgG antibody titres to H11 correlated with the degree of protection conferred. H11 also significantly protected pregnant ewes with evidence of antibody-mediated protection being transferred from the mother to unborn lambs and to newborns in the colostrum. Lambs born to immunised ewes showed moderate levels of protection against challenge, manifested as lower egg counts and lower ratios of female to male worms (Andrews, Hole, Munn et al., 1995). This was thought to be due to transfer of detectable levels of anti-H11 antibody, likely to be predominantly IgG1, from ewe to lamb (Andrews, Hole, Munn et al., 1995). Moreover, aminopeptidase activity of H11 is significantly inhibited by immunoglobulins from the sera of sheep vaccinated with H11, inhibition being correlated with the level of protection (Smith, Graham, Munn et al., 1997). Together, these data strongly suggest that antibody ingested by *H. contortus* affects H11 in such a way as to impair the parasite’s survival although as the exact role of H11 is not yet known, the mechanism of this impairment is unclear.

Other hidden antigens from *H. contortus* have been identified. H-gal-GP (*Haemonchus* galactose-containing protein complex) is a large (1,000 kDa) protein complex isolated from *H. contortus* membrane-bound proteins (S3) by affinity chromatography with peanut lectin and localised to the intestinal brush border of the gut (Smith, Smith, & Murray, 1994). Protection trials with native H-gal-GP have given promising results, with reductions in egg counts of up to 90% and worm counts of 70% (Smith, Smith, & Murray, 1994). Characterisation of the complex by sequence and substrate gel analysis showed it to contain several proteases (Smith, Pettit, Newlands et al., 1999). A major protective component of H-gal-GP is a family of four zinc metalloendopeptidases (MEP 1-4). Native MEPs, dissociated from H-gal-GP, have given egg reductions of up to 33%, with MEP3 being the most effective (Smith, Newlands, Smith et al., 2003). However, trials with recombinant MEP1 and MEP3 have yet to induce significant levels of protection (Smith, Newlands, Smith et al., 2003). The cysteine protease-enriched fraction, thiol sepharose binding proteins (TSBP), is also expressed in the adult intestinal cells and gives reductions in egg output of 77% and worm numbers of 47% in 3 to 10-month old lambs (Knox, Smith, & Smith, 1999). The dominant component of *H. contortus* TSBP, strongly
recognised by sera from TSBP-immunised sheep, was identified as a mitochondrial enzyme, glutamate dehydrogenase (GDH), and was expressed almost exclusively in the blood-feeding stages of the parasite (Skuce, Stewart, Smith et al., 1999). TSBP was further purified, by affinity chromatography with recombinant *H. contortus* cystatin, a cysteine protease inhibitor, and the eluted bound material contained 100% of TSBP cysteine protease activity (Redmond & Knox, 2004). Immunisation with the bound fraction gave substantial reductions in egg count (48 and 28%) and worm burden (44 and 46%) against a challenge infection over two trials (Redmond & Knox, 2004). This evidence suggests that cysteine proteases are at least partly responsible for the protective capacity of *H. contortus* S3 TSBP. Immunisation with a cocktail of all three recombinants resulted in a significant reduction in worm burden (38%), although no reduction in egg output was observed (Redmond & Knox, 2004).

Similar fractions from the gut of *T. circumcincta* have been extracted and are being evaluated in protection trials. *H. contortus* is a blood-feeder and will therefore ingest great quantities of antibody. *T. circumcincta* browses on the surface of the intestine, rarely consuming blood unless from an existing wound, but has been shown to ingest some immunoglobulin (Murray & Smith, 1994). A cross-protection trial showed that vaccination with *H. contortus* H11 and H-gal-GP did not confer any significant protection against *T. circumcincta* challenge (Smith, Pettit, & Smith, 2001). In the same trial, lambs immunised with *T. circumcincta* gut membrane glycoproteins (Peanut and ConA lectin binding fractions) were partially protected against challenge with *H. contortus* but not *T. circumcincta* even though both produced measurable circulating antibody responses (Smith, Pettit, & Smith, 2001). This suggests that *T. circumcincta* did not take in enough antibody to cause significant impairment of intestinal function and this may mean that a gut protein may not be suitable for vaccination against this species. A trial involving vaccination with TSBP from the gut membrane of *T. circumcincta* did generate consistent highly
significant protection values in young animals but a further trial gave more variable results (D. Knox, personal communication). The nature of the proteins in this fraction remains undefined and this topic is one of the major objectives of the research described in this thesis.

### 1.8.4 Recombinant Vaccines

In order to develop and manufacture a commercially viable vaccine against GI nematodes, alternatives to native antigenic material are necessary due to the expense of production of such material. Recombinant technology may provide the answer to this problem due to the opportunity afforded by bacterial and eukaryotic expression systems to produce antigenic material inexpensively and in bulk. cDNA for specific proteins, that have been identified as immunogenic, can be isolated using several methods including cDNA library screening with immune serum. This cDNA can then be inserted into a vector, which is used to transform a host cell. Host cells expressing the protein can then be produced in large-scale cultures and the protein purified from cell extracts. However, this system can have its problems. Firstly, cloning and expression can be tricky and lengthy operations. Secondly, the protein produced is unlikely to be identical to the native protein due to differences in conformation and lack of glycosylation when produced in bacterial cells. If these aspects are crucial for inducing protection then the recombinant protein will not be suitable for vaccination. However, new techniques involving eukaryotic expression systems such as yeast, insects cells or *C. elegans* may help overcome this (Knox, Redmond, Skuce et al., 2001). The options available for recombinant protein expression have recently been extensively reviewed (Dalton, Brindley, Knox et al., 2003).

This approach has given promising results against other parasites. For example, several protective proteins from the cestode parasites *Taenia ovis*, *Taenia saginata* and *Echinococcus granulosus* have been cloned and expressed in *Escherichia coli* (Lightowlers, Colebrook, Gauci et al., 2003). These gave consistent high levels of protection and are now being developed as commercial vaccines.
1.9 AIMS OF PROJECT

The general aims of the work presented here were to learn more about the biology of *T. circumcincta*, through characterisation of proteins expressed in the different life stages, and to answer the following questions:

- Are the antigenic protein mixtures, *T. circumcincta* S3 TSBP and ES material, sources of potential vaccine candidates?
- What is the nature of the TSBP components recognised by antibodies from immunised animals?
- Does TSBP immunisation stimulate a host serum antibody response and does this response relate to protection?
- What are the potential functions of proteins released by *T. circumcincta* during *in vitro* culture?
- Are these *in vitro* ES proteins representative of 'true' *in vivo* ES material?

The methods employed to this end are detailed in Chapter Two. Chapter Three presents the results of an immunoscreen of an adult *T. circumcincta* cDNA library with anti-TSBP serum and subsequent analysis of the positive clones identified. The further characterisation of three TSBP components identified by this immunoscreen, an antioxidant and two metabolic enzymes, is described in Chapters Four, Five and Six, and the relevance of their presence in this fraction is discussed. Chapter Seven details the analysis of serum antibody responses in TSBP-immunised sheep and their relationship with protection against a subsequent challenge infection. The results are considered in relation to the immunological factors implicated in protection against such infections and the suitability of such a fraction as a source of vaccine candidates is discussed. Chapter Eight describes the harvesting of *in vitro* ES material, from L4 and adult *T. circumcincta*, and its proteomic analysis. The putative functions and significance of the components identified are discussed.
CHAPTER TWO - MATERIALS AND METHODS

2.1 LABORATORY REAGENTS

2.1.1 General Solutions

10X Tris-buffered saline (TBS): 24.22 g Tris base was dissolved in 800 ml dH₂O and the pH adjusted to 7.5 with 10 M HCl. 87.66 g of NaCl was then added and the total volume made up to 1 l with dH₂O.

TBS with Tween (TBST): 100 ml of 10X TBS was made up to 1 l with dH₂O and 0.5 ml Tween 20 added.

SM buffer: 5.8 g NaCl, 2.0 g MgSO₄, 50 ml 1 M Tris-HCl (pH 7.5) and 5 ml 2% (w/v) gelatin were made up to 1 l with dH₂O. The solution was autoclaved (121°C for 15 min) before use.

RPMI culture medium: 10.4 g RPMI-1640® (Sigma) and 2.0 g sodium hydrogen carbonate were made up to 1 l with dH₂O and autoclaved. Penicillin and streptomycin were added (0.5 units/ml and 50µg/ml respectively) along with 0.2 g gentamicin and the solution warmed to 37 °C before use.

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

4X separating gel buffer (1.5 M Tris-HCl, pH 8.8): 27.23 g Tris base was dissolved in 80 ml of dH₂O and the pH adjusted to 8.8 by slow addition of hydrochloric acid. The buffer was then made up to 150 ml with dH₂O.
4X stacking gel buffer (0.5 M Tris-HCl, pH 6.8): 6 g Tris base was dissolved in 60 ml dH$_2$O and the pH adjusted to 6.8 by slow addition of hydrochloric acid. The buffer was then made up to 100 ml with dH$_2$O.

2X reducing loading buffer: Loading buffer was prepared by mixing 4 ml dH$_2$O, 1 ml 0.5 M tris-HCl (pH 6.8), 0.8 ml glycerol, 1.6 ml 10% (w/v) SDS, 0.4 ml 2-β mercaptoethanol and 0.2 ml 0.05% (w/v) bromophenol blue to give a total volume of 8 ml.

2X non-reducing loading buffer: As above but without addition of 2-β mercaptoethanol.

5X SDS-PAGE electrode running buffer: 9 g Tris base, 43.2 g glycine and 3 g SDS were dissolved in 600 ml dH$_2$O. This stock was diluted 1/5 with dH$_2$O for electrophoresis.

Coomassie blue stain: Coomassie brilliant blue was made up to 0.23% (w/v) in 30% methanol, 10 % (v/v) acetic acid and filtered through a funnel lined with a Whatman® filter paper circle.

Coomassie blue destain: 30% (v/v) methanol, 10% (v/v) acetic acid.

2.1.3 Western Blotting

Anode buffer 1: 18.72 g Tris base was dissolved in 500 ml dH$_2$O. The pH was not adjusted.
Anode buffer 2: 1.51 g Tris base was dissolved in 500 ml dH₂O. The pH was not adjusted.

Cathode buffer: 1.51 g Tris base was dissolved in 500 ml dH₂O. The pH was adjusted to 9.4 by addition of 0.1 to 0.5 g glycine.

2.1.4 Periodate Treatment

Acetate buffer: 50 mM sodium acetate was made by dissolving 2.05 g in 400 ml of dH₂O, adjusting the pH to 4.5 with glacial acetic acid and making up to a final volume of 500 ml with dH₂O.

Sodium periodate (50 mM): 1.07 g sodium periodate was dissolved in 100 ml acetate buffer.

Sodium borohydride (50 mM): 0.189 g sodium borohydride was dissolved in 100 ml TBST. This solution was made immediately prior to use.

2.1.5 Agar plates and bacterial cultures

Luria-Bertani (LB) Broth: 1.0% (w/v) tryptone and 0.05% (w/v) yeast extract were dissolved in 0.17 M sodium chloride. The pH was adjusted to 7.5 with 5 M sodium hydroxide and the solution autoclaved before use.

LB Agar: 20 g agar (Bacto™ Agar, Becton Dickinson) was added to 1 l LB broth and autoclaved before use.
LB Top Agarose: 7 g agarose (0.7% w/v) was added to 1 l LB broth and autoclaved before use.

LB/Ampicillin plate: 100 μg ampicillin added per ml of agar before pouring.

LB/Ampicillin/IPTG/X-gal plate: 100 μg ampicillin added per ml of agar before pouring. After plates set and air-dried briefly, 100 μl 100 mM IPTG was added to agar surface and distributed with a sterile glass spreader. 20 μl 50 mg/ml X-gal in N, N-dimethyl-formamide was then added and spread in the same way. Plates were incubated at 37 °C for 30 min to allow IPTG/X-gal to sink in.

SOC medium: 1 M NaCl with 2.0% (w/v) tryptone and 0.5% (w/v) yeast extract. Autoclave, then add 1 M MgSO₄ (filter-sterilised) and 1 M MgCl₂ (filter-sterilised), both to a final concentration of 10 mM. Add filter-sterilised 1 M glucose to a final concentration of 20 mM and store 1 ml aliquots at -20°C until required.

2.1.6 DNA/RNA separation and visualisation

Ethidium bromide solution: 50 mg ethidium bromide was dissolved in 5 ml dH₂O and stored in a foil-covered tube at 4 °C.

50X Tris-Acetic acid-EDTA Buffer (TAE): 2 M Tris base, 5.71% (v/v) glacial acetic acid and 0.05 M EDTA in a final volume of 1 l. The pH was adjusted to 8.0 with 10 M HCl. This solution was diluted 1/50 with dH₂O before use.

0.8% agarose gel: 2.4 g agarose was dissolved in 300 ml 1X TAE by heating in a microwave and swirling occasionally. The solution was cooled to approximately 60°C and 15 μl ethidium bromide was added before pouring.
2% agarose gel: 6.0 g agarose was dissolved in 300 ml 1X TAE by heating in a microwave and swirling occasionally. The solution was cooled to approximately 60°C and 15 µl ethidium bromide was added before pouring.

20X standard sodium citrate (SSC, 3 M NaCl/300 mM sodium citrate): 175.3 g NaCl and 88.2 g sodium citrate dissolved in 1 l dH₂O and autoclaved. Dilute with dH₂O as desired.

Blue juice loading buffer: 40% (w/v) sucrose, 0.25% (w/v) bromophenol blue in dH₂O.

Xylene cyanol loading buffer: 40% (w/v) sucrose, 0.25% (w/v) xylene cyanol in dH₂O.

2.1.7 Protein Extract Buffers

S1 buffer: 1.25 mM EDTA (0.186 g in 500 ml 1X PBS) with pH adjusted to 7.4 with 10 M HCl. 100 mM PMSF was added to a final concentration of 1 mM (1 ml/100 ml) just prior to use.

S2 buffer: 1.25 mM EDTA (0.372 g in 1 l 1X PBS) with 0.1% (v/v) Tween 20 and pH adjusted to 7.4 with 10 M HCl. 100 mM PMSF was added to a final concentration of 1 mM (1 ml/100 ml) just prior to use.

S3 buffer: 2% (v/v) Triton X-100 was dissolved in 1X PBS (6 ml/300 ml) and adjusted to pH 7.4 with 10 M HCl. 100 mM PMSF was added to a final concentration of 1 mM (1 ml/100 ml) just prior to use.
2.1.8 Antibody Elution Buffers

**Elution buffer (5mM glycine/ 500 mM NaCl/ 0.2% Tween 20):** 0.037 g glycine was dissolved in 500 ml dH2O and the pH adjusted to 2.3 with 10 M HCl. 2.922 g NaCl and 0.2% (v/v) Tween 20 were then added.

**Neutralisation solution (1 M Tris-HCL, pH 7.4):** 60.55 g Tris-HCl was dissolved in 500 ml dH2O and the pH adjusted to 7.4 with 10 M HCl.

2.1.9 2-D Electrophoresis Solutions

**Rehydration buffer:** 8 M urea (120 g in 250 ml dH2O) with 2% (w/v) CHAPS, 0.2% (w/v) DTT and 0.2% (v/v) Pharmalyte™ (Broad range pH 3-10, Amersham) added.

**Equilibration Buffer:** 6 M urea (90 g in 250 ml dH2O) with 30% (v/v) glycerol and 2% (w/v) SDS. Tris-HCl was added to 1.5 M and pH adjusted to 8.8 with HCl.
2.2 WESTERN BLOT ANALYSIS OF SERUM ANTI-TSBP ANTIBODIES

2.2.1 TSBP preparation

TSBP were prepared previously by Dr Stuart Smith according to previously published methods (Knox, Smith, & Smith, 1999). Briefly, adult *T. circumcincta* were successively solubilised in PBS, PBS plus Tween 20 and, finally, in PBS containing Triton X-100 with the insoluble material being pelleted by centrifugation at each step. The Triton X-100 soluble protein extract (S3), containing the integral membrane protein fraction of the parasite, was then applied to a Thiol-sepharose column, unbound material washed through and bound material eluted by the addition of 50 mM DTT to the wash buffer. TSBP were concentrated 20 fold and stored at −80 °C until required.

The protein concentration of the neat TSBP preparation was estimated by Beckman DU® 650 spectrophotometer. The eluting buffer (10 mM Tris-HCl, pH 7.4/1% (v/v) Triton –X 100) was used as a blank and absorbance at λ 260 (A) and at λ 280 (B) with UV light was measured. The following formula, from Layne (1957), was used to calculate the concentration: 

\[
\text{concentration (mg/ml)} = \frac{(1.55 \times A_{280}) - (0.76 \times A_{260})}{1.47}
\]

This gave an approximate protein concentration of 2.8548 mg/ml.

2.2.2 Protein fractionation by SDS-PAGE

SDS-PAGE gels were prepared using the methods of Laemmli (1970), and cast using Mini Protean® II Dual Slab Cell apparatus (Biorad). 10% separating gels were poured as follows: 4.05 ml dH2O, 2.5 ml 4X separating buffer, 100 μl 10% (w/v) SDS and 3.3 ml 30% (w/v) acrylamide/ bis-acrylamide stock (Severn Biotech) were mixed and 25 μl TEMED (N, N, N', N'-Tetramethylethylenediamine - Sigma) and 75 μl 10% (w/v) ammonium
persulphate were added to facilitate polymerisation. 3.6 ml of this mixture were poured into the casting apparatus, carefully overlaid with dH₂O and left at room temperature for approximately 1 h to allow polymerisation to occur.

A 4% stacking gel was prepared by mixing 6.05 ml dH₂O, 2.5 ml stacking buffer, 100 μl 10% SDS and 1.3 ml 30% acrylamide/bis-acrylamide then adding 25 μl TEMED and 75 μl 10% ammonium persulphate. The water above the separating gel was removed and stacking gel was added to the top of the apparatus. An appropriate gel comb was immediately inserted to create wells in the stack. Protein samples were diluted with an equal volume of either reducing or non-reducing sample buffer before loading in sample wells along with a single well containing 10 μl of protein standards (Mark 12™, Invitrogen) and the gel was run at 200V for approximately 45 min in 1X SDS-PAGE electrophoresis buffer. Gels were stained with Coomassie Blue and destained with Coomassie Blue destain until protein bands were clearly visible against the background.

2.2.3 TSBP Trial Details

An experimental group of seven sheep were vaccinated with adult *T. circumcincta* TSBP and challenged with *T. circumcincta* (D Knox, unpublished). The sheep were outbred Greyface/Suffolk crosses and were seven months old at the start of the trial. The experimental and control groups were balanced for sex and weight. The experimental sheep were immunised with three intra-muscular injections of 100 μg TSBP in 5 mg Quil A adjuvant (Brentag Biosector, Denmark), with a period of three weeks between boosts. Seven control sheep received three injections of Quil A adjuvant only. A week after the final immunisation, each animal was then challenged orally with 5,000 infective *T. circumcincta* L3. The pre-challenge sera, taken just before challenge in order to gauge the animals’ response to immunisation, from six experimental animals and one control were used to probe the blot.
2.2.4 Western blotting of TSBP proteins

TSBP (0.28 mg/100 μl) was mixed with an equal volume of reducing sample buffer and heated at 95 °C for 4 min. After cooling to room temperature, the sample was applied to each of two 10% SDS-PAGE gels poured using a comb with one large sample well (capacity 100 μl) plus one small well for markers. The gels were run in 1X SDS-PAGE running buffer at 200 V for 45 min then blotted onto Immobilon™-P transfer membrane using the MilliBlot™ Graphite Electroblotter (Millipore) according to the manufacturer’s instructions.

The western blot markers were removed and stained briefly with Coomassie blue. The remainder of the blot was then cut into strips vertically. One set of strips was periodate treated by washing in acetate buffer for 10 min followed by incubation for 60 min in the dark (covered in foil) at room temperature with 50 mM sodium periodate. Strips were washed twice in acetate buffer for 10 min and twice in TBST for 10 min, incubated for 30 min at room temperature in 50 mM sodium borohydride and given a final rinse for 30 min in TBST. Both sets of strips were blocked in 5% (v/v) horse serum in TBST overnight.

One strip each, from the periodate and non-periodate treated blots, was incubated in a 1:500 dilution in TBST of each of the seven sera overnight. All strips were washed extensively in TBST, incubated at room temperature with 1:500 Donkey Anti-sheep IgG Horse Radish Peroxidase (HRP) conjugate (Sigma) in 5% (v/v) horse serum and developed with FAST™ 3, 3′-dianinobenzidine tablet sets (Sigma), according to the manufacturer’s instructions.
2.3 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) OF SERUM ANTI-TSBP ANTIBODY ISOTYPES

ELISAs for IgG1 and 2, IgE and IgA isotypes against TSBP in the pre-challenge sera of sheep were carried out. This was to determine whether serum anti-TSBP antibody titre was correlated to the degree of protective immunity exhibited by individual lambs to a challenge *T. circumcincta* infection following immunisation with TSBP. These sera were from the same trial as those used to probe western blots. The sheep from which the sera were collected showed variable degrees of protection after challenge so comparison of antibody titre with egg and worm counts could demonstrate whether a relationship exists. Periodate treatment of the antigen was carried out to differentiate whether any apparent relationship was attributable to the protein or carbohydrate component of TSBP.

2.3.1 Antigen-coating and periodate treatment of plates

A 1:1000 dilution (0.0028 mg/ml) of the TSBP preparation in 50 mM bicarbonate buffer (pH 9.6) was used to coat Dynatech M129B 96 well ELISA plates for each antibody isotype to be tested. 50 μl of the dilution was added to each well and the plate was tapped gently to distribute the liquid before incubating overnight at 4 °C. The excess liquid was then poured off and the plate rinsed x6 with TBST.

Periodate treatment of some antigen-coated plates was used to block carbohydrate reactivity. The wells were filled with acetate buffer (50 mM sodium acetate, pH 4.5), shaken briefly then emptied. 100 μl of 50 mM sodium periodate in acetate buffer was then added to each well. The plates were wrapped in tin foil to block out light and incubated at room temperature for 1 h. The plates were rinsed x2 in acetate buffer and x2 in TBST. 100 μl of 50 mM sodium borohydride in TBST was added to each well. The plates were incubated at room temperature for 30 min then rinsed x3 in TBST. All six plates were then screened for antibody activity.
2.3.2 ELISA Procedure

Serial dilutions of six experimental sera, from 1:20 to 1:20,480, and a 1:20 dilution of the control sera were made up in 1X PBS. The experimental sera were the same as those used for the Western blot. The controls were pre-challenge sera from six sheep given adjuvant only then challenged with *T. circumcincta*.

Serum (50 μl) was added to each active well, as shown in Table 2.3.2, a well for each of the eleven dilutions of the experimental sera and one well for each of the control sera. The first column of wells contained 1X PBS only while all other wells received all treatments except serum.

The plates were incubated at room temperature for 1 h. The serum was poured off and the plates rinsed x6 in TBST. Monoclonal antibody was diluted appropriately in TBST: IgG2 (2Rshx2 anti-ovine IgG2, ICR London): 1/32, IgG1 (anti-bovine IgG1, ILRI Kenya), IgA (IL-A71 anti-bovine IgA, ILRI Kenya) and IgE (2F1 anti-ovine IgE, CSIRO Australia): 1/1000. 50 μl was added to every well on each of the six plates. The plates were incubated at room temperature for 1 h. The anti-serum was poured off and the plates rinsed x6 in TBST. 50 μl of HRP-conjugated Mouse Immunoglobulin (DAKO), diluted 1:2,000 in TBST, was added to every well on all six plates and incubated at room temperature for 1 h.

The plates were washed six times in TBST and 100 μl of o-Phenylenediamine dihydrochlorate substrate (Sigma), prepared according to the manufacturer’s instructions, was added to every well. The plates were incubated at room temperature for 25 min before adding 25 μl 2.5 M sulphuric acid per well to halt colour development. The absorbance of each plate was then read on a Dynatech MR5000 spectrophotometer at 490 nm and the resulting data analysed.
### Table 2.3.2. ELISA plate layout. Exp = Experimental sera 1 to 6. Con = Control sera 1 to 6. 1/n = serum dilution.

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### 2.4 IMMUNOSCREENING ADULT T. CIRCUMCINCTA cDNA LIBRARIES

Two adult *T. circumcincta* Uni ZAP-XR cDNA libraries were constructed previously by Dr D.L. Redmond, using a Stratagene kit. Library #1 had a titre of $1.1 \times 10^5$ plaque-forming units (pfu)/ml and Library #2 of $0.7 \times 10^5$ pfu/ml. Plaques were plated as described below and screened with serum from a sheep vaccinated with *T. circumcincta* TSBP as before. This sheep showed a significant level of protection against *T. circumcincta* infection, as measured by post-challenge egg and worm counts compared with controls.
2.4.1 Plating and Membrane Procedure

XL1 MRF cells (Stratagene) were grown by inoculating a single colony into a sterile solution of LB broth supplemented with 10 mM MgSO$_4$ and 0.5% (w/v) maltose and incubating overnight at 30 °C with shaking at 225 rpm. Bacteria were harvested by centrifugation (500xg for 10 min) and the resultant pellet resuspended in 10 mM MgSO$_4$ to give an OD$_{600}$ of 0.5.

The cDNA libraries were plated at 250 pfu/plate (90 mm plates) or 5,000 pfu/plate (150 mm plates) following incubation with host bacterial cells for 15 min as described in the manufacturer’s instructions. Plates were incubated at 42 °C for approximately 4 h until clear pin-prick size plaques were just visible in the bacterial lawn. Hybond C-extra nitrocellulose membranes (Amersham) of the appropriate dimensions were impregnated with 10 mM IPTG by soaking briefly and overlaid onto the surface of the agar. IPTG induces expression of the lacZ gene, into which the cDNA is inserted in the Bluescript vector, and thus induces expression of the recombinant protein. The plates were inverted and incubated overnight at 37 °C.

Each plate was cooled at +4 °C for 2 h. Before removing the membrane from the plate, it was ‘punch-orientated’ by pushing a sterile needle through the membrane and the agar to give a pattern of holes that could be matched up later. The membrane was then carefully peeled from the surface of the agar. The plate was stored at +4 °C.

2.4.2 Blocking of non-specific immunoreactivity and Positive control

The membranes were rinsed for 10 min in TBST three times and blocked overnight in 5% (v/v) horse serum in 1X TBS at room temperature with constant agitation. A positive control was created by spotting a small amount of whole adult *T. circumcincta* S3 onto a strip of membrane and this was incubated with the membrane blocking solution as described above.
2.4.3 Screening with anti-TSBP serum

After blocking, each membrane and positive control was washed for 3 x 10 min in TBST and incubated with a 1:500 dilution of anti-TSBP serum in TBST for 2 h. After washing again in TBST, membranes were incubated in 1:500 dilution of Donkey Anti-sheep IgG HRP-conjugate (Sigma) in 5% (v/v) horse serum in TBS for 1 h. All these stages took place at room temperature. The membrane and control were thoroughly washed again and stained with FAST™ 3, 3’-dianinobenzidine tablet sets (Sigma) to visualise immunopositive plaques. Membranes were dipped in cold water to stop colour development and dried on filter paper.

Positive plaques, i.e. those that stained noticeably darker than background plaques, were located on the original agar plate by lining up the punched holes in the membrane with those in the agar with the aid of a light box. These plaques were picked using sterile pipette tips. Each positive plaque was placed in a 1.5 ml tube containing 500 µl of SM buffer and 20 µl of chloroform, vortexed briefly and stored at +4 °C.

2.4.4 Titring immunopositives – Grid method

The titre of immunopositives (POs) was determined by spotting different dilutions of the phage stocks in SM buffer onto a bacterial lawn in a grid formation. After overnight incubation at 37 °C, plaques were counted at the dilutions where discreet plaques were visible and the stock titre calculated.

2.4.5 Plaque Purification of POs

All of the POs collected were plaque-purified by plating and re-screening until all of the plaques present on a plate were positive and sufficiently spaced to allow an individual plaque to be picked. Isolated positive plaques was stored in SM buffer and chloroform as before.
2.5 PRELIMINARY SEQUENCE ANALYSIS OF POs

2.5.1 PCR amplification of POs

PO inserts were amplified by PCR using buffers, nucleotides and BIOTAQ DNA polymerase from Bioline. Primers are short double-stranded segments of DNA, complementary to the template sequence, that initiate DNA synthesis in a PCR reaction. Primers directed at the regions of Bluescript vector sequence on either side of the clone inserts were used for this amplification (Table 2.5.1). All primers were ordered as HSPF® (High Purity Salt Free) oligonucleotides from MWG Biotech (www.mwg-biotech.com).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction</th>
<th>Sequence</th>
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<tr>
<td>T3</td>
<td>Sense</td>
<td>AAT TAA CCC TCA CTA AAG G</td>
</tr>
<tr>
<td>T7</td>
<td>Antisense</td>
<td>TAA TAC GAC TCA CTA TAG GG</td>
</tr>
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</table>

Table 2.5.1. Primers for PCR amplification of PO inserts

For use as PCR template, freeze-thaw lysates of all POs were made by diluting the phage stock 1:1 in dH2O and subjecting this to three cycles of freezing at −80 °C for 30 min and thawing at 37 °C for 30 min.

A stock PCR mix was made by adding the following, multiplied by the number of 50 μl reactions required: 5 μl 10X NH₄ buffer (160 mM (NH₄)₂SO₄, 670 mM Tris-HCl, 0.1% (v/v) Tween 20, pH 8.8), 1.5 μl 50 mM MgCl₂, 10 μl 5X dNTPs, the latter with a final reaction concentration of 10 μM, 18 μl dH₂O and 1 U BioTaq polymerase. To 35 μl of this mixture in a 0.5ml PCR tube was added 5 μl T3 primer and 5 μl T7 primer, both from 10 μM stock, and 5 μl of template.
Amplification was carried out according to the following program on the GeneAmp 2400 PCR System (Applied Biosystems):

Stage 1: 95 °C for 5 min – 1 cycle

Stage 2: 95 °C for 30 s (Denaturation) 
52 °C for 30 s (Primer annealing) 
72 °C for 1 min (DNA synthesis) 
\{ 30 cycles \}

Stage 3: 72 °C for 8 min – 1 cycle

PCR products were purified using the Qiagen QIAquick spin kit as described in the manufacturer’s instructions (Qiagen, U.K.), fractionated in 0.8% (w/v) agarose gels and visualised by ethidium bromide staining using an Amersham Pharmacia Biotech ImageMaster® to check the insert sizes. The products were stored at —20 °C.

2.5.2 Sequencing of inserts with vector primers

The inserts of an initial fourteen POs were sequenced with the standard 5' T3 primer to the Bluescript vector sequence. This was carried out in the Moredun Research Institute Functional Genomics in-house sequencing lab, using the ABI 377 XL DNA sequencer and fluorescent labelling reagent Big-Dye Terminator Chemistry, both from Applied Biosystems.

