Haemonchus contortus and hookworms - parallels in vaccine development

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DECLARATION

The work reported in this thesis was carried out under the supervision of Dr David Knox at the Moredun Research Institute and Dr Collette Britton at the Faculty of Veterinary Medicine, University of Glasgow. All results presented, unless otherwise stated, are the sole work of this author, as is the composition of this thesis.

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ABBREVIATIONS

Ac	*Ancylostoma caninum*
AEBSF	4-2-Aminoethyl benzenesulphonyl fluoride
Ag	Antigen
Amp	Ampicillin
ANOVA	Analysis of variance
ASP	*Ancylostoma* secreted protein
BSA	Bovine serum albumin
bp	Base pair
CD	Cluster of differentiation
cDNA	Complementary DNA
Contigs	Contiguous sequences
CRISPs	Cysteine rich secreted proteins
DALYs	Disability-adjusted life years
DAB	Diaminobenzidine tetrahydrochloride
DIG	Digoxygenin
dH_2O	Distilled water
dNTPs	Deoxynucleoside 5´-triphosphates
DNA	Deoxyribonucleic acid
ds	Double stranded
DTT	dithiothreitol
EBSS	Earle's Balanced Salt Solution
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetra-acetic acid

EF	Exsheathing fluid
ES	Excretory/ secretory
EST	Expressed sequence tag
Ex	Exsheathed
FAR	Fatty acid and retinol binding proteins of nematodes
FEC	Faecal egg count
fc	final concentration
g	Gram
gsp	Gene-specific primers
GM-CSF	Granulocyte macrophage colony stimulating factor
xg	G-force
Hc	*Haemonchus contortus*
HRP	Horse-radish peroxidase
HHVI	Human Hookworm Vaccine Initiative
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IDA	Iron-defiency anaemia
IPTG	Isopropyl-β-D-thiogalactoside pyranoside
kDa	Kilo Dalton
L	Larval stage
LAP	Leucine amino peptidase
LB	Luria-Bertani broth
LN_2	Liquid nitrogen

LWBT	Lectin Wash Buffer + 0.5% Triton-X-100
MALDI-TOF M/S	Matrix Associated Laser Desorption Ionisation –Time of Flight Mass Spectrophometry
mRNA	Messenger ribonucleic acid
min	Minute(s)
MEP	Metallendopeptidase
MCS	Multiple cloning site
MOSI	Moredun Ovine Susceptible Isolate
MRI	Moredun Research Institute
MTP	Metalloprotease
NAS	Nematode astacin-like family
NIF	Neutrophil inhibitor factor
NFDM	Non-fat dried milk
OD	Optical density
OPD	o-phenylenediamine dihydrochloride
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVDF	polyvinylidene fluoride
peLB	Periplasmic leader sequence
PMSF	phenylmethylsulphonylfluoride
RNAi	RNA interference
RT	Room temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction

s	Second(s)
SCID	Severe combined immunodeficient
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
STH	Soil transmitted nematode helminths
Th	T helper cell
Tm	Melting temperature
TBSP	Thiol Sepharose Binding Protein
TE	Tris-EDTA
TIMP	Tissue inhibitor of metalloproteases
TBS	Tris-buffered saline
TBST	TBS containing 0.1% Tween 20
TNF-α	Tumour necrosis factor alpha
TRPs	Transthyretin-related proteins
TTR	Transthyretin-like protein
U	Unit (s)
UPM	Universal primer
UV	Ultraviolet
V	Volts
VAP	Venom allergen-like proteins
v/v	Volume/volume
w/v	Weight/volume
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indoyl-β-D-thiogalactopyranoside

ZSF	Zinc salts fixative

ABSTRACT

The recognition of hookworm infection as a leading cause of global morbidity has led to the establishment of a major initiative to develop a vaccine against human hookworms. Lead candidate hookworm antigens have been selected on the basis of either their association with the dog hookworm attenuated larval vaccine or because they are homologues of the major vaccine candidates from the related species of veterinary importance, *Haemonchus contortus*. Some of these hookworm antigens have been established as modestly protective in recombinant form, but have not been tested as native proteins. Higher levels of protection against homologous challenge have been achieved using the corresponding native *H. contortus* antigen in sheep trials. The feasibility of obtaining native protein from *H. contortus* and the convergence of vaccine targets in hookworms and *H. contortus* render the latter a useful model to aid vaccine development against the former.

Homologues of the major larval hookworm vaccine candidate antigens thought to play a role in the transition to parasitism (*Ancylostoma* secreted proteins, an astacin-like metalloprotease and a transthyretin-like protein) have been sought in *H. contortus* with a view to assessing their efficacy as protective antigens against this infection in sheep. *H. contortus* larval excretory/secretory proteins were collected and fractionated by affinity chromatography into lentil lectin-binding and non-binding fractions. The former was purportedly enriched for an *Ancylostoma* secreted protein-1 homologue and the latter shown to contain metalloprotease activity. A sheep protection trial demonstrated the lectin-bound fraction had a modest protective effect indicated by a trend towards lower faecal egg count, a significant 16% reduction in worm burden and a reduction in the length of female worms. Vaccination with the non-binding fraction showed a trend towards reduced faecal egg count and worm burden and sera from vaccinated sheep inhibited larval establishment in an *in vitro* abomasal challenge model.

The full length sequence of the *H. contortus* larval ASP-1 homologue was established, and the transcript shown to be expressed by exsheathed larvae and adult

worms. However, expression of the recombinant protein was not possible thus definitive establishment of the protein within the lectin-binding ES fraction not confirmed. Novel homologues of the hookworm transthyretin-like protein and astacin-like metalloprotease were identified in *H. contortus*, cloned and characterised. The former was expressed in all life stages and immunolocalised to the lining of the uterus wall and on the surface of the eggs *in utero* as well as in the sub-cuticular muscle. Degenerate PCR followed by rapid amplification of the cDNA ends identified full length sequence of the latter, a novel astacin-like metalloprotease, the protein product of which was immunolocalised to foci within the exsheathed larvae immediately sub-cuticular to the lateral alae, possibly labelling the hypodermal chords.

This work has established the modest protective effects of fractionated larval ES thus indicating the proteins therein to be of protective potential. Homologues of the lead hookworm larval vaccine candidates in *H. contortus* have been identified and these may have potential as larval vaccine candidates against homologous challenge.

Chapter 1

General Introduction

1.1 Introduction

The soil transmitted nematode helminths (STH) *Ascaris lumbricoides*, *Trichuris trichiura* and the hookworms *Necator americanus* and *Ancylostoma duodenale,* are estimated to infect more than one third of the World's population. In 1999 a report commissioned by the World Health Organisation (WHO) estimated that in combination with Schistosomiasis, soil transmitted nematode infections account for forty percent of all tropical disease burden excluding malaria (WHO World Health Report, 1999). Hookworm accounts for a large proportion of this disease burden, infecting an estimated 700 million. The numbers infected, combined with the iron deficiency and protein malnutrition associated with hookworm disease, earned it a reputation as "the great infection of mankind" (Stoll, 1962).

Disease caused by hookworm manifests itself as an intestinal helminthiasis caused by either *N. americanus* or *A. duodenale,* though zoonotic infections with *A. ceylanicum*, *A. caninum* and *A. braziliense* are also reported (Prociv and Croes, 1996). Morbidity caused by hookworm infection is attributed to the voracious blood-feeding nature of the intestine dwelling adults; intestinal blood loss from the host can lead to iron deficiency and protein malnutrition.

Unlike, for example, Schistosomiasis, infection with hookworm is not necessarily associated with dramatic clinical symptoms and mortality. Perhaps due to this, hookworm disease has been somewhat overlooked as a major public health problem in comparison to its more dramatic cousin. It is now recognised that, in terms of morbidity, hookworm outranks all other major tropical diseases with the exception of malaria, leishmaniasis and filariasis (WHO, 2002). For this reason, and many more, a major initiative has been set up to develop a vaccine against the human hookworms: the Human Hookworm Vaccine Initiative (HHVI) (Hotez *et al.*, 2003).

The nematode order Strongylida contains parasites which infect all classes of vertebrates and is divided into superfamilies which include the Strongyloidea and Trichostrongyloidea. The hookworms fall into the former and the latter include *Haemonchus contortus*, an economically important parasite of sheep and goats.

Both *H. contortus* and the hookworm species have well-developed buccal capsules and fourth stage larvae and adults are blood feeders. As well as similarities in morphology and the pathology of infection, *H. contortus* and the hookworms share a number of other characteristics; studies have demonstrated a number of proteins and enzymes common to both species. Research over the last two decades on vaccine development against *H. contortus* has discovered several highly protective protein complexes that line the worm gut (Knox, 2000). Contained within these protective fractions are proteases common to both *H. contortus* and the hookworm species (Hotez *et al.*, 2003). Studies focusing on antigens excreted or secreted by adult *H. contortus* have also identified protective fractions that contain proteins with homologues in the hookworm species (Schallig *et al.*, 1997). The similarities between the protective antigens of *H. contortus* and the hookworm antigens and the wealth of knowledge gained from sheep vaccine studies in the veterinary field combine to suggest that *H. contortus* could be used as a model for vaccine candidate selection and evaluation for hookworm.