The resulting sequences were analysed using the DNAStar computer package (Lasergene, DNASTAR Inc.). Databases on the EBI (European Bioinformatics Institute – www.ebi.ac.uk) and NCBI (National Center for Biotechnology Information – www.ncbi.nlm.nih.gov) websites were searched using the nucleotide sequences to identify homologues.
2.5.3 Screening dot-bLOTS with DIG-labelled probes

As described in Chapter 3, two highly represented sequences, homologues of methyl malonate-semialdehyde dehydrogenase (MMSD) and 10-formyltetrahydrofolate dehydrogenase (FTDH), were identified during the preliminary sequencing. Gene-specific probes were prepared to screen dot blots of all the POs for clones with inserts sharing similarity with either of these sequences. The probes were labelled and visualised using the Boehringer Mannheim Biochemica DIG Luminescent Detection kit according to the manufacturer’s instructions. Digoxigenin (DIG) binds to uridine nucleotides in the probe DNA and is enzymatically incorporated into the probe by a process of random primed labelling. After hybridization and blocking of the blot, high affinity anti-DIG-antibody Fab-fragments conjugated with alkaline phosphatase detect the labelled DNA. The alkaline phosphatase reacts with the chemiluminescent substrate AMPPD®, which decomposes and emits light that can be recorded by X-ray film.

Probe construction and labelling

Gene-specific primers (Table 2.5.3) were designed and used to amplify a region of approximately 1 kb from each of these sequences. This was done using the same PCR protocol described previously, with freeze-thaw lysate of an appropriate clone as template, and the products were cleaned as before. The probes were denatured by boiling for 10 min then freezing and DIG-labelled according to the manufacturer’s instructions.

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<th>Primer Name</th>
<th>Primer Sequence (5’ to 3’)</th>
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<td>P45S (sense)</td>
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<td>P24A (antisense)</td>
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Table 2.5.3. Gene-specific primer sequences for probe amplification.
**Dot-blot preparation and hybridisation**

Two grids of 45 cm$^2$ were drawn in pencil on Hybond-N nylon membrane (Amersham) and the membranes soaked briefly in 10X standard sodium citrate (SSC). For each positive, a 1:1 mixture of phage stock and 20X SSC was boiled for 5 min then frozen to denature. Four μl of each mixture was dotted onto a square on each of the dot blot grids. The blots were then denatured and neutralised according to kit protocol. Blots were wrapped in clingfilm and exposed to UV light on a Hybaid Crosslinker set to 0.4 J/cm$^2$ to fix the DNA to the membrane. The blots were pre-hybridised for 1 h in DIG Easy Hyb solution (Boehringer) at 42 °C. The appropriate probe was then added to the membrane buffer of each blot and hybridisation allowed to proceed in a vertical rotating incubator at 42 °C overnight.

**DIG-labelled probe detection**

Each probe plus buffer was poured off. High stringency washes of the blots were performed twice with 2X SSC/0.1% (w/v) SDS for 15 min at room temperature and twice with 0.2X SSC/0.1% (w/v) SDS for 15 min at 65 °C. Probe detection was carried out according to manufacturer’s instructions and the blots were then exposed to X-ray film and developed. Multiple exposures were taken in order to find the length of exposure giving the clearest result. This was 30 s.

**2.5.4 Clone Excision**

POs that were not identified as homologues of either MMSD or FTDH were sequenced with vector primers as described in section 2.5.2. The PCR products of several of these POs gave ‘messy sequence’ sequence data i.e. short length of coding sequence and many unspecified bases (denoted N) so an *in vivo* excision was carried out as described in the manufacturers instructions (Stratagene). XL1 MR$^F$ cells were cultured as described before and resuspended in MgSO$_4$ to give an OD$_{600}$ of ~ 1.0. SOLR cells (Stratagene) were grown overnight on LB agar-kanamycin (50 μg/ml) plates and an LB broth culture prepared according to the protocol in the cDNA Synthesis Kit manual (Stratagene). The Single-Clone Excision protocol was then
followed for each PO to be excised. Colonies from each PO were picked and grown overnight in 10 ml LB broth/20 μl ampicillin at 37 °C with shaking. Each 10 ml culture was purified using the centrifugation protocol of the Wizard Plus SV Minipreps DNA purification system (Promega) and eluting in 50 μl dH2O. Each purified product was diluted to 1:40 in nuclease-free water and the DNA concentration measured on a spectrophotometer. This was done using the following formula:

\[ \text{OD}_{260} \times 50 \times \text{dilution faction} = \text{concentration (μg/ml)} \]

They were then amplified using the same PCR reaction mixture and cycling as described before (15 μl rather than 50 μl reactions) and the product run on a 0.8% (w/v) agarose gel to check the inserts. The neat purified product was re-sequenced with vector primers.

2.6 SEQUENCE ANALYSIS OF SELECTED POS

2.6.1 Further sequencing with gene-specific primers

Clones with insert sequences that shared considerable similarity with either catalase, MMSD or FTDH were selected for further analysis as these enzymes have not been described in this species previously. Where a full-length coding sequence could not be obtained using vector primers alone, gene-specific primers were designed to allow extension of the sequence. Where the combined insert sequences from several clones still did not provide a full-length coding sequence, PCR reactions using gene-specific primers in combination with vector primers were used in an attempt to amplify the missing sections of sequence from cDNA library. The primers used are listed in table 2.6. PCR products were purified as described previously and sequenced with the primers used to generate them or were cloned into pGEM-T Easy vector (Promega) according to the manufacturers instructions and sequenced using primers to the vector sequence (T3/T7).
2.6.2 Translation and analysis of protein sequence

Further sequence analysis, such as nucleotide sequence alignment and translation, was carried out using the DNASTar software. As before, online databases were searched with both the nucleotide and translated protein sequences in order to establish the level of similarity shared with sequences from other species. Alignments of the translated sequences with homologues from other species were performed using the ClustalW tool on the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw/). The Prosite database on the ExPASy (Expert Protein Analysis System) website (http://us.expasy.org/prosite) was used to scan the translated sequences for conserved motifs or biologically significant domains in order to confirm putative families or functions for each protein.

<table>
<thead>
<tr>
<th>Homologue</th>
<th>Primer name</th>
<th>Direction</th>
<th>Primer sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMSD</td>
<td>MMSD</td>
<td>Sense</td>
<td>GCC TCA ACA TTA TCC ATG G</td>
</tr>
<tr>
<td></td>
<td>MMSD-S2</td>
<td>Sense</td>
<td>TAT GAC ATG CTA AAG GAG G</td>
</tr>
<tr>
<td>FTDH</td>
<td>FD1</td>
<td>Sense</td>
<td>CCG ATT GAT GTC ATA GAG G</td>
</tr>
<tr>
<td></td>
<td>FD2</td>
<td>Sense</td>
<td>GTG GCG ATA CAT GGC TG</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>Sense</td>
<td>AAG GTG CCA GGA GCA TGG G</td>
</tr>
<tr>
<td></td>
<td>F1-S</td>
<td>Sense</td>
<td>GTG TTC ACG ATC CCT GAC</td>
</tr>
<tr>
<td></td>
<td>F2-S</td>
<td>Sense</td>
<td>TGG GCC GAC GAT GGC CTC</td>
</tr>
<tr>
<td></td>
<td>F3-S</td>
<td>Sense</td>
<td>GGA GCA GTA TAT ACG CTA GCG</td>
</tr>
<tr>
<td></td>
<td>F3-A</td>
<td>Anti-sense</td>
<td>CGG TAG CGT ATA TAC TGC TCC</td>
</tr>
<tr>
<td></td>
<td>F4-A</td>
<td>Anti-sense</td>
<td>ATT TTG TGG TCC ATG GTT GGT</td>
</tr>
</tbody>
</table>

Table 2.6. Gene-specific primers used for sequence extension
2.7 WESTERN BLOTS WITH SPECIFIC ELUTED ANTIBODY

2.7.1 Production of *T. circumcincta* adult and L4 extracts

**S1 extract**

An aliquot of L4 or adult worms, harvested and stored in liquid nitrogen as described in section 2.9, was thawed at room temperature. From this point, all equipment and buffers were chilled or kept on ice where possible. The worms were transferred to a sterile 20 ml tube and an appropriate volume of chilled S1 buffer added (8 ml for ~ 0.5 g adults, 6 ml for ~ 0.2 g L4). They were then homogenised using an Ultra-Turrax T25 homogeniser (IKA) at top speed (24,000 rpm) for 30 s, chilled for 30 s, homogenised again and chilled again. The mixture was then decanted into a glass homogeniser and further homogenised for 5 min. The mixture was split equally between an even number of 2 ml glass centrifuge tubes for use with the Beckman TL-100 ultracentrifuge, pre-chilled to +4°C. Each tube was weighed to ensure a good level of balance in the centrifuge. Tubes were spun at 25,000 xg for 30 min then placed on ice. Supernatant was carefully removed by pipette and stored as S1 at -80°C. Pellets were retained on ice.

**S2 extract**

The pellets retained from S1 preparation were re-suspended in an appropriate volume of chilled S2 buffer (6 ml for ~ 0.5 g adults, 4 ml for ~ 0.2 g L4) and transferred to a glass homogeniser. This material was homogenised to give a smooth mixture for 5 min and then transferred to glass centrifuge tubes as before. After centrifugation at 25,000 xg for 30 min at + 4°C, the supernatant was carefully removed by pipette and stored as S2 at -80°C. Pellets were retained on ice.

**S3 extract**

The pellets retained from S2 preparation were re-suspended in an appropriate volume of chilled S3 buffer (4 ml for ~ 0.5 g adults, 2 ml for ~ 0.2 g L4) and
transferred to a glass homogeniser. This material was homogenised and transferred to centrifuge tubes as before. These were spun at 100,000 x g for 1 h at +4°C then placed on ice. Supernatant was carefully removed by pipette and stored as S3 at -80°C. Pellets were discarded.

The protein content of extracts

This was visualised using 4-15% Tris-HCl Ready Gels (Biorad). 15 µl of each fraction was run with either 15 µl of non-reducing or 15 µl of reducing sample buffer. The gels were then stained with Coomassie Blue. These are shown in Figure 2.7.1 – 2.7.3.

2.7.2 Production of L3 extracts

Infective \textit{T. circumcincta} larvae (L3) were obtained from the faeces of experimentally infected animals by previously published methods (Knox & Jones, 1990). Due to the resilient nature of the sheath of the L3, this stage required a more aggressive form of homogenisation as provided by a ribolyser. Samples were added to special 1.8 ml tubes containing small glass beads (lysing matrix, Q-BIOGENE) and then placed in the ribolyser, which vigorously shakes the tubes, homogenising the sample. The instrument used was the FastPrep® FP120 (Savant).

S1 extract

Approximately 1g (wet weight) of L3 worms, stored at -80°C, were thawed at room temperature then split between two ribolyser tubes on ice. Chilled 1X PBS was added to the tubes until less than 1 mm from the top to prevent foam formation. Tubes were placed in ribolyser and shaken for 20 s at speed 6.5 then chilled on ice for 10 min. This was repeated another 3 times. Five µl was extracted from one tube, placed on a slide and examined using a microscope at a magnification of x100 to confirm that the worms were sufficiently disrupted. Tubes were then ribolysed and chilled once more then spun at 13,000 rpm for 10 min in a bench top centrifuged pre-
chilled to +4°C. The tubes were placed on ice and supernatant carefully removed and stored as S1 extract at -80°C. Pellets were retained on ice.

**S2 extract**

The tubes containing pellets retained from S1 preparation were topped up with S2 buffer, ribolyzed, chilled and spun in the same manner as S1. The supernatant was removed and stored as S2. Pellets again retained on ice.

**S3 extract**

The tubes containing pellets retained from S2 preparation were topped up with S3 buffer and ribolyzed, chilled and spun as before. The supernatant was removed and stored as S3.

**The protein content of extracts**

This was visualised using 4-15% Tris-HCl Ready Gels (Biorad). 15 μl of each fraction was run with either 15 μl of non-reducing or 15 μl of reducing sample buffer. The gels were then stained with Coomassie Blue. These are shown in Figure 2.7.1 – 2.7.3.

As can be seen from Figures 2.7.1 – 2.7.3, very little L4 material was available. The remaining L4 extracts were concentrated x 100 using Centricon® YM-10 centrifugal filter devices from Amicon Bioseparations as described later in section 2.10.1. The concentrated extracts were used for blotting and screening (section 2.7.7).
Figure 2.7.1. S1 extract from L3, L4 and adult *T. circumcincta*, run on 4%-15% Tris-HCl gels under non-reducing (NR) and reducing (R) conditions and coomassie-stained.

Figure 2.7.2. S2 extract from L3, L4 and adult *T. circumcincta*, run on 4%-15% Tris-HCl gels under non-reducing (NR) and reducing (R) conditions and coomassie-stained.
2.7.3 Expression of recombinant protein in liquid culture

By referring to the original library screen filters, clones that gave a strong signal after immuno-screening were selected for each protein (MMSDH, FTDH and catalyse) of interest. Excised Bluescript plasmid (see section 2.3.4) from these clones was then used to transform JM109 competent *E. coli* cells (Stratagene).

50 μl of JM109 cells were added to 3 μl of plasmid in a 1.5 ml eppendorf and incubated on ice for 30 min. The cells were then heat-shocked in a 42 °C water bath for 40 s, 200 μl of SOC medium added and the tubes incubated at 37 °C with shaking for 2 h. The transformed cells were then spread onto LB/Ampicillin/IPTG/X-gal plates and incubated overnight at 37 °C. The *lacZ* gene in Bluescript codes for beta-galactosidase, when un-interrupted by an insert. X-gal is a substrate for beta-galactosidase, forming a blue precipitate when hydrolysed. Therefore, X-gal is used, in conjunction with IPTG, in order to distinguish recombinant colonies from non-recombinants colonies, as non-recombinants will be blue in colour.

Figure 2.7.3. S3 extract from L3, L4 and adult *T. circumcincta*, run on 4%-15% Tris-HCl gels under non-reducing (NR) and reducing (R) conditions and coomassie-stained.
White colonies were inoculated into 10 ml LB/Ampicillin cultures and incubated overnight at 37 °C with shaking. Fresh 10 ml LB/Ampicillin cultures were inoculated with 0.5 ml of overnight culture and incubated at 37 °C with shaking for 2.5 h. One ml from each new culture was removed, spun at full speed for 10 min in a microcentrifuge and the pellet stored at —20 °C. The remainder of the culture was supplemented with 1 M IPTG to a final concentration of 1 mM, in order to induce recombinant protein expression, and incubated at 37 °C for a further 3 h. One ml aliquots of cell culture were spun at full speed for 10 min and the pellets stored at —20 °C. This process was carried out for one clone from each sequence of interest and also with non-recombinant Bluescript to act as a negative control.

2.7.4 Preparation of soluble and insoluble fractions from cell cultures

Each cell pellet was thawed and resuspended in 1/10 the original volume 50 mM Tris-HCl pH 8.5/ 2 mM EDTA (fraction buffer). Freshly made lysosyme was added to 100 µg/ml from a 10 mg/ml stock in fraction buffer and Triton-X-100 to 0.1% from a 1% (v/v) stock in fraction buffer. The resuspended pellet was incubated at room temperature for 30 min, supplemented with MgCl2 to 8 mM (from 80 mM stock in dH2O) and DNase and RNase both to 10 µg/ml (from 1 mg/ml stock in dH2O) and incubated for a further 30 min. The insoluble fraction was pelleted by centrifugation at full speed for 10 min in a microcentrifuge. The soluble fraction was carefully pipetted off into a fresh tube and the insoluble pellet resuspended in the desired volume of buffer. Both fractions were stored at —20 °C until further use.

2.7.5 Screening cell fractions for expressed recombinant proteins

As the protein expressed by the cultured clones is immunogenic, blots of the cell fractions were screened with serum from the same trial as that used for the library screen in order to localise the protein, identifying its presence in either the soluble or insoluble fraction and determining its molecular weight.
Serum from TSBP-vaccinated sheep was pre-absorbed with *E. coli* cells in order to reduce non-specific antibody binding. XL1 MRF cells were cultured and diluted to OD$_{600}$ ~ 0.5 as described before. Two ml of cells were freeze-thaw lysed by incubating at −80 °C for 30 min then 37 °C for 30 min, repeated twice. Four hundred µl serum was diluted 1/100 in TBST and the lysed cells added. The serum was incubated overnight at room temperature with gentle agitation, spun at 500 xg for 15 min to pellet the cell debris and the serum supernatant stored at +4 °C.

Soluble and insoluble fractions of the induced and uninduced cultures for each clone were run on SDS-PAGE gels under reducing conditions and blotted onto Immobilon membrane as described previously. The blots were then screened with the pre-absorbed anti-TSBP serum, diluted to a final concentration of 1/300 in TBST, and 1/500 anti-sheep IgG as described before and developed with DAB stain.

### 2.7.6 Elution of specific antibody from expressed recombinant proteins

10% SDS-PAGE gels were poured with a 4% stack containing one large well capable of holding up to 500 µl of sample and one small well for markers. Two hundred µl of the appropriate cell fraction was mixed with 200 µl of 2X reducing sample buffer, incubated in boiling water for 4 min and loaded on the gel along with markers. The gel was run and blotted onto Immobilon-P nylon membrane as described before. The markers were removed and Coomassie-stained and the rest of the blot was blocked overnight in 10% (w/v) Marvel® in TBST. A thin vertical strip of the blot was cut off and screened with 1/300 pre-absorbed serum as before to localise the recombinant protein band. This strip was then lined up with the rest of the blot and a horizontal strip containing the protein band cut out. This blot strip was incubated with 5 ml of 1/100 pre-absorbed serum with gentle agitation for 4 h at room temperature. The serum was poured off and the strip rinsed thoroughly in TBST. The strip was then incubated for exactly 2 min in 2 ml elution buffer before immediately adding neutralisation buffer to 10% (v/v) and horse serum to 5% (v/v) to prevent degradation of the antibody. The resulting eluted antibody was stored at −20 °C.
2.7.7 Screening worm extracts with specific antibody

S1, S2 and S3 extracts from L3, L4 and adult *T. circumcincta* were run on 10% SDS-PAGE gels under reducing and non-reducing conditions and blotted onto Immobilon-P nylon membrane as described before. The blots were blocked overnight in 10% (w/v) Marvel® and then screened with either eluted specific anti-CAT, anti-MMSD or anti-FTDH antibody or with whole pre-absorbed anti-TSBP serum. The eluted antibody was diluted 1/3 in TBST before use. Whole serum was used at 1/500. HRP-conjugated anti-sheep IgG, diluted 1/500, was used as 2' antibody and the blots were developed with FAST™ 3, 3'-dianinobenzidine tablet sets (Sigma) as before.
2.8.1 Total RNA extraction from worms

Total RNA extraction was carried out using the Nucleospin® RNA II kit from Macherey-Nagel. Worms were harvested as described in sections 2.7 (L3) and 2.9 (L4 and adult) and stored in N2. They were then broken down and ground to a powder in N2 as described in the previous section. As this kit is designed for small samples, the protocol was scaled up depending on the volume of starting material. Therefore, for ~1 g (wet-weight) L3, \( n = 6 \), for ~0.2 g L4, \( n = 6 \) and for ~0.15 g adults, \( n = 4 \).

After grinding the worms, \( n \times 350 \mu l \) of RA1 (Lysis Buffer) /1% (v/v) β-mercaptoethanol was pipetted over the frozen sample. The sample was then broken down with the pestle, while allowing it to thaw to a liquid, and was then divided between \( n \) sterile 1.5 ml eppendorfs and vortexed briefly. The sample was then divided between \( n \) filter units and spun at 12 000 x g to clear the lysate. One volume 70% (v/v) ethanol was added to each of the lysates and mixed by vortexing. Each lysate was then applied to one of \( n \) sterile RNA column/tube assemblies and spun for 30 s at 8 000 x g. Membrane Desalting Buffer (350 μl) was added to each column and spun at 12 000 x g for 1 min. Ninety five μl DNase reaction mixture was then applied directly onto the membrane of each column and incubated at room temp for 15 min. The membrane was then washed once with RA2 (DNase Stop Solution) and twice with RA3 (Wash Buffer II) and spun at 12 000 x g for 2 min to dry. The RNA from each column was eluted in 60 μl RNase-free water.

The eluates were pooled in a sterile 1.5 ml eppendorf. RNA was concentrated by ethanol-precipitation. One tenth volume 3 M sodium acetate and 3 volumes 100% ethanol were added, vortexed and centrifuged at 14, 000 x g for 30 min. The supernatant was removed and the pellet dried in an air cupboard for 5 min then resuspended in the desired volume of RNase-free water. Spectrophotometric and electrophoretic analyses were carried out to confirm the quality of the RNA.
2.8.2 First-Strand cDNA synthesis

First-strand cDNA was synthesised from total RNA with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). One μl of a 10 mM dNTP mix and 0.5 μg oligo (dT) primer were added to 5 μl total RNA in a sterile 0.5 ml eppendorf and the total volume made up to 10 μl with DEPC-treated water. This was incubated at 65 °C for 5 min then chilled on ice for 2 min. Two μl 10X Reaction buffer, 4 μl 25 mM MgCl₂, 2 μl 0.1 M DTT and 1 μl RNase OUT were then added and the mixture incubated at 42 °C for 2 min. One μl Superscript Reverse Transcriptase was added and the mixture incubated at 42 °C for 50 min and 70 °C for 15 min. The mixture was spun briefly to collect the tube contents, 1 μl RNase H added and incubated at 37 °C for 20 min. Two μl of the mixture was run on 0.8 % (w/v) agarose to visualise the cDNA, which should appear as a smear on the gel. The first strand cDNA was stored at —20 °C till further use.

2.8.3 PCR from first-strand cDNA

A pair of gene-specific primers for each of the three genes of interest was used to amplify different sized regions by PCR. A pair of primers designed to target conserved regions of *T. circumcincta* and *O. ostertagi* actin EST sequences, available online, were used as a positive control to ensure that the PCR reaction from cDNA was viable (Table 2.8.3). These were used in PCR reactions with approximately equivalent amounts of cDNA from each life stage as template. A negative control, containing the PCR mix and primers but lacking the cDNA template was also added to detect any contamination.
<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequences (5’ to 3’)</th>
<th>Expected size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>P40A (sense)</td>
<td>ATT CGG GAT CCA ATC AAT TCC CG</td>
<td>613bp</td>
</tr>
<tr>
<td></td>
<td>P40B (antisense)</td>
<td>TCT GTG TAC GAG AAT AGT CGA CCC</td>
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<tr>
<td></td>
<td>ACT-RTC2 (antisense)</td>
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<td></td>
</tr>
</tbody>
</table>

Table 2.8.3. Primer pairs for RT-PCR and expected size of product.
2.8.4 Detection of gene-specific products with DIG-labelled probes

In order to confirm their specificity, the RT-PCR products were blotted and probed with gene-specific DIG-labelled probes.

Southern blot of RT-PCR products

Roughly equivalent amounts of each PCR product were run on a 2% (w/v) agarose gel along with negative and positive controls suitable for each gene. The negative control was the template-free PCR from above. The positive control was a PCR product from an original P0 template from each gene to ensure that the probes bound to that sequence. This gel was blotted onto positively-charged membrane (Boehringer Mannheim) using alkaline southern blotting as shown in Figure 2.8.4. A platform was created by balancing a glass plate on two glass tubes lain over a glass dish. NaOH (0.4 M) was used as the alkaline blotting buffer. A wick was made with a long strip of filter paper, the same width as the gel, soaked in buffer then draped over the glass plate with the ends dipping into the buffer in the glass dish. The gel and a piece of membrane cut to the same size were lain on top of the wick followed by three layers of NaOH-soaked filter paper and three layers of dry filter paper. A thick pile of paper towels was then added and a weight placed on top. The blot was left overnight at room temperature.

Fig. 2.8.4. Southern blotting apparatus.
DIG-labelled DNA probing

This was carried out using the Biochemica DIG Luminescent Detection kit (Boehringer Mannheim) as before. Three specific DNA probes, approximately 500 bp in length, were created by PCR using freeze-thaw lysates from plaque stock of the original CAT, MMSD and FTDH POs as template. Each of the PCR products was cleaned using the QIAQuick method as described before.

Ten µl of each probe was added to 5 µl dH₂O in a sterile tube and denatured by boiling for 10 min then chilling on ice. The probes were DIG-labelled and stored according to the manufacturers instructions.

The blot was cut into appropriate sections, in this case one each for CAT, MMSD and FTDH. Each piece was placed in a Hybaid bottle and rinsed for 30 min at room temperature with 10 ml 2X SSC on a rotator. The SSC was removed and the membrane pre-hybridised with 10 ml pre-warmed DIG Easy Hyb buffer for 4h at 42 °C. Each 50 µl probe was diluted in 40 ml pre-warmed DIG Easy Hyb, 20 ml of which was added to the membrane. Hybridisation was carried out at 42 °C overnight.

Following hybridisation, the membrane was washed twice in 20 ml 2X SSC/0.1% (w/v) SDS at room temperature and twice in 20 ml 0.1X SSC/0.1% (w/v) SDS (pre-warmed) at 68 °C. Probe detection was carried out according to the manufacturers instructions and the blots were exposed to X-ray film for an appropriate length of time and developed.
2.9 IN VITRO CULTURE OF T. CIRCUMCINCTA

2.9.1 Culture of L4

Infection regime and harvesting of worms

Donor lambs (6 to 10 months old) were given approximately 100,000 infective L3 orally. L4 parasites were harvested from the abomasa at necropsy, 7 days post infection, using established procedures. Briefly, the abomasa were incubated in physiological saline (0.85% (w/v) NaCl) to release the L4 from the glands. The larvae were then cleaned either by sedimentation or agar migration, both of which are performed at 37 °C for 4 h.

Cleaning and counting worms

The above process for harvesting L4 worms did not remove all contaminating host stomach contents so the worms were cleaned further using a Baerman apparatus. A funnel, the outlet fitted with a clamped tube, was placed in a clamp stand. A sieve, over which a piece of muslin was secured, was placed in the bowl of the funnel. Physiological saline, warmed to 37 °C, was poured into the funnel (with tube clamp closed) until the level was just over the muslin. The worms were poured onto the muslin and teased apart gently with a needle. The whole assembly was placed in a warm room at 37 °C. The worms migrated through the sieve and collected above the tube clamp. To harvest the clean worms, the clamp was slowly loosened and the worms, plus approximately 25 ml of saline, were poured into a clean plastic tube. The clamp was then closed, the funnel refilled with saline and the process repeated until it appeared that most of the worms had been collected. All the worms were pooled together and most of the saline carefully poured off. A 50 μl aliquot of worms was pipetted onto a circular grid plate and the worms counted under a dissecting microscope in order to estimate the total yield. Several aliquots were removed and stored in liquid N2, for use in protein extract preparation and RNA extraction, and the remaining worms were cultured.
Culturing worms

Approximately 50,000 L4 worms were rinsed briefly in RPMI-1640 (Sigma) then transferred aseptically to a sterile 250 ml conical flask containing 200 ml of RPMI-1640, pre-warmed to 37 °C. The flask was then incubated at 37 °C with gentle agitation. After 2 h, the culture fluid was carefully poured off aseptically into sterile 50 ml tubes which were then stored at -80 °C. This was done with as little disturbance to the clump of worms as possible. Another 200 ml of RPMI-1640 was added to the flask aseptically and the worms incubated again at 37 °C. Further harvesting of the culture medium took place at 21 h, 40 h and 48 h. At 40 h, a 100 μl aliquot of culture was removed and the worms observed under a dissecting microscope to check their integrity.

2.9.2 Culture of adults

Infection regime and harvesting of worms

For adult worms, donors were again given approximately 100,000 L3 orally. Donor necropsy and harvesting was carried out at 21 days post infection. Adults are found in the contents and also associated with the abomasal surface. The abomasal mucosa was sloughed off manually and the worms were again cleaned by sedimentation in physiological saline.

Cleaning and counting worms

Adult worms, harvested as described above, were largely free of contaminating debris and so did not require Baermanisation. They were rinsed briefly in dH2O, a 50 μl aliquot was counted as before and the estimated total yield calculated. Most of the excess liquid was removed and small clumps of worms were separated off for storage in N2. The remaining worms were cultured.
Culturing worms

Approximately 67,000 adult worms were rinsed briefly in RPMI-1640 then cultured in the same way as the L4 but in 400 ml of RPMI-1640 in a 1 l conical flask. Again, at 40 h, an aliquot of worms was observed under the microscope to check their integrity.

2.10 ANALYSIS OF ES MATERIAL

2.10.1 Concentration of culture fluids

Culture fluids from adult and L4 worms were concentrated x250 using Centricon® YM-10 centrifugal filter devices from Amicon Bioseparations. The culture fluid is concentrated as centrifugal force drives solvents and low molecular weight solutes (<10 kDa) through low-adsorption, hydrophillic membranes. Larger proteins are retained in the sample reservoir and concentrated as the sample volume is reduced. Centricons were spun at 3000 rpm, in a Jouan CR 4.22 centrifuge pre-chilled to 4 °C, until the desired reduction in volume was achieved. Initial concentration was carried out in 15 ml total volume centricons and further concentration of smaller volumes in 2 ml centricons. The red pH indicator present in RPMI can interfere with spectrophotometer reading so in order to measure the protein concentration, a small volume of each concentrated sample was dialysed with 1X PBS to remove this dye. Dialysis was carried out by adding 3X the sample volume of PBS to the concentrated sample then concentrating down to the original volume in a Centricon as above. This was repeated until the sample appeared clear to the eye.
2.10.2 BCA Protein Assay

The BCA protein assay (Pierce) was used to determine the protein content of L4 and adult culture fluids that had been concentrated x250 and dialysed with PBS. A series of BSA (Bovine serum albumin – Sigma) dilutions in 1 x PBS were used as standards for the assay. An original 2000 μg/ml solution was made up then diluted to give 1,500, 1,000, 750, 500, 250, 125, 25 and 5 μg/ml dilutions. One in ten dilutions of x250 culture fluid from each time point for L4 and adults were also made up in 1 x PBS. A flat-bottom 96-well ELISA plate were set up with 25 μl of standard or sample added to each well as shown in Figure 2.10.2.

![BSA Standards](image)

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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
</tbody>
</table>

**Figure 2.10.2. Plate set-up for BCA assay.**

The BCA working reagent was made up as described in the manufacturer’s instructions and 200 μl added to each active well. The plate was placed on a shaker for 30 s to mix, covered in cling film and incubated at 37 °C for 30 min. The OD_{562} for each well was read using an EL_{x}808 Ultra Multiplate Reader (BIO-TEK) which automatically compares samples with standards and calculates the blank-corrected
protein concentration for each. This value was then multiplied by ten to give the
ccentration for each neat sample.

2.10.3 One dimensional (1-D) electrophoresis of ES material

The concentrated culture fluids were run on 4-15% Tris-HCl ready gels
(Biorad) under reducing and non-reducing conditions and stained with Coomassie
blue in order to visualise the proteins present.

2.10.4 Two dimensional (2-D) Electrophoresis of ES material

Preparation of ES material for 2-D Electrophoresis

Concentrated ES material was prepared for 2-D electrophoresis using the
PlusOne™ 2-D Clean-Up Kit (Amersham). This kit precipitates the proteins present,
leaving behind any substances that might interfere with the electrophoresis, such as
detergents, salts or lipids. The proteins are then resuspended in a solution suitable for
the first dimension.

The clean-up was performed according to the manufacturer’s instructions.
One hundred µl of x50 concentrated ES was transferred to a 1.5 ml eppendorf. Three
hundred µl precipitant was added and the mixture was vortexed and incubated on ice
for 15 min. 300 µl of co-precipitant was added and the mixture vortexed. The tube
was then centrifuged at 13 000 x g for 5 min and the supernatant removed carefully.
The tube was spun again briefly and any remaining supernatant removed with a fine
pipette tip. Twenty five µl dH₂O was added to the pellet and vortexed for 10 s. One
ml of wash buffer and 5 µl of wash additive were added and the tube vortexed. The
resuspended pellet was incubated at −20 °C for 30 min, vortexing for 30 s every 10
min, then spun at 13 000 x g for 5 min. The supernatant was discarded and the pellet
allowed to air-dry for 5 min before resuspending in 100 µl Rehydration buffer.
First Dimension

The first step in 2-D electrophoresis is Isoelectric Focussing (IEF) which is described as 'electrophoresis in a pH gradient set up between a cathode and anode, with the cathode at a higher pH than the anode' (Texas A&M University website – www.ntri.tamuk.edu/if/if.html). Proteins present will migrate and concentrate at their isoelectric points, depending on the amphoteric properties of their component amino acids. IEF of *T. circuncincta* ES proteins was carried out using 11 cm, pH 3-10 Immobiline™ DryStrips (Amersham). Two hundred µl of ES material, resuspended in Rehydration buffer, was loaded between the electrodes in an 11 cm IPG tray. An 11 cm, pH 3-10 DryStrip was carefully placed, gel-side down, in the tray and overlain with PlusOne DryStrip cover fluid (Amersham) before replacing the tray lid. The tray was then placed on an Ettan IPGphor (Amersham) and IEF was run overnight using the following program:

12 h - 30 V, 1 h - 150 V, 1 h - 300 V, 1 h - 600 V, 1 h -1200 V, 1 h - 2400 V, 4 h - 800 V.

The strip was placed along the side of a petri dish, gel side out. Two hundred mg DTT in 20 ml Equilibration buffer was added to the dish, incubated at room temperature with gentle agitation for 10 min and then the buffer poured off. Eight hundred mg of iodoacetamide in 20 ml Equilibration buffer (vortexed to dissolve) was then added and the dish incubated for 10 min as before. The strip was removed and carefully blotted to remove excess buffer.

Second Dimension

The second step in 2-D electrophoresis is SDS-PAGE. The proteins that have undergone IEF are further separated by molecular weight. This was performed using the horizontal Multiphor II (Amersham) flatbed system with precast ExcelGel 12-14% gradient SDS gels.

Three ml of DryStrip cover fluid was dropped on to the surface of the multiphor and the SDS gel placed, acetate side down, over this. Bubbles under the gel
were carefully smoothed out. The plastic cover was removed from the gel and the positive (yellow) and negative (white) buffer strips were carefully laid over the edges of the gel. Two equilibrated DryStrips were placed along the inside edge of the white buffer strip, gel side down, with a small space between their ends. One μl of protein standards (Mark 12™, Invitrogen) were dropped onto a 50 mm square of filter paper and laid between the two strips. The electrodes were positioned directly over the buffer strips and the lid was replaced. The multiphor was run at 20 mA for 20 min. The strips and marker paper were then removed, the white buffer strip moved to cover the position previously occupied by the DryStrips and the electrode moved to this position also. The lid was then replaced and the multiphor run at 40 mA for 90 min or until the blue dye front had reached the yellow buffer strip. The buffer strips were carefully removed and the gel stained.

Automatic Staining

2-D SDS-PAGE gels were stained using the PlusOne Protein Silver Staining Kit and Hoefer Processor Plus automatic stainer (Amersham), according to the manufacturer’s instructions. The gel image was then captured using an Amersham ImageScanner.

2.10.5 Liquid Chromatography and Tandem Mass Spectrometry/N-terminal sequencing

Concentrated 21 h culture fluids from adult (x250) and L4 (x500) cultures were run individually on 4-15% gradient Tris-HCl gels as before and stained with Coomassie-blue stain. All visible bands were individually labelled and excised, taking care to include as little unstained gel as possible. Each band was placed in a labelled 1.5 ml eppendorf tube and submitted to the Sir Henry Wellcome Functional Genomics Facility (SHWFGF), at the University of Glasgow, for Tandem Mass Spectrometry and N-terminal sequencing.

The details of the methods involved in producing the peptide mass fingerprints and sequences are available on the SHWFGF website.
Briefly, each band was washed in 100 mM ammonium bicarbonate followed by 50% (v/v) acetonitrile/100 mM ammonium bicarbonate. The gel was then shrunk in 100% acetonitrile and dried in a vacuum centrifuge. In-gel digestion of the protein was carried out by reswelling the gel, in 0.2 mg trypsin in 10ml of 25 mM ammonium bicarbonate, then covering in 20 ml of 25 mM ammonium bicarbonate and incubating at 37°C overnight. Each sample was cleaned-up using a C18 ziptip (Millipore), according to the manufacturer’s instructions. A Nano-Liquid Chromatography module, coupled to the mass spectrometer, was employed to further clean, concentrate and separate the peptides. The resulting peptides for each band were then analysed using the Q-Star Pulsar Mass Spectrometry/Mass Spectrometry (MS/MS) system from Applied Biosystems. This system consists of a Quadropole and TOF (Time of Flight) mass spectrometer. In the first step (MS1), peptides are ionised and the time required for each accelerated ion to travel a defined distance in a field free zone, which is directly related to the mass of the peptide, is measured to produce a peptide mass spectrum or fingerprint. The Quadropole, which acts as a mass filter, can select a peptide ion from this spectrum, which is then fragmented by collision with inert gas. This produces a number of fragment ions, the mass of which are again measured (MS2). The resulting spectrum is used to deduce the peptide sequence, as shown in Appendix 3. The results were analysed using the MS/MS Ion Search function of the Mascot search engine (www.matrixscience.com).

The advantage of using this tandem MS/MS system, especially for organisms for which protein mass data is limited, is that sequence databases may also be searched. This greatly increases the chance of identifying a particular protein.
CHAPTER THREE – ISOLATION OF cDNA CLONES ENCODING PUTATIVE COMPONENTS OF T. CIRCUMCINCTA TSBP

3.1 INTRODUCTION

Extensive studies have shown that an integral membrane protein fraction, enriched for cysteine protease activity (S3 TSBP), from adult H. contortus is a highly effective vaccine candidate (Knox & Smith, 2001). Protection trials evaluating the equivalent fraction from T. circumcincta have resulted in variable degrees of protection against infection with this parasite. The best protection effects observed to date were 63% and 83% reductions in worm burdens and cumulative faecal egg output respectively (Knox, Smith, Smith et al., 1995), protective effects which would be adequate to control infection under field conditions. However, other trials have given reductions in worm burden as low as 20% and the reasons for this variation are unclear.

TSBP consist largely of membrane-bound proteins expressed in the gut of the parasite. These were purified by applying Triton X-100 soluble worm extracts to a thiol-sepharose column and eluting the bound material (Knox, Smith, & Smith, 1999). As noted above, they are enriched for cysteine protease activity. Vaccination stimulates the host to produce specific antibodies against proteins within the vaccine and immunolocalisation studies have shown that serum antibody solely targeted proteins on the microvillar surface of the intestine (Knox, Smith, Smith et al., 1995). Specific antibody ingested by the parasite may somehow impair parasite digestion by either mechanically disrupting the gut, binding to the luminal surface and preventing absorption, or by inhibiting the action of functional proteins such as enzymes expressed on the surface of the gut, resulting in worm damage or death. Certainly, the latter mode of action is thought to mediate protective immunity when the same vaccination strategy is employed against H. contortus (Knox & Smith, 2001). It has been shown that, although T. circumcincta does ingest some antibody in vivo, it is at
a much lower level than *H. contortus* (Murray & Smith, 1994). Successful vaccination against the latter is, to an extent, dependent on antibody titre to the target protein (Murray & Smith, 1994). Therefore, a vaccine against *T. circumcincta* directed at proteins expressed on the surface of the gut may need to target individual proteins to stimulate a sufficiently high titre of specific antibody for a protective effect to be observed. If the constituent or constituents of the TSBP fraction responsible for inducing a protective response could be identified, it may be possible to refine the purification strategies employed to yield a simpler protein mixture, which would produce a more consistent host response.

cDNA library immunoscreening is a technique that allows the identification of the target organism genes encoding the proteins that are recognised by antibody present in the serum of an immunised sheep. A cDNA library is produced by extracting mRNA from the organism, in this case adult *T. circumcincta*, which is then used as a template to create single-stranded cDNA. Each eukaryotic mRNA sequence has a 3' poly A region and can therefore be reverse transcribed with a reverse transcriptase enzyme and a poly dT primer, resulting in mRNA/cDNA hybrids. The mRNA is then ‘nicked’ to form primers with which DNA polymerase synthesises the second strand cDNA. This gives a double-stranded DNA copy of every gene expressed in the worms at the time the extract was prepared. In this case, the cDNA inserts, prepared using a ZAP®-cDNA Synthesis kit (Stratagene), were cloned into Lambda Uni-ZAP XR vector phage, containing the Bluescript phagemid with sequence for ampicillin resistance and blue-white colour selection. The library was packaged with a ZAP®-cDNA Gigapack III Gold Cloning kit (Stratagene) to form phage particles and used to infect XL1 MRF⁻ *E. coli* cells. These cells were grown on agar plates and, in areas where phage gene transcription was activated, the cells were lysed to form plaques releasing the expressed recombinant protein. The expressed protein was absorbed by overlain nitrocellulose membranes, which were then screened with serum to identify plaques expressing immuno-reactive proteins. The serum used for screening was pre-challenge serum from an animal that showed a high level of protection after vaccination with TSBP, as judged by reduction in egg and final worm burdens post-challenge.
The nucleotide sequence of the cDNA insert in POs can be determined, translated into a predicted amino acid sequence and these sequences can then be used to search all the available databases for homologues. This procedure allows the nature of the unknown protein to be identified in most cases. Where homologues are not identified, information on the likely function of the unknown protein can be derived by identifying regions of amino acid conservation in the sequence that have been associated with a particular function in other proteins. In this way, the proteins recognised by the host immune response can be characterised.
3.2 RESULTS

3.2.1 Western blot analysis of TSBP

A vaccination trial with *T. circumcincta* TSBP proteins gave variable levels of protection against a single challenge infection between individuals. Details of this trial are given in Chapter 2. Table 3.2.1 shows the protection data for the vaccinated animals in this trial.

<table>
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<tr>
<th>Sheep</th>
<th>% protection eggs</th>
<th>% protection worms</th>
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<tr>
<td>9</td>
<td>56.8</td>
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</tr>
<tr>
<td>10</td>
<td>74.8</td>
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<tr>
<td>14</td>
<td>4.7</td>
<td>-77</td>
</tr>
</tbody>
</table>

*Table 3.2.1.* Percentage of protection of animals vaccinated with TSBP proteins and given a single challenge infection of *T. circumcincta*. Protection was measured by individual reductions in faecal egg counts and abomasal worm numbers, compared with adjuvant-only controls.

Three of the vaccinated animals (No. 9, 10 and 13) showed considerable reductions in both egg and worms numbers. However, the remaining three animals showed no reduction or higher egg or worm counts than controls. This degree of variation was not present in the previous trial, mentioned in this chapter’s introduction, where consistently high levels of protection were observed. However, this trial involved the use of Freund’s complete adjuvant for the first injection and Freund’s incomplete for the boosts, whereas the adjuvant in the current trial was QuilA. TSBP preparation also differed between the two trials. The TSBP in the first
trial were prepared from mixed stages whereas in the current trial they were only from adult *T. circumcincta*.

*T. circumcincta* TSBP were prepared from adults in the same manner as that used in the trial. Adult *T. circumcincta* were successively solubilised (Chapter 2, Section 2.7.1), as shown in Figure 3.2.1, to give the S3 fraction, composed predominantly of membrane-bound proteins. S3 was then applied to a Thiol-sepharose column and bound material was concentrated 20-fold. TSBP were visualised on a Coomassie-stained 10% SDS-PAGE gel. TSBP comprise a number of proteins with one at ~55 kDa being particularly concentrated in the thiol-sepharose bound material (Figure 3.2.2, Panel A). As can be seen from the substrate gel (Figure 3.2.2, Panel B, lane C), TSBP contains protease activity at approximately 36.5 and 45 kDa. This activity disappeared in the presence of the cysteine protease inhibitor, E64. Western blots were carried out, as described in Chapter 2, to visualise the recognition of proteins in the TSBP fraction by serum from the vaccinated animals and a control animal.

Sodium periodate oxidation is commonly used to disrupt carbohydrate epitopes present in a protein sample. One blot was periodate-treated in order to block binding of immunoglobulins to areas of glycosylation so that only reactivity against the protein epitopes could be detected. The results of this can be seen in Figure 3.2.2. It is evident that periodate treatment does decrease reactivity of the serum with the blot, with some faint bands disappearing altogether. The prominent band, at approximately 57 kDa, is strongly recognised by serum from all 6 immunised animals both before and after periodate treatment (Fig. 3.2.2, panels C and D, 9-14). There is also a faint recognition with serum from the control animal (Fig. 3.2.2, panels C and D, 14), but this may be non-specific binding of antibodies to this prominent protein band. A fainter band at about 66 kDa is also present in all immunised animals but is absent in both blots for the control. A weaker band can also be seen at 35 kDa but this is greatly depleted after periodate treatment and can only be seen in animals 11, 12 and 13. Although there do appear to be differences in the pattern of protein recognition between individuals, there are no consistent differences between animals showing high and low levels of protection.
**Figure 3.2.1.** Coomassie-stained SDS-PAGE gel of adult *T. circumcincta* fractions. S1 = Soluble fraction, S2 = Membrane-associated fraction, S3 = Membrane-bound fraction.

**Figure 3.2.2.** Coomassie-stained SDS-PAGE gel (Panel A), gelatin substrate gel (Panel B) and western blots of TSBP (Panels C and D). Blots were probed with sera from TSBP-vaccinated sheep (# 9-14) and one control animal (# 15). Blot 2 was periodate-treated prior to probing. Bands marked * are artefacts from the blot-mounting method.
3.2.2 cDNA Library Screen

Approximately 40,000 plaques of the adult *T. circumcincta* Uni ZAP-XR cDNA library were screened with pre-challenge serum from a TSBP-vaccinated animal. This animal is not present in Table 3.2.1 as it was used only for library screening. Post-challenge, it exhibited protection levels, in reduction of FEC and in estimated worm numbers, of 47%. Serum from animals in the same trial recognised a number of *T. circumcincta* proteins (Figure 3.2.3), in the size range 24 to 66 kDa. Incubating over-lain membranes, firstly in serum, then in HRP-conjugated anti-sheep IgG and finally developing with DAB, identified recombinant plaques expressing immuno-reactive proteins. Plaques expressing proteins recognised by the serum are known as immunopositives or POs and are stained visibly darker than the background plaques, as can be seen in Figure 3.2.3.

![Filter with clear immunopositive plaque (circled).](image)

Forty-five POs were identified by immuno-screening in this manner and were picked and stored for further analysis.
3.2.3 Visualisation of PO inserts

POs were plaque-purified and the insert DNA was amplified by PCR with vector primers as described in Materials and Methods. The distance between the T7 and T3 primer sites in the wild-type vector is 130 bp. This must therefore be subtracted from the size of the PCR product to give the true insert size. The products, ranging in size from 0.7 to over 4.0 kb, were visualised in agarose gels as shown in Figure 3.2.4. Clone inserts, therefore, ranged from approximately 0.67 to 3.87 kb in size.
Figure 3.2.4. Insert DNA, amplified by PCR of freeze-thaw lysates from PO plaque stocks and visualised on 0.8% (w/v) agarose gels.
3.2.4. Initial sequencing and probing of clones

The first 14 PO inserts to be amplified and purified were submitted for a single run of sequencing with the 5' T3 vector primer. The resulting sequences were used to search online databases for homologues. In this manner, it was established that clones sharing a high level of identity (> 70%) with either MMSD or FTDH sequences were highly represented in this subset. The eight MMSD-like clones all had inserts of approximately 1.75 kb in size and are represented by bands 16, 20, 23, 25, 27, 28, 29 and 38 in figure 3.2.4. The three FTDH-like clones had inserts ranging from approximately 1.75 to 2.65 kb in size and are represented by bands 15, 19 and 40 in Figure 3.2.4.

On the basis of these results, it was decided to screen the remaining unsequenced POs for further MMSD or FTDH-like clones. Sequence-specific primers were used to create DIG-labelled DNA probes, which were used to screen dot-blots of insert DNA. The developed dot-blots can be seen in Figure 3.2.5. By comparing the strength of staining of each spot with known positives (circled) and negatives (underlined), it was possible to identify a further six MMSD-like sequences. These are represented by bands 8, 9, 18, 34, 35 and 36 in Figure 3.2.4, and all had inserts of approximately 1.75 kb, save PO 35 which gave a PCR product of less than 1.0 kb. No further FTDH positives were identified. This analysis established that of the original 45 POs, 14 contained inserts encoding MMSD-like proteins, 3 contained inserts encoding FTDH-like proteins and 28 contained inserts encoding other proteins.
Figure 3.2.5. Dot blot of POs incubated with DIG-labelled MMSD and FTDH probes.
3.2.5 Sequencing of unidentified clones

Clones not identified as either MMSD or FTDH-like were submitted for an initial single run of sequencing with the vector primer T3. As before, the resulting nucleotide sequences were used to search online databases for homologues. The results from these as well as those from section 3.2.3 are summarised in Figures 3.2.6 and 3.2.7.

![Bar chart](image)

**Figure 3.2.6.** Results of preliminary sequence analysis of 45 POs.

The majority of the newly sequenced inserts showed significant similarity to galectin. These sixteen inserts were all approximately 0.6 kb in size. Thirteen of these sequences aligned together to give a 503 base consensus. On translation, this consensus sequence showed highest identity (51%, 6e-30) with a 16 kDa *H. contortus* galectin. Interestingly, although several *T. circumcincta* galectin sequences are present in the databases searched, these clones shared a significantly higher level of identity with *H. contortus* galectin. This *H. contortus* galectin is of a different type to galectins previously identified from parasitic nematodes as will be discussed later. This suggests that a novel *T. circumcincta* galectin gene may have been isolated. The
remaining three galectin sequences aligned together to give a consensus that, on translation, also shared a high level of identity with the same *H. contortus* protein.

Two sequences aligned to give a consensus that showed significant similarity with vitellogenin from *C. elegans* and other species. These clones each had a large insert of approximately 5.0 kb. One PO, with an insert size of approximately 1.65 kb, had a nucleotide sequence that shared over 70% identity in a 1.5 kb overlap with *C. elegans* mRNA for 'catalase 2'. It also gave highly significant scores with *Ascaris suum* mRNA for catalase and catalases from many other species. Translation of the partial nucleotide sequence gave a continuous predicted protein sequence of 148 amino acids that again shared the highest level of identity with *C. elegans* 'catalase 2'. Surprisingly, considering that TSBP is enriched for cysteine protease activity, no cysteine protease homologues were identified. Clones encoding sequences similar to MMSD, FTDH and catalase were chosen for further analysis as these enzymes have not been described previously in this species.

Two POs, classified as unidentified above, gave low scoring database hits as they shared only limited similarity over a short overlap, one with a *Caenorhabditis briggsae* hypothetical protein, and one with a *C. elegans* nuclear anchorage protein. Several clones did not produce any significant database hits. These results are summarised in Figure 3.2.7.
<table>
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<th>Homologue identity</th>
<th>POs</th>
<th>% Identity</th>
<th>E-value</th>
<th>Organism</th>
<th>NCBI Accession No.</th>
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</thead>
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<td>51</td>
<td>6e-30</td>
<td>H. contortus</td>
<td>CAB71314</td>
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<tr>
<td>Proto-type Galectin</td>
<td>14,22,31</td>
<td>50</td>
<td>1e-25</td>
<td>H. contortus</td>
<td>CAB71314</td>
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<tr>
<td>Vitellogenin 5</td>
<td>3,7</td>
<td>31</td>
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<tr>
<td>MMSD</td>
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<td>71</td>
<td>e-180</td>
<td>C. elegans</td>
<td>MMSA_CAEEL</td>
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<tr>
<td>FTDH</td>
<td>19</td>
<td>71</td>
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<td>69</td>
<td>e-118</td>
<td>C. elegans</td>
<td>NP_502054.1</td>
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<tr>
<td>(Similar to FTDH)</td>
<td></td>
<td></td>
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<tr>
<td>Catalase</td>
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<tr>
<td>Hypothetical protein</td>
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<td>48</td>
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<td>C. briggsae</td>
<td>CAE63044.1</td>
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<tr>
<td>Nuclear anchorage protein 1</td>
<td>39</td>
<td>40</td>
<td>4e-42</td>
<td>C. elegans</td>
<td>DAA04553.1</td>
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</tbody>
</table>

**Figure 3.2.7.** Summary of top database hits for immunopositive sequences. PO numbers refer to Figure 3.2.3.
cDNA library screening can be used to identify parasite-derived proteins that are recognised by antibodies in host serum and may be important factors in the development of protective immunity. In this study, approximately 40,000 plaques of an adult *T. circumcincta* cDNA library were screened with serum from an animal showing a high level of protection against challenge with the same parasite. This animal was part of a group, each of which had been vaccinated with a parasite extract in the same way but showed variable degrees of protection to a single challenge infection with *T. circumcincta* compared with non-vaccinated controls. The library was constructed using the Lambda Uni-ZAP XR kit (Stratagene), which produces recombinant cDNA inserts that are directionally cloned, easily excised within the Bluescript plasmid and can be isolated by blue/white colour selection. The intention of this study was to identify clones encoding *T. circumcincta* proteins of potential importance in stimulating the host protective immune responses associated with vaccination. Such proteins could have a function in maintenance of the parasite within the host, be instrumental in the pathogenesis of the infection or be important triggers in the development of protective immunity by the host stimulated by exposure to infection.

As stated earlier, the antigen extract (TSBP) prepared from adult parasites was obtained by passage of membrane-bound proteins over a Thiol-Sepharose affinity column. This medium has affinity for proteins carrying free sulphhydryl (SH) groups, amongst which are the cysteine proteases. A relatively small subset of parasite proteins bound to the matrix amongst which were cysteine proteases and several of these appeared to be recognised by the serum used for the immunoscreen (Figure 3.2.2).

A total of 45 POs were identified by the immunoscreen. These POs were plaque-purified and their inserts amplified by PCR with primers directed at the vector. Preliminary analysis of selected inserts was carried out using a single run of sequencing with the T3 vector primer. This identified eight POs with similarity to MMSD. The closest homologue was *C. elegans* MMSD (MMSA_CAEEL), sharing
71% identity at the amino acid level. Probing of dot-blots, with DNA amplified from a region of sequence common to these inserts, identified six more MMSD-like clones. Three POs with similarity to FTDH were also identified by sequence analysis but no further were found by probing dot-blots. Of the remaining unidentified POs, the majority showed similarity to a _H. contortus_ galectin.

Galectins or β-galactoside-binding lectins have been identified in a range of vertebrates and invertebrates including several parasitic nematodes. However, their functions are not yet clear as they appear to be involved in many biological processes such as differentiation and morphogenesis (Kasai & Hirabayashi, 1996). They are characterised by the presence of a carbohydrate recognition domain (CRD) and have an affinity for β-galactoside sugars (Greenhalgh, Beckham, & Newton, 1999). Galectins can be divided into three groups: proto-type (single CRD); chimera-type (CRD fused to a collagen-like repeating domain); and tandem repeat-type (two fused CRDs in tandem) (Kasai & Hirabayashi, 1996). Several tandem repeat-type and proto-type galectins have been identified in _C. elegans_ (Hirabayashi, Ubukata, & Kasai, 1996). However, only tandem repeat-type galectins have been identified in _T. circumcincta_ to date (Newton, Monti, Greenhalgh et al., 1997; Greenhalgh, Loukas, Donald et al., 2000). The _H. contortus_ galectin, to which the _T. circumcincta_ clones in the present study show the highest level of similarity, has been identified as a proto-type galectin according to its database entries (CAB71314, Q9NG17), as it only possesses a single CRD domain. It is likely, therefore, that the clones identified in this library screen represent a novel _T. circumcincta_ galectin. Further analysis, of the full coding sequence for this _T. circumcincta_ galectin, would be required in order to confirm the number of CRD domains present. The cloning of a tandem repeat-type galectin from the gut of _H. contortus_, was described by Newlands _et al_ (1999). The recombinant protein was identified by immuno-screening a cDNA library with serum from sheep vaccinated with _Haemonchus_ galactose-containing glycoprotein complex (H-gal-GP) derived from the surface of the parasite’s gut. Vaccination with native H-gal-GP results in highly significant reductions in egg and worm count post-challenge (Smith, Smith, & Murray, 1994). It was postulated, therefore, that this galectin may be the protective constituent of this complex. However, vaccination with the native _H. contortus_ galectin did not induce a protective immune response.
On the basis of these results, it was decided not to further characterise the *T. circumcincta* galectin homologues identified here. However, these putative galectin sequences, and others isolated during this study, will be discussed further in Chapter 8.

The sequence of two POs showed 31% identity (2e-6) with a vitellogenin from *C. elegans* (NP_508589). Vitellogenin homologues were also highly represented in the results of an immunoscreen of an *O. ostertagi* cDNA library with anti-ES serum raised in rabbits (Vercauteren, Geldhof, Peelaers et al., 2003). Vitellogenins are the major constituents of the egg yolk in most oviparous animals and are highly conserved among vertebrates, insects and nematodes (Chen, Sappington, & Raikhel, 1997). These proteins are released when egg production is already underway rather during the establishment of an infection. If used as a vaccine, they are therefore unlikely to reduce worm burdens. However, stimulation of an immune response directed at the eggs may be effective at reducing any further pasture contamination.

Of the unidentified PO sequences, one shared limited similarity (48% identity, 9e-15) with hypothetical protein CBG07311 from *C. briggsae* (Accession no. CAE63044.1). Another shared low level similarity (40% identity 4e-42) over a small region with *C. elegans* nuclear anchorage protein 1 (ANC-1). Others did not provide any significant hits with sequence data available at present. These sequences were not investigated further in this study.

MMSD-like sequences were highly represented in this immuno-screen. MMSD is an enzyme involved in valine and pyrimidine metabolism. Mammalian MMSDs have been extensively characterised but their importance to nematode survival has not been investigated. The same is true of FTDH, another metabolic enzyme. FTDH is involved in folate metabolism, a pathway that has proven an effective target for chemotherapeutic treatment of filarial disease (Subrahmanyam, 1987). In the case of both MMSD and FTDH, the data here represent the first description of sequences encoding these proteins from parasitic nematodes. These enzymes are unlikely to be the protective components involved in TSBP vaccine-
induced immunity but, because of their presumed importance in nematode metabolism, are clearly prospective chemotherapy targets.

Finally, a single PO encoding a catalase homologue was identified. Catalase is an antioxidant enzyme and may play an important role in protection of the parasite against host-derived free radicals (Henkle-Duhrsen & Kampkotter, 2001). Recent in vitro studies with H. contortus have shown that catalase activity is upregulated in the presence of hydrogen peroxide and that catalase inhibition reduces the survival of the parasite (Kotze & McClure, 2001; Kotze, 2003). These three homologues were therefore selected for further characterisation, the results of which are presented in Chapters Five, Six and Seven.

As shown (Figure 3.2.2), TSBP contained cysteine protease activity yet the immunoscreen failed to identify any POs encoding this class of enzyme. An extensive immunoscreen of an adult H. contortus cDNA library identified only 3 cysteine protease-encoding POs, despite the fact that these sequences represent approximately 10% of the mRNA present in the adult worm as judged by expressed sequence tag analysis (Skuce, Redmond, Liddell et al., 1999). These sequences are considerably less abundant in the equivalent EST dataset from T. circumcincta. In addition, subsequent work examining the immune response to H. contortus cysteine proteases, which is induced by vaccination of lambs with TSBP, suggests that they are only weakly immunogenic (D.L. Redmond and D.P. Knox, personal communication). It is possible that very weak POs are overlooked during library immunoscreening. It is also worth considering that these proteases may be involved in the protective immune response but this response may have a cellular component. Unpublished work has indicated that serum antibody from H. contortus TSBP-vaccinated sheep does not inhibit the activity of the cysteine proteases present in that fraction (D.P. Knox, personal communication). However, this work utilised, as a substrate, a low molecular weight peptide that may have been able to access the protease active site despite any antibody binding in the vicinity. Investigation of the ability of antibody to inhibit TSBP cysteine protease activity against a larger substrate, such as gelatin or azocasein, may give a different result.
In conclusion, this chapter has described the outcome of screening an adult *T. circumcineta* cDNA library with serum from a lamb protected against homologous challenge by vaccination with TSBP. The level of variability in protection in this trial suggests that the protective component of TSBP may not have been present in sufficient quantities and that the difference in antigen preparation and adjuvant between this and a previous more successful trial may have effected the outcome.

The analysis described here identified two novel nematode enzyme sequences, namely MMSD and FTDH, as well as a catalase. These, and other proteins identified such as galectin, are unlikely to stimulate protective immune responses by analogy to studies in other parasites but may be suitable drug targets. The unidentified POs are intriguing and worthy of further definition. The full-length transcript size could be determined by Northern blotting and full length sequences sought by PCR with cDNA as target. These sequences and the sequences identified here can be used to search the databases at regular intervals because the volume of nematode sequence data available online is continually expanding.
4.1 INTRODUCTION

Antioxidant enzymes are essential to aerobic organisms to reduce the damage caused to a large range of biological molecules by reactive oxygen species (ROS). ROS are often generated as by-products of various normal metabolic pathways but can also be produced as part of the mammalian immune response to parasites (Henkle-Duhrsén & Kampkötter, 2001). Catalases (CATs) are part of a group of antioxidant enzymes that convert hydrogen peroxide ($\text{H}_2\text{O}_2$) into oxygen and water, as shown in Fig. 4.1. $\text{H}_2\text{O}_2$ is an oxidative agent but more importantly is a source of the highly reactive $\cdot\text{OH}$ group, for which no effective antioxidant exists (Henkle-Duhrsén & Kampkötter, 2001).