Support for the feasibility of vaccination against hookworm comes from the collective knowledge that protective immunity can be stimulated by antigens secreted by living larvae; dogs can be highly protected by vaccinating with radiation attenuated *A. caninum* infective larvae (Miller, 1971), and the evidence from the veterinary field that protection against *H. contortus* can be elicited using antigens of the adult parasite gut and antigens secreted by the adult parasite.

1.2 Parasite Biology

1.2.1 Classification and morphology

1.2.1.1 Hookworm classification

The hookworms belong to the Superfamily Strongyloidea and the family Ancylostomidae. This family is characterised by the large buccal capsule, usually armed with teeth, lancets, cutting plates or a dorsal cone, indicative of the blood feeding nature of the adult worms. The site of predilection is the mucosa of the small intestine, where the adults attach and feed on tissue fluids and blood. The most important species in terms of potential infection to man are *N. americanus,* the only member of this genus thought to infect humans, and *A. duodenale*, the only member of this genus known to cause significant morbidity. Infection with *A ceylanicum* causes insignificant pathology and is rarely found other than when it has constituted a small proportion of worms in a mixed infection (Miller, 1979). Other species can cause zoonotic infection; including *A. braziliense*, the cat hookworm and *A. caninum* the dog hookworm.

1.2.1.2 *Haemonchus* species classification

Within the Strongylida and Superfamily Trichostrongyloidea is the family Trichostrongylidae to which the *Haemonchus* genera belong. Trichostrongylids parasitise all the major classes of vertebrates and are normally small worms with a simple buccal capsule that feed on the mucosa or blood in the host gut. *Haemonchus* species have a single tooth within the buccal capsule, which is used to pierce the mucosal lining of the abomasum (the "true stomach") and allows blood to be sucked from the wound. There are four species of *Haemonchus* found in domestic ruminants: *H. contortus*, the ovine and caprine species; *H. longistipes,* from camels; *H. similis,* a bovine species; and *H. placei*, a bovine species (Jacquiet *et al.*, 1997). The species of greatest importance in terms of economic and welfare cost, and the particular focus in this body of work, is *H. contortus*. Female worms are approximately 3 cm long and of a pale cream appearance; males are slightly smaller. When blood feeding, the white ovaries of the female wind around the blood filled intestine giving the worm its characteristic barber's pole appearance.

1.2.2 Hookworm life cycle

The life cycle of the hookworm species is of a direct nature in that it only involves one host species (Looss, 1901) (**Figure 1A**): man in the case of *N. americanus* and *A. duodenale*; the dog in the case of *A. caninum*; the dog or cat in the case of *A. braziliense*; and certain carnivorous species in the case of *A. ceylanicum.*

Eggs laid by the adult female are passed in the faeces and onto the soil. To develop, eggs require a moist and warm (at least 17°C; 23-30°C is optimal) environment and are sensitive to both frost and direct sunlight. The aerobic nature of the hatching larvae's metabolism also necessitates a damp but not waterlogged soil (Schmidt and Roberts, 1996). First stage larvae (L1) hatch from eggs within 24-48 hours under conditions as those described above and feed on surrounding faecal matter. The shedding of the L1 cuticle and moult to the second stage larvae (L2) occurs within two to three days. L2 continue to feed and undergo a second moult (the second stage cuticle is often retained as a loose fitting sheath) to the infective third larval stage (L3) within three to five days. L3 do not feed and are sustained by stored food ingested as earlier larval stages. Under optimal conditions the time taken from eggs hatching to L3 can be as little as five days. Infective L3 can survive on the ground for several weeks under optimal conditions, though freezing or desiccation kills them quickly. At temperatures of 35°C and above, larvae die. However, when the ambient temperature is greater than this, larvae are able to survive by migration to areas of shade or below the soil (Brooker *et al.*, 2005). L3 live in the layer of moisture surrounding soil particles and under damp conditions (for example morning dew) a short vertical migration to the top of the soil in is undertaken where many worms may gather, swaying to and fro to maximise exposure to passing potential hosts (Schmidt and Roberts, 1996).

Upon contact with the host, L3 larvae penetrate the skin epidermis directly. Larvae are capable of penetrating any part of the skin, though those parts that are most often in contact with the ground are most susceptible. In the case of *N.*

americanus skin penetration must occur for the parasite to successfully infect whereas, *A. duodenale* can penetrate the oral mucosa as well as the skin (Brooker *et al.*, 2005). During skin penetration the sheath is normally shed, although the biochemical basis of this is poorly understood (Hawdon and Schad, 1990). Following successful skin penetration, feeding and development are resumed [this can be achieved *in vitro* by stimulating L3 with host serum (Hawdon and Schad, 1990)]. This resumption in development is concomitant with the expression of proteins likely required for infection (Zhan *et al.*, 2002; Hawdon *et al.*, 1996; Hotez *et al.*, 2003).

L3 enter the vasculature and are carried through the circulation to the heart and then lungs. Once in the lungs, L3 emerge from the capillaries and migrate up the alveoli, are carried up the trachea to the glottis from where they are swallowed and pass through the stomach to the site of predilection, the small intestine. Here, L3 attach to the gut mucosa and moult to the fourth larval stage (L4) which has a fully developed buccal capsule and blood feeds. Following a final molt to the adult, worms become sexually mature and mate and females begin egg laying. Female worms are capable of laying thousands of eggs daily. The whole process from infection to gravidity takes at least five weeks and once established, adult *A. duodenale* can live in the human intestine for up to three years; *N. americanus* for three to ten years (Hoagland and Schad, 1978).

There is evidence for the vertical transmission of *A. duodenale*, although the exact mechanism has not been established. This would not prove surprising as the resumption of development of *A. caninum* L3 following a period of dormancy in muscle tissues, and transmission of L3s across the mammary glands to newborn pups, is a well documented phenomenon (Schad *et al.*, 1982). *A. duodenale* infection has been observed in children likely to be too young to have picked it up by environmental transmission. In these cases, the only hookworm species is *A. duodenale* despite there being a local prevalence of *N. americanus* (Schad, 1991).

1.2.3 *Haemonchus* life cycle

Like the hookworm species, *Haemonchus contortus* is dioecious and undergoes a direct lifecycle (**Figure 1B**). The adult females are prolific egg layers and eggs are passed in the faeces onto pasture. Eggs hatch to L1 within a couple of days and feed on microbes within the faecal pat. L1 undergo a moult to L2 which in turn moults to the infective L3. Like some of the hookworms, the L2 cuticle is retained by the infective L3 as a loose fitting protective sheath; this acts as a protective barrier against moisture fluctuations on pasture (Ellenby, 1968). L3s are non-feeding and have to survive on energy stored in the early larval stages. The sheathed L3 are fairly resistant to desiccation and can survive for up to three months on pasture under conditions of reasonable humidity (Urquhart *et al.*, 1987). The L3 migrate from the faeces and onto the herbage where they are ingested by their ruminant host (sheep or goats). Once in the rumen, the L3 exsheath. Exsheathment is thought to occur in response to a combination of temperature rise to 39°C, optimal pH of between 6.5 and 7.5 (Rogers and Sommerville, 1957) and exposure to CO_2 and H_2CO_3 in the rumen (Rogers and Sommerville, 1968). L3 release a zinc metalloprotease that acts to digest a refractory ring approximately 20 μm from the anterior end allowing release of a cuticular cap and escape of the L3 from within the sheath (Gamble *et al.*, 1989; 1989). Following exsheathment, L3 pass through to the abomasum where they become closely associated with the mucosa and burrow into the gastric glands. Here the L3 moult to L4 within about two days and gain the buccal lancet that allows the onset of blood feeding. Following a final moult to the adults, worms exit the gastric gland and move freely upon the mucosal surface. Upon achievement of sexual maturity, females begin egg laying approximately 17-20 days post infection, and can each lay between 5000-10,000 eggs per day.

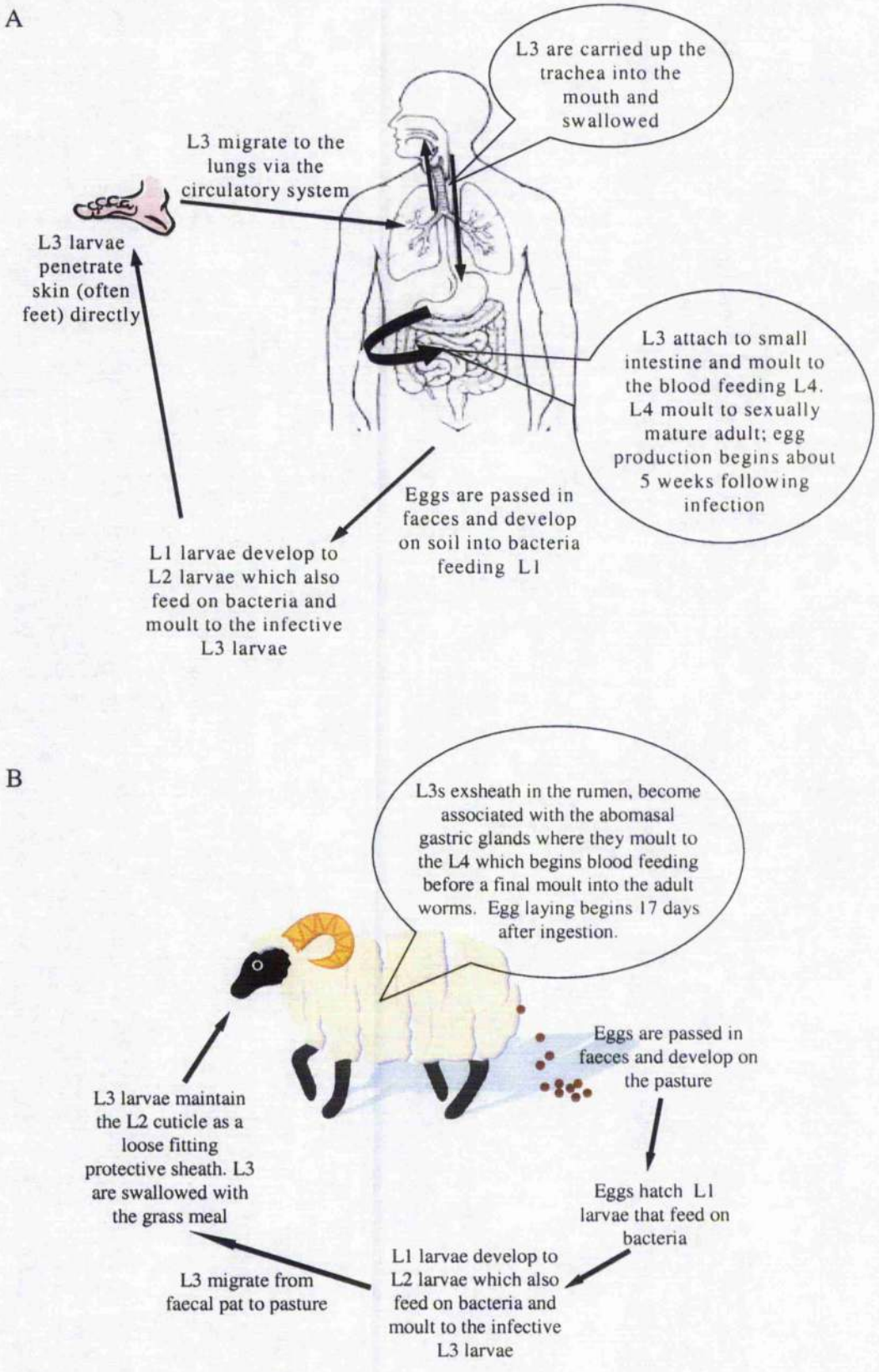


Figure 1: The lifecycle of human Hookworm and *H. contortus*. **A** - Lifecycle of *N. americanus* in the human host. **B** - Lifecycle of *H. contortus*.

1.3 Epidemiology

1.3.1 Epidemiology of Hookworm

Despite a dearth in actual organised surveillance programmes, a recent and extensive study of the literature (de Silva *et al.*, 2003) estimated the numbers infected by hookworm globally, at 740 million. Human hookworm infection is found throughout South America, Africa, the Middle East, Central, East and South East Asia, China, Indonesia and the Pacific islands (de Silva *et al.*, 2003). Areas of highest prevalence are sub-Saharan Africa, Southern China and East Asia and Papua New Guinea; high transmission levels are found within areas of rural poverty throughout the tropics including China, Central and South America (Hotez *et al.*, 2003) and the Indian subcontinent (Yadla *et al.*, 2003). Throughout all these areas, there is a conspicuous relationship between levels of hookworm frequency and socioeconomic status; the poorest economies have the highest worm prevalence (de Silva *et al.*, 2003).

N. americanus is the predominant species globally. Areas where *A. duodenale* occur include Egypt, Northern Argentina and Paraguay (Brooker *et al.*, 2005). *A duodenale* infection is also found in the more northerly latitudes of South and West China and India where *N. americanus* cannot survive the harsher conditions. There is evidence of hypobiosis in *A. duodenale* (Scahd, 1991) and this ability of *A. duodenale* L3 to undergo arrested development within the human host may allow survival during cold periods where development on the ground is not viable (Schad *et al.*, 1973).

Hookworm infection can occur in children as young as six months (Brooker *et al.*, 1999). Typically hookworm epidemiology was considered similar to other major STH, *A. lubricoides* and *T. trichiura;* children become infected at a young age with a peak of infection intensity between five and ten years old followed by a decline in intensity in adults, giving a convex age-intensity profile (Bundy, 1990) (though prevalence remains constant in adults). This, however, is now recognised as not the case with hookworm. Recent data from studies carried out in China (Bethony *et al.*, 2002; Gandhi *et al.*, 2001) shows both hookworm prevalence and infection intensity to increase with age with the highest levels

shown in middle age with a peak prevalence and infection intensity occurring in those aged greater than fifty in the former study; a concern in countries such as China where they are the most rapidly expanding age group (Hotez *et al.*, 2002). Data from a similar cross sectional study in Brazil demonstrated an analogous monotonic profile (Hotez *et al.*, 2003). Interestingly, this does not seem to be the case in Mali: a study looking at age-intensity profiles carried out by Behnke and colleagues (2000) found a convex pattern, with intensity decreasing in adulthood. A possible explanation for the variation in age-intensity profiles may be higher levels of exposure to hookworm among adults and the elderly in Asia (Brooker *et al.*, 2004), though age-intensity profiles will also reflect larval establishment and adult mortality.

An analysis of the literature carried out by Brooker *et al.* (2004) looked at the prevalence of infection with STH in males and females of a school age. They found no differences between male and females with regard to prevalence of *A. lubricoides* and *T. trichiura*. However, males showed a significantly higher prevalence of hookworm infection than females, a finding likely to reflect a difference in exposure. When considered in conjunction with prevalence in adults, occupational exposure is thought to be important (Brooker *et al.*, 2004). This is consistent with Behnke *et al.* (2000) findings that in Mali, where there was a significantly higher incidence and intensity of hookworm infections amongst males. Human faecal matter is often incorporated into building materials to strengthen housing, and it is predominantly men who undertake this work.

A further compounding variable in hookworm epidemiology is that of worm burden heterogeneity within the population. Reviewed by Anderson and May in 1991, it is a well established trend that a minority of the infected population harbour very heavy worm burdens, whereas the majority of an infected population have few worms [sometimes 10% of the human population carries 70% of the worms (Anderson and May, 1985)]. A study in Mali found that, when age and sex were controlled for, it was apparent that a subset of the population appeared relatively worm free, whilst another subset seemed predisposed to heavy infections, and became quickly re-infected following

treatment (Behnke *et al.*, 2000). This is in line with findings from work carried out in Papua New Guinea that found a significant predisposition to hookworm re-infection following drug treatment, that did not vary with age or sex (Quinnell *et al.*, 1993). This predisposition to re-infection remained detectable up to eight years following drug treatment (Quinnell *et al.*, 2001). However, should individuals have received repeated chemotherapy treatments during this time, predisposition to re-infection was not detectable. This led Quinnell *et al.* (2001) to suggest that differences in host susceptibility are involved, but variation over the long term in exposure to infection or susceptibility limits the detection of predisposition. Interestingly they also report a correlation between male hookworm weight and individual host following measurement of hookworms upon expulsion after two doses of chemotherapy at a two year interval (Quinnell *et al.*, 2001). This would suggest that individual hosts are predisposed to infection with light or heavy worms, perhaps indicating a host genetic aspect to susceptibility.

Early studies in the southern states of the USA indicated a far higher prevalence of hookworm in individuals of European versus African origin further suggesting a genetic basis (see Quinnell, 2003 and references therein). Familial clustering of predisposition may also be indicative of a genetic factor though this could be explained by exposure levels. Quantitative measurements of heritability give the most convincing case for a genetic component to hookworm susceptibility. A quantitative genetic analysis of 289 infected individuals carried out in Zimbabwe by Williams-Blangero *et al.* (1997) demonstrated that, after controlling for confounding variables, 37% of the variation in hookworm eggs per gram in the study population could be attributable to genetic heritability factors. Unpublished work carried out in Brazil by Bethony and co-workers (described in Brooker *et al.*, 2004) looked at the role genetic factors played in regulating infection and humoral immune responses to hookworm infection using "multi-household extended pedigrees". They found that 49% and 26% of the variation in faecal egg output was accounted for by additive genetic factors when modelled with or without household effects, respectively. Further to this, they found that 45% of the variation in the specific humoral response (Immunoglobulin isotypes G1, G4 and E) directed to L3 and adult worm antigens could be explained by

additive genetic factors. These studies all indicate the importance of the underlying genetics of susceptibility.