![Antioxidant pathway showing ROS and antioxidant enzymes involved. SOD = superoxide dismutase, PRX = peroxiredoxin, GPX = glutathione peroxidase. Adapted from Henkle-Dührsen and Kampkötter (2001).](image)

Figure 4.1.
CATs can be separated into three groups: true or haem catalases; catalase-peroxidases and manganese catalases (Zamocky & Koller, 1999). The monofunctional haem catalases comprise the largest group, thought to be common to all aerobic prokaryotes and eukaryotes. They tend to form tightly interacting homotetramers and their biological activity is dependent on a highly conserved distal histidine residue and the proximal haem-ligand, tyrosine, in each 60 kDa subunit (Zamocky & Koller, 1999).

Catalase activity has been detected in various parasitic nematodes but full-length CAT sequences are only available for a few species such as *A. suum* (Eckelt, Liebau, Walter et al., 1998). Much work has been done on the role of free radicals released by the host in damage and expulsion of parasitic worms (Brophy & Pritchard, 1992; Lo Verde, 1998; Kotze & McClure, 2001). Certain radicals may cause disruption of the parasite’s lipid membranes, intracellular proteins and nucleic acids. Recent studies with *H. contortus* have shown that CAT expression is up-regulated in response to H$_2$O$_2$ exposure *in vitro* and that inhibition of the enzyme results in increased susceptibility to oxidative damage (Kotze & McClure, 2001; Kotze, 2003). It is possible, therefore, that the parasite-derived catalase neutralises the threat of ROS released locally by the host and therefore prevents its subsequent expulsion or death. For this reason, antioxidant enzymes may make suitable vaccine candidates or good targets for novel anthelmintic drugs.

An initial run of sequencing on a single PO from *T. circumcincta*, isolated by the cDNA library screening, revealed a sequence sharing a high level of identity with catalase from other species available online. Due to the potential importance of parasite-derived antioxidant enzymes in maintaining infection, further characterisation of this clone was carried out. The full-length coding sequence was obtained, translated and then scanned for the conserved regions necessary for a functional catalase enzyme. Stage-specificity and location of the native protein in worm extracts were also examined.
4.2 RESULTS

4.2.1 Sequence Analysis of CAT clone

Preliminary sequence analysis of a single PO, identified by cDNA library screen, revealed an insert sharing a high level of identity with CAT sequences from other species. Further analysis of this *T. circumcincta* CAT homologue (TcCAT) revealed the presence of a full-length coding insert with a 1.524 kb open reading frame (ORF). On translation, this gave a continuous predicted amino acid sequence of 506 residues with a predicted molecular weight of approximately 54 kDa.

The PROSITE database was used to scan the translated sequence for recognisable domains, identifying several features characteristic of CATs, as shown in Figure 4.2.1. A proximal CAT heme-ligand signature is present, including a conserved tyrosine, known to serve as the heme proximal side ligand, and a conserved arginine involved in heme-binding. A proximal CAT active site signature containing a conserved histidine residue, associated with catalase activity, was also identified. The sequence also contains one potential N-linked glycosylation site, defined on PROSITE as N-X-[TS]-X, where X is any residue but proline in most cases.

As can be seen from the multiple alignment (Figure 4.2.2), TcCAT shares a high level of identity with CAT sequences from other nematodes, prokaryotes and with bovine liver CAT. For example, TcCAT shares 73% identity and 83% similarity (E value: 2.0e-206) with *C. elegans* 'catalase-2' (GenBank Accession No. O61235) and 70% identity and 80% similarity (E value: 3.3e-199) with *A. suum* CAT (P90682). Of particular interest are the residues highlighted in red in Figure 4.2.2. These are involved in the binding of the cofactor NADPH by bovine liver CAT and the majority are conserved in TcCAT and in the *C. elegans* sequence but are less so in the other parasitic nematodes. Common to all sequences are a conserved serine residue thought to be involved in the proper folding of the enzyme and a conserved asparagine associated with binding of the substrate H2O2. The sequence does not contain a signal peptide or transmembrane domain.
Figure 4.2.1. Protein sequence of T. circumcincta CAT showing domains and active sites identified by Prosite scan. Key: □ - Catalase proximal active site signature and ▢ - Catalase proximal heme-ligand signature, present in all known catalase sequences; ■ - potential N-linked glycosylation site; underlined residues - amino acids conserved in catalase sequences; ☑ - active site amino acid; ☐ - heme proximal side ligand; numbers at end of lines indicate number of residues.
**Figure 4.2.2.** Alignment of TcCAT with sequences from *A. suum* (P90682), *C. elegans* (O61235), *Onchocerca volvulus* (S49465), *Toxoplasma gondii* (Q9XZD2) and bovine liver (P00432). * denotes residues conserved over all six sequences. CAT signatures are highlighted as in Figure 4.2.1. □ denotes residues associated with binding of NADPH in bovine liver catalase. ▌ denotes conserved serine associated with folding. ▲ denotes conserved asparagine associated with H₂O₂ binding.

A predicted three-dimensional model of TcCAT was created using the SWISS-MODEL server ([http://swissmodel.expasy.org](http://swissmodel.expasy.org)). This program performs automated comparative modelling of 3D protein structures (Schwede, Kopp, Guex et al., 2003). SWISS-MODEL was used in ‘first approach mode’ where only the target amino acid code is required. The server automatically selects suitable templates, from a database of known protein structures (Protein Data Bank – PDB: [http://www.rcsb.org/pdb/](http://www.rcsb.org/pdb/)), on the basis of sequence identity with the submitted target sequence. In the case of TcCAT, the template with the highest level of identity (64.7%) was chain B of bovine liver catalase (P00432, PDB code: 8CAT). A local pair-wise alignment of the target and template is performed and the most stable model is created. The result is shown in Figure 4.2.3. As can be seen, the pattern of folding is very similar between the two proteins and the conserved residues, of known importance in catalase activity and heme-binding, occupy a similar region of the structure. There are visible differences however. For example, the TcCAT model has a shorter N-terminal α-helix, extra β-pleats in the centre but fewer on the N-terminal extension and an overall structure that is ‘looser’ in appearance than bovine liver catalase. Further geometric analysis of the model would be required in order to quantify these differences but was not attempted here.
Figure 4.2.3. 3D secondary structure of bovine liver catalase and predicted TcCAT structure, modelled by SWISS-MODEL.

Residues conserved in catalase sequences are highlighted in both:

- Histidine in active site
- Tyrosine heme side ligand
- Arginine involved in heme-binding

= β-pleated sheet
= α-helix
4.2.2 Stage Specificity of CAT expression

Gene-specific sense and anti-sense primers (Table 2.7.3) were designed against the TcCAT cDNA sequence to amplify a 0.6 kb fragment of this gene. These were used in PCR reactions, with first-strand cDNA from L3, L4 and adult worms as template, in order to detect expression of this gene. A product of the correct size was amplified for each stage as shown in Figure 4.2.4. These products were blotted onto nylon membrane and screened with DIG-labelled DNA probes made by PCR from the original TcCAT clone. As can be seen from Figure 4.2.5, all three products were confirmed as TcCAT. This shows that mRNA encoding this protein is transcribed to some degree in both larval stages and in the adult parasite. In the Figures, (-) is a negative control with no exogenous DNA added to the PCR. Primers against actin sequence were used in the positive PCR control, not shown in the figure. (+) is a PCR of the full-length TcCAT coding sequence, from the excised original clone, as a positive control for the DNA probe.
Figure 4.2.4. 2% (w/v) agarose gel showing products of RT-PCR with TcCAT primers for each stage (→), template-free negative PCR control (−) and a PCR product of the full-length TcCAT coding sequence as a positive control for probe hybridisation (+).

Figure 4.2.5. Southern blot of products from TcCAT RT-PCR (Figure 4.2.4), probed with DIG-labelled CAT-specific DNA probes.
4.2.3 Visualisation of CAT in worm extracts

The excised TcCAT clone, in Bluescript plasmid, was used to transform E. coli cells. These cells were grown in liquid culture and recombinant expression was induced by addition of IPTG. Soluble and insoluble cell fractions were produced from the culture, before and after induction of expression. These fractions were run on SDS-PAGE gels, Western blotted and screened with anti-TSBP serum. The results of this are shown in Figure 4.2.6. There is a strong band of recognition around 60 kDa in the soluble fraction from the induced recombinant culture. This band is present to a lesser degree in the insoluble fraction but not in the uninduced or non-recombinant controls. The band was used to elute specific antibodies from the serum as described in Materials and Methods section 2.7.6. These antibodies were then used to screen SI, S2 and S3 extracts from L3, L4 and adult worms (Figures 2.7.1 – 2.7.3). The blots were Ponceau-stained in order to check protein transfer before screening. The results are shown in Figures 4.2.7, 4.2.8 and 4.2.9. The predicted size of TcCAT from the coding sequence, minus post-translational modifications, is approximately 54 kDa. A band at approximately 57 kDa is detected with anti-TcCAT antibodies in the adult SI extract only.

Figure 4.2.6. Cell fractions from liquid culture of cells transformed with plasmid containing the T. circumcincta CAT clone (UN – uninduced, I – induced) or with non-recombinant plasmid (NR – induced), western blotted and screened with anti-TSBP serum.
Figure 4.2.7. S1 protein extracts from three stages of *T. circumcincta*, blotted and probed with eluted anti-TcCAT antibodies.

Figure 4.2.8. S2 protein extracts from three stages of *T. circumcincta*, blotted and probed with eluted anti-TcCAT antibodies.

Figure 4.2.9. S3 protein extracts from three stages of *T. circumcincta*, blotted and probed with eluted anti-TcCAT antibodies. + = positive cell fraction, = NR cell fraction.
4.3 DISCUSSION

A PO of a full-length haem catalase homologue was identified by screening an adult *T. circumcincta* cDNA library with anti-TSBP serum. The ORF was translated and the predicted amino acid sequence scanned for conserved regions of interest and compared with catalase sequences from a range of other animals. TcCAT shares a high level of identity with other catalase sequences and possesses several features characteristic of a functional catalase. A proximal CAT active site signature is present from residues 62 to 78. This includes the conserved histidine residue (His-73) essential for catalase activity (Zamocky & Koller, 1999). Asn-146, conserved in all sequences studied, is located in the active site of the enzyme and is also important for catalysis due to its role in binding the H$_2$O$_2$ substrate (Loewen, 1996). A proximal CAT heme-ligand signature is also present. This involves a conserved tyrosine residue (Tyr-355), known to serve as the proximal ligand to the heme iron, and a conserved arginine (Arg-351) thought to be involved in heme-binding (Loewen, 1996). Correct folding of the enzyme is thought to be partly dependent on a conserved serine residue, represented by Ser-112 in TcCAT, which is part of a complex involving a side chain on the heme group and a water molecule (Loewen, 1996). As can be seen from Figure 4.2.3, modelling of the secondary structure of TcCAT gives a folding pattern very similar to that of a known catalase.

Studies with mammalian catalases have shown that the enzyme is gradually converted to an inactive form by its substrate, H$_2$O$_2$ (Kirkman, Galiano, & Gaetani, 1987). This inactivation can be prevented and reversed in the presence of NADPH at concentrations likely to be found *in vivo* (Kirkman, Galiano, & Gaetani, 1987). It has been demonstrated that bovine liver catalase (BLC) and human catalases strongly bind NADPH, which is then oxidised and the bound NADP$^+$ displaced again by NADPH. This cycle somehow prevents inactivation of the enzyme although the exact mechanism of this action is not fully understood (Kirkman, Galiano, & Gaetani, 1987). In fact, catalases from the majority of species studied have been shown to bind NADPH. There are exceptions that do not, however, such as a catalase isolated from the fungus, *Penicillium vitale* (reviewed by (Zamocky & Koller, 1999). Fluorescence studies with an enzymatically active recombinant catalase (rAsCAT) of
another parasitic nematode, *A. suum*, have shown that it too binds NADPH (Eckelt, Liebau, Walter et al., 1998). Several residues have been implicated in the binding of NADPH (highlighted in red in Figure 4.2.2) in BLC (Eckelt, Liebau, Walter et al., 1998; Zamocky & Koller, 1999). All but one of these residues are conserved in TcCAT. It is likely, therefore, that TcCAT does bind the cofactor NADPH and, by doing so, has the ability to prolong its own activity.

The majority of eukaryotic catalases are found within peroxisomes, small organelles containing a range of proteins (Zamocky & Koller, 1999). These structures do not contain DNA or ribosomes and proteins must therefore be translocated across the peroxisomal membrane, a process which requires the presence of a peroxisomal targeting sequence (Zamocky & Koller, 1999). These sequences vary between species but fit a consensus pattern of three residues and tend to be located close to the C-terminus of the protein. The first residue in the consensus must be one of six possible residues; the second, one of three; and the third, one of seven to give the following pattern: [STAGCN]-[RKH]-[LIVMAF'] (Zamocky & Koller, 1999). Such a sequence is not present in TcCAT. Also absent is a signal peptide (Nielsen, Engelbrecht, Brunak et al., 1997). This suggests that, like AsCAT, TcCAT is a cytosolic enzyme.

As described before, the monofunctional haem or true catalases are present in almost all aerobic organisms (Zamocky & Koller, 1999). They tend to form homotetramers made up of four subunits of approximately 60 kDa in size, each with a prosthetic haem group (Zamocky & Koller, 1999). BLC exists as a 240 kDa homotetramer composed of four identical 57 kDa subunits (Murthy, Reid, Sicigano et al., 1981). Purified recombinant *A. suum* rAsCAT gave a band of approximately 60 kDa on an SDS-PAGE gel but the active form of the recombinant, as measured by gel filtration, was 240 kDa in size (Eckelt, Liebau, Walter et al., 1998). Screening of *T. circumcincta* worm extracts with anti-TcCAT antibodies identified a band of approximately 57 kDa in size in adult S1. This band was visibly stronger when the protein extract was loaded with reducing sample buffer (Figure 4.2.6). By analogy to BLC and the *A. suum* catalase, this band is therefore likely to represent the
dissociated TcCAT monomer. The gel conditions used here would dissociate the tetramer into the monomeric subunits.

That this band is recognised in adult S1 extract but not in the S2 or S3, indicates that TcCAT is soluble rather than membrane-associated or membrane-bound, providing further evidence that TcCAT is a cytosolic enzyme. This is an anomalous result given that TSBP are a subfraction of the integral membrane protein enriched S3 extract. Because the pellet remaining after solubilisation of membrane-associated proteins is not washed extensively prior to Triton X-100 extraction (Knox, Smith, & Smith, 1999), it is possible that water soluble proteins remain trapped within it. Alternatively, catalase is a free-radical scavenging enzyme with a prime function of protecting cell membranes from damage and, hence, it would be appropriate for the enzyme to be intimately associated with cell membranes. However, TcCAT does not contain a predicted transmembrane domain and all other evidence suggests that it is a cytosolic enzyme. It is therefore likely to be involved in detoxification of intracellular H₂O₂. It has been shown that H₂O₂ is miscible with water and can freely cross cell membranes (Halliwell, Clement, & Long, 2000). This means that a cytosolic catalase may be important in protecting the parasite against host-derived H₂O₂ that has defused into the cells. The issue of localisation could be addressed by immunogold staining of parasite sections using antibody raised to bacterially-expressed recombinant *T. circumcincta* catalase.

As discussed previously, antioxidant enzymes protect an organism against oxidative stress. In the case of helminths, this stress can be caused not only by free radicals produced by the parasite as metabolic by-products but also by host-derived ROS. Effector cells of the host immune system, such as macrophages and eosinophils, activated in the presence of parasite antigens, are able to generate ROS by a respiratory burst (Callahan, Crouch, & James, 1988). These toxic molecules can cause considerable damage in several ways. They may cause inactivation and unfolding of intracellular proteins and degradation of nucleic acids. They may also disrupt cell membranes via lipid peroxidation, resulting in release of lipid hydroperoxides and cytotoxic substances into the cells (reviewed by Brophy &
Pritchard, 1992). The relationship between host-derived ROS and parasite rejection has been demonstrated for several species of helminth. Upregulation of free radical generation by leukocytes in the small intestine of rats infected with *N. brasiliensis* correlates with worm expulsion (Smith & Bryant, 1989). In rodents infected with *F. hepatica*, only rats can develop resistance to the parasite while mice cannot. It was shown that the leukocytes of infected rats generate significantly higher levels of free radicals than those of mice (Smith, Ovington, & Boray, 1992). The potential of ROS to kill GI nematodes *in vitro* has also been established. For example, incubation with H$_2$O$_2$ results in dose-dependent death of *Heligmosomoides polygyrus, N. brasiliensis* and *T. spiralis* (Ben-Smith, Lammas, & Behnke, 2002; Batra, Srivastava, Gupta et al., 1993; Kazura & Meshnick, 1984). Neutralisation of these toxins by intracellular and secreted antioxidant enzymes can increase the parasite’s survival. *H. contortus* feeding is reduced *in vitro* in the presence of ROS and this effect increases with exposure to aminotriazole, a catalase inhibitor (Kotze & McClure, 2001). Catalase activity in adult and L4 worm homogenates was shown to increase in *H. contortus* incubated with H$_2$O$_2$ and this upregulation continued after the parasite was transferred to an environment free from the toxin (Kotze, 2003). It is apparent, therefore, that upregulation of catalase production protects *H. contortus* from external sources of H$_2$O$_2$ *in vitro* and may therefore an important defence mechanism *in vivo*. This catalase activity was measured in whole worm homogenates and its location or whether the enzyme was secreted were not investigated (Kotze, 2003). Antioxidant production also appears to be an important defence mechanism in filarial nematodes. *Brugia malayi* adults secrete significant quantities of superoxide dismutase (SOD) into culture medium whilst maintained *in vitro* and there is evidence that they also do so *in vivo* (Tang, Ou, Henkle-Duhrsen et al., 1994; Ou, Tang, McCrossan et al., 1995). Secretion of SOD has also been demonstrated in *Dirofilaria immitis* adults (Callahan, Hazen-Martin, Crouch et al., 1993). Glutathione peroxidase secretion has also been detected in both these filarial species (reviewed by Selkirk, Smith, Thomas et al., 1998). This suggests that these nematodes may have the ability to neutralise exogenous ROS.
RT-PCR demonstrates that TcCAT mRNA is present in the infective and parasitic stages of *T. circumcincta*. However, studies with specific anti-TcCAT antibodies could only detect the protein in adult extracts. This may indicate that only small amounts of TcCAT mRNA were present in the L3 and L4, as this technique was not quantitative. There has been some investigation of the stage-specificity of catalase expression in other parasitic nematodes. As discussed previously, catalase activity was detectable in the L4 and adults of *H. contortus* and *H. polygyrus* (Kotze, 2003; Pleass & Bianco, 1996), but no information regarding catalase in the L3 of either species is currently available. In *T. spiralis*, the newborn larvae are highly susceptible to external sources of H2O2, whereas the adults and muscle larvae are resistant (Kazura & Meshnick, 1984). Also, the larvicidal effect could be blocked by addition of exogenous catalase, suggesting that a lack of this enzyme is responsible for H2O2-mediated killing (Kazura & Meshnick, 1984). On exposure to H2O2 *in vitro*, *H. contortus* L4 catalase activity increased by up to 4.6 fold whereas in adults the increase was only 2.3 fold (Kotze, 2003). The *H. contortus* L4 described were developed from L3, exsheathed *in vitro*, and had, therefore, had no contact with the host environment. The *T. circumcincta* L4 used in the present study were extracted from the host abomasal glands after developing naturally. However, it is likely that the *T. circumcincta* L4 may also have had little exposure to H2O2, due to the inability of the immune effector cells, responsible for ROS generation, to enter the glands. It appears, therefore, that the L4 of both species may have the capacity to elevate the production of active catalase in response to the presence of its substrate. This burst of catalase activity may be the result of a build-up of TcCAT mRNA, detected by RT-PCR, which is not translated to TcCAT protein until the worms have neared adulthood where they may become exposed to ROS on leaving the abomasal glands. TcCAT protein may be undetectable in the L3 for similar reasons. These infective larvae have not had any contact with the host and so will not have encountered host-derived ROS. The L3 are also not undergoing growth and reproduction, processes that, in adults, may result in elevated parasite-derived ROS levels due to their production as metabolic by-products.
In conclusion, this chapter has described the identification and characterisation of a novel *T. circumcincta* catalase. The recombinant clone was identified by screening an adult cDNA library with anti-TSBP serum. Although the TSBP fraction has been shown to contain predominantly membrane-bound gut-expressed proteins, TcCAT appears to be a cytosolic enzyme. It would be interesting to determine whether an extracellular form of the enzyme, such as that identified for *H. contortus* SOD, is secreted by *T. circumcincta*, using PCR techniques as described by Liddell and Knox (1998). A cytosolic protein is unlikely to be a suitable vaccine candidate but a secreted form may be, as it will be exposed to effectors of the host immune response. If a full-length extracellular *T. circumcincta* catalase clone could be obtained, it might be worthwhile to attempt to express the recombinant in a system suitable for purifying larger quantities of protein. This recombinant protein could then be tested in vaccination trials. Further investigation of the importance of TcCAT in protecting the parasite from external sources of H$_2$O$_2$ *in vitro* could be performed using the H$_2$O$_2$ consumption assay and inhibitor studies as described by Kotze (2003) and Kotze and McClure (2003).
CHAPTER FIVE – FURTHER ANALYSIS OF A T. CIRCUMCINCTA MMSD HOMOLOGUE

5.1 INTRODUCTION

As described in the general introduction (Chapter 1), the ongoing search for new methods of control for GI nematodes is becoming increasingly important. This is due to the rising number of instances of anthelmintic-resistant nematode strains worldwide. In regions of Australasia, South Africa and South America, H. contortus is resistant to the three major anthelminthic classes available for worm control. Of major concern, a similar resistance problem has recently been identified in T. circumcincta populations in lowland Scotland (Sargison, Scott, & Jackson, 2001; Bartley, Jackson, Johnston et al., 2003).

As well as the hunt for a suitable vaccine candidate, the search for new drug targets is another possible avenue to pursue. To this end, an increased understanding of the metabolism of these parasites would be beneficial. Although the primary mode of action of the benzimidazoles is now widely accepted to be the binding to tubulin with resultant disruption of microtubule formation, it was at one point believed to be their affect on various metabolic pathways that led to worm death (Sanchez-Moreno, Ortega, & Valero, 1989). The action of malate dehydrogenase, involved in the breakdown of and release of energy from carbohydrates, for example, was shown to be inhibited by a range of benzimidazoles (Sanchez-Moreno, Ortega, & Valero, 1989). The group of drugs known as salicylanilides, of which Closantel is a member, are highly effective against the liver fluke, F. hepatica. Their primary mode of action is thought to be disruption of energy metabolism via their ability to uncouple oxidative phosphorylation, disturbing the electrochemical gradient and resulting in reduced ATP synthesis (Fairweather & Boray, 1999). It is possible therefore that investigation of other metabolic pathways about which little is known may identify new targets for drug therapy.
MMSD belongs to a superfamily of aldehyde dehydrogenases. It has a unique CoA-dependency and plays a role in pyrimidine and valine metabolism (Kedishvili, Popov, Rougraff et al., 1992). Nematodes in which pyrimidine metabolism has been studied, including *N. brasiliensis* and *T. muris*, have been found to possess the enzymes required for pyrimidine biosynthesis in crude worm extracts (Berens, Krug, & Marr, 1995). Nematodes also have the ability to salvage pyrimidines from their surroundings but this varies with species (Berens, Krug, & Marr, 1995). Valine is one of the ten ‘essential’ amino acids required in the diet of humans. Studies have indicated that this requirement is common to other mammals and to birds, insects and protozoans, although additional amino acids may be required depending on the species (reviewed by Barrett, 1981). The limited information available suggests that the ability to synthesise valine varies with nematode species (Fitzgerald & Szmant, 1997), so it is unclear whether it is essential for *T. circumcincta*. MMSD is involved in catabolic, rather than synthetic, pathways. The major role of such pathways is the production of energy but they may also generate reducing power and intermediates for synthetic pathways and provide a means of turnover of macromolecules (Barrett, 1981).

The enzyme MMSD has not been described in *T. circumcincta* previously and its importance to the parasite’s survival is not known. POs sharing a high level of similarity with available MMSD sequences were highly prevalent in the cDNA library screen. It was therefore decided to characterise this protein more extensively. Further sequencing was performed in order to obtain the full coding sequence. The translated sequence was then analysed in order to annotate conserved regions thought to be essential for the action of the enzyme. Alignment of *T. circumcincta* MMSD with other MMSD sequences was used to compare levels of variation between closely and more distantly related species. The stage specificity of expression of the enzyme was also investigated using RT-PCR and the location of the native protein was examined in worm extracts screened with specific antibodies.
5.2 RESULTS

5.2.1 Extension of MMSD sequence

Initially, eight POs exhibiting a high level of similarity to MMSD were identified. This was done by sequencing the 5' end of each insert with the T3 vector primer and searching online databases with the nucleotide sequences. These eight sequences aligned together to give an overall consensus sequence of 840 bp. A full-length insert was not present among these POs. PCR was used to amplify the 3' end of the sequence from an aliquot of cDNA library freeze-thaw lysate by priming with sequence from the 3' end of the partial cDNA alignment. This fragment was added to the alignment, completing the coding region, to give a final ORF of 1.569 kb. This was translated to give a contiguous predicted protein sequence of 523 aa.

5.2.2 Sequence analysis of T. circumcincta MMSD

The predicted protein sequence for this T. circumcincta cDNA, known as TcMMSD, was scanned for regions of interest using PROSITE. This identified several features of interest as shown in Figure 5.2.1. An aldehyde dehydrogenase (ALDH) active site including a conserved cysteine residue is present. This residue is conserved in all members of the ALDH family and is implicated in their catalytic activity (Kedishvili, Popov, Rougraff et al., 1992). Most ALDUs also possess a conserved glutamic acid active site (Kedishvili, Popov, Rougraff et al., 1992), but such a site is not present in this sequence. Interestingly, the scan identified a crystallin ‘Greek key’ motif signature, the significance of which is discussed later. Also present were two putative N-linked glycosylation sites. Neither of these are present in the rat or Pseudomonas sequences and only one is conserved in C. elegans.

Mammalian MMSDs are located in the mitochondria but are encoded by nuclear DNA and synthesized cytoplasmically (Deichaite, Berthiaume, Peseckis et al., 1993). The predicted TcMMSD protein sequence was therefore analysed using the MitoProt II program (link available on the MIPS website: http://www.mips.biochem.mpg.de). A survey of a large set of mitochondrial protein
sequences, available for a range of organisms, has revealed that proteins that are imported into the mitochondria often possess a cleavable N-terminal signal sequence. Although these signals are very variable in length and aa sequence, several common properties have been identified and this program examines a number of parameters to identify whether such a sequence is present (Claros & Vincens, 1996). MitoProt II calculates the N-terminal region that can support a Mitochondrial Targeting Sequence, the predicted cleavage site and the probability that the protein is exported to the mitochondria (Claros & Vincens, 1996). For TcMMSD, this identified a putative cleavage site at position 26, giving the cleavage sequence: MLSRVGRFSAYSVGQQYAFFSTSRA. The probability of export to the mitochondria is 0.9199, suggesting that TcMMSD is also a mitochondrial enzyme. This signal sequence should facilitate the transportation of the protein through the outer and inner mitochondrial membranes, where it will be cleaved by a signal peptidase to leave the mature mitochondrial protein.

Full-length TcMMSD was identified as a putative MMSD by database searches and the sequence was aligned with other MMSD sequences as shown in Figure 5.2.2. TcMMSD shares high levels of identity with these other sequences, for example, 72% identity (1e-180) with a probable C. elegans MMSD and 61% identity (1e-149) with rat MMSD. The cysteine active site residue is conserved in all 4 sequences. Also of interest are two conserved glycine residues (highlighted in red). These are thought to be involved in nucleotide binding in ALDHs (Vedadi, Vrielink, & Meighen, 1997).
Figure 5.2.1. Protein sequence of *T. circumcincta* MMSD showing domains and active sites identified by Prosite scan. Key: Aldehyde dehydrogenase cysteine active site, present in all known aldehyde dehydrogenase sequences; potential N-linked glycosylation site; underlined residues - aa conserved in aldehyde dehydrogenase sequences; active site aa; omega-crystallin motif; conserved glycines implicated in nucleotide binding; putative mitochondrial targeting sequence; numbers at end of lines indicate number of residues.
Figure 5.2.2. Alignment of TcMMSD (top line) with sequences from other species (NCBI accession numbers in brackets): CeMM - C. elegans putative MMSD (P52713), RaMM - Rat MMSD (AAA41638), PsMM - Pseudomonas aeruginosa MMSD (AAA25891). * denotes residues conserved over all four sequences. Domains of interest are highlighted as for Figure 5.2.1.
5.2.3 Stage specificity of MMSD expression

Gene-specific sense and anti-sense primers (listed in Materials and Methods, Table 2.7.3) were designed against the TcMMSD cDNA sequence to amplify a 0.2 kb fragment of the gene. These were used in PCR reactions, with first-strand cDNA from L3, L4 and adult worms as template, in order to detect expression of the gene. A negative control and a positive PCR control were added as before. A product of the correct size was amplified for each stage as shown in Figure 5.2.3. These products were blotted onto nylon membrane and screened with DIG-labelled DNA probes made by PCR from an original partial TcMMSD clone as described in Materials and Methods. A PCR product from this clone was also run on the gel as a positive control (+) for probe hybridisation. As can be seen from Figure 5.2.4, all three products were confirmed as TcMMSD sequence. This technique is not quantitative but shows that TcMMSD mRNA is expressed to some degree in both larval stages and in the adult parasite.
Figure 5.2.3. 2% (w/v) agarose gel showing products of RT-PCR with TcMMSD primers for each stage (→), template-free negative control (−) and positive control for probe hybridisation (+).

Figure 5.2.4. Southern blot of products from TcMMSD RT-PCR (Figure 5.2.3), probed with DIG-labelled MMSD-specific DNA probes.
5.2.4 Visualisation of MMSD in worm extracts

A partial TcMMSD clone was shown by sequence analysis to be "in frame" for expression in Bluescript plasmid. This plasmid was used to transform *E. coli* cells. Cells were cultured, recombinant expression induced by addition of IPTG and fractions made and screened with whole anti-TSBP serum as described in Materials and Methods. The results of this are shown in Figure 5.2.5. There is a strong band of recognition around 60 kDa in the insoluble fraction from the induced recombinant culture which is not present in the other fractions. This band was used to elute specific antibodies from the serum as described in Materials and Methods section 2.7.6. These antibodies were then used to screen S1, S2 and S3 extracts from L3, L4 and adult worms (Figures 2.7.1 – 2.7.3). The results are shown in Figures 5.2.6 – 5.2.8. The predicted size of TcMMSD from the coding sequence, minus post-translational modifications, is approximately 51 kDa. A band just below 55 kDa was detected with anti-TcMMSD antibodies in all three stages. This agrees with the RT-PCR results, showing that gene encoding TcMMSD is not only transcribed but is expressed in all three parasitic stages. The protein was detected in the water-soluble S1 and membrane-associated S2 extracts from all stages suggesting that native MMSD is soluble. However, it also appears to be present in the membrane-bound extracts from L4 and adult parasites but not in L3s.

![Soluble and Insoluble Fractions](image)

**Figure 5.2.5.** Western blot of soluble and insoluble fractions from liquid culture of cells transformed with plasmid containing a partial TcMMSD clone (UN – uninduced, I – induced) or with non-recombinant plasmid (NR – induced). The blot was probed with anti-TSBP serum.
Figure 5.2.6. S1 protein extracts from three stages of *T. circumcincta*, blotted and probed with eluted anti-TcMMSD antibodies.

Figure 5.2.7. S2 protein extracts from three stages of *T. circumcincta*, blotted and probed with eluted anti-TcMMSD antibodies.

Figure 5.2.8. S3 protein extracts from three stages of *T. circumcincta*, blotted and probed with eluted anti-TcMMSD antibodies. + = positive cell fraction, − = NR cell fraction.
5.3 DISCUSSION

The aim of this section was to further characterise a putative *T. circumcincta* MIMSD identified by screening with serum from an animal immunised with TSBP. The full-length coding sequence for this gene was obtained by PCR. The deduced aa sequence was scanned for peptide domains associated with the protein function and compared with MIMSD sequences from a range of other animals.

TcMIMSD is very similar in length to and shares a very high level of identity with other MIMSD sequences studied previously, particularly with a putative *C. elegans* MIMSD. MIMSD sequences possess several features characteristic of ALDHs, a family of enzymes involved in the oxidation of aldehydes to their corresponding acids. An ALDH active site between residues 300 and 311, including a conserved cysteine residue at position 308, was identified in TcMIMSD. This active site motif is common to all members of the ALDH family and is conserved in MIMSD sequences (Kedishvili, Popov, Rougraff et al., 1992). Mutated bovine MIMSD, where the active site cysteine was replaced with a serine residue, was not enzymatically active, providing further evidence of its importance (Berthiaume, Deichaite, Peseckis et al., 1994). It has also been suggested that MIMSD activity may be regulated in vivo via fatty acid acylation of this cysteine residue (Berthiaume, Deichaite, Peseckis et al., 1994). Several glycine residues are considered to be important for the binding of nicotinamide adenine dinucleotide (NAD)$^+$ in ALDHs. TcMIMSD Gly-252 and Gly-257 align with conserved glycines in all other MIMSD sequences in this comparison. Studies with a marine bacterium, *Vibrio harveyi*, have shown that mutation of these two glycine residues resulted in a complete loss of nucleotide affinity (Vedadi, Vrielink, & Meighen, 1997). They are, therefore, likely to be essential for the energy-releasing role of MIMSD. In addition to a cysteine active site residue, a glutamic acid active site is conserved in most members of the aldehyde dehydrogenase family. However, this site is not present in TcMIMSD or in other MIMSD sequences studied. Kedishvili *et al* (1992) concluded that there is one set of residues critical to all ALDHs, including the MIMSDs, and a second set only
required by certain groups of ALDHs. Interestingly, a crystallin ‘Greek key’ motif signature was also identified in TcMMSD and was conserved in the *C. elegans* and rat MMSD but not in *Pseudomonas aeruginosa*. Omega-crystallins are structural components present in the eye lens of octopus and squid. They are evolutionarily related to the ALDHs and are thought to have originated from the duplication of an ancestral ALDH gene which then became further adapted for use in the eye (Zinovieva, Tomarev, & Piatigorsky, 1993). They share a high level of identity with these enzymes, but do not exhibit any ALDH activity (Zinovieva, Tomarev, & Piatigorsky, 1993). In mammals, MMSD is a mitochondrial protein but is synthesized outside of the mitochondria (Deichaite, Berthiaume, Peseckis et al., 1993). Using the online MitoProt II program (Claros & Vincens, 1996), a 26 aa long putative N-terminal mitochondrial targeting sequence was identified in TcMMSD. The probability that the protein was exported to the mitochondria was over 90%, suggesting that, in *T. circumcincta* also, MMSD is located here.

As described above, MMSD is a member of the ALDH superfamily but is unique in several ways, including its CoA-dependency. In mammals, the degradation of the pyrimidines, uracil and thymine, produces β-alanine and β-aminoisobutyric acid which can be further converted to malonate semialdehyde and methylmalonate semialdehyde, respectively (Barrett, 1981). MMSD catalyses the oxidative carboxylation of these molecules, in a reaction involving the reduction of NAD⁺, and couples them with CoA to form acetyl- and propionyl-CoA (Kedishvili, Popov, Rougraff et al., 1992; Popov, Kedishvili, & Harris, 1992). It is presumed that pyrimidine degradation occurs similarly in nematodes, particularly as pyrimidines have not been detected in nematode excretions (Barrett, 1981). MMSD is also involved in valine degradation, a pathway which has proven identical, in terms of the enzymes involved, in all species investigated (Barrett, 1991). Valine is ultimately broken down to give succinyl-CoA (Barrett, 1991). In bacteria, this involves the conversion of methylmalonate semialdehyde to propionyl-CoA by MMSD (Barrett, 1991).

In mammalian tissues, the catabolism of branched-chain aa, such as valine, is an important source, not just of energy, but also of intermediates for biosynthesis
(Berthiaume, Deichaite, Peseckis et al., 1994), and has been studied in much detail. The equivalent pathways in nematodes are less well-understood. Barrett (1981) suggested that aa were unlikely to be an important energy source in parasitic nematodes as, at that time, few had been shown to catabolise them to any significant degree. Since then, however, the complete breakdown of valine to carbon dioxide has been described in several species of parasitic nematode (reviewed by Barrett, 1991). For example, *H. polygyrus*, a parasite of the murine intestine, was shown to catabolise valine by a pathway very similar to that found in mammals and MMSD activity was present at detectable levels in this nematode (Grantham & Barrett, 1986).

The prevalence of TcMMSD amongst POs identified by the present cDNA library screen suggests that this enzyme is a major component of *T. circumcincta* TSBP. RT-PCR analysis showed TcMMSD mRNA expression at detectable levels, not just in the adult but in the L3 and L4 also, and this was confirmed by the appearance of TcMMSD protein in extracts from all three stages. It is possible that the role of certain catabolic pathways may differ in each life stage. In the L3 stage, energy will be the main requirement. The parasite is not growing and cannot feed due to the presence of the protective L2 cuticle. However, it must remain mobile in order to migrate to a suitable location for ingestion by the host to occur. This is made possible by the complete breakdown of stored molecules with concomitant energy release. It is worthy of note that MMSD protein was detected not just in the soluble extract but also in the membrane-associated and membrane-bound extracts. This is perhaps due to its mitochondrial location. If all the mitochondria present are not sufficiently disrupted by the method used for S1 extract production, MMSD may only be gradually released from the pellet over the successive extractions.

Previous studies have focused on the role of lipids as the primary energy source during transmission of the infective larvae of GI nematodes (Medica & Sukhdeo, 1997). However, it is possible that the parasite may use alternative sources of energy in order to supplement its lipid reserves and, therefore, remain active for as long as is necessary. This may explain the presence of TcMMSD in the infective larvae. If the equivalent enzyme in *C. elegans* serves a similar function, it might be
expected that MMSD expression is upregulated in the dauer stage of this species, but this data is not currently available. In the parasitic stages of *T. circumcincta*, energy will be supplied by food ingested by the worm and biosynthesis will become more important for growth in the L4 and egg production in the mature adult. TcMMSD may, therefore, be involved in production of intermediates that, rather than being further degraded, are used for synthesis of new molecules. If this is the case, the disruption of such a catabolic pathway could have serious consequences for the parasite, depriving it of the necessary nutrition to reach maturity or to produce viable eggs.

Being a metabolic enzyme involved in pathways that take place within the mitochondria, MMSD is unlikely to be exposed to the host immune system. It is not suitable, therefore, as a potential vaccine candidate. TcMMSD may, however, be a suitable chemotherapeutic target. An anthelmintic drug targeted at a metabolic pathway can be effective in one of two ways. Its mode of action can involve a pathway unique to the parasite in some respect, either in its importance or in the enzymes it involves. Alternatively, it can involve a common pathway but one in which the enzymes differ in their sensitivity to chemical treatment between the host and the parasite. For many anthelmintics in use today, the mode of action is not entirely clear but they often involve pathways common to the parasite and host. For example, oxidative phosphorylation is the last step in aerobic respiration and an essential process for all aerobic organisms. The enzymes involved are therefore likely to be highly conserved over a wide range of species. Closantel is a member of the salicylanides, effective drugs against *Fasciola* spp. and against some nematode species. These chemicals are thought to act by disrupting the electrochemical gradient necessary for energy production by oxidative phosphorylation (Fairweather & Boray, 1999). They induce a rapid paralysis in *F. hepatica* at a dosage that has little or no deleterious effect on the host (reviewed by Fairweather & Boray, 1999). Closantel can be extremely harmful to host species but only at dose levels significantly higher than those required for an anthelminitic effect (Van Cauteren, Vandenberghe, Herin et al., 1985). Chemicals that disrupt aa metabolism are available. For example, the primary mode of action of Imazethapyr, a commercially available herbicide, is the inhibition of acetolactate synthase, an enzyme involved in
the synthesis of branched-chain aa such as valine (Gaston, Zabalza, González et al., 2002). To my knowledge, a selective inhibitor of MMSD or other enzymes involved in valine or pyrimidine catabolism is not currently available, but screening for such substances to test might be of value. It may also be interesting to investigate the effects of ‘anti-metabolic’ chemicals already available, such as Imazethapyr, on nematodes if this has not been tried already.

In conclusion, this chapter has described the identification and characterisation of a novel MMSD homologue in the infective and parasitic stages of *T. circumcincta*. This provides further evidence that valine and pyrimidine catabolism take place by a pathway similar to that characterised in mammals. The recombinant protein was identified by screening a cDNA library with serum raised against a nematode fraction thought to consist of predominantly membrane-bound gut-expressed proteins. However, the native TcMMSD protein is likely to be a soluble enzyme synthesized in the cell cytoplasm and exported to the mitochondria. This suggests that the TSBP fraction contains intracellular as well as membrane bound proteins, agreeing with the identification of a putative cytosolic catalase by the same method. Although TcMMSD is unlikely to be a suitable vaccine candidate, the quantity of the protein present in worm extracts may imply its importance to the parasite. It may therefore prove a potential drug target, should a suitable chemical become available.

Further characterisation of TcMMSD might involve an assay for levels of native MMSD activity in the parasite. Such an assay, that utilizes the CoA-binding properties of the enzyme by investigating the incorporation of radiolabeled myristryl-CoA probes, has been described (Deichaite, Berthiaume, Peseckis et al., 1993). It would be very interesting to carry out RNA interference (RNAi) in order to establish how crucial the enzyme is to the nematode. There was no obvious phenotype when RNAi was carried out on a probable *C. elegans* MMSD gene (Kamath, Fraser, Dong et al., 2003). However, it is possible that blocking expression of MMSD may have a subtle or slow-acting effect on the organism or that the enzyme may be of greater importance to a parasite.
ALDHs are NAD(P)^+-dependent enzymes which oxidise a wide range of aldehydes. Like MMSD (Chapter 5), FTDH is a member of the ALDH superfamily (Sophos, Pappa, Ziegler et al., 2001). FTDH is categorised as one of the ALDH-like proteins, evolutionarily related to the ALDHs and containing either complete or almost complete ALDH sequences (Sophos, Pappa, Ziegler et al., 2001). FTDH possesses both hydrolase and dehydrogenase activities, the catalytic centres of which are thought to reside in two discrete domains of the enzyme, bridged by a linking region, but act in sequence to oxidise 10-formyltetrahydrofolate (10-FTF) (Krupenko & Wagner, 1999; Krupenko, Vlasov, & Wagner, 2001). In mammals, the native protein exists as a homotetramer of four identical subunits, each 90 to 100 kDa in size (Schirch, Villar, Maras et al., 1994). The proposed structure of FTDH, adapted from Schirch et al (1994), is shown in Figure 6.1.

FTDH is a metabolic enzyme, involved in folate metabolism, and is associated with two reactions. It catalyses the NADP^+-dependent oxidation of 10-FTF to tetrahydrofolate (THF) and carbon dioxide (Case, Kaisaki, & Steele, 1988) and, in the absence of NADP^+, 10-FTF is hydrolyzed to give THF and formate (Case, Kaisaki, & Steele, 1988). THF is the active form of folic acid, a carrier of hydroxymethyl, formyl and methyl groups in certain biological pathways, and 10-FTF is particularly important for purine synthesis (Barrett, 1981). FTDH recycles THF, allowing it to re-enter the folate pool. It may also be involved in mitochondrial protein synthesis, through the formylation of methionyl-tRNA, and may prevent formate intoxication of the cell by clearing it as CO₂ (Krupenko & Wagner, 1999).
As discussed previously, the continuing rise in instances of anthelmintic resistance has lead to a search for alternative methods of control, either by vaccination or novel anthelmintic drugs. Enzymes involved in folate metabolism are an important target for chemotherapy in bacterial and protozoan infections and folic acid antagonists have been employed to treat a range of diseases. For example, pyrimethamine, used to treat *Toxoplasma gondii* and malaria in humans, inhibits the enzyme dihydrofolate reductase in bacteria and protozoans but does not effect the mammalian form (Barrett, 1981). These parasitic organisms cannot metabolise folate and must therefore synthesise dihydrofolate *de novo* and reduce it to tetrahydrofolate, a process that requires the action of dihydrofolate reductase (Barrett, 1981). Folate metabolism is also an important target for the control of filarial worms and several of the enzymes involved in this pathway have been identified in these parasites (reviewed by Subrahmanyam, 1987). Suramin is an antifilarial drug used in the
treatment of onchocerciasis. Grossly, it affects the gut epithelium of the nematode, disrupting uptake of nutrients, and this may be a result of its influence on several enzymes involved in carbohydrate and folate metabolism. It has been shown to inhibit dihydrofolate reductase in *Onchocerca volvulus* and FTDH in *Brugia pahangi* (Subrahmanyam, 1987). However, it is not clear which enzyme is the main site of action of the drug or whether it is the combination of effects that is responsible for worm death. Folate metabolism in other parasitic nematodes has not received as much attention. Dihydrofolate reductase has been detected in *N. brasiliensis* (Barrett, 1981), but no literature regarding FTDH activity in gastrointestinal nematodes is available.

Screening of an adult *T. circumcincta* cDNA library, with serum from a TSBP-vaccinated animal, identified three POs that were shown to have similarity to other FTDH sequences after preliminary sequence analysis. Further characterisation of this putative *T. circumcincta* FTDH was undertaken. Further sequence data was acquired using PCR and sequencing with gene-specific primers. The acquired sequence was translated and then scanned for the conserved domains and active sites characteristic of FTDH. Stage-specificity of mRNA and protein expression for this enzyme were also examined.
6.2 RESULTS

6.2.1 Sequence Analysis of FTDH clones

Preliminary sequence analysis of three POs, identified by cDNA library screening, revealed inserts sharing a high level of similarity with FTDH sequences from other species. Further investigation, and comparison with other FTDH sequences, revealed that none of these clones contained a full-length coding sequence. Further PCR with gene-specific primers and cDNA library template failed to complete the sequence. The available sequence was translated and aligned with sequences from other species that shared a high level of homology.

The available *T. circumcincta* sequence shared 65% identity and 75% similarity (9.7e-116) with *C. elegans* aldehyde dehydrogenase 3 (NP_502054) and 49% identity and 62% similarity (3.3e-8.5) with rat FTDH (P28037), over 712 aa residues. The alignment is shown in Figure 6.2.1. By analogy with these sequences, it would appear that the TcFTDH sequence is missing approximately 100 aa residues from the N-terminal. There is also a section of around 55 aa residues from the centre of the sequence (740 –794 in *C. elegans*) that could not be recovered by PCR. As can be seen from the alignment, there are some further regions present in both the *C. elegans* and rat sequences that appear to be deleted from TcFTDH. These ‘gaps’ could represent true deletions but are more likely a result of the presence of an incomplete coding sequence.

The PROSITE database was used to scan the translated sequence for recognisable domains, identifying several features characteristic of FTDH, highlighted in Figure 6.2.1. FTDH has three putative domains (Cook, Lloyd, & Wagner, 1991), all of which are present here. Firstly, there is an N-terminal region showing a high level of similarity with phosphoribosylglycinamide formyltransferase (GART) and other 10-FHF utilising enzymes. Although the full N-terminal sequence is not available for the *T. circumcincta* sequence, a GART active site with conserved aspartate residue was identified. Secondly, there is a region sharing little identity with any known sequences. This linker region was present but did contain a putative phosphopantetheine attachment site, common to acyl carrier proteins (ACPs).
Thirdly, there is a region sharing considerable similarity with many ALDH sequences and containing two ALDH active sites. The cysteine and glutamic acid sites are conserved in most members of the ALDH family.

All of these features were also present in the *C. elegans* ALDH 3. This suggests that this may also be a FTDH sequence although no further information on this is currently available.
Figure 6.2.1. Alignment of TcFTDH (Tcirc) with aldehyde dehydrogenase 3 sequence from *C. elegans* (Celeg, NP_502054) and FTDH sequence from *Rattus norvegicus* (Rat, P28037).

* denotes residues conserved over all three sequences.

- Red = Phosphoribosylglycinamidine formyltransferase active site with D active site residue.
- Blue = Acyl carrier protein phosphopantetheine domain.
- Pink = Aldehyde dehydrogenase glutamic acid active site with conserved E active site residue.
- Green = Aldehyde dehydrogenase cysteine active site with conserved C active site residue ('Cysteine 707').
- Yellow = Potential N-linked glycosylation sites.
6.2.2 Stage Specificity of TcFTDH Expression

Gene-specific sense and anti-sense primers were designed against conserved TcFTDH cDNA sequence to amplify a 0.7 kb fragment of this gene. These were used in PCR reactions, with first-strand cDNA from L3, L4 and adult worms as template, in order to detect expression of this gene. In the Figures, (-) is a negative PCR control with no added exogenous DNA. A product of the correct size was amplified for each stage as shown in Figure 6.2.2. These products were blotted onto a membrane and screened with DIG-labelled DNA probes made by PCR from an original TcFTDH clone. (+) is a positive control for probe hybridisation, a product from a PCR using an excised original FTDH P0 as target. As can be seen from Figure 6.2.4, all three products were confirmed as TcFTDH. This shows that TcFTDH mRNA is expressed to some degree in both larval stages and in the adult parasite.
Figure 6.2.2. 2% (w/v) agarose gel showing products of RT-PCR with TcFTDH primers for each stage (→), template-free negative control (-) and positive control for probe hybridisation (+).

Figure 6.2.3. Southern blot of products from TcFTDH RT-PCR (Figure 6.2.2), probed with DIG-labelled FTDH-specific DNA probes.
6.2.3 Visualisation of FTDH in worm extracts

An excised TcFTDH clone, in Bluescript plasmid, was used to transform *E. coli* cells. These cells were grown in liquid culture and recombinant expression was induced by addition of IPTG. Soluble and insoluble cell fractions were produced from the culture and screened with anti-TSBP serum as before (Figure 6.2.4). There is a band of recognition around 90 kDa in the induced recombinant culture which is particularly strong in the insoluble fraction. This band is present to a lesser degree in the soluble fraction but not in the uninduced or non-recombinant controls. The insoluble band was used to elute specific antibodies from the serum as described in Materials and Methods section 2.7.6. These antibodies were then used to screen S1, S2 and S3 extracts from L3, L4 and adult worms. The results are shown in Figures 6.2.5 – 6.2.7. The predicted size of TcFTDH, by analogy with other known FTDH proteins, is 90-100 kDa. A band at approximately 90 kDa is detected with anti-TcFTDH antibodies in the reduced adult S1 extract only. Native FTDH has been shown to exist as a homotetramer in mammals (Krupenko, Wagner, & Cook, 1995), but it is likely that only the dissociated monomer, represented by this band, is detectable under these conditions. This suggests that the native TcFTDH protein is water-soluble and is present in larger quantities in the adult worms than in the larvae.

![Figure 6.2.4](image)

**Figure 6.2.4** Cell fractions from liquid culture of *E. coli* cells transformed with plasmid containing a *T. circumcincta* FTDH clone (UN – uninduced, I – induced) or with non-recombinant plasmid (NR – induced), western blotted and screened with anti-TSBP serum.
Figure 6.2.5. S1 protein extracts from three stages of *T. circumcincta*, blotted and probed with eluted anti-TcFTDH antibodies.

Figure 6.2.6. S2 protein extracts from three stages of *T. circumcincta*, blotted and probed with eluted anti-TcFTDH antibodies.

Figure 6.2.7. S3 protein extracts from three stages of *T. circumcincta*, blotted and probed with eluted anti-TcFTDH antibodies. + = positive cell fraction, - = NR cell fraction.
6.3 DISCUSSION

Three POs, identified by screening an adult *T. circumcincta* cDNA library with anti-TSBP serum, contained inserts sharing a high level of similarity with an ALDH from *C. elegans*. This enzyme, in turn, shared significant identity with mammalian FTDH sequences. None of the clones contained the full length coding sequence for TcFTDH and further PCR with gene-specific primers failed to obtain the 5' end and a region from the centre of the sequence. The DNA sequence available was translated and the fragments of predicted aa sequence were aligned with *C. elegans* ALDH3 and rat FTDH sequences. The predicted partial TcFTDH sequence shared 65% identity (9.7e-116) with ALDH3 (NP_502054) and 49% identity (3.3e-8.5) with rat FTDH (P28037), over 712 aa residues.

A PROSITE search revealed the presence of several recognised domains within TcFTDH that are common to known FTDH sequences (Figure 6.2.1). Cook *et al* (1991) proposed that mammalian FTDH, isolated from the rat liver, is composed of three putative domains with discrete functions. The authors of a later study, with rabbit FTDH, proposed that FTDH consists of two catalytic domains bridged by a linking sequence (Schirch, Villar, Maras et al., 1994). The first 300 or so residues from the NH2 terminus form what is known as the amino-terminal domain or Nr-FDH (Krupenko & Wagner, 1999). Nr-FDH shares considerable identity with other enzymes that use 10-FTF as a substrate, such as phosphoribosylglycinamid formyltransferase (GART) and L-methionyl-tRNA formyltransferase (MTRF) (Krupenko, Wagner, & Cook, 1997). Although the full N-terminal coding sequence could not be isolated for TcFTDH, a GART active site was identified, including a aspartate residue conserved in all known GART sequences and required for GART activity (Inglese, Smith, & Benkovic, 1990). Asp142 (Rat) is strictly conserved in all enzymes utilising 10-FTF, and site-directed mutagenesis studies with recombinant mammalian FTDH have shown that its substitution with a different aa results in a complete loss of both the hydrolase and dehydrogenase activities of the enzyme (Krupenko & Wagner, 1999). Nr-FDH is also thought to be involved in 10-FTF
binding. It was shown that replacement of Asp142 results in a decrease in the enzyme's affinity for folate, suggesting that this residue is involved in binding the folate portion of 10-FTF. An Asp residue is conserved in the equivalent position in TcFTDH.

The second catalytic domain runs, in rat FTDH, from around residue 420 to the COOH-terminal and is known as C$_r$-FDH. This region shares a high level of identity with known NAD$^+$-dependent ALDH sequences [47% identity, 81% similarity (Cook, Lloyd, & Wagner, 1991)]. Alignment of TcFTDH with rat FTDH shows that this region is highly conserved between the two sequences (Figure 6.2.1). Analysis of the TcFTDH sequence revealed the presence of two ALDH active sites. Firstly, there is a region fitting the pattern for a glutamic acid active site, including a conserved glutamic acid implicated in mammalian ALDH catalytic activity. Secondly, there is an ALDH cysteine active site pattern. The conserved cysteine residue in this region corresponds to Cys707 in rat FTDH. Site-directed mutagensis, to substitute alanine for cysteine at this position, results in complete loss of the dehydrogenase activity of rat FTDH (Krupenko, Wagner, & Cook, 1995). This substitution had no effect on the folding or stability of the resulting mutant enzyme or on its hydrolase activity (Krupenko, Wagner, & Cook, 1995). This suggests that Cys707 is the key residue in the dehydrogenase active site.

The linker region runs between the two catalytic domains, from around residue 300 to 420 in the rat enzyme. This region is enriched in the negatively charged aa, glutamic acid and aspartic acid (Krupenko, Wagner, & Cook, 1997), and this is also true for TcFTDH. Krupenko et al (1997) suggested that this intermediate domain may provide electrostatic interactions that contribute to the orientation of the two catalytic domains of the native enzyme and may even play a regulatory role in response to physiological conditions. Previous studies had shown it to share little identity with any known sequences (Cook, Lloyd, & Wagner, 1991). However, more recent work has revealed that residues 384 to 405 share up to 45.5% identity with an $\alpha$-helix linker region between two globular domains of a rice calmodulin-like protein.
(Reuland, Vlasov, & Krupenko, 2003). Disruption of this region, by site-directed mutagenesis of full-length recombinant FTDH, resulted in retention of hydrolase and aldehyde dehydrogenase activities but loss of FTDH activity (Reuland, Vlasov, & Krupenko, 2003). This suggests that the flexibility of this region is required in order to bring together the two catalytic domains, allowing the events required for oxidation of 10-FTF to occur. The sequence analysis in the present study revealed the presence of an ‘ACP’ domain with a putative phosphopantetheine attachment site in TcFTDH and in C. elegans ALDH3 and rat FTDH. Phosphopantetheine is the prosthetic group of the acyl carrier proteins [ACPs (Majerus, Alberts, & Vagelos, 1965)], and has been shown to be covalently linked to the hydroxyl group of a serine residue within this attachment domain (Pugh & Wakil, 1965). ACPs are members of multienzyme complexes, such as the fatty acid synthetases. The phosphopantetheine group was originally thought to be solely responsible for allowing interaction between the various active sites in such complexes, by acting as a flexible ‘arm’ for the attachment of fatty acid or aa groups, but recent work has implied that considerable conformational changes must also be involved (Smith, Witkowski, & Joshi, 2003). The evidence presented here that the T. circumcincta FTDH contains a putative phosphopantetheine attachment site is the first description of this domain in FTDH from any source. It may play a similar role to that of the ACP group i.e. allowing interaction between the two catalytic domains of FTDH and thus facilitating the sequential hydrolase and dehydrogenase activities of the enzyme (Krupenko, Vlasov, & Wagner, 2001). However, it is also possible that this region’s resemblance to such a domain is simply a coincidence and this pattern does not represent a prosthetic group attachment site.

The recombinant TcFTDH protein was identified by screening a cDNA library with serum raised against a T. circumcincta worm fraction. As discussed in previous chapters, this fraction is thought to consist of predominantly membrane-bound gut-expressed proteins. However, analogy with the location and properties of FTDH in other organisms, plus the detection of TcFTDH protein in T. circumcincta S1 fraction only (Figure 6.2.6), suggest that the native TcFTDH protein is likely to
be a soluble enzyme, located in the cytosol and in the mitochondrial matrix (Cook, Lloyd, & Wagner, 1991). This provides further evidence that the *T. circumcincta* TSBP fraction contains intracellular as well as membrane bound proteins. As discussed above, Cys707 is a conserved active site residue in FTDH. In TcMMSD (Chapter Five), Cys308 is the equivalent conserved ALDH active site residue. A free sulfhydryl, or thiol, group is the functional group of cysteine and is likely to be involved in catalysis when that cysteine is a conserved active site residue. The TSBP fraction is prepared by passing nematode material over a thiol-sepharose column. Such a column is used to enrich for cysteine proteases as it specifically binds the free sulfhydryl groups involved in the catalytic mechanism of these enzymes. This may explain why the metabolic enzymes described here were present in TSBP. As discussed in Chapter Three, the pellet remaining after solubilisation of membrane-associated proteins is not washed extensively prior to Triton X-100 extraction (Knox, Smith, & Smith, 1999), which may explain the retention of some cytosolic and mitochondrial matrix enzymes in the integral membrane protein S3 fraction.

Again, TcFTDH is unlikely to be a suitable vaccine candidate. However, like TcMMSD, it may prove a potential drug target, should a suitable chemical become available. Present chemotherapeutic approaches to several bacterial and protozoan infections already target folate metabolism. For example, protozoan parasites are unable to metabolise folate and must synthesize dihydrofolate *de novo* then reduce it to tetrahydrofolate (Barrett, 1981). Specific dihydrofolate reductase inhibitors, pyrimethamine and cycloguanil, are commonly used against the malaria parasite, *Plasmodium falciparum* (Lemcke, Christensen, & Jørgensen, 1999). These drugs disrupt protozoan DNA synthesis by preventing the reduction of dihydrofolate to tetrahydrofolate in these organisms (Lemcke, Christensen, & Jørgensen, 1999).

Several enzymes involved in folate metabolism have been identified in filarial nematodes and this pathway in the parasites has been shown to differ from that of vertebrates (reviewed by Subrahmanyam, 1987). Filarial worms do possess FTDH and the enzyme has been shown to be more active in *B. pahangi* than in mammalian liver (Subrahmanyam, 1987). The antifilarial drug, Suramin, is thought
to act through effects on folate metabolism. Studies of the effects of the drug on *B. pahangi* have shown that its influence is restricted to the intestinal lumen (Howells, Mendis, & Bray, 1983). There, it causes ultrastructural changes that result in disruption of the permeability and absorptive capability of the parasite’s intestinal epithelium, interrupting the uptake of nutrients. As well as affecting several enzymes involved in carbohydrate metabolism, Suramin has been shown to inhibit dihydrofolate reductase in *O. volvulus* and FTDH in *B. pahangi* (Jaffe & Chrin, 1980; Subrahmanyam, 1987). The exact mode of action of Suramin is not clear and it is possible that a combination of effects, on different metabolic enzymes, is responsible for its anthelmintic properties.

Data on the effects of Suramin on other parasitic nematodes, such as members of the family Trichostrongylidae like *T. circumcincta*, are not available so it is not clear whether this has been investigated. Dihydrofolate reductase has been detected in the closely related strongyle *N. brasiliensis* (Barrett, 1981) and the present study has identified a putative FTDH homologue in *T. circumcincta*, multiple clones of which were present in a library screen. This implies that the drug could be effective against these species, if the structure and role of these enzymes were similar to those in filarial worms. A tblastn search of the NEMBASE *B. malayi* EST dataset (www.nema.cap.ed.ac.uk) with the TcFTDH sequence hits a cluster containing a single microfilariae sequence (BMC010103, 56% similarity, 6e-13). This implies that there is considerable sequence conservation between these species. Testing the effect of Suramin on GI nematodes, if this has not already been undertaken, may be of interest.