1.3.2 Epidemiology of *H. contortus*

Due to optimal larval development of *H. contortus* occurring at higher temperatures, the species is most prevalent in tropical and sub tropical regions, although it can still be a problem in temperate areas such as Northern Europe and Canada. The epidemiology of infection within the tropics and subtropics is quite distinct to that in temperate zones. Larval development is highly dependent on high humidity, thus disease outbreak often follows periods of high rainfall (Urquhart *et al.*, 1987). The very high fecundity of *H. contortus* (a faecal egg count of between 5000 and 20,000 is not uncommon, even in moderate infections) allows large pasture contamination to build up quickly leading to the appearance of acute disease. In certain areas of the subtropics, such as the Middle East, South America and Australia, *H. contortus* can undergo hypobiosis as arrested L4 larvae, within the host. The cues for this are unknown, though an environmental trigger has been suggested (Blitz and Gibbs, 1972). Hypobiosis occurs at the beginning of the dry season and allows survival within the host, when environmental conditions on the pasture would cause desiccation of the L3 and break the infective cycle. Often both adult worms and hypobiotic larvae coexist and although the existence of hypobiotic larvae may not be absolutely crucial to worm survival during the dry summer season, it is thought to play a major role (Gatongi *et al.*, 1998). Resumption of development begins prior to the rainy season and allows completion of the cycle to gravid adult (Urquart *et al.*, 1987). Interestingly, hypobiosis does not occur in all areas of the tropics; this has been explained by more frequent rainfall negating any need to survive prolonged dry spells.

In temperate zones, such as the UK, the infective cycle is normally described as a single annual cycle. Infected larvae that have developed on pasture from eggs in the spring are ingested in the early summer and many undergo hypobiosis as early L4 and remain arrested until the following spring (Urquart *et al.*, 1987). Although either immune mechanisms or environmental stimuli may contribute to

stimulation of hypobiosis, it has been suggested that in temperate areas, over winter hypobiosis may be an obligatory survival mechanism that occurs regardless of external stimuli (Capitini *et al.*, 1990) and is likely regulated by the worms themselves (Blitz and Gibbs, 1972). Developmental resumption occurs the following spring and in non pregnant animals it may not occur *en masse* but over a couple of months (Blitz and Gibbs, 1972). In pregnant ewes, development is often associated with lambing, though not necessarily in a peri-parturient synchronous fashion as rapidly as is seen in the development of *Teladorsagia circumcincta* (Connan, 1967). During this period of hypobiotic larval maturation, acute disease may be seen in ewes post lambing and grazing lambs.

Clinical haemonchosis is sometimes seen in grazing lambs in late summer, however the epidemiology behind this is not well understood; it has been suggested that infection may be with those ingested larvae that did not undergo developmental arrest in the late spring, and have become gravid adults (Urquart *et al.*, 1987).

1.3.3 Pathology and impact of Hookworm

In areas of very high transmission of either *N. americanus* or *A. duodenale*, the first sign of pathology associated with hookworm may be ground itch: a cutaneous condition of the feet caused by high numbers of L3 penetrating the epidermis characterised by a pruritic maculo-papular eruption. Should this occur by zoonotic infection with *A. braziliense*, the cat hookworm, L3s penetrate the skin epidermis but are unable to develop any further in their lifecycle. This can result in a *larva migrans* and creeping eruption causing an intensely itchy pruritis characterised by a serpiginus tract that may fall foul of a secondary bacterial infection, normally occurring on the lower limbs or feet (Blackwell and Vega-Lopez, 2001). In contrast, there is evidence that zoonotic infection by *A. caninum* can result in the dog hookworm completing its lifecycle in a human host, and this has been associated with human eosinophilic enteritis (Prociv and Croese, 1996).

Following host entry and pulmonary migration, human hookworm L3s exit the lungs, as previously described, and this may be accompanied by production of eosinophilic infiltrates that can cause a mild, dry cough. Other upper respiratory tract complications may include laryngitis, pharyngitis and pain when speaking or swallowing (Miller, 1979). Upon entry to the gastrointestinal tract, development to adults is commonly accompanied by epigastric and abdominal pain (Anyaeze, 2003). In experimentally infected volunteers, abdominal pain, nausea and flatulence just prior to the appearance of eggs in stool samples were found, the pain sufficiently acute in one circumstance to warrant chemotherapy administration prior to the proposed trial endpoint (Maxwell *et al.*, 1987). Severe abdominal pain has been described as commonplace in twenty to fifty percent of chronic infections (Miller, 1979).

As prolific and voracious blood feeders, the most common and important clinical sign of hookworm infection is intestinal blood loss; volumes of blood loss have been estimated at 0.04 ml per worm per day for *N. americanus* and 0.20 ml per worm per day for *A. duodenale* resulting in 25 worms causing the loss of 1 ml blood per day in the former species and only 5 worms causing 1 ml blood loss per day in the latter (Martinez-Torres *et al.*, 1967; Crompton, 2000). As long ago as 1947, the eminent parasitologist Norman Stoll estimated that the volume of blood lost to hookworms in a single day was equivalent to the complete exsanguination of one and a half million people (Stoll, 1947). More recently it has been suggested that this may even be an underestimate (Schad, 1991). A consequence of this blood loss is either the cause, or exacerbation, of anaemia. In acute canine hookworm infection (with *A. caninum*) massive blood loss can cause an immediate, acute and often fatal anaemia; however, though reported, this type of acute haemorrhagic anaemia is very uncommon in human hookworm infection (Miller, 1979). The cardinal symptom of human hookworm infection is iron-deficiency anaemia (IDA).

Chronic IDA develops gradually following primary infection and is usually associated with a continuous exposure to hookworm infection. Development of IDA is also dependent upon a number of sequelae including the level of infection and species of hookworm, iron-reserve status of the host, dietary iron intake and

its absorption and on other sources of iron loss from the body (Miller, 1979). Thus, a malnourished population with little dietary iron intake is likely to be at an increased risk of IDA associated with hookworm infection. Groups at highest risk include those whose iron status is impaired; adolescent girls, women of a child bearing age and pregnant women are particularly vulnerable (Crompton, 2000).

A negative association between hookworm eggs counts and serum ferritin levels (a measure of IDA) has been reported by several authors (Gilgen and Mascie-Taylor, 2001; Pritchard *et al.*, 1991; Crompton and Whitehead, 1993). Compelling evidence that intensity of hookworm infection is associated with blood loss and IDA was demonstrated in a study of school children carried out in Zanzibar. A positive relationship was found between faecal egg count (a measure of intensity) and blood loss (as measured by faecal haeme) and this was negatively correlated with serum ferritin concentration. Thus, the prevalence of IDA increased with hookworm infection intensity and blood loss (Stoltzfus *et al.*, 1996). Interestingly, a recent study on the affect of daily low dose iron supplementation and quarterly anthelmintic treatment on anaemia in young children with light helminth infections found that, in toddlers, iron supplementation did not improve mild to moderate anaemia, however anthelminthic treatment did (Stoltzfus *et al.*, 2004). This suggests that in these young children, the affect of iron supplementation on anaemia was constrained by intestinal worm infection (albeit light) of which hookworms made a significant proportion.

Due to the difficulties faced in speciating hookworm eggs, there is a dearth of evidence relating the iron status of infected individuals to the specific hookworm species. By culturing eggs collected from individuals' faeces and speciating the L3, Albonico *et al.* (1998) surveyed hookworm species prevalence and infection intensity and related it to individuals iron status and community burden of IDA. Infection with *A. duodenale* was clearly associated with a heavier burden of IDA, adding epidemiological weight to the concept that a difference in blood loss translates to a greater extent of IDA.

Thus IDA associated with hookworm infection is governed by a variety of complicating cofactors, including the iron status of the individual, the dietary iron intake, the level of infection and the hookworm species responsible for infection. The general consensus is that hookworm chemotherapy can improve IDA, and this can be further progressed by dietary iron supplementation, particularly important in very poor quality diets.

IDA has an adverse effect on the growth and development of children, and there is a general consensus (though it remains a topic of some debate and beyond the scope of this review) that IDA is closely associated with impaired academic performance at school. The relationship of infection with IDA infers hookworm will contribute to this effect (Crompton and Nesheim, 2002). Sakti *et al.*, (1999) demonstrated an association between hookworm infection and lower scores in tests aimed to measure cognitive abilities, however infection intensity was not measured and there was no link found between levels of anaemia and cognitive ability. It would certainly seem that improving severe anaemia by iron supplementation and anthelminthic treatment is associated with a positive effect on motor and language development in pre-school children (Stoltzfus *et al.*, 2004). IDA is associated with a fatigue and listlessness that is thought to affect physical labour, productivity and work capacity (Guyatt, 2000). Due to the nature of much of the work undertaken in countries where hookworm is endemic (often land based physical labour), it can be assumed that IDA associated with hookworm infection may be responsible for some loss of productivity. In her extensive review, Guyatt (2000) also points out that a link between adverse growth and development of children (both cognitive and physical) associated with helminth infection and a loss of productivity in adulthood, although not proven, is certainly suggested. Crompton and Nesheim (2002) cite the productivity losses due to IDA in South Asia to have a value of five billion U.S. dollars annually.