In conclusion, this chapter has described the isolation and characterisation of a partial sequence encoding a putative FTDH homologue from adult *T. circumcincta*. Further analysis of TcFTDH would involve obtaining the full-length coding sequence, using PCR with further gene-specific primers and cDNA template. This was attempted in the present study but time constraints meant that it could not be completed. It would also be of value to study the effect of suramin on this species *in vitro*, if in fact this has not been done. This might involve an assay of FTDH activity, using techniques adapted from those outlined by Jaffe and Chrin (1980), and
examination of the structural integrity of the parasite’s intestine before and after treatment with the drug. Again, RNAi in order to establish this enzyme’s importance to the worm’s development and survival might be of use. According to the RNAi report for *C. elegans* ALDH3 (Kamath, Fraser, Dong et al., 2003), there was no obvious phenotype when worms were fed the genomic PCR product from this gene but FTDH may play a more crucial role in *T. circumcincta.*
CHAPTER SEVEN - SERUM ANTIBODY RESPONSES TO TSBP VACCINATION

7.1 INTRODUCTION

As described in Chapter One, antibody production is often induced in response to parasitic infection and serum antibody levels have been linked to parasite rejection in various rodent and ruminant gastrointestinal nematode infections. This has been supported by work involving transfer of immune serum to susceptible *N. brasiliensis* or *H. polygyrus*-infected mice, resulting in the development of a species-specific protective immunity (Brindley & Dobson, 1983). In rodent models, GI nematode infections tend to be associated with elevated IgE and IgG1 levels. A typical Th2-type response involves induction of IL-4 and upregulation of IgG1 and IgE production (Miller, 1996). Inhibition of IL-4 function in *T. muris*-infected mice results in a large increase in IgG2 levels leading to a chronic infection whereas IFN-γ depletion raises IgG1 secretion resulting in expulsion (Else, Finkelman, Maliszewski et al., 1994). During secondary infection of calves with *O. ostertagi* (Canals & Gasbarre, 1990), and sheep with *H. contortus* (Schallig, Van Leeuwen, & Hendrikx, 1995), a rise in serum IgG1 and, to a lesser extent, IgG2 is generally observed.

IgE has also been strongly implicated in protective immunity against helminths, including *H. contortus*, with a negative correlation between worm burden and serum total IgE levels being recorded (Kooymen, Van Kooten, Huntley et al., 1997). A similar study with *T. circumcincta* did not produce the same results. A secondary infection did induce a marked L3-specific IgE antibody response in the gastric lymph but this was relatively short-lived, peaking around 5 days post-challenge (Huntley, Schallig, Kooymen et al., 1998). IgE levels were much lower in the serum and did not correlate with worm numbers (Huntley, Schallig, Kooymen et al., 1998).

Another study, examining immune responses during a natural infection with *T. circumcincta*, revealed an association between reduced worm length and higher serum levels of L4-specific IgA (Stear, Strain, & Bishop, 1999). It was shown in a
previous study that there is a strong positive phenotypic correlation between female worm length and the number of eggs present in the uterus (Stear, Bairden, Duncan et al., 1997). After a challenge infection of previously exposed sheep with *T. circumcincta*, a correlation between worm length and IgA levels in the gastric lymph was also observed (Smith, 1988). This suggests that resistance may be a result of reduced worm growth, and therefore fecundity, and may involve IgA. Further evidence for the role of IgA has been provided by investigation of the basis of genetic resistance to GI nematodes. Anti-*T. circumcincta* IgA activity has been shown to be a heritable trait in sheep breeding programs (Strain, Bishop, Henderson et al., 2002). However, there is some dispute over the role of serum IgA. Only low levels of anti-*H. contortus* or anti-*T. colubriformis* IgA were found in the serum of animals immune to these species after a challenge infection and it was suggested that local, rather than systemic, IgA responses may be responsible for immunity (Schallig, Van Leeuwen, & Hendrikx, 1995; Douch, Green, & Risdon, 1994).

In order for a vaccine to be successful, it must induce an appropriate response by the host. *T. circumcincta* S3 TSBP has been shown, by immunolocalisation in worm sections, to be expressed predominantly on the luminal surface of the gut i.e. it is a ‘hidden antigen’ fraction (Knox, Smith, Smith et al., 1995). The mechanism by which hidden antigen vaccines are thought to act is by stimulating a specific antibody response to proteins expressed in the gut. When such antibody is ingested by the parasite, it binds to the surface of the gut, possibly inhibiting the action of proteins that are essential for worm survival. For example, the *H. contortus* antigen H11, an integral membrane aminopeptidase expressed on the gut surface of the parasite, has been successfully used to immunise sheep, conferring significant protection against challenge infections (Tavernor, Smith, Langford et al., 1992; Smith, 1988). Serum antibody titres to H11 correlated to the degree of protection conferred and pregnant ewes were also protected, with evidence of antibody-mediated protection being transferred from the mother to unborn lambs and to newborns in the colostrum (Andrews, Hole, Munn et al., 1995). Transfer of detectable levels of anti-H11 antibody from ewe to lamb was thought to be responsible (Andrews, Hole, Munn et
al., 1995). The authors predicted that this antibody would be predominantly IgG1 as this is the major class transferred in this manner (Reynolds & Griffin, 1990). It was also shown that the aminopeptidase activity of H11 is inhibited by immunoglobulins in the sera from H11-vaccinated sheep, suggesting that anti-H11 antibody-mediated inhibition of the enzyme impairs the parasite’s survival in some way (Smith, Graham, Munn et al., 1997).

_H. contortus_ TSBP is also expressed on the surface of the adult gut and gives reductions in egg output of 77% and worm numbers of 47% in 3-10 month old lambs (Knox, Smith, & Smith, 1999). TSBP is enriched for cysteine protease activity (Knox, Smith, & Smith, 1999). These enzymes may be partly responsible for the digestion of the blood meal and their inhibition by serum from TSBP-vaccinated animals suggests that, again, ingested antibody is the effector responsible for protection (Knox, Smith, & Smith, 1999).

As described in Chapter Three, _T. circumcincta_ TSBP is also enriched for cysteine protease activity and protection trials have resulted in variable degrees of protection against a single challenge infection with this parasite (Knox, Smith, Smith et al., 1995). _T. circumcincta_ browse on the surface of the intestinal mucosa, rarely consuming blood unless from an existing wound, but has been shown to ingest some immunoglobulin (Murray & Smith, 1994). It is therefore predicted that vaccination with this fraction would need to elicit a similar response to that induced by _H. contortus_ TSBP in order to prove effective. The aim of this chapter was to determine whether any correlation between protection and serum antibody titres resulted from vaccination with _T. circumcincta_ TSBP. Individual serum anti-TSBP IgG1, IgG2, IgE and IgA titres were measured by ELISA and plotted against percentage protection as measured by reductions in post-challenge FEC and worm burden for each sheep in the group (Chapter 3, Table 3.2.1).
7.2 RESULTS

All ELISAs were performed as described in Chapter Two. Briefly, 96 well plates were coated with *T. circumcincta* S3 TSBP, prepared earlier by a colleague as described previously, diluted to 0.0028 mg/ml in bicarbonate buffer (pH 9.6). Serial dilutions of six experimental sera, from 1/20 to 1/20480, and a 1/20 dilution of the control sera were made up in 1X PBS. After incubating with sera, the plate was rinsed and monoclonal isotype-specific anti-sheep antibody, diluted appropriately, was added to each well. After further incubation, the plate was again rinsed and Mouse Immunoglobulin HRP conjugate (DAKO), diluted 1/2000, was added to every well. The plate was incubated for 1 h, rinsed and stained with o-Phenylenediamine dihydrochloride before reading by spectrophotometer at 490 nm. This was carried out in triplicate for each antibody isotype investigated. Comparison of the OD\(_{490}\) for the experimental sera with that of the control sera was used to calculate the antibody titre for each individual. Titre is expressed as the dilution factor for an experimental serum sample that gave an equivalent OD\(_{490}\) to the control sera assayed at a 1/20 dilution. The arithmetic mean titre, from the three plates, and the estimated levels of protection for each animal, as shown in Table 3.2.1 (Chapter 3), were used in the analysis discussed below.

7.2.1 IgG1 and IgG2 ELISA

Figure 7.2.1 summarises the ELISA data for all antibody isotypes investigated. It shows that, in all but one sheep, an increase in IgG1 and, to a lesser extent, IgG2 titre was observed. There was considerable variation between individuals. Vaccination appeared to have induced a much greater increase in IgG of both isotypes in sheep 9 in particular. However, the standard deviation is very large for these values, suggesting that there was also between-plate variation and implicating experimental error. These data were plotted against protection values as measured by reductions in egg and worm counts compared with adjuvant-only controls for each experimental individual. The correlation coefficient (R\(^2\)) is given
for each plot. $R^2$ can be used to indicate what proportion of the variability in $Y$ (% protection) is accounted for by its association with $X$ (antibody titre), should a relationship between the two exist. If serum levels of either isotype were associated with protection, we would expect to see a positive trend between titre and % protection, with an $R^2$ value close to 1.0. However, as shown in Figures 7.2.2 - 7.2.5, this was not the case for either IgG1 or IgG2. It is apparent just by observing the distribution of points that no obvious positive relationship is present, particularly when the out-lying point, (sheep 9) is discounted. For IgG1, both $R^2$ values are less than 0.1. For IgG2 they are slightly higher but still do not indicate a positive relationship. Individuals with both high and low levels of protection are shown to have similar IgG1 and IgG2 titres.

![Graph showing mean serum antibody titres](image)

**Figure 7.2.1.** Comparison of mean serum antibody titres, from ELISAs performed in triplicate, between individual TSBP-vaccinated animals. Black bars denote the standard deviation for each titre.
for each plot. $R^2$ can be used to indicate what proportion of the variability in $Y$ (% protection) is accounted for by its association with $X$ (antibody titre), should a relationship between the two exist. If serum levels of either isotype were associated with protection, we would expect to see a positive trend between titre and % protection, with an $R^2$ value close to 1.0. However, as shown in Figures 7.2.2 – 7.2.5, this was not the case for either IgG1 or IgG2. It is apparent just by observing the distribution of points that no obvious positive relationship is present, particularly when the out-lying point, (sheep 9) is discounted. For IgG1, both $R^2$ values are less than 0.1. For IgG2 they are slightly higher but still do not indicate a positive relationship. Individuals with both high and low levels of protection are shown to have similar IgG1 and IgG2 titres.

![Graph showing mean serum antibody titres comparison](image)

**Figure 7.2.1.** Comparison of mean serum antibody titres, from ELISAs performed in triplicate, between individual TSBP-vaccinated animals. Black bars denote the standard deviation for each titre.
Figure 7.2.2. Mean IgG1 Titre vs % protection as measured by reduction in faecal egg count, $R^2 = 0.0512$.

Figure 7.2.3. Mean IgG1 Titre vs % protection as measured by reduction in estimated worm count, $R^2 = 0.0918$. 
Figure 7.2.4. Mean IgG2 Titre vs % protection as measured by reduction in faecal egg count, $R^2 = 0.3436$.

Figure 7.2.5. Mean IgG2 Titre vs % protection as measured by reduction in estimated worm count, $R^2 = 0.2584$. 
7.2.2 IgE ELISA

Increases in serum anti-TSBP IgE levels were comparatively low, as shown in Figure 7.2.1. The titres were plotted against individual protection values and the results are presented in Figures 7.2.6 and 7.2.7. Again, there is no apparent positive relationship between the magnitude of IgE increase and protection. The titres for all but one animal are clustered between 2 and 6.3 but these animals show highly varied levels of protection. The $R^2$ values are less than 0.1.
Figure 7.2.6. Mean IgE Titre vs % protection as measured by reduction in faecal egg count, $R^2 = 0.0582$.

Figure 7.2.7. Mean IgE Titre vs % protection as measured by reduction in estimated worm count, $R^2 = 0.0913$. 
7.2.3 IgA ELISA

Increases in serum anti-TSBP IgA levels over the baseline were similar in magnitude to those for IgE, as shown in Figure 7.2.1. The titres were plotted against individual protection values and the results are presented in Figures 7.2.8 and 7.2.9. As with the other isotypes investigated, there does not appear to be a relationship between serum IgA and protection.
Figure 7.2.8. Mean IgA titre vs % protection as measured by reduction in faecal egg count, $R^2 = 0.0473$.

Figure 7.2.9. Mean IgA titre vs % protection as measured by reduction in estimated worm count, $R^2 = 0.0954$. 
7.2.4 Effect of Periodate Treatment on IgG ELISA

TSBP antigen-coated plates were periodate-treated as described in Chapter Two. This method is commonly used to disrupt carbohydrate epitopes in the antigen preparation, thus allowing the measurement of antibody specific for TSBP protein epitopes only. They were then incubated with serum followed by either monoclonal anti-IgG1 or IgG2 and developed as before. Due to the limited volume of serum available, this was only carried out for IgG1 and IgG2 as rises in pre-challenge serum IgG have been associated with protection by hidden antigen vaccination in previous studies (Andrews, Hole, Munn et al., 1995). As can be seen from Figure 7.2.10, it appears that the bulk of both the IgG1 and IgG2 responses are directed at carbohydrate epitopes in TSBP, resulting in a large drop in titre after periodate treatment of the antigen. Again, titres were plotted against individual protection data and the results are presented in Figures 7.2.11 - 7.2.14. Periodate treatment also caused sheep 9, previously an outlier on other figures, to become grouped with the other animals. Again, all animals were clustered over a small range of titres despite variation in the level of protection observed and $R^2$ values were low.

![Figure 7.2.10. Between sheep comparison of anti-TSBP IgG isotype titres with and without periodate treatment of TSBP. Black bars denote the standard deviation for each titre.](image_url)
**Figure 7.2.11.** Mean periodate-treated IgG1 titres vs % protection as measured by reduction in faecal egg count. $R^2 = 0.0347$.

**Figure 7.2.12.** Mean periodate-treated IgG1 Titre vs % protection as measured by reduction in estimated worm count, $R^2 = 0.2422$. 
Figure 7.2.13. Mean periodate-treated IgG2 titre vs % protection as measured by reduction in faecal egg count, $R^2 = 0.2681$.

Figure 7.2.14. Mean periodate-treated IgG2 titre vs % protection as measured by reduction in estimated worm count, $R^2 = 0.0026$. 
The aim of work described in this chapter was to investigate the relationship between pre-challenge serum antibody titres, after vaccination with *T. circumcincta* S3 TSBP, and protection against a single challenge infection with the same species. As discussed previously, antibody ingested by the parasite is thought to be the main effector involved in the protective immunity induced by vaccines composed of hidden antigens. Animals in a trial evaluating the protective capacity of TSBP showed considerable individual variation in the level of protection observed, as measured by reductions in FEC (-33 to 75% protection) and worm burden (-77 to 60% protection) post-challenge. In order to discern whether this variation was due to the magnitude of the serum antibody response generated in response to TSBP-vaccination, serum anti-TSBP IgG1, IgG2, IgE and IgA titres were measured by ELISA for each experimental animal and plotted against that animal's protection data.

In rodent models, immunity to GI helminth infections is generally associated with a Th2-type response, involving upregulation of IgG1 and IgE production (Miller, 1996). There is some evidence that acquired immunity to such infections in ruminants may operate by a similar mechanism. For example, secondary infections with *O. ostertagi* or *H. contortus* have been associated with a rise in serum IgG1 (Canals & Gasbarre, 1990; Schallig, Van Leeuwen, & Hendrikx, 1995). As discussed earlier, *T. circumcincta* TSBP is enriched for cysteine protease activity. It has been demonstrated that elevated levels of anti-cysteine protease IgG1, and to a lesser extent IgE, are present in the sera of *N. brasiliensis*-infected rats (Kamata, Yamada, Uchikawa et al., 1995). IgG1 has also been associated with protection against *H. contortus*, induced by vaccination with a hidden antigen. Vaccination of pregnant ewes with H11 resulted in protection being transferred from the mother to unborn lambs and to newborns in the colostrum (Andrews, Hole, Munn et al., 1995). Detectable levels of anti-H11 were present in the sera of lambs and, as IgG1 has been shown to be the predominant class of antibody transferred from ewe to lamb (Reynolds & Griffin, 1990), it is thought to be the effector responsible for protection in this case.
In the present study, a rise in serum levels of anti-TSBP IgG1 and, to a lesser extent, IgG2 was observed in all but one vaccinated animal. However, when plotted against protection values, no relationship was discernible (Figures 4.2.2-4.2.5). In other work where a correlation between serum antibody levels and protection has been observed, this relationship has been linear (Munn, Smith, Graham et al., 1993; Hotez, Ashcorn, Zhan et al., 2003). A simple linear regression was therefore performed on the data discussed here. This produced very low $R^2$ values i.e. a significant linear relationship was not present. Titres tended to be clustered over a small range of values despite the variation in protection between individuals.

Periodate treatment of the antigen prior to ELISA of IgG1 and IgG2 resulted in a substantial drop in the subsequent titres, suggesting that a considerable proportion of circulating antibody of these isotypes is directed at carbohydrate moieties in the TSBP. This was expected as periodate treatment of a TSBP Western Blot, before screening with anti-TSBP sera and anti-sheep IgG secondary antibody, resulted in a drop in reactivity and the disappearance of some bands of recognition (Chapter 3). Blocking of reactivity with carbohydrate did not reveal a relationship between antibody, directed at protein epitopes, and protection. It is possible that sodium periodate treatment may affect the integrity of the antigen preparation further than just disrupting carbohydrate epitopes, which could mask such a relationship. Work by other investigators, involving sodium periodate treatment to study carbohydrate epitopes from the gut of *H. contortus*, included a control for antigen integrity (Jasmer, Perryman, Conder et al., 1993). However, this work was carried out using monoclonal antibodies against carbohydrate epitopes with a polyclonal antibody as a control. A suitable control was unfortunately not available for the work described here.

Such high antibody reactivity with the carbohydrate portion of parasite glycoproteins has been reported in other infections. For example, resistance to the trematode, *Schistosoma mansoni*, strongly correlates with titres of specific antibody. It has been shown that the majority of the antibody response to schistosomiasis is directed at carbohydrate or glycan antigens from the parasite and that the antibodies produced are predominantly of classes IgG and IgM (Nyame, Lewis, Doughty et al., 2003). Further investigation identified several specific complex N-glycans, expressed
in all stages of S. mansoni, that were immunogenic in both infected mice (Nyame, Leppanen, Bogitsh et al., 2000) and humans (Nyame, Lewis, Doughty et al., 2003). LDNF [GalNAcβ1-4(Fucα1-3)ClcNAc-R] is a carbohydrate epitope expressed on S. mansoni N-glycans and Th2-type responses to LDNF were shown to be generated by S. mansoni-infected mice (Nyame, Lewis, Doughty et al., 2003). Recent work with H. contortus has shown that, after vaccination with ES proteins, a considerable proportion of the antibody response was directed at glycan epitopes (Vervelde, Bakker, Kooyman et al., 2003). In the same study, it was shown that vaccination with ES proteins, in an adjuvant that induces a strong Th2-type response (Alhydrogel), increased the levels of antibody directed at the LDNF epitope and that production of anti-LDNF IgG was associated with protection (Vervelde, Bakker, Kooyman et al., 2003). It is possible, therefore, that areas of glycosylation on the proteins present in T. circumcincta TSBP may play a role in inducing protective immunity.

IgE is also an integral component of the Th2-type response and has been strongly implicated in protective immunity against helminths. A number of studies with rodent and human nematode infections have reported a correlation between IgE levels and protection (reviewed by Negrao-Correa, 2001). However, these studies did not provide evidence for a direct mechanism involving IgE. There have also been suggestions that IgE responses, and associated immediate hypersensitivity reactions, may actually cause more harm than good in such infections. It has been suggested that high levels of non-parasite specific IgE may result in increased pathogenesis due to increased mucosal permeability and could actually benefit the parasite by blocking receptors on effector cells (Pritchard, 1993; Miller, 1996).

Previously exposed sheep given a challenge infection of H. contortus exhibited elevated IgE levels in the serum 2-4 weeks post-infection and a negative correlation between worm burden and serum total IgE levels was recorded (Kooyman, Van Kooten, Huntley et al., 1997). However, while a similar regime with T. circumcincta did induce a marked L3-specific local IgE antibody response in the gastric lymph, this was relatively short-lived, peaking around 5 days post-challenge (Huntley, Schallig, Kooyman et al., 1998). In addition, IgE levels were much lower.
in the serum and did not correlate with worm burden (Huntley, Schallig, Kooyman et al., 1998). Vaccination with *T. circumcincta* TSBP did induce an increase in serum IgE levels but this was of a much lower magnitude than those observed for IgG1 and IgG2. Again, there was no apparent relationship between the increase in serum IgE and protection.

Investigation of natural infections with *T. circumcincta* have revealed an association between reduced worm length, and therefore fecundity, and higher serum levels of L4-specific IgA (Stear, Strain, & Bishop, 1999). Further evidence for the role of IgA has been provided by investigation of the basis of genetic resistance to gastrointestinal nematodes. Sheep breeding programs have also shown anti-*T. circumcincta* IgA activity to be a heritable trait in genetically resistant animals (Strain, Bishop, Henderson et al., 2002). However, only low levels of anti-*H. contortus* or anti-*T. colubriformis* IgA were found in the serum of animals immune to these species after a challenge infection (Schallig, Van Leeuwen, & Hendrikx, 1995; Douch, Green, & Risdon, 1994). There is, therefore, some dispute over the role of serum IgA in such infections and it has been suggested that local, rather than systemic, IgA responses may be responsible for the immunity observed. This theory is supported by other studies where levels of specific IgA tended to be low in the serum but elevated locally in the mucus, tissues and lymph nodes at the site of infection (reviewed by Balic, Bowles, & Meeusen, 2000a). It has also been suggested that IgA detected in the serum during GI nematode infections may actually be a 'spill-over’ of IgA secreted into the gastrointestinal lumen (Claerebout & Vercruysse, 2000). This may explain why no discernible relationship between increases serum anti-TSBP IgA levels and protection was observed in the present study as the serum titres may not reflect the local response. This would agree with a study that showed only a weak association between parasite-specific IgA responses in the mucus and the plasma in previously exposed sheep, challenged with *T. circumcincta* (Sinski, Bairden, Duncan et al., 1995).
No apparent relationship between the serum levels of any of the antibody isotypes investigated and the degree of protection exhibited post-challenge was observed. There may be several explanations for this. As discussed previously, *T. circumcincta* is not a blood feeder. The exact composition of the diet of non-blood-feeding gastrointestinal nematodes is unknown but they are thought to feed on host tissue and tissue fluids, mucus, digesta or a combination of these materials (Murray & Smith, 1994). The principle of vaccination with gut-expressed parasite proteins is thought to rely on ingestion of specific antibody that binds to the proteins contained within the vaccine and blocks their action, affecting the viability of the parasite. It has been shown that non-blood-feeding parasites do ingest host immunoglobulin. ELISA detected the presence of ovine immunoglobulin in homogenates of fourth stage and adult *T. circumcincta*, although, as expected, this was at a much lower concentration than for *H. contortus* (3.4 μg/g adult *T. circumcincta* compared with 22.8 μg/g adult *H. contortus*) (Murray & Smith, 1994). As the nematode cuticle is impermeable to molecules of this size and the worms were thoroughly washed before assaying, the authors concluded that any immunoglobulin detected must have been ingested *in vivo* (Murray & Smith, 1994). Studies with *T. colubriformis* have shown that gastrointestinal mucus from immune sheep contains specific IgG1 and IgA directed at antigens expressed on the surface of the parasite (Harrison, Pulford, Hein et al., 2003a). It has also been suggested that the observed negative correlation between local IgA production and worm length in *T. circumcincta*-infected sheep reflects the impairment of parasite feeding by antibody (Miller, 1996). In trials with an equivalent S3 TSBP fraction from *O. ostertagi*, vaccination resulted in elevated parasite-specific antibody levels in the mucosa (Geldhof, Claerebout, Knox et al., 2002). It is possible, therefore, that sheep vaccinated with *T. circumcincta* TSBP release anti-TSBP antibody in the mucus or other local secretions, which is then ingested by the parasites. Serum antibody titres would therefore be unlikely to correlate with protection unless they closely mirror antibody titres at the site of infection and this does not appear to be the case (Sinski, Bairden, Duncan et al., 1995). Only serum was available from this trial but it may be worth investigating local responses in any future *T. circumcincta* TSBP trials. This may be achieved by cannulation of the lymph nodes draining the abomasum in order to analyse the
mucosal immune response. The presence of anti-TSBP antibodies in the abomasal mucus could also be investigated post-mortem.

Another possibility is that, in this study, a cellular component, not correlated with serum antibody titres, is involved in protection. As detailed in Chapter One, mucosal eosinophilia, mastocytosis and local antibody production have all been associated with immunity to GI helminth infections. There is some evidence that hidden antigen vaccination regimes also result in cellular responses at the site of infection. It has been shown that depletion of CD4+ T lymphocytes partially abrogates gut-antigen induced immunity against *H. contortus* (Karanu, McGuire, Davis et al., 1997), suggesting that these cells work simultaneously with serum antibody in expelling the parasite. TSBP is described as a hidden antigen preparation and immunolocalisation studies have shown that anti-TSBP antibody targets the luminal surface of the parasite gut (Knox, Smith, Smith et al., 1995). This means that, during infection of a vaccinated animal, the proteins present in TSBP should not stimulate an immune response as they are not accessible to the immune system. However, the nature of these so-called ‘hidden antigen’ fractions has been questioned by some recent findings. Assays with abomasal lymph node lymphocytes, from goats given a primary infection with *H. contortus*, have shown that their proliferation can be induced by intestinal fractions from the parasite (Jasmer, Karanu, Davis et al., 2003). This implies that the host’s immune system has had some previous contact with these antigens and therefore has a memory of them. TSBP consists of a largely undefined mixture of antigens and the library screen results, presented in Chapter Three, demonstrate that TSBP does not appear to consist entirely of membrane-bound proteins as the POs identified did not fit into this category. It is possible, therefore, that TSBP also contains proteins that are expressed on the external surface of the parasite or released in the ES products. These components of the vaccine, rather than the ‘hidden’ proteins, may be responsible, at least in part, for inducing a protective response. Also, investigation of *in vitro* ES material from parasitic nematodes has revealed the presence of some integral membrane proteins. For example, among the proteins identified by proteomic analysis of adult *H. contortus* ES material was H11, the protective integral membrane aminopeptidase discussed previously (Yatsuda, Krijgsveled, Cornelissen et
This may be a consequence of degradation of the worms in culture but it is also possible that such proteins are released in vivo. Another possibility is that, if the parasite's gut were to become damaged in some way, possibly a result of ingestion of specific antibody present in a TSBP-immunised host, then this may cause some of these membrane-bound proteins to be released into the environment. One or all of these factors may have the consequence of inducing further local responses in the TSBP-primed host.

Further studies with *T. colubriformis* have demonstrated that the major antigen recognised by intestinal mucus antibodies (IgG1 and IgA) from immune sheep is actually carbohydrate, rather than a protein (Harrison, Pulford, Hein et al., 2003b). As discussed above, periodate treatment, to block antibody reactivity with carbohydrate in TSBP, greatly reduced the subsequent serum IgG1 antibody titre. If locally produced antibody is directed at carbohydrate to a similar magnitude then the inhibition of parasite gut-expressed proteins, by ingested antibody, may be strongly dependent on areas of glycosylation in the vaccine. This has considerable implications for the production of recombinant vaccines. A recombinant protein produced in a bacterial expression system is unlikely to be identical to the native protein due to a lack of post-translational modifications. This means that, for antigens where conformation and glycosylation are essential for inducing a protective response, a prokaryotically-expressed recombinant will not be suitable. This has been illustrated by the extensive work on immunisation with recombinant forms of protective antigens from *H. contortus*, virtually all of which are glycosylated in their native form (Knox, Redmond, Newlands et al., 2003). For example, the glycoprotein H11 is highly protective in its native form. Vaccination with recombinants of defined extracellular domains of H11, expressed in *E. coli*, did not confer significant protection (Knox, Redmond, Newlands et al., 2003). This suggested that correct folding of the enzyme was required. However, vaccination with the full-length, enzymatically active recombinant H11, expressed in the baculovirus-insect cell system, was also unsuccessful (Knox, Redmond, Newlands et al., 2003). One of the reasons for this failure may be that glycosylation of the native antigen is largely responsible for its protective capacity. Recently, another candidate *H. contortus* vaccine antigen, pepsinogen, from the protective H-gal-GP complex,
has been expressed in transgenic *C. elegans* (Redmond, Clucas, Johnstone et al., 2001). This demonstrates the potential of this free-living nematode as a system for antigen expression, which could have several advantages including the presence of ‘nematode-like’ post-translational modifications. Different organisms process glycans in different ways (Dalton, Brindley, Knox et al., 2003), so expressing a protein in an organism closely related to the parasite, i.e. a nematode, may produce glycosylation closer in nature to that found on the native parasite protein. Further optimisation of this technique is required to maximise the level of protein expression in this system.

In conclusion, this chapter has demonstrated that, although TSBP-vaccination is thought to protect through an antibody-mediated mechanism, there is no apparent correlation between serum antibody titres and protection. This evidence combined with the inconsistent level of protection observed in this trial suggests that the efficacy of this method of vaccination against non-blood-feeding parasites may be dependent on one or more as yet unresolved factors. The library screen described in previous chapters identified several components of TSBP but did not turn up any sequence for cysteine proteases or for any putative membrane-bound proteins. Further analysis of this protein fraction from *T. circumcincta* could be performed using proteomic techniques such as those employed for ES material in the subsequent chapter. Further investigation of the immune response in TSBP-vaccinated sheep to *T. circumcincta* challenge may also be of interest. Characterisation of abomasal antibody expression, both before and after challenge, and histological analysis of cellular infiltration in the infected abomasal mucosa post-mortem may be of particular benefit.
8.1 INTRODUCTION

ES proteins are those released during *in vitro* culture by the parasite. It is thought that some of these substances may be essential *in vivo* for parasite maintenance within the host due to various functions such as feeding, evasion of the host immune response or alteration of the gastrointestinal environment (Knox, 2000). True ES proteins are derived from the body surface or from specialised external excretory-secretory glands and are often released in a stage-specific manner (Knox, 2000). Proteins released into the parasite’s environment will be readily accessible to the host’s immune system. This means that, in addition to providing further insight into the biology of a parasite, characterisation of ES material may identify potential vaccine candidates.

Previous studies evaluating helminth ES proteins as vaccine candidates are reviewed in Chapter One. Briefly, mixed ES proteins obtained from *in vitro* culture of *O. ostertagi* adults, *H. contortus* L3 and *T. colubriformis* L3 and adults have given promising results in protection trials (Emery, 1996). Several low molecular weight antigens further purified from *T. colubriformis* adult ES conferred up to 50% protection in guinea pig models (Emery, 1996). Recent work with adult *H. contortus* ES has also given promising results. The protective capacity of a cysteine protease enriched fraction of adult ES (ES TSBP) was evaluated in sheep. Reductions of 52 and 50% in egg output and worm burden, respectively, were observed after a single challenge infection (Bakker, Vervelde, Kanobana et al., 2004). A family of promising cross-protective vaccine candidates, ASPs, have been isolated from several hookworm species and are currently under evaluation in protection trials (Sen, Ghosh, Bin et al., 2000; Zhan, Liu, Badamchian et al., 2003). A protective 24 kDa ES antigen, isolated from *H. contortus*, was found to be an ASP homologue (Schallig, Van Leeuwen, Verstrepen et al., 1997; Yatsuda, Krijgsfeld, Cornelissen et al., 2003). Protection levels of over 70% reduction in mean egg output and abomasal worm burden were observed in immunised sheep (Schallig, Van Leeuwen, &
Corneissen, 1997). ASP homologues have also been identified in ES material from adult *O. ostertagi* (Vercauteren, Geldhof, Peelaers et al., 2003), and are represented in *T. circumcincta* EST datasets.