Chan *et al.* (1994) used a method of estimating potential global morbidity based on the observed infection prevalence. The global numbers infected by hookworm were estimated at 1297 million; 96 million of whom were estimated to suffer from a developmental morbidity, 61 million a more severe clinical

morbidity. An update of the picture was delivered by de Silva *et al.* in 2003 who found a marked decline in total global numbers infected to approximately 740 million. The decline is most dramatic in some Asian and Latin American countries and reasons suggested for this decline are an increase in national control activities combined with economic development and a shift away from a largely agricultural and rural economy to a more suburban one (de Silva *et al.*, 2003). On the other hand, no change in hookworm prevalence combined with an increase in population at risk has lead to an actual increase in the numbers infected from sub-Saharan Africa (de Silva *et al.*, 2003).

Recently, the measurement of choice to quantify the burden of disease, injuries and other risk factors, and allow comparisons between these different factors, has been the disability-adjusted life years (DALYs). This measure is based on both the economic and ethical principles that can guide healthcare, treatment and financial policy and is particularly designed as a quantifier of burden of non-fatal disease (Murray and Acharya, 1997). DALYs provide a summary of population health by combining the years of life lost from premature death and the years of life lived with a disability. According to a WHO World Health Report in 2002, hookworm causes the loss of 1.8 million DALYs worldwide, more than Schistosomiasis, Chagas disease, Trypanosomiasis and Onchocerciasis. (WHO. Reducing Risks, Promoting Healthy Life, 2002).

1.3.4 Pathology and economic burden of *H. contortus*

Like the pathogenic human hookworm species described, the pathology of *H. contortus* is almost entirely attributable to the blood feeding nature of the adult worms and manifests as anaemia. In acute *H. contortus* infection, a pronounced anaemia presents about two weeks post infection characterised by a prominent fall in haematocrit levels. Over the following weeks haematocrit stabilises at a low level, and this is associated with a visible pallor of the mucous membranes of the eye that can be used as a measure of clinical anaemia (Van Wyk *et al.*, 1997- cited in Vatta *et al.*, 2001). This drop in haematocrit is coupled with a compensatory expansion of erythropoiesis. However, this expansion in red blood cells cannot be kept up indefinitely and when compounded by a continual blood,

iron and protein loss and an increasing inappetance, the bone marrow becomes exhausted, haematocrit further falls and death may occur (Urquart *et al.*, 1987). A feature of haemonchosis and other gastro-intestinal (GI) nematode infections is a loss of protein into the GI tract causing hypoalbuminaemia (Holmes, 1987). A clinical consequence of this is a reduction in the colloid osmotic pressure within the blood capillaries which forces fluid out of the capillaries into the interstitial spaces causing oedema. This often occurs in the submandibular area leading to a condition known as bottle jaw. Loss of protein leads to reduced nitrogen retention and is characteristic of GI nematode infection. This is associated with a decrease in growth rates and protein deposition in muscle and wool. In the case of *H. contortus*, nearly all the loss of nitrogen into the GI tract is attributable to blood loss (Holmes, 1987). In very heavy infestations, up to 30,000 worms, a very acute severe haemorrhagic gastritis can occur. Though rare, this can kill apparently healthy sheep very suddenly (Urquart *et al.*, 1987).

In tropical areas, when infection with blood feeding adults takes place all year round, chronic haemonchosis can occur. Less clinically obvious than acute disease, it is nonetheless economically important. During the dry season, when the nutrient value of the pasture is inferior, but fresh reinfection is unlikely, a small burden of blood feeding adults can cause clinical signs associated with weight loss and inappetance rather than anaemia (Urquart *et al.*, 1987).

There is very little information regarding the global economic losses caused by parasitic nematodes of livestock. Within the UK, production losses due to G I parasites cost the sheep farming industry approximately £60 million annually (F. Jackson, personal communication) and the total annual expenditure on endoparasticides is approximately £44 million (source: http://www.noah.co.uk/). On a global scale, Coles (2002) estimated the amount of money spent on antiparasitic drugs in 1999 to be in the region of 3.5 billion $US and of that, approximately 379 million $US are spent on the sheep industry. Despite a dearth of exact figures for the economic impact of *H. contortus*, there is no question that it runs into hundreds of millions of $US.

1.4 The immunology associated with gastro-intestinal helminth infection

Whilst much work has been carried out to characterise the immune response associated with GI nematodes and their persistence or expulsion in rodent models, it is always a contentious issue as to whether one can extrapolate these results to humans (or ruminants). As such, the cell mediated, humoral and cytokine responses associated with generalised GI helminth infection will be discussed first, followed by a focus on the immune responses associated with hookworm and *H. contortus* infection specifically. The majority of the murine work has focused on four nematode infection models, *Nippostrongylus brasiliensis, Heligomosoides polygyrus, Trichinella spiralis* and *Trichuris muris*. The former three species reside in the small intestine and the latter, *T. muris* inhabits the proximal colon; primary or secondary infection with these species often results in expulsion of worms in immune animals, thus this is commonly used as a measure of immunity (Artis and Grencis, 2001).

1.4.1 T cell mediated immunity

An essential function of T cells in the immune response to nematodes was confirmed by the requirement of a thymus for mice to expel *T. muris* (Koyama *et al.*, 1995). Convincing evidence for a pivotal role of $CD4^+$ T helper cells in resistance to nematode infection comes from adoptive transfer experiments (Grencis *et al.*, 1985) and *in vivo* depletion experiments using murine models (Katona *et al.*, 1988). Furthermore, $CD4^+$ cells from an immune mouse were demonstrated to confer immunity to infection in severe combined immunodeficient (SCID) mice that would not normally be able to mount any immune response (Else and Grencis, 1996).

The highly influential work by Mosmann *et al.* (1986) describing two individual subsets of $CD4^+$ T helper (Th) cells defined by the distinct cytokine profiles secreted by, and associated with, them has been central to understanding the T cell responses to nematode infection. T helper 1 (Th1) cells are associated with cell mediated inflammatory reactions and produce type 1 cytokines: interferon gamma (IFN-γ), lymphotoxin and interleukin (IL) 2 and are associated with

Immunoglobuliin (Ig) G2a and cell mediated responses. T helper 2 (Th2) cells are associated with antibody production and allergic responses and produce type 2 cytokines: IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 and drive production of IgE, IgG1, mastocytosis and eosinophilia (Mosmann and Sad, 1996). Both subtypes can produce tumour necrosis factor alpha (TNF-α), IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF). There is considerable evidence for a cross-regulation of Th1 and Th2 cells; cytokines produced by one subtype can inhibit proliferation or cytokine production by the other cell type. IFN-γ will inhibit Th2 cell proliferation and IL-10 can inhibit cytokine synthesis by Th1 cells and this cross regulation can lead to the skewing of either a Th1 or Th2 type response to a pathogen. When first stimulated by an antigen presenting cell, the undifferentiated naive Th cell produces principally IL-2 (Mosmann and Sad, 1996). Following this, the T helper cell goes through an intermediate stage (Th0) where a mixed profile of cytokines is produced prior to differentiating into the polarised Th1 or Th2 phenotype cells (Janeway *et al.*, 1999). A critical factor in influencing T helper cell polarisation is the cytokine environment at the moment of antigen presentation (IL-12 promotes Th1 and IL-4, Th2), and the type of antigen presenting cell/pathway involved (Else and Finkelman, 1998). It would appear that antigen presented by macrophages favour a Th1 response, and B cell antigen presentation favours a Th2 response (Gajewski *et al.*, 1991). The costimulatory ligands and receptors involved is also likely to play a role, however this is really beyond the scope of this thesis (see Else and Finkelman, 1998 for a comprehensive review). This Th1/Th2 paradigm, based on the type 1 or type 2 response profiles is now regarded as oversimplified, but it does prove useful as a basis for looking at the response to GI nematodes (Hayes *et al.*, 2004). Utilising the aforementioned murine infection models, a wealth of experimental evidence indicates a Th2 and associated type 2 immune response as resistant to nematode infection, and a Th1, type 1 response as susceptible (see reviews by Hayes *et al.*, 2004; Artis and Grencis, 2001; Else and Finkelman, 1998). The roles some of the key cytokines play in infection in murine models are summarised in **Table 1.1** and **Figure 1.2**

Table 1.1: A summary of the role of some of the key cytokines indicated in promotion of resistance or chronicity in murine rodent models.

Model System	Summary of key cytokines involved and infection outcome	Key References
T. muris	IL-4, IL-5, IL-9, IL-13 and eosinophilia associated with resistance. IL-12, IL-18 and IFN-γ associated with promotion of chronicity. Th2 type of response necessary for resolving infection.	Else and Grencis, 1991; Else *et al.*, 1992; Else *et al.*, 1994; Bancroft *et al.*, 2000; Helmby *et al.*, 2001
N. brasiliensis	IL-12 and IFN-γ associated with enhancement of adult worm survival. IL-4 not required for resistance but may play a role. IL-13 required for worm expulsion, even if a Th2 type response exists. Th1 response promotes chronicity, aspects of a Th2 response are protective	Finkelman *et al.*, 1991; Finkelman *et al.*, 1994; McKenzie *et al.*, 1998
T. spiralis	IL-18 downregulates Th2 responses and promotes chronic infection. Th2 type of response required for worm expulsion	Helmby and Grencis, 2002;
H. polygyrus	IL-4 required for resistance. IL-9 indicated in resistance, by maintaining mastocytosis	Urban *et al.*, 1991; Faulkner *et al.*, 1997 ; Hayes *et al.*, 2004.