Most work on *T. circumcincta* ES proteins has concentrated either on their effects on the host tissues or on characterisation of proteases released in vitro. Stage-specific protease activity at a range of pH optima has been demonstrated in ES material from L3, L4 and adult *T. circumcincta* (Young, McKeand, & Knox, 1995). The bovine parasite *O. ostertagi* is closely related to *T. circumcincta*. ES material from the parasitic stages of *O. ostertagi* has been characterised using various techniques. Substrate gel analysis again demonstrated the activity of stage-specific proteases with the ability to degrade a variety of substrates, including bovine IgG, in a pH-dependent manner (Geldhof, Claerebout, Knox et al., 2000). Vercauteren *et al.* (2003) identified a range of proteins by screening *O. ostertagi* cDNA libraries with rabbit serum raised against *in vitro* ES material from the parasite. Some of these were not considered to be true ES proteins and may be present due to cellular damage and leakage. These included structural proteins such as actin and intracellular metabolic enzymes such as cytochrome-c-oxidase. Many of the proteins identified were confirmed as ES proteins by western blot analysis or by the presence of signal peptides and these included homologues of a globin-like protein, vitellogenin, heat shock protein and protein disulphide isomerase (Vercauteren, Geldhof, Peelaers et al., 2003).

Other components of *T. circumcincta* ES have received less attention. A group of stage-specific 31 kDa antigens from *T. circumcincta*, located in secretory organelles of the oesophageal glands and abundant in L3 ES material, was identified by immunoscreening with serum from resistant sheep (McGillivery, Yong, Adler et al., 1992). Initial protection trials in six-month old lambs gave promising results but these were not reproducible (McGillivery, Yong, Adler et al., 1992; Morton, Yong, Riffkin et al., 1995). The nature and function of this protective antigen is unknown and it may have been missing from the 31 kDa complex used in subsequent trials due to problems with the purification process.
The aim of the present study was to culture *T. circumcincta* fourth stage larvae and adults and to characterise some of the proteins released *in vitro*. The culture fluids were collected at several time points and concentrated in centrifugal filter devices. The proteins present were then analysed by 1D and 2D electrophoresis and Tandem Liquid Chromatography Mass Spectrometry/N-terminal sequencing.
8.2 RESULTS

8.2.1 Mini-gel analysis of ES material

*T. circumcincta* were cultured *in vitro* in RPMI medium, containing amino acids, salts, vitamins and glucose. Proteins released during culture of L4 and adult *T. circumcincta* were concentrated x 250, separated by electrophoresis on 4-15% Tris-HCl gels and visualised with coomassie stain. The results are shown in Figure 8.2.1.

![Figure 8.2.1](image)

**Figure 8.2.1.** L4 and adult *T. circumcincta* ES proteins concentrated x250, run under reducing conditions on a 4-15% gradient Tris-HCl mini-gel (Biorad) and Coomassie-stained. Media were harvested at the times indicated.

In L4 ES, around 12 distinct bands, ranging from 18 to over 200 kDa were present while the adult profile was more complex. Most protein accumulated up to 21 h in L4 and up to 40 h in adult cultures. In both cases very little protein was released into the culture media after 40 h. This suggests that, in both L4 and adult...
worms, either the ability to release proteins is reduced over time or the stimulus necessary to induce their synthesis or secretion is not present \textit{in vitro}.

In L4 ES, one particularly strong band of approximately 18 kDa is present at 2 and 21 h. In the adult ES, a pattern of >15 distinct bands ranging from 18 to 115 kDa can be seen at 2, 21 and 40 h. A particularly prominent doublet is present at around 18 and 20 kDa. Most other bands are not visible in culture fluids harvested after 40 h \textit{in vitro} maintenance.
8.2.2 Samples for Tandem MS Analysis

Concentrated 21 h culture fluids were run on 4-15% gradient Tris-HCl gels, as shown in Figure 8.2.2. All visible bands were labelled, excised and analysed as described in Chapter Two and Appendix 3.

The results for each sample were analysed using the MASCOT database as described. MOWSE (molecular weight search) searches compare the experimental peptide masses from a digested protein sample with the calculated peptide masses, on digestion with the same enzyme e.g. trypsin, for each database entry (Pappin, Hojrup & Bleasby, 1993). When a sample mass value is within a given mass tolerance of a database entry mass value, this counts as a match. MOWSE then assigns a statistical weight or score to each individual peptide match. MASCOT uses a probability-based form of the MOWSE algorithm that allows the combination of peptide mass and fragment ion (see Appendix 3) matching. The total Probability Based MOWSE Score is the absolute probability that the observed match is a random event. Scores are given as $-10 \times \log_{10}(P)$, where $P$ is the absolute probability. Those peptides giving database hits with a Probability Based MOWSE Score of 50 ($p<0.05$) or more are considered significant in this case, sharing identity or extensive similarity with one or more database entries. Hits against trypsin were discarded as contamination due to the method of sample degradation. The remaining results are summarised in Tables 8.2.3 and 8.2.4.
Figure 8.2.2. L4 ES (concentrated x 500) and adult ES (concentrated x 250), run on a 4-15% Tris-HCl mini-gel and Coomassie-stained. Labelled bands (L1-L15 and A1-A14) were excised for further analysis.
8.2.3 Tandem MS/MS results for L4 ES Material

The results for Tandem LC/MS/MS analysis of proteins present in L4 culture fluids are summarised in Table 8.2.3. This technique gives both a peptide mass fingerprint for each sample and sequence data for selected peptide ions, allowing a more thorough database search. Not all bands present gave significant hits. Significant hits will be briefly described here and further discussed at the end of this chapter. Peptide fragment sequence data for L4 and adult ES are compiled in Appendices I and II.

Many of the proteins identified in L4 ES were homologues of metabolic enzymes, involved in various stages of energy metabolism. Present are proteins showing similarity to a hypothetical *C. elegans* fructose-1, 6-biphosphate aldolase and a *C. elegans* enolase, both involved in glycolysis, and to mammalian fumarate hydratases, part of the Tricarboxylic Acid Cycle. Also identified was a protein similar to a *C. elegans* mitochondrial carrier protein, ATP/ADP translocase.

Apparent structural proteins were also present. Band L7 (Figure 8.2.2) contains a protein sharing considerable similarity with actin 2, a major component of the cytoskeleton, from the plant-parasitic nematode, *Globodera rostochiensis*. Proteins showing similarity to *C. elegans* muscle components, tropomyosin and paramyosin, were present at approximately 42 kDa and 97 kDa respectively. The presence of structural proteins and several intracellular enzymes indicates that a certain degree of cell damage and protein leakage may have occurred during the culture of the worms. However, this argument is confounded by the observation that protein accumulation in the culture fluids was markedly reduced after 40 h. It might be expected that this would increase if worms were dying and breaking down.

Some of the proteins identified are more likely to be true secretory products, being similar to those identified from the ES products of a closely related nematode. A homologue of a detoxifying enzyme, thioredoxin peroxidase, isolated from the ES products of adult *O. ostertagi* (Vercauteren, Geldhof, Peelaers et al., 2003), was present at approximately 27 kDa. A small protein of approximately 20 kDa with
similarity to a globin-like protein isolated from *O. ostertagi* L4 ES (Vercauteren, Geldhof, Peelaers et al., 2003), was present in band L1 (Figure 8.2.2). A secreted protein disulphide isomerase (PDI), around 55 kDa in size, has been identified in the ES products from L4 and adult *O. ostertagi* (Vercauteren, Geldhof, Peelaers et al., 2003; Geldhof, Vercauteren, Knox et al., 2003). *T. circumcincta* L4 ES contained a protein at approximately 60 kDa showing considerable similarity to this PDI.

Cysteine protease activity has been demonstrated in the ES products of several parasitic nematodes including *T. circumcincta, O. ostertagi* and *H. contortus*. A protein of approximately 30 kDa in size showed similarity to an EST sequence from adult *T. circumcincta* that, in turn, shared considerable similarity with several cysteine proteases.

Finally, a protein similar to a heat shock protein from *Panagrellus redivivus* and a heat shock protein precursor from *C. elegans* were identified. The expression of such proteins is often upregulated in response to stress (Hartman, Cottee, Savin et al., 2003).

**Table 8.2.3.** Results of Mass Spectrometry/N-terminal sequence analysis of ES material from the fourth larval stage of *T. circumcincta*. Significant hits are those with a total score greater than 50. The identity and source organism for the highest ranked hit for each group of peptides is given. Band reference numbers and approximate sizes refer to Figure 8.2.2.
<table>
<thead>
<tr>
<th>Band Ref.</th>
<th>Band size (kDa)</th>
<th># Peptides with significant hits</th>
<th>Total Score</th>
<th>Homologue identity</th>
<th>Organism</th>
<th>Homologue Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>20</td>
<td>2</td>
<td>83</td>
<td>Globin-like ES protein F6</td>
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<td>CAD20463</td>
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<tr>
<td>L2</td>
<td>27</td>
<td>3</td>
<td>85</td>
<td>Thioredoxin peroxidase (fragment)</td>
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<td>CAD207373</td>
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<tr>
<td>L3</td>
<td>30</td>
<td>3</td>
<td>125</td>
<td>EST similar to cysteine protease precursor</td>
<td><em>T. circumcincta</em></td>
<td>CB037400</td>
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<tr>
<td>L5</td>
<td>34</td>
<td>6</td>
<td>293</td>
<td>Galectin</td>
<td><em>H. contortus</em></td>
<td>O44126</td>
</tr>
<tr>
<td>L5</td>
<td>34</td>
<td>4</td>
<td>170</td>
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<td>64</td>
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<tr>
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<tr>
<td>L8</td>
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<td>1</td>
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<tr>
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<td>1</td>
<td>64</td>
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<td>97</td>
<td>5</td>
<td>290</td>
<td>Paramyosin</td>
<td><em>C. elegans</em></td>
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</table>
8.2.4 *T. circumcincta* Galectins

Galectins from several parasitic nematodes have been extensively characterised. In the present study, as described earlier, *T. circumcincta* sequences sharing identity with galectins were identified by cDNA library immunoscreening. In the ES also, a protein sharing a high level of identity with galectins is present in band L5 at approximately 34 kDa. Interestingly, like the sequences discussed in Chapter 3, this ES protein appears to share a higher level of identity with a *H. contortus* galectin, rather than with *T. circumcincta* galectins for which sequence is available. In order to compare all these sequences, the two translated consensus sequences from the immunoscreen were aligned with confirmed galectin sequences from *H. contortus* and *T. circumcincta*. Peptide sequences, derived from ES band L5, are highlighted also. The results are presented in Figure 8.2.4.

The putative galectin sequences from the immunoscreen (designated TSBP1 and TSBP2 in Figure 8.2.4) contain only one potential carbohydrate recognition domain (CRD) and align with the *H. contortus* proto-type galectin. TSBP1 and TSBP2 only have small differences between them. They were derived from a single read of sequencing for each clone and further reads may reveal these differences to be errors. The sequence presented here represents the isolation of a novel *T. circumcincta* proto-type galectin.

Two of the *T. circumcincta* L4 ES peptide sequences share identity with the central region of the tandem repeat-type galectins. This region contains a second CRD and is not present in the proto-type galectin. Several of the peptide sequences are identical to either the *H. contortus* or the *T. circumcincta* tandem-repeat type galectin whereas others are identical to both. This implies that there may be more than one galectin present in band L5. One of these might be the tandem repeat-type galectin (AAC47547), isolated from *T. circumcincta* L3 by Newton *et al* (1997). The other might be a novel *T. circumcincta* galectin that shares a higher level of identity with the *H. contortus* tandem repeat-type galectin isolated by Greenhalgh *et al* (2000).
Figure 8.2.4. Alignment of putative partial galectin sequences from Chapter 3 with confirmed galectin sequences.

TSBP1 = consensus of 13 clones from anti-TSBP immunoscreen.

TSBP2 = consensus of 3 clones from anti-TSBP immunoscreen.

H-CAB71314 = *H. contortus* proto-type galectin

T-AAC47547 = *T. circumcincta* tandem repeat-type galectin

H-AAF63406 = *H. contortus* tandem repeat-type galectin

- ES peptide identical to *T. circumcincta* tandem repeat-type sequence
- ES peptide identical to *H. contortus* tandem repeat-type sequence
- ES peptide identical to both tandem repeat-type sequences

Underlined residues = CRD domains
8.2.5 Tandem MS/MS results for Adult ES Material

The results for Tandem MS/MS analysis of proteins present in adult culture fluids are summarised in Table 8.2.5. As with the L4 ES material, not all bands present gave significant hits.

Several proteins are common to L4 and adult ES. A globin-like protein is present in both stages but appears as a doublet of bands at 18 and 20 kDa in the adult. Proteins similar to fructose-1, 6-biphosphate aldolase, enolase, actin and disulphide isomerase also appear in both.

In addition to those mentioned above, additional metabolic enzymes are present among proteins identified in the adult ES material. A protein of approximately 20 kDa in size is similar to *C. elegans* nucleoside diphosphate kinase. This enzyme is involved in purine nucleotide synthesis (Barrett, 1981). Also present are proteins similar to a *C. elegans* aldo/keto reductase and a possible glycogen phosphorylase, arginine kinase from *H. glycines* and a putative glutamate dehydrogenase from *H. contortus*.

A protein of approximately 29 kDa shows considerable similarity to a member of the 14-3-3 protein family isolated from *C. elegans*. These proteins have been ascribed a number of functions, particularly in signal transduction and development (Wang & Shakes, 1997).

Table 8.2.5. Results of Mass Spectrometry/N-terminal sequence analysis of ES material from the adult stage of *T. circumcincta*. Significant hits are those with a total score greater than 50. The identity and source organism for the highest ranked hit for each group of peptides is given. Band reference numbers and approximate sizes refer to Figure 7.2.2.
<table>
<thead>
<tr>
<th>Band Ref.</th>
<th>Band size (kDa)</th>
<th># Peptides with significant hits</th>
<th>Total Score</th>
<th>Homologue identity</th>
<th>Organism</th>
<th>Homologue Accession (NCBI)</th>
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<tbody>
<tr>
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<td>18</td>
<td>1</td>
<td>72</td>
<td>Globin-like ES protein F6</td>
<td>O. ostertagi</td>
<td>CAD20463</td>
</tr>
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<td>2</td>
<td>216</td>
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<td>CAD20463</td>
</tr>
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<td>NP 492761</td>
</tr>
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<td>51</td>
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<td>1</td>
<td>71</td>
<td>Hypothetical protein similar to glycogen phosphorylase</td>
<td>C. elegans</td>
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8.2.6. 2D Electrophoresis of Adult ES Proteins

The analysis of bands excised from 1-D electrophoresis gels of ES material demonstrated that a single band could contain more than one protein. For example, band A11 in adult *T. circumcincta* ES (Figure 8.2.2) contained possible homologues of both disulphide isomerase and glutamate dehydrogenase. Two h and 21 h adult ES showed very similar banding patterns on a 1-D gel as shown in figure 8.2.1. To demonstrate the complexity of these bands, these two samples were further separated by 2-D electrophoresis, as described in Chapter Two, and the results can be seen in Figure 8.2.6.

Although the two samples have similar 1-D banding patterns, 21 h ES contains many more spots than 2 h. It is apparent, when comparing the number of spots in adult 21 h ES with the number of putative proteins established for this sample, that there are many proteins present that have not been identified. This is supported by the fact that much of the peptide data from the analysis above did not produce significant database hits.
Figure 8.2.6. Adult 2 h and 21 h ES material, cleaned and concentrated using PlusOne 2-D Clean-Up Kit (Amersham) then separated by 2-D electrophoresis using 11 cm, pH 3-10 IEF strips and ExcelGel XL SDS 12-14 gel (Amersham). The gel was stained using the PlusOne Silver Stain Kit (Amersham).
8.3 DISCUSSION

The proteins expressed on the surface of or released into the environment by GI nematodes are a potential source of antigens. Vaccination with whole in vitro ES from different stages of the parasite has induced significant levels of protection against infection with several species of helminth (reviewed by Emery, 1996). Vaccination trials with fractionated ES in *O. ostertagi* and *H. contortus* (Geldhof, Claerebout, Knox et al., 2002; Bakker, Vervelde, Kanobana et al., 2004) or with discrete antigens purified from ES in *H. contortus* (Schallig, Van Leeuwen, & Cornelissen, 1997; Schallig & Van Leeuwen, 1997) and *T. colubriformis* (Frenkel, Dopheide, Wagland et al., 1992) have also yielded promising results. In *T. circumcincta*, a stage specific 31 kDa glycoprotein from L3 ES induced significant levels of protection in early trials but these results were not reproducible (McGillivery, Yong, Adler et al., 1992; Morton, Yong, Rifikin et al., 1995). Therefore, although the protective potential of ES material is apparent, the nature of the antigens responsible is still largely unknown. This chapter describes the results of the application of several techniques in order to further characterise ES material from the fourth larval stage and adults of *T. circumcincta*.

*In vitro* culture of *T. circumcincta* was performed in order to harvest the ES material released by the parasitic stages of worm. Fourth larval stage and adult *T. circumcincta* were cultured in a sterile protein-free medium supplemented with antibiotics for a total of 48 h. The culture medium was removed and fresh medium added at 2, 21, 40 and 48 h. The culture fluids were filtered, concentrated and stored at –80 °C. Preliminary analysis of the proteins released was carried out on gradient mini-gels. As can be seen from Figure 7.2.1, proteins of a range of sizes were released by both L4 and adult worms. ES from the later stages of the culture period contained few bands visible with Coomassie-staining, indicating that, in both stages, the rate of protein release declines after a certain period of time. To my knowledge this phenomenon has not been reported before. Similar studies have tended to involve collection of ES material at only one time point, at the end of the culture period (Young, McKeand, & Knox, 1995; Geldhof, Claerebout, Knox et al., 2000), or the pooling of material from more than one collection (Yatsuda, Krijgveld,
Cornelissen et al., 2003). This reduction in the release of ES proteins could be due to several factors. The culture medium contains a range of nutrients, such as amino acids, glucose and sources of calcium and magnesium, which should enable the worms to synthesise proteins. However, the culture environment is unlikely to provide any stimuli, present in the host environment, which may be necessary for the continued induction of such synthesis. It is possible therefore, that the majority of proteins released were synthesised and stored before the culture period. Another possibility is that the worms have begun to degrade, resulting in a cessation of normal biosynthesis pathways. However, if this were the case, the adult worms would be expected to decrease protein output earlier as they exhibited decreased integrity at 40 h, as judged by reduced mobility and ‘kinked’ appearance of the body, whereas the L4 still appeared healthy at this time. Also, a reduction in integrity might be expected to result in an increase in protein release due to leakage of intracellular and structural proteins into the medium. Whatever the reason, this tailing off of protein secretion may suggest that the worms are not behaving as they would do in vivo. However, it is possible that the protein profile released while secretion is underway is similar to that expressed in vivo, as it may be a result of stimuli encountered by the parasite within the host before the culture period. To address this question, it would be worthwhile to probe the ES material at the different time points with sera, lymph and gastric mucus from lambs rendered immune to infection by continual exposure to T. circumcincta. Genuine ES proteins are likely to be recognised by local or systemic antibody responses. It might also be possible to add radiolabelled amino acids to the culture media in order to detect their incorporation into proteins should synthesis be occurring.

Concentrated ES proteins from L4 and adult T. circumcincta, after 21 h in culture, were separated on Tris-HCl gradient gels under reducing conditions. Bands visible with Coomassie-staining were excised and submitted for LCMS/N-terminal sequence analysis and the resulting peptide mass and sequence data were used to search the online GenBank database. There were a large number of positive hits for trypsin of porcine origin. However, as trypsin was used to digest the samples, these results were regarded as contamination. A large proportion of the peptide sequences generated by this method did not produce any significant database hits. This is most
an FBA (Ov-fba-1) from the L3 stage of a filarial nematode, *O. volvulus*, by immunoscreening a cDNA library with serum from putatively immune humans. The nature of this enzyme means that it is likely to be located intracellularly. It is also part of a pathway conserved in host and parasite. However, protection trials with recombinant Ov-fba-1 in a murine model resulted in significant reductions in surviving larvae and did not result in host autoimmunity, suggesting that the immunogenic component of Ov-fba-1 is not conserved in the host FBA (McCarthy, Wieseman, Tropea et al., 2002). Proteins showing similarity to another glycolytic enzyme, enolase or 2-phosphoglycerate dehydratase, from *C. elegans* were also present in both stages. Again, enolase has not been studied in GI nematodes but has recently received attention in filarial nematodes. Experiments with a recombinant *O. volvulus* enolase (Ov-ENO) have shown the enzyme to be highly concentrated in the musculature of all stages of the parasite and to be recognised by serum antibodies from infected individuals (Jolodar, Fischer, Bergmann et al., 2003). The authors suggest that these results, in addition to the plasminogen-binding properties of enolase, may intimate its involvement in plasmin-mediated degradation of the host’s extracellular matrix, facilitating migration of the parasite through the tissues. Homologues of enolase and aldolase have also been identified by proteomic analysis of ES material from *H. contortus* (Yatsuda, Krijgveld, Cornelissen et al., 2003).

In L4 ES only, a protein of approximately 52 kDa shows similarity to fumarate hydratase or fumarase from several mammals. This enzyme is part of the Tricarboxylic Acid Cycle (TCA). In the past, there has been some disagreement over the presence of a fully functional TCA cycle in helminths (Singh, Katiyar, & Srivastava, 1992). However, studies with *A. ceylanicum* and *N. brasiliensis* have demonstrated the presence of the complete set of TCA cycle enzymes, including fumarase, in these species (Singh, Katiyar, & Srivastava, 1992). L4 ES also contains a protein sharing limited similarity with a *C. elegans* mitochondrial carrier protein, ATP/ADP translocase, which is involved in transport of metabolites to and from the mitochondrial matrix (Barrett, 1981).

Several apparent metabolic enzymes were identified exclusively in adult ES material. A 20 kDa protein showing similarity to *C. elegans* nucleoside diphosphate

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likely to be due to the limited nematode sequence data available online at present although it cannot be discounted that some of the proteins are entirely novel. The entire *C. elegans* genome is available for searching but this is a free-living nematode and many of the proteins secreted by *T. circumcincta* may be parasite-specific, being responsible for functions at the host-parasite interface. In fact, initial comparisons of the various parasitic nematode EST datasets with the *C. elegans* proteome have shown that up to 60% of the gene sequences available for each species do not share any similarity with *C. elegans* (Parkinson, Mitreva, Hall et al., 2003). At the beginning of 2003, there were more than 400,000 nematode expressed sequence tag (EST) sequences, originating from over 30 species of nematode, available online (Parkinson, Mitreva, Hall et al., 2003) and that number is constantly rising as more data are added. However, a large proportion (~190,000) of these current sequences are derived from *C. elegans*. At the time of writing, around 4,300 *T. circumcincta* EST sequences, arranged in 1,671 clusters, are available on NEMBASE. The *C. elegans* genome is predicted to contain more than 19,000 ORFs (Brooks & Isaac, 2002) and parasitic nematodes are thought to possess a similar number. It is apparent, therefore, that only a small percentage of *T. circumcincta* genes will be represented in online datasets. As more data becomes available, further searches with the unidentified peptide sequences may be performed. Despite this problem, putative functions were ascribed to several proteins from each stage as will be discussed below.

Several of the proteins identified in L4 and adult ES appeared to be homologues of metabolic enzymes, involved in various stages of energy metabolism. Little is known about the role of many of these enzymes in GI nematodes and whether they are involved in the host-parasite interaction. However, most have been extensively investigated in other parasites such as filarial worms and this work will be discussed here. ES from both stages contains a protein similar to a hypothetical *C. elegans* fructose-1, 6-biphosphate aldolase (FBA). This enzyme is involved in the early stages of glycolysis and is thought to be common to helminths and their mammalian hosts (Barrett, 1981). FBA activity has been demonstrated in several helminths, such as *A. lumbricoides* and *F. hepatica* (Barrett, 1981), but the enzyme has not been further characterised in these species. McCarthy *et al* (2002) identified
kinase (NDK) was present. NDKs are ubiquitous enzymes involved in phosphate transfer during the maintenance of intracellular reserves of dNTPs (Gounaris, Thomas, Najarro et al., 2001). An NDK was identified in a filarial nematode, B. malayi but this was achieved by immunoscreening a cDNA library with serum raised against somatic extracts of the parasite and the sequence did not contain a signal peptide (Ghosh, Raghavan, Fitzgerald et al., 1995). Until recently, secretion of NDKs had only been described in pathogenic prokaryotes. Gounaris et al (2001) demonstrated that the infective larvae of the nematode T. spiralis secrete NDK. However, T. spiralis is an intracellular parasite and is therefore likely to employ secreted NDK to the same ends as intracellular bacteria i.e. promotion of infected-cell survival (Gounaris, Thomas, Najarro et al., 2001). It might be expected that NDK is more likely to play a purely metabolic role in T. circumcincta. As described in Chapter One, the presence of T. circumcincta in the abomasum can result in a range of morphological and biological changes and substances secreted by the parasite may be in part responsible. NDK has been shown to play a role in extracellular nucleotide metabolism in human airways (Donaldson, Picher, & Boucher, 2002). In this system, NDK is expressed on the epithelial surface and in gland secretions and is thought to affect actions such as liquid secretion and goblet cell degranulation, through regulation of luminal nucleotide concentrations (Donaldson, Picher, & Boucher, 2002). If a similar system were to operate on the surface of the abomasum, it is possible that T. circumcincta-derived NDK could play a role in manipulating the abomasal environment.

Other possible metabolic enzymes were identified in adult ES. Proteins similar to a C. elegans aldo/keto reductase family member and glutamate dehydrogenase (GDH) from H. contortus, both of which may be involved in aa catabolism (Barrett, 1981), were present at 40 and 60 kDa respectively. GDH, the dominant component of H. contortus TSBP, is strongly recognised by sera from TSBP-immunised sheep, and is expressed almost exclusively in the blood-feeding stages of the parasite (Skuce, Stewart, Smith et al., 1999). Another possible glycolytic enzyme, glycogen phosphorylase, responsible for the first step in glycolysis (Barrett, 1981), and a protein similar to an arginine kinase from the soybean cyst nematode H. glycines were also identified.
Galectins are discussed in more detail in Chapter Three. Here, a protein sharing a high level of identity with galectin is present in L4 ES at approximately 34 kDa. As with galectin sequences identified in the library screen, this protein gave a higher score with *H. contortus* galectins than with *T. circumcincta* galectins for which sequence data is available. Alignment of these sequences, and localisation of the regions identical to the ES peptides, indicates the presence of two tandem repeat-type galectins in this ES band. One of these may not have previously been sequenced and is more closely related to the *H. contortus* GAL-3 galectins, rather than the GAL-1 or GAL-2 galectins isolated from *T. circumcincta* (Greenhalgh, Loukas, Donald et al., 2000). The other may be one of the galectins isolated previously from *T. circumcincta* L3 by Newton *et al.* (2000). However, further sequence analysis would be required in order to confirm this. This might also represent stage-specificity of expression as the *T. circumcincta* GAL-1 and GAL-2 galectins were cloned from the L3 (Newton, Monti, Greenhalgh *et al.*, 1997). As discussed previously, vaccination with native *H. contortus* galectin, purified from a protective worm fraction, did not confer protection in sheep (Newlands, Skuce, Knox *et al.*, 1999).

Proteins showing similarity to several structural proteins were identified in ES from both stages. Both contained a possible actin at approximately 47 kDa. This structural protein is a major component of the cytoskeleton, and, although also identified in the ES material from *O. ostertagi*, is unlikely to be actively secreted by the worm (Vercauteren, Geldhof, Peelaers *et al.*, 2003). Proteins showing similarity to *C. elegans* tropomyosin and paramyosin are present in L4 ES. These are both components of muscle and are again unlikely to be secreted. However, tropomyosin has been shown to be a potential protective antigen in other parasite infections. A novel 41 kDa antigen, not recognised by antibodies in sera from a naturally infected host, was isolated from a detergent-soluble fraction of *T. colubriformis* and induced a post-challenge reduction in worm counts of 43-51% when used to vaccinate guinea pigs (O'Donnell, Dineen, Wagland *et al.*, 1989). Incubation of *T. colubriformis* L4 sections with monoclonal antibody raised against this protein caused staining predominantly of the musculature. Proteolytic digestion and partial aa sequence analysis of resulting peptides confirmed that the protein was very similar to tropomyosin (O'Donnell, Dineen, Wagland *et al.*, 1989). In addition to this, host
antibody responses to parasite tropomyosin have been implicated in limiting microfilarial densities in natural infections with *O. volvulus* (Jenkins, Taylor, Gilvary et al., 1998). Tropomyosin in the musculature is unlikely to be exposed in living worms. It has been suggested that tropomyosin leaked from dying worms may induce a response that leads to cross-reactivity with a non-muscle form, resulting in parasite destruction (O'Donnell, Dineen, Wagland et al., 1989). Whatever the mechanism of immunity, these studies demonstrate that vaccination with a structural protein may induce significant levels of protection. These ubiquitous metabolic enzymes and structural proteins are predominantly intracellular or the product of worm degradation rather than true secretory proteins. Like many of the proteins detected in ES material from *O. ostertagi* and *H. contortus*, they are likely to be present due to cellular damage or leakage due to a reduction in the integrity of the cultured worms.