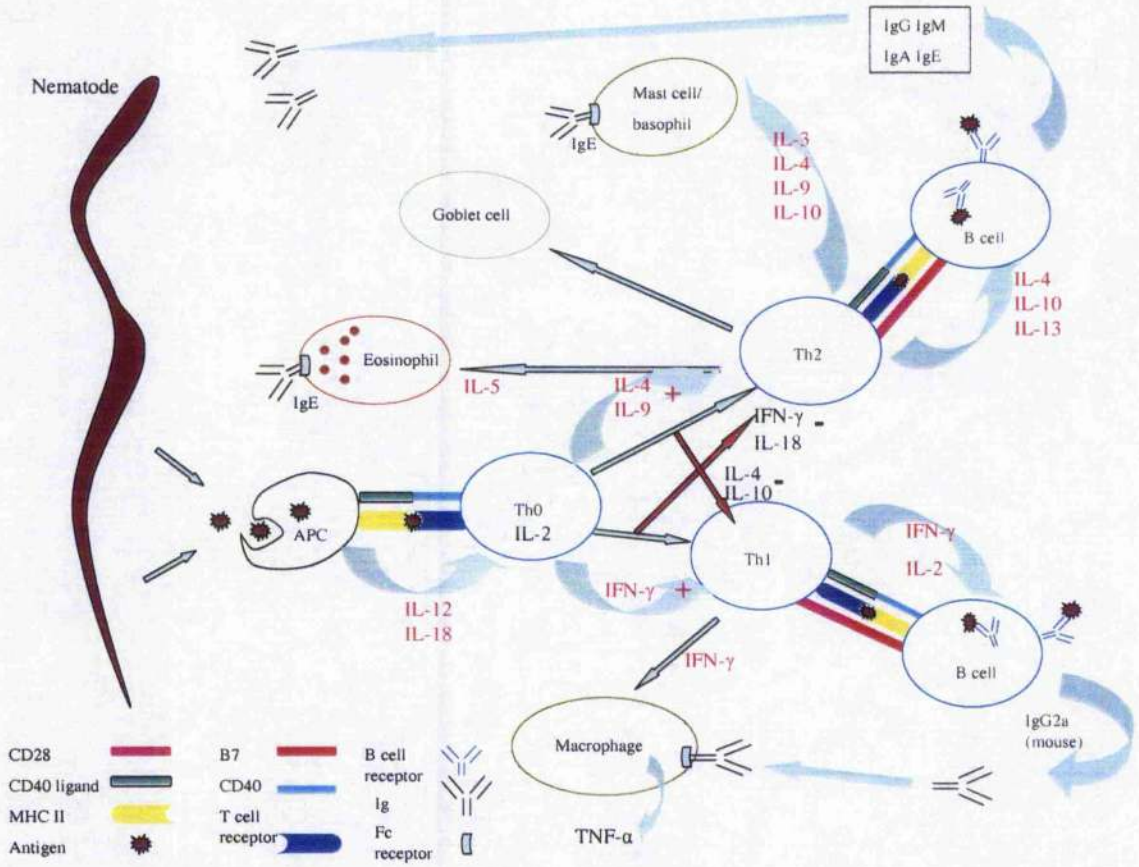


Figure 1.2: A simplified schematic overview of the major cytokines involved in the Th1/Th2 response to nematode infection in the mouse. The Th1 polarisation is associated with susceptibility and the Th2 polarisation with resistance (adapted and redrawn from Else and Finkelman, 1998).

Aside from the rodent models, the role of the Th1 and Th2 response in parasite-host interactions remains ill-defined. One recent study focused on the cytokines expressed by cells in abomasal lymph nodes or mucosa from calves following vaccination with a host protective fraction and challenge with *Ostertagia ostertagi* L3. Although vaccination itself had no effect on cytokine profiles, cells from the lymph nodes showed a significant decrease in Th1 type cytokines and an increase in Th2 type cytokines following challenge infection suggesting that polarisation to a Th2 profile following GI nematode infection occurs in ruminants. Intriguingly, this polarisation was not observed at the mucosal level (Claerebout *et al.*, 2005). Experiments looking at the cytokine profile in

abomasal tissue of immune and non immune sheep challenged with *H. contortus* found an increase in IL-4 associated with rapid expulsion of worms and an increase in IL-5 and IL-13 in animals that expelled worms after they had reached the tissue (Meeusen *et al.*, 2005). The authors suggest that this might imply IL-4 to play a role in the mast cell and globular leukocyte-associated rapid expulsion, whilst IL-5 and IL-13 are important in delayed worm rejection. In humans, there is evidence that susceptibility to STH infection is associated with a weak Th2 response, with a negative correlation between IL-5 and IL-13 levels and STH infection (Jackson *et al.*, 2004).

1.4.2 Effector mechanisms

The exact bases of the effector mechanisms that underlie a protective Th2 response are still not well understood. As already discussed, the hallmarks of the type 2 response associated with GI helminth infection are eosinophilia, intestinal mastocytosis, goblet cell hyperplasia, IgE and IgG1. A direct roll for all or some of these cells and/or antibodies has been difficult to establish.

1.4.2.1 Eosinophils

Cells of the polymorphonuclear leukocyte family, eosinophils are produced in the bone marrow from where they migrate via the blood to the tissues or site of predilection. Eosinophilia (the upregulated production of eosinophils in the bone marrow and increase in numbers in the blood and tissues) is characteristic of allergic reactions, inflammation and helminth parasitoses (Jones, 1993). Demonstration of eosinophilia inhibition *in vivo* using antibody to IL-5 confirmed this cytokine's role as a key growth and differentiation factor (Coffman *et al.*, 1989). In 1979, Butterworth *et al.* showed that eosinophils could cause direct damage and kill schistosomula of *Schistosoma mansoni*. More recently, it was shown that, in the presence of specific antibody, eosinophils could kill *H. contortus* L3 *in vitro* (Rainbrid *et al.*, 1998). Despite the demonstration of an *in vitro* affect, direct *in vivo* evidence of a protective role for eosinophils is limited to correlative evidence of an association between eosinophils and dead parasites (Meeusen and Balic, 2000), and work citing an association between sheep bred for resistance to GI nematodes and an increased

level of eosinophilia compared to non responders or those selected for high faecal egg count (Dawkins *et al.*, 1989; Buddle *et al.*, 1992).

Antibody inhibition of IL-5, leading to eosinophilia depletion, had no effect on immunity against *N. brasiliensis* (Coffman *et al.*, 1989). Similar depletion experiments found no effect on immunity to *T. spiralis* (Herndon and Kayes, 1992), *H. polygyrus* (Urban *et al.*, 1991) or *T. muris* (Else and Finkelman, 1998). These experiments would suggest that eosinophils are perhaps not important effector cells against GI nematodes. In contrast there is some evidence that in unnatural or non-permissive helminth infections, often characterised by an increased non specific or innate inflammatory response, eosinophils may play a protective role (Meeusen and Balic, 2000).

Upon larval challenge of sheep several weeks after they had cleared a previous infection, *H. contortus* larvae found within the tissues of the abomasum 24 hours post challenge are surrounded by eosinophils (Meeusen and Balic, 2000). Though not reflected by an increase in circulating eosinophil numbers, eosinophils seemed to be specifically recruited to the site of larval gastric penetration, and larval damage could be detected microscopically. Thus the role of eosinophils as effector cells in GI nematode infection is unclear; it seems likely that they are more important in some infections than others. A recent development is the demonstration that ruminant GI nematode species produce a potent chemotactic factor for eosinophils and that this activity is not seen in the related, but free living, species *Caenorhabditis elegans*. This suggests that these ovine nematodes may actively encourage eosinophil recruitment (Wildblood *et al.*, 2005); local eosinophil-mediated tissue damage might actually prove beneficial to the parasite.

1.4.2.2 Mast cells, globule leucocytes and mucous

Prominent intestinal mastocytosis is a characteristic of GI nematode infections. Mast cells contain basophilic granules with histamine as a constituent, have on their cell surface high affinity IgE receptors and are a major component of hypersensitivity reactions (Huntley, 1992). Historically, mucosal mast cell hyperplasia has been shown a feature of rodent infections with *N. brasiliensis*

(Miller and Jarrett, 1971), *T. spiralis* (Ruitenberg and Elgersma, 1976) and in ruminants with *T. circumcincta* (Murray *et al.*, 1968) and *H. contortus* (O'Sullivan and Donald, 1973). During maturation, and after stimulation with antigen, mast cells migrate to the epithelia (for example, the GI epithelium) and can degranulate, becoming globule leucocytes. In animals subjected to a continuous parasite burden, mast cell secretion is likely to be under a continuous stimulation, thus globule leucocytes will be common (Huntley *et al.*, 1984). Antigen cross-linking of bound IgE on mast cells leads to degranulation and release of a number of inflammatory mediators including histamine, leukotriene and serine proteases (mast cell protease) and cytokines (Janeway *et al.*, 1999). Indeed, IgE has been shown to promote mast cell protease release and enhance parasite expulsion clearance, although is not essential for intestinal mastocytosis (Gurich *et al.*, 2004). As the level of mast cell protease correlates with numbers of tissue mast cells and globule leucocytes, it can be used as a serological measure of mast cell hyperplasia (Huntley, 1992).