Some of the ES proteins identified are likely to be true secretory products. A small protein of approximately 20 kDa with similarity to a globin-like protein isolated from *O. ostertagi* L4 ES was identified (Vercauteren, Geldhof, Peelaers et al., 2003). This protein is present in *T. circumcincta* L4 and adult ES but appears as a doublet of bands at 18 and 20 kDa in the adult. Globin-like sequences are also highly represented in the *T. circumcincta* EST dataset (8 clusters), with sequences being identified from adult and L4 libraries. A secreted 18 kDa globin-like protein isolated from the ES products of *T. colubriformis* induced 60-84% protection against challenge with this species in guinea pigs (Frenkel, Dopheide, Wagland et al., 1992). However, no data has been published on this antigen’s protective capacity in sheep. Screening of *O. ostertagi* somatic extracts with rabbit anti-globin serum identified a 17 kDa globin-like protein thought to be a potential vaccine candidate (de Graaf, Berghen, Moens et al., 1996). This protein was located in the body wall musculature and cuticle of the adult worm. Sequence analysis of an *O. ostertagi* cDNA encoding the globin-like protein identified in ES showed that it contained a signal peptide and had a different tryptic peptide fingerprint from the somatic globin (Vercauteren, Geldhof, Peelaers et al., 2003). Vaccination with globin purified from somatic extracts did not induce a consistent protective response (de Graaf, Berghen, Moens et al., 1996). The protective capacity of globin purified exclusively from *O. ostertagi* ES has not been evaluated (P. Geldof, personal communication).
A protein of approximately 30 kDa in size in L4 ES showed similarity to an EST sequence from adult *T. circumcincta* that, in turn, shared considerable similarity with several cysteine proteases. Protease activity in *T. circumcincta* L4 and adult ES has been partially characterised by substrate gel analysis (Young, McKeand, & Knox, 1995). This study demonstrated several zones of proteolysis between 32 and 100 kDa in L4 ES. Apart from a possible serine protease at 90 kDa, the nature of the proteases responsible was unclear (Young, McKeand, & Knox, 1995). In adult *T. circumcincta* ES, proteolysis was predominantly attributed to metallo- and serine proteases (Young, McKeand, & Knox, 1995). Cysteine protease activity has been demonstrated in the ES products of several other closely related nematodes. In *O. ostertagi*, substrate gel analysis at pH 5 detected cathepsin L-like activity in adult ES, at 42 kDa but not in L4 ES (Geldhof, Claerebout, Knox et al., 2000). Vaccination with *O. ostertagi* adult ES, enriched for cysteine proteases by thiol-sepharose chromatography, induced a 60% reduction in post-challenge egg counts (Geldhof, Claerebout, Knox et al., 2000). There was also a reduction in worm numbers and a higher percentage of inhibited L4 in vaccinated animals. The presence of cathepsin B-like cysteine proteases in *H. contortus* in membrane-bound gut-expressed fractions and their protective capacity has been discussed in previous chapters. *In vitro* culture of *H. contortus* identified cathepsin L-like activity in both L4 and adult ES between 30 and 41kDa (Rhoads & Fetterer, 1995). More recently, immunisation with a *H. contortus* ES fraction, enriched for cysteine protease activity, has been shown to significantly reduce egg and worm burdens after a single challenge infection (Bakker, Vervelde, Kanobana et al., 2004). Cathepsin L proteases are also secreted by all developmental stages of the liver fluke, *F. hepatica* (Jeffries, Campbell, van Rossum et al., 2001; Mulcahy & Dalton, 2001). Two native cathepsin L proteases, purified from adult *F. hepatica* ES products, have been evaluated in vaccine trials and gave levels of protection of over 50% in cattle (Dalton, McGonigle, Rolph et al., 1996), and from 30-60% in sheep (Piancenza, Acosta, Basmadjian et al., 1999). In addition, a greater proportion of flukes in cathepsin L-immunised animals failed to reach maturity and their fecundity was greatly reduced (Dalton, Neill, Stack et al., 2003).
In both adult and L4 *T. circumcincta*, a 60 kDa protein similar to a protein disulphide isomerase (PDI), identified in the ES from the parasitic stages of *O. ostertagi*, was present (Vercauteren, Geldhof, Peelaers et al., 2003). This ubiquitous enzyme is a catalyst of protein folding in eukaryotes via disulphide bond formation, a process that is rate-limiting during the generation of many secretory and outer membrane proteins (Pirmeskoski, Klappa, Lobell et al., 2003). In nematodes, it is thought to play a crucial role in cuticle synthesis. PDI exists as the β-subunit of a multi-enzyme complex in *C. elegans* and is expressed in collagen-synthesising ectodermal cells (Winter & Page, 2000). Disruption of the *C. elegans* PDI gene by RNAi produces an embryonic lethal phenotype, which the authors attribute directly to cuticular defects (Winter & Page, 2000). Western blot analysis with *O. ostertagi* ES has demonstrated recognition of parasite PDI by serum antibodies from naturally *O. ostertagi*-infected calves (Geldhof Vercauteren, Knox et al., 2003). This suggests that PDI is released *in vivo* by the parasite, either by active secretion or by dying worms.

A 75 kDa protein similar to heat shock protein (HSP) 70-C from the *Panagrellus redivivus*, a small soil-dwelling nematode commonly known as the microworm, was identified in L4 ES. In *C. elegans*, HSPs of around 70 kDa are coded for by the hsp70 multigene family, made up of at least nine genes (Heschl & Baillie, 1990). HSPs of 70 kDa have been isolated from a range of vertebrates and invertebrates, including several parasitic nematodes. For example, expression of a 70 kDa HSP was identified during the *in vitro* transformation from free-living larva to the parasitic stage, induced by an increase in temperature, in *Strongyloides venezuelensis* (Tsuji, Ohta, & Fujisaki, 1997). Smaller HSPs are also common. Moreover, a 20 kDa HSP has recently been identified and characterised in *H. contortus* (Hartman, Cottee, Savin et al., 2003). The protein was expressed in the L4 and adult worms and, in adults, was located in the intestine and reproductive organs. 15 clusters representing homologues of HSPs of various sizes are present in the *T. circumcincta* EST dataset. All of these clusters are composed of sequences from adult libraries. The exact function of heat shock proteins in these parasites is not known. They are thought to act as molecular chaperones within cells and their expression is often upregulated in response to stressful conditions. It has been
suggested that they may somehow assist the worm's survival during dramatic changes in its environment, such as that experienced by free-living infective larva on entering the mammalian host or by maturing worms as they leave the abomasal glands. This suggestion is supported by the fact that their expression is often developmentally regulated (Jecock & Devaney, 1992). It is possible, therefore, that the protein identified here is a novel HSP, expressed stage-specifically in L4 *T. circumcincta*, and may play some role in the transition from non-feeding larva to feeding adult.

A protein of around 27 kDa in L4 ES was similar to a fragment of thioredoxin peroxidase (TPx), isolated from the ES products of adult *O. ostertagi* (Vercauteren, Geldhof, Peelaers et al., 2003). TPx, a member of the peroxiredoxin (Prx) gene superfamily, was discovered quite recently and is part of the antioxidant pathway that protects the parasite from oxidative damage, either from by-products of the parasite's metabolism or from host-derived ROS. This enzyme reduces hydrogen peroxide with thioredoxin as a hydrogen donor (Chae, Chung, & Rhee, 1994). The role of antioxidants in evasion of the host immune response was discussed in Chapter Four. Prx have been described in several species of parasitic nematode. A cDNA encoding a TPx with a predicted molecular mass of 22.1 kDa has been isolated from an L4 library of the filarial heartworm, *Dirofilaria immitis*, by screening with serum from immune dogs (Klimowski, Chandrashekar, & Tripp, 1997). TPx was detected in L3, L4 and adult *D. immitis* and was also present in the adult ES products. Its sequence lacked a signal sequence, suggesting that it is not actively secreted but the authors suggest that it may be transported by an alternative mechanism (Klimowski, Chandrashekar, & Tripp, 1997). cDNAs encoding TPx made up 2.5% of an *O. volvulus* L3 library and the protein was detected in the larvae and adults, implying that TPx is an important detoxifying enzyme in this species (Lu, Egerton, Bianco et al., 1998). A cDNA encoding a Prx of around 22 kDa has also been isolated from *A. suum* (Tsuji, Kasuga-Aoki, Isobe et al., 2000). Immunoblot analysis with anti-Prx antibodies identified two proteins of 23 and 25 kDa in extracts from all life stages of the parasite. A Prx is implicated in the defence of the potato cyst nematode, *G. rostochiensis*, against plant immune responses, being expressed on the surface of the parasite and in its secretions (Robertson, Robertson, Sobczak et al., 2000).
Recently, it has been suggested that TPx may be the major detoxifying enzyme in the protoscoleces of the cestode, *Echinococcus granulosus*, as they lack catalase and glutathione peroxidase (Li, Zhang, Loukas et al., 2004). The enzyme is expressed at similar levels in several stages of the parasite and is recognised by serum antibodies from some infected hosts (Li, Zhang, Loukas et al., 2004). TPx was also among several antioxidant enzymes present in ES material from adults of the liver fluke, *F. hepatica* (Jefferies, Campbell, van Rossum et al., 2001). The identification of TPx in *T. circumcincta* ES suggests that Prxs may also play an important role in defence against free radicals in this species. ESTs from adult *T. circumcincta* libraries encoding a homologue of TPx are present on NEMBASE, suggesting that this enzyme is expressed in both parasitic stages.

The 14-3-3 proteins make up a ubiquitous and highly conserved family, which have been identified in a variety of eukaryotes but have not been characterised in parasitic nematodes. A protein of approximately 29 kDa in adult *T. circumcincta* ES shows considerable similarity to a member of the 14-3-3 protein family isolated from *C. elegans*. These proteins have been ascribed a number of functions, particularly in signal transduction and development (Wang & Shakes, 1997). In *C. elegans*, the protein kinase Raf is a signalling protein essential for larval viability and fertility (Hsu, Zobel, Lambie et al., 2002). Sequence analysis of Raf has revealed a C-terminal 14-3-3-binding domain. Mutation of this domain reduces Raf activity, indicating that binding of 14-3-3 protein positively regulates Raf (Hsu, Zobel, Lambie et al., 2002). Studies involving mutation of *C. elegans* 14-3-3 genes so far have not resulted in defects in Raf function but not all genes have been analysed (Hsu, Zobel, Lambie et al., 2002). ESTs sharing a high level of similarity with the same *C. elegans* 14-3-3 protein (NP_509939) have been identified from several species of parasitic nematode including *N. brasiliensis* and *A. suum* but not from *T. circumcincta*.

In conclusion, this chapter has described the use of proteomic techniques to carry out a preliminary characterisation of the proteins released during in vitro culture of *T. circumcincta* L4 and adults. ES contained proteins with a wide range of
putative functions including carbohydrate and aa metabolism, oxygen transport, proteolysis, protein folding and detoxification. Many of these proteins are represented in the *T. circumcincta* EST dataset. Two proteins, the L4 70 kDa HSP and the 14-3-3 homologue, have not been described in this species before and may have important functions in parasite development. A large proportion of the proteins gave significant hits with intracellular metabolic enzymes. Analysis of *in vitro* ES from *O. ostertagi* and *H. contortus* gave similar results. This evidence suggests that the worms may begin to degrade after less than a day in culture and that what we know as ES contains many proteins that cannot be described as ES material and may not be released by healthy worms *in vivo*. However, ES material prepared by the same culture method has induced significant levels of protection in various trials so it is apparent that some of its components are accessible to effectors of the immune system. As stated above, the resources for identifying and ascribing functions to nematode proteins are continually expanding. The data collected from this experiment can therefore be expanded upon in several ways. As new parasitic nematode nucleic acid and protein sequences and peptide fingerprint data become available online, the peptide data from this study can be re-examined to possibly identify further ES proteins. Also, proteins of interest identified here could be investigated further. For example, the importance of antioxidant enzymes has been discussed at length previously. It might be interesting, therefore, to isolate a full-length cDNA for the putative thioredoxin peroxidase, using nucleic acid sequence data from the ESTs, in order to further characterise this enzyme in *T. circumcincta*. As a cysteine protease-enriched ES fraction has proven protective against *H. contortus*, further work on the putative cysteine protease in L4 ES may also be of benefit. Overall, the results presented in this chapter demonstrate that current proteomic technology provides a useful tool for characterisation of complex protein mixtures and is sure to be of enormous benefit to this area of research in future.
CHAPTER NINE - GENERAL DISCUSSION

Infection of the ovine abomasum with the nematode, *T. circumcincta*, causes changes in the gastrointestinal environment that result in diarrhoea and reduced appetite, leading to diminished fleece and carcass quality. This results in considerable economic losses to the sheep and goat farming industries. At present, infections with this and other such nematode parasites are combated with a combination of grazing management and treatment with anthelmintic drugs. However, isolation of nematode strains resistant to multiple anthelmintics worldwide has prompted a search for alternative forms of control, such as vaccination. Sheep acquire immunity to infection with *T. circumcincta* after multiple infections, implying that vaccination against the species should, in principle, be possible. The aim of the work presented here was to further characterise two potential sources of antigens from the ovine GI parasite. In the current project, these consisted of a ‘hidden antigen’ fraction, known to stimulate a degree of protective immunity, and ES material from *T. circumcincta*, the nature of which was largely undefined. The prime aim of this project was to further define these compartments at the molecular level with a view to defining the proteins present, expanding knowledge of the biology of the parasite and identifying potential vaccine targets on the basis of function.

The integral membrane protein fraction S3 TSBP has been shown, by immunolocalisation studies, to be composed predominantly of proteins expressed on the luminal surface of the gut and is enriched for cysteine protease activity. In this study, serum from a *T. circumcincta* TSBP-vaccinated animal, that exhibited a high level of protection to a single homologous challenge with the same species, was used to screen a cDNA library. The aim of this was to identify components, by homology searches of nucleic acid and protein databases, of the fraction recognised by antibodies produced by the host immune system in response to vaccination. Due to the nature of the fraction, it was expected that these would consist chiefly of
membrane-bound proteins and cysteine proteases. However, as detailed in Chapter 3, this was not the case. The majority of the POs isolated shared a high level of similarity with galectin sequences. Galectins have been isolated from several vertebrate and invertebrate species, including *T. circumcincta*. The exact functions of these galactoside-binding proteins are not yet clear but they are thought to play important roles in differentiation and morphogenesis. Interestingly, the POs, and a galectin-like protein identified in *T. circumcincta* L4 in vitro ES material (Chapter 8), shared a higher level of similarity with *H. contortus* galectins than with any *T. circumcincta* galectins sequenced to date. This suggests that these are novel *T. circumcincta* galectin sequences. The top hit for the PO sequence was a *H. contortus* prototype galectin, a type not previously identified in *T. circumcincta*. The top hit for the ES protein was a *H. contortus* tandem-repeat type galectin, suggesting that this *T. circumcincta* galectin is different from the two tandem-repeat type galectins previously sequenced from *T. circumcincta*. Further analysis of the full coding sequence for each would be required to confirm this. Galectins have proven unsuccessful as nematode vaccine candidates but a further understanding of their purpose may provide important information on the biology of the worms.

Many metabolic pathways have been studied widely in mammals but little is known of their conservation in gastrointestinal nematodes. An increased knowledge of the metabolism of these parasites is likely to be useful in focussing the search for novel anthelmintic drug targets. Of the remaining POs identified with anti-TSBP serum, clones encoding two metabolic enzymes, both members of the aldehyde dehydrogenase superfamily, predominated. Chapters 5 and 6 discuss the first sequence data for MMSD and FTDH available for helminths, and the first report of the presence of FTDH in a GI nematode. This provides further evidence that the pathways for pyrimidine and valine catabolism characterised in mammals, in which MMSD is involved, are conserved in strongyloid nematodes and suggests that the same may be true of folate metabolism. Neither of these proteins possessed putative transmembrane domains and, hence, both are likely to be intracellular. In mammals, MMSD activity is located in the mitochondria and this would appear to be true also
of TcMMSD, due to the presence of a mitochondrial targeting sequence with a high calculated probability of export to this organelle. FTDH activity has also been detected in the mitochondrial matrix in other organisms but is predominantly a cytosolic enzyme. mRNA for both enzymes was present in both the infective and parasitic stages of the parasite. A band representing the native TcMMSD protein could be detected in the soluble (S1), membrane-associated (S2) and membrane-bound (S3) extracts for all stages examined. This demonstrates that non-membrane-bound proteins are present in the T. circumcincta S3 extract, prepared by the same protocol as the S3 extract fractionated to give the TSBP vaccine. Evidence from the literature concerning these enzymes and comparison of their sequences with those from other species confirmed that both possess a putative aldehyde dehydrogenase cysteine active site, of which a free sulphhydryl group is the functional group. TSBP are prepared by passing S3 over a column with an affinity for such groups so, in addition to enriching for cysteine proteases, this process is likely to favour other enzymes involving a catalytic cysteine residue. This explains why antibodies raised against a predominantly membrane-bound protein fraction have identified such intracellular enzymes. As discussed previously, vaccine candidates must be accessible to effectors of the host immune system in order to be effective. These two enzymes can therefore be ruled out as potential vaccine candidates, as they are not expressed on the parasite external or intestinal surface. However, enzymes involved in related pathways have been shown to be the site of action of chemicals effective against other species of parasitic worms, so they could be prospective drug targets.

Proteomic analysis of material excreted or secreted by T. circumcincta during in vitro culture revealed the presence of several more enzymes involved in apparently conserved metabolic pathways. Proteins with similarity to glycolytic enzymes, such as fructose-1, 6-biphosphate aldolase, glycogen phosphorylase and enolase, were identified in both L4 and adult material. In L4 ES, a Tricarboxylic Acid Cycle enzyme was identified and, in adults, proteins similar to enzymes involved in amino acid catabolism were present.
The ubiquitous metabolic enzyme NDK is involved in maintenance of dNTP reserves and was thought to act only intracellularly. However, two recent studies have demonstrated potential extracellular functions for NDKs. Firstly, in humans they have been shown to be expressed on the epithelial surface and in gland secretions of the airways, and are thought to regulate goblet cell degranulation and gland secretions (Donaldson, Picher, & Boucher, 2002). Secondly, the infective larvae of an intracellular parasitic nematode, \textit{T. spiralis}, have been shown to secrete NDK (Gounaris, Thomas, Najarro et al., 2001). A protein similar to \textit{C. elegans} NDK was present in adult \textit{T. circumcincta} ES.

In haematopoietic cells, signalling through nucleotide receptors stimulates a multitude of responses including platelet aggregation and mediator release, degranulation of mast cells, neutrophils and eosinophils, cytokine production by T cells, monocytes and macrophages, and activation, differentiation and T cell-polarising properties of dendritic cells (Di Virgilio, Chiozzi, Ferrari et al., 2001; Linden, 2001; Ohta & Sitkovsky, 2001; Panther, Corinti, Idzko et al., 2003). Nucleotides may be secreted in a regulated manner or released upon mechanical stimulation. Tissue damage results in massive release into extracellular fluids, and thus extracellular nucleotides are archetypal activators of the innate immune system (Linden, 2001; Haskó & Cronstein, 2004). Adenosine 5'-diphosphate (ADP) has long been known to be a major agonist of platelet aggregation. Thus, the discovery of an apyrase, that hydrolysed ADP in the saliva of the tick, \textit{Ixodes dammini} (Ribeiro, Makoul, Levine et al., 1985), made sense in that a prime consideration for blood-feeding ectoparasites is inhibition of haemostasis. Subsequent studies identified a variety of other nucleotide metabolising enzymes in arthropod saliva that were postulated to suppress the proinflammatory effects of these molecules, in addition to inhibiting pain and promoting vasodilation (Ribeiro & Francischetti, 2003).

Both adenosine and inosine exhibit potent immunomodulatory effects. Adenosine not only inhibits platelet aggregation, but suppresses the production of inflammatory cytokines, such as tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and IL-12, and enhances IL-10 production by monocytes and macrophages (Haskó & Cronstein, 2004). In turn, inosine has been demonstrated to suppress synthesis of IL-1, IL-12,
MIP-1 \( \alpha \), TNF-\( \alpha \) and IFN-\( \alpha \) in macrophages and IL-8 by epithelial cells, and inhibit neutrophil activation. Nucleotides also induce mucin release from hamster surface tracheal cells (Kim, Park, Shin et al., 1996), and it is possible that NDKs may inhibit this action.

A secreted form of NDK, derived from \textit{T. circumcincta}, could therefore be responsible for some of the morphological and chemical changes that occur during infection with this species, through its influence on abomasal luminal nucleotide levels. However, it cannot be presumed that this \textit{T. circumcincta} protein, with similarity to NDK, is actively secreted on the basis of its appearance in \textit{in vitro} ES material. In order to investigate this further, the full-length \textit{T. circumcincta} NDK sequence could be obtained and the presence of a signal sequence confirmed or dismissed. It might also be possible to express an active recombinant form of the enzyme and observe its effect on abomasal tissue \textit{in vitro}. As described in Chapter 1, the outcomes of many studies imply mucus release is an important factor in the clearing of gastrointestinal nematode infections. If the parasite were able to curb this release, it may be able to impair expulsion. If this were the case, a secreted \textit{T. circumcincta} NDK may be a potential vaccine candidate.

The importance of detoxifying enzymes, in the evasion of the host’s immune response by nematode parasites, has been well documented. Effector cells, such as macrophages and eosinophils, have been shown to release toxic reactive oxygen species in the presence of parasite antigens. These molecules can bring about a wide range of deleterious effects and are thought to contribute to a host’s ability to expel parasites. To protect themselves from such an attack, many species of nematode are thought to produce antioxidant enzymes to neutralise the oxidative agents. Such enzymes were identified in both sources of antigenic material from \textit{T. circumcincta} studied in this project. Chapter 4 describes the analysis of a single P0 clone, isolated by cDNA library screening with anti-TSBP serum. This clone contained a full-length coding sequence for a protein sharing a high level of similarity with confirmed catalase sequences from a range of organisms, including other parasitic nematodes and mammals. Further analysis of the sequence confirmed the presence of a conserved catalase active site and other features characteristic of catalases. H\(_2\)O\(_2\) is
an oxidative agent but, more importantly, is a source of the highly reactive hydroxyl group, for which no effective antioxidant has been identified. Catalase is part of a series of enzymes that convert hydrogen peroxide into oxygen and water, thus protecting the organism from damage. The TcCAT sequence did not contain a signal peptide or a putative peroxisomal targeting sequence and also lacked a transmembrane domain. This, combined with the identification of the native protein in the soluble worm extracts only, led to the conclusion that TcCAT is a cytosolic enzyme. It is likely to protect intracellularly against H$_2$O$_2$ released by the parasite itself as a by-product of metabolism. However, the fact that H$_2$O$_2$ appears to have the ability to freely diffuse across cell membranes suggests that this enzyme could also neutralise exogenous H$_2$O$_2$ that enters the cells. This might be instrumental in preventing disruption of the luminal surface of the nematode intestine by ingested H$_2$O$_2$. Kotze (2003) reported the upregulation of catalase activity in *H. contortus* exposed to H$_2$O$_2$ and demonstrated a positive correlation between catalase activity and worm survival. This apparent catalase activity was calculated by measuring the disappearance of H$_2$O$_2$ from homogenates of worms previously exposed to the toxin. As discussed below, other enzymes have the ability to neutralise H$_2$O$_2$. Consequently, a specific catalase inhibitor was used to demonstrate that a large proportion of the activity was attributable to catalase and that its inhibition increased the worm’s susceptibility to H$_2$O$_2$. As the cellular location of the catalase activity was not explored, it is possible that a cytosolic *H. contortus* catalase, similar to TcCAT, was responsible. Another detoxifier of H$_2$O$_2$, thioredoxin peroxidase (TPx), has been found in ES material from *O. ostertagi*, the heartworm *D. immitis* and the liver fluke *F. hepatica*, suggesting that hydrogen peroxidase is an important antioxidant in parasitic worms. The peroxiredoxins (Prxs), of which TPx is a member, have also been implicated in the defence against plant immune responses by *G. rostochiensis*, the potato cyst nematode. The identification of a protein similar to TPx in *T. circumcincta* ES suggests that Prxs may be important in defence against exogenous free radicals in this species.

Further investigation of TcCAT and TPx activity *in vitro* would be advantageous in understanding the role that these enzymes play. As TcCAT appears to be a cytosolic enzyme, it is unlikely to be suitable as a vaccine. ESTs encoding
T. circumcincta homologues of TPx could be used for further analysis of its sequence and to investigate whether a secreted form, and possible vaccine candidate, is expressed. At present, two clusters representing putative T. circumcincta homologues of TPx are present on NEMBASE. At present, only one of these appears to include the N-terminal sequence. On entering the translated sequence for this cluster into the Signal P program, the signal peptide probability was 0.150 and the signal anchor probability 0, leading to the prediction that this is not a secretory protein. However, it is worth noting that no Prx sequences with a signal peptide have been isolated from parasitic nematodes. The Prx cloned from G. rostochiensis appears to be transported to the parasite surface and released in secretions without the presence of a signal sequence (Robertson, Robertson, Sobczak et al., 2000). It may also be useful to investigate other antioxidant enzymes produced by T. circumcincta and whether these enzymes are capable of protecting the parasite from external sources of oxidative damage. Should this be the case, the ever-expanding EST database will again be of great use in extracting the coding sequence for such proteins. This would allow possible immunisation studies with recombinant antioxidants, should the native proteins be deemed potential vaccine candidates. Indeed, a recent report demonstrated that lambs vaccinated with enzymically active recombinant SODs from H. contortus were partially protected against homologous single challenge infection (Liddell & Knox, 1998).

It is apparent from the data presented here that the components of particular antigenic preparations cannot be taken for granted. Although TSBP was shown to be composed of predominantly intestinal membrane-bound proteins, the results of cDNA library screening clearly show that several components not matching this description were present. MMSD and FTDH both possess cysteine active sites that would explain their affinity for the thiol-separose lectin column. Catalase, however, does not posses such a site. An explanation might be that TcCAT is closely associated with cell membranes, to protect them from oxidative attack, and that it remains in the membrane-bound fraction due to insufficient washing of the pellet before applying to the column. This does not explain, however, why cysteine
proteases or proteins possessing transmembrane domains were not prevalent in the immunoscreen. It could be argued that the major components of the fraction, while responsible for any resulting protection to the host, may be only weakly immunogenic. In fact, this has proven to be the case with cysteine proteases isolated from the gut of *H. contortus* (D Knox, personal communication). This might make them difficult to identify by immunoscreening, where immunopositive plaques are picked on the basis of a visible contrast with the background. If this were the case, weakly stained plaques might be overlooked, explaining the lack of cysteine proteases isolated. It might therefore be worthwhile to employ the putative cysteine proteases available in the *T. circumcincta* EST dataset, for which there are currently 16 clusters, to obtain full coding sequences from this species in order to investigate their antigenic properties and vaccine potential. A starting point here would be to identify the closest homologues to cysteine proteases known to be localised in the gut of *Haemonchus* and then to examine localisation using in situ hybridisation or immunolocalisation with antibody probes.

To further elucidate the components of *T. circumcincta* TSBP, proteomic techniques, such as those employed for ES material in Chapter 8, might be of use. TSBP-vaccination is thought to protect through an antibody-mediated mechanism but analysis of serum antibody responses to vaccination did not reveal any apparent relationship with protection. Therefore, investigation of the local immune response in TSBP-vaccinated sheep may also be of interest. However, the decision to proceed further down this route would depend on the assessment of whether, on the basis of the results presented here, TSBP is still considered a viable source of vaccine candidates. The level of protection observed in the TSBP trial was highly variable, suggesting that this method of vaccination, i.e. hidden antigens, may not be suitable for non-blood-feeding parasites. However, the early successful vaccination trial with TSBP was conducted with TSBP produced from mixed stages of *T. circumcincta* while material used in later trials was purified from adults alone. It is possible that TSBP from e.g. L4s may have a potent stage-specific effect given that this stage of the parasite grows rapidly in the abomasal glands.
The search for vaccine targets from the bovine parasite *O. ostertagi*, closely related to *T. circumcincta*, has focused on ES material. This has produced some promising results, particularly the recent work with ES polyprotein allergens (Vercauteren, Geldhof, Vercruysse et al., 2004). The analysis of proteins released by *T. circumcincta* during *in vitro* culture demonstrated the difficulty of isolating true ES material, i.e., that which would be secreted *in vivo*, from a GI parasite. The presence of multiple putative structural and cytosolic proteins implies that the worms, although checked visually for viability, may begin to deteriorate after as little as 21 h in such an environment. Obtaining full-length sequence to scan for signal peptides can aid the search for actively secreted proteins. However, as demonstrated by work with a peroxiredoxin from the plant parasite, *G. rostochiensis*, cleavable N-terminal signal peptides are not always present in proteins expressed on the surface or in secretions of nematodes (Robertson, Robertson, Sobczak et al., 2000). This suggests that an alternative mechanism for transport of proteins to the parasite surface and its surroundings is in operation and has yet to be characterised. Another way of identifying true ES proteins might be to screen two-dimensional gels of *in vitro* culture fluids with serum from naturally or experimentally *T. circumcincta*-infected animals. Such screening should only identify proteins released into the host environment during infection, presenting promising subjects for further investigation.

As reviewed recently in Science, methods of drug design are continually being revised, with various disciplines, such as computational and organic chemistry and high-throughput screening of targets, utilised (MacCoss & Baillie, 2004; Jorgensen, 2004). However, the path from potential drug target to clinical trials through to a commercially available product is complex. Firstly, a suitable target must not only be essential to some aspect of the parasite’s ability to survive and reproduce, it must also be either unique to the parasite or adequately different in some respect from the equivalent host molecule. This difference could be manifested in the aa sequence, post-translational structure or site of expression but must result in the parasite molecule being more susceptible to inhibition by a particular chemical.
This allows the drug to affect the parasite without being significantly detrimental to the host. For example, the macrocyclic lactone anthelmintics are thought to exert their effect by binding to a family of α-type subunit-containing glutamate-gated chloride channels that are unique to invertebrates. After a potentially suitable target is identified, there can then follow a myriad of screening and computational modelling methods in order to identify chemicals that might interact with this target, in order to hinder its purpose in some way.

The evaluation of potential vaccine targets is a similarly arduous process. This is often due to the requirement for an antigen whose manufacture can be scaled-up to a commercially-viable level. This is not possible with native antigens from most parasites as donor animals are required and the material obtained from each donor is very limited. This makes the harvesting of such antigens expensive, time-consuming and certainly not marketable. This is where the advent of recombinant DNA technology is hoped to provide the answers. However, as discussed in earlier chapters, the choice of expression system is crucial if an antigen that is protective in its native form is also to prove so as a recombinant. It is becoming apparent that bacterially-expressed recombinants, of nematode proteins, often do not possess the correct properties required to stimulate the response induced by the native protein. As mentioned above, vaccine trials with a native O. ostertagi polyprotein allergen have given promising results, with considerable reductions in FEC (Vercauteren, Geldhof, Vercruysse et al., 2004). However, vaccination with a recombinant polyprotein allergen, expressed in E. coli, did not provide any protection. To this end, alternative expression systems that might produce more appropriate post-translational modifications, such as insect cells and transgenic C. elegans, are being developed.

The work presented here has advanced the search for vaccine candidates against T. circumcincta by defining numerous components of the host-protective TSBP fraction and also providing the first comprehensive definition of the components in ES from L4 and adult stages. As discussed, many of these proteins have putative functions that could aid parasite survival in the host, be it in normal metabolism or in immuno-evasion. These proteins are clearly worthy of further study.


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## APPENDIX 1 - L4 ES PEPTIDE DATA

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### APPENDIX 2 - ADULT ES PEPTIDE DATA

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A single peptide ion is selected from the spectrum generated by MS1.

This peptide ion is fragmented by collision with inert gas. This creates a series of fragment ions.

The mass of the fragment ions is measured by MS2. The amino acid sequence is then deduced from the differences in mass of the fragment ions.

Adapted from [www.gla.ac.uk/departments/ibls/ASU/fgf/](http://www.gla.ac.uk/departments/ibls/ASU/fgf/)