Although associated with the hypersensitivity reactions that may play a part in immunity to nematodes, evidence for a role of mast cells in parasite protection remains unclear, and seems to depend on the species involved. In ruminant infection experiments, mast cell protease concentrations in local tissue were significantly raised in sheep immune to challenge infections with *H. contortus* or *T. circumcincta* compared to the naïve challenge controls (Huntley *et al.*, 1987) suggesting a role for mast cells in immunity of ruminants to GI helminths. Additional experiments demonstrated infected then treated animals to have a higher mast cell protease level, but lower worm burden than naive controls upon challenge with a mixed *T. circumcincta* and *Trichostrongylus vitrinus* infection (Huntley *et al.*, 1995).

Despite evidence of mast cell proteinase secretions detectable in the gut of rats during expulsion of *N. brasiliensis* and *T. spiralis*, indicating a role for functionally active mucosal mast cells in worm rejection (Woodbury *et al.*, 1984), some studies have questioned the importance of mast cells during immunity to the former infection. Mice treated with anti IL-3 and IL-4 antibody showed an 85% decrease in mastocytosis but expelled *N. brasiliensis* as normal

(Madden *et al.*, 1991). Likewise, stem cell factor receptor (*c-kit*) knockout mice, deficient in mast cells, expelled this infection normally (Crowle and Reed 1981). However, evidence for a role of mast cells in immunity to *T. spiralis* is more persuasive. Alizadeh *et al.*, (1984) showed that prolongation of *T. spiralis* infection in mice occurred in strains that had a delayed appearance of mucosal mast cells and levels of mast cell protease were found to be higher, and mast cell response quicker in fast responder mice (those able to clear infection quickly) than in intermediate and low responder strains (Tuohy *et al.*, 1990). Furthermore, mice with antibody neutralised stem cell factor (or *c-kit*) showed a complete abrogation of mast cell response and a significant delay in worm expulsion; this delay, and the mast cell response, could be reversed by restoration of stem cell factor (Donaldson *et al.*, 1996).

The mechanism behind mast cell hyperplasia-associated worm expulsion is still not well understood. Worm infection is associated with an increase in mast cell activity which is accompanied by an increased permeability of the GI duct wall; this has been hypothesised to create a leaky membrane that might allow circulating or locally produced antibody or other mediators access to the parasites and cause expulsion, termed the leak-lesion hypothesis (Murray *et al.*, 1971). Recently, using the *T. spiralis* infection model, it has been demonstrated that, under control by $CD4^+$ T helper cells, mast cells are directly responsible for the increase in permeability of the gut epithelial cells. It seems likely that this permeability is a result of degradation of the tight junction protein occludin, most probably by a protease, possibly by the mast cell protease itself (McDermott *et al.*, 2003). Interestingly, *N. brasiliensis* expulsion from rats is also associated with intestinal permeability, and detectable levels of mast cell protease in the intestine (King and Miller, 1984), despite mast cells not being required for expulsion, thus indicating an as yet unresolved cell type or effector mechanism in this system.

Another role that mast cells may play is to allow the accumulation of substances toxic to invading worms in the intestinal or gastric mucous (Miller, 1996). As well as playing a physical role in nematode immunity, by providing a thick, sticky barrier, mucous can contain histamine, leukotriene and antibody and show

a distinct anti-nematode property (Douch *et al.,* 1983; Miller, 1987). Mucous from the small intestine of immune sheep cause *T. colubriformis* larvae to clump *in vitro*, and can prevent larval establishment *in vivo* (Harrison *et al.*, 1999). The recently elucidated mechanism responsible for this phenomenon is likely to be worm specific IgG1 and IgA in the mucous (Harrison *et al.*, 2003).

1.4.2.3 Role of antibody

Of the different antibody isotypes, those associated with immunity to GI nematode infection are IgA, IgG1 and IgE with evidence suggesting that antibody secreted at the mucosal surfaces may play an important role (Else and Finkelman, 1998). Adaptive transfer of purified IgG1 from immune serum can confer resistance to naïve mice infected with *H. polygyrus* (Pritchard *et al.,* 1983) suggesting a role for this isotype. A comparison of antibody levels in guinea pigs genetically determined as high responders and low responders found the high responders to generate a significantly higher anti *T. colubrifomris* IgG1 upon homologous challenge. Serum from high responders did not transfer passive immunity to low responders (Manjili *et al.,* 1999) suggesting that, albeit important in immunity, IgG1 in this system is not sufficient an effector on its own to confer immunity.

The adoptive transfer of immunity against *A. caninum* from vaccinated pups to susceptible pups was demonstrated using both lymphoid cells and serum (Miller, 1967). Immunity against both *H. contortus* and *T. circumcincta* in sheep can be conferred by passive transfer of lymphocytes from immune animals and the authors suggest, although other cell types may play a role, this may be due to the IgA producing cells transferred (Smith *et al.,* 1984; Smith *et al.,* 1986). Mucosal IgA in the abomasum appears a feature of *H. contortus* infection (Smith, 1977). IgA is also thought to be important in the immune response to *T. circumcincta*; IgA has been shown to regulate the growth of worms *in vivo* (Stear *et al.,* 1995) and IgA levels negatively correlated with worm size (Smith *et al.*, 1985). Levels of specific IgA directed against antigens of the L4 have been demonstrated statistically to account for over 90% of the variation in worm length (Stear *et al.,* 1999).

IgE is a prominent antibody isotype and is classically related with GI nematode infection. However, despite the almost universal increase in IgE associated with nematode infection, evidence of a protective role is limited. IgE was found to enhance *T. spiralis* clearance in mice and to regulate the mast cell response (Gurish *et al.*, 2004). Mice, though a non-permissive laboratory model host for hookworms, do allow tissue migration of the L3 stage and heavy infections can be fatal. After several doses, mice become resistant to larval challenge; this immunity coincides with significantly increased levels of IgG1 and IgE (Hotez *et al.*, 1999) suggesting a role for IgE in host protection. Rats transfused with IgE from immune animals rapidly expel *N. brasiliensis* infection (Ahmad *et al.*, 1991); however, despite this, earlier evidence would suggest that in rats unable to mount an antibody response worm expulsion is not necessarily affected (Jacobson *et al.*, 1977). Although adoptive transfer from immune animals may be unsuccessful, significant immunity against *T. spiralis* can be transferred to normally non-immune mice by infusing with serum from vaccinated animals (Robinson *et al.*, 1995). In contrast, experiments using SCID mice infected with *T. muris* have questioned a role for antibody: the adoptive transfer of $CD4^+$ T helper cells reconstituted immunity to worms in the total absence of antibody (Else and Grencis, 1996).

During infection with *T. circumcincta* there appears to be a marked contrast between the levels and specificity of worm-specific IgE levels of primary and secondary infected sheep. In primary infections, gastric lymph had low levels of anti L3 and early adult specific IgE; however, a few days post challenge of previously infected sheep, a pronounced IgE response was detected against L3, but not adult, antigens. IgE levels in the serum were significantly lower than in the local lymph supporting the importance of this antibody in the mucosa (Huntley *et al.*, 1998). Also reported were high levels of non-specific IgE. More recently a *T. circumcincta* allergen that reacts with IgE and is localised to the surface of the L3 has been discovered; in grazing lambs, levels of specific IgE to this allergen are significantly higher in animals with low faecal egg counts compared to those with high faecal egg counts (Huntley *et al.*, 2001). These studies provide evidence of a protective function of specific IgE in this infection. Moreover, high numbers of IgE-bearing cells (lymphocytes and monocytes) are

present in the blood of lambs selected for resistance to *T. circumcincta* compared to susceptible animals (Pettit *et al.*, 2005).

Of significance is the fact that much of the IgE generated during worm infection is not specific, raising questions about the benefit of this response to the host or parasite; some authors postulate that non specific IgE may benefit the parasite by blocking the effects of specific IgE through a saturation of IgE Fc receptors (Pritchard, 1993) whilst others have suggested it may be of benefit to the host, reducing a hypersensitivity reaction to parasite allergens (Hagan, 1993).

1.4.3 Immune response to hookworm

Because of a lack of suitable animal models for human hookworm infection, much of the information on the immune response to hookworm infection comes from immunoepidemiological studies in human populations; however, uncontrollable variables are inherent problems within this sort of study (Loukas and Prociv, 2001). The most noticeable trait of hookworm immunology is that the response is highly heterogeneous. In terms of antibody isotypes, levels and specificity differ within the population (Brooker *et al.*, 2004; Pritchard *et al.*, 1992). Another feature is the long-lived infection and lack of intestinal inflammation compared to other intestinal nematodes, and lack of clear evidence for the development of a protective immunity (Loukas and Prociv, 2001).

Within a *N. americanus* infected population in Papua New Guinea a strong response of all Ig isotypes (IgG, IgM, IgA, IgD, and IgE) was found against larval and adult worm antigens (Pritchard *et al.* 1992); however, despite the vigorous humoral response there is little evidence that these antibodies actually play a protective role. As discussed, non-specific IgE is upregulated in response to helminth infections, but there is a strong specific IgE response directed against *N. americanus*, and in fact this IgE is more specific than any other class of Ig (Pritchard and Walsh, 1995). A study conducted over two years of the *N. americanus* endemically infected population in Papua New Guinea looked at the levels of circulating total IgE and the fecundity and weight of recovered adult parasites (post chemotherapy) whilst controlling for the confounding variables of

host age and worm burden. A highly significant negative correlation between total IgE levels and worm weight and fecundity was found both in the first experiment and in the second, two years later (Pritchard *et al.,* 1995). Curiously, worm-specific IgE levels were only weakly (not significantly) negatively correlated with female worm weight, and were not correlated with male worm weight. The authors suggest that the correlations are unlikely to reflect a direct effector role for non-specific IgE, but that this is indicative of a Th2-dependent immune response that acts to reduce parasite weight and fecundity. Quinnel *et al.*, (1995) report that from the same sample population there was a negative correlation, as age progressed, between anti-adult excretory/secretory antigens IgE and IgM, and parasite burden; a result indicative of these isotypes playing a role in host immunity. Further evidence for an antibody mediated role in immunity comes from cross sectional epidemiological studies in China and Brazil that found patients exhibiting high titres of circulating IgE specific to *Ancylostoma* secreted protein 1 of L3, harboured a less intense worm burden (Bethony, unpublished results reported in Hotez *et al.*, 2003).

There is a strong peripheral eosinophil response associated with *N. americanus* infection (Maxwell *et al.,* 1987) but although eosinophils adhere to L3 in the presence of complement and antibody *in vitro,* there is no evidence that this is harmful to the worms (Desakorn *et al.,* 1987). Indeed, IL-5 knockout mice with no eosinophilia showed no increase in tissue numbers of *A. caninum* (Loukas and Prociv, 2001), though the mouse is a non permissive host. Little is known about the mast cell response in human hookworm infections, though in the *A. ceylanicum/* hamster model, animals become quickly resistant to challenge and this is characterised by a mucosal mast cell hyperplasia (Brooker *et al.*, 2004).

Recently, two separate studies carried out in Papua New Guinea and Brazil looked at the cytokine response in *N. americanus* infected populations. In the former study, looking at the cytokines release by lymphocytes, the authors found an expected upregulation of Th2 cytokines IL-4 and IL-5 in response to hookworm antigen. However, they also report detectable IFN-γ released with a negative relationship between IFN-γ levels and worm burden (Quinnell *et al.,* 2004). This relationship could be explained as evidence of a protective effect or

of immunomodulation by the worms; the increase of IFN-γ levels found 5 weeks after chemotherapy and worm expulsion might indicate the latter. However, pre-treatment IL-5 levels were shown to be negatively correlated with the intensity of reinfection, providing clear evidence for a protective role of IL-5 and indicating that this response is directed against incoming larvae and that it may involve eosinophils. Interestingly, hookworms release products that cleave eotaxin, an eosinophil chemokine (Culley *et al.*, 2000). A study carried out in school children in Brazil found antigen specific IFN-γ expression levels higher in egg negative endemic control patients than in hookworm patients but found IL-10 levels higher in patently infected patients (Geiger *et al.*, 2004). These results would suggest that both Th1 and Th2 arms of the immune response play a role in protection against hookworms.

Despite the ability of dogs to develop immunity to *A. caninum* (Miller, 1965), there is still limited evidence to suggest that humans develop immunity to hookworms. Indeed there is evidence that chronic human infection with hookworm actually leads to a hyporesponsiveness of cellular immune response, to both hookworm and non-hookworm antigens (Loukas *et al.*, 2005). The observed higher IL-10 production by stimulated peripheral blood mononuclear cells from *N. americanus* infected children compared to non-infected controls suggests a T regulatory cell response that might dampen a proinflammatory response, possibly as a mechanism to regulate pathology due to inflammation (Geiger *et al.*, 2004). Loukas *et al.*, (2005) reported that, in the presence of IL-2 and IL-12, excretory/ secretory (ES) products from *N. americanus* can stimulate IFN-γ production by natural killer (NK) cells; as such, hookworms may be cross regulating a potentially host protective Th2 immune response, therefore, allowing long term survival of *N. americanus* (Loukas *et al.*, 2005).

From an epidemiological point of view, the monotonic age-intensity profile of hookworm infection would not at first appear consistent with a development of immunity in adults (Quinnell *et al.*, 2004). However, mathematical models suggest that because of the confounding variable of constant exposure (or increasing exposure) to infection with age, a partial immunity would not necessarily buck the monotonic trend (Woolhouse, 1992); therefore, this linear

pattern may actually be consistent with a degree of immunity developing (Quinnell *et al.*, 2004).

In summary, the protective immune response to human hookworm is complicated and remains unclear but is likely to involve a combination of several effector cell types and mediators and is likely regulated to a degree by the secretion of immunomodulatory hookworm proteins.

1.4.4 Immune response to *H. contortus*

Following repeated infection, sheep can become resistant to *H. contortus* infection. Experimentally-infected hyperimmune sheep can rapidly expel infective larvae; invading L3 become briefly associated with the abomasal mucosa but, rather than enter the gastric glands, are expelled by means of an immune exclusion mechanism (Miller *et al.*, 1983). These sheep show little increase in lymphocyte recruitment to the mucosal tissue (Gorrell *et al.*, 1988) and the mechanisms behind immunity to infection are still not well understood. Both primary and secondary infection with *H. contortus* is accompanied by an increase in serum antibody (Smith, 1977; Schallig *et al.*, 1994; Schallig *et al.*, 1995); however, a direct relationship between these antibody levels and the immune status of the sheep has been questioned (Schallig, 2000). If antibody does play a role in immunity, it may be that the local mucosal response is more important than the systemic. Interestingly, following a secondary challenge infection, circulating antibody levels rose slightly, but not until after a fall in faecal egg count associated with immunity. In contrast, local mucosal IgA levels rose rapidly following the secondary challenge, suggesting this to be a mediator of immunity (Charley-Poulain *et al.*, 1984). By cannulating the gastric lymph duct, Gill *et al.* (1992a) measured the number of cells containing the various antibody isotypes before and during *H. contortus* infection. Post infection, an increase in Ig containing cells was found, with a particularly marked increase in IgG1 and IgA positive cells, the latter being the most frequently observed with a peak 2-4 weeks post infection. Strain and Stear (2001) have suggested that IgA is a particularly important isotype with regard to control of fecundity and worm size in *T. circumcincta*. Although, despite IgA levels being inversely associated

with worm burden and faecal egg count in *H. contortus* infection, IgA levels were not found to differ between resistant and non-resistant breeds (Amarante *et al.*, 2005). As with other helminth infections, elevated serum IgE levels are a feature of *H. contortus* infection; a negative correlation of total IgE with worm burden was found but, in a situation analogous to that in hookworm infection (Pritchard *et al.*, 1995), specific IgE did not correlate with protection (Kooyman *et al.*, 1997).

An investigation of the cytokine profiles associated with infection in resistant and non resistant breeds found infected sheep of both breeds to have lower IFN-γ and higher IL-5 levels than their non infected counterparts. Resistant sheep had higher IL-5 levels, increased levels of cultured cell derived IgE and IgG1 and a higher density of local mast cells and eosinophils than non resistant sheep, implying a Th2 type response in genetically resistant sheep, and supporting the notion of a Th1/Th2 dichotomy in sheep (Gill *et al.*, 2000). Elevated levels of IL-5 might suggest a role for eosinophils, concurring with the discovery that eosinophils can kill L3 *in vitro* in the presence of antibody and complement and that this killing is enhanced by IL-5 (Rainbird *et al.*, 1998).

Smith *et al.* (1984) demonstrated transfer of immunity to naïve sheep by efferent gastric lymph from immune animals though this was only evident when the lymph was taken 3 and 5 days post challenge. The cell type responsible for this is likely to be $CD4^+$ helper cells. The importance of this cell type in resistance against *H. contortus* was confirmed by the ability of anti-$CD4^+$ T cell monoclonal antibody to abrogate resistance *in vivo* to normally resistant animals (Gill *et al.*, 1993).

A recent study has provided evidence that there may be two distinct types of immunity elicited against invading *H. contortus* larvae. In two separate experiments, hyperimmune sheep were challenged and killed 3 and 5 days post-challenge to allow investigation of larval-mucosal association and of cell types present (Balic *et al.*, 2002). In the first study sheep had no larvae present at either time point and no lymphocyte or eosinophil recruitment was found. In the second experiment (of identical design) larvae were present within the abomasal

tissue and there was an increase in activated $CD4^+$ T cells, and an increase in γδ-T cells, (but not $CD8^+$ T cells) and B cells. Higher levels of eosinophils were also observed, as was a close association between this cell type and the larvae. Balic and colleagues suggest that immune sheep show two distinct responses to invading L3, either a delayed or rapid expulsion, and that different responses are associated with the different expulsion kinetics. The reason for the different outcome in the two experiments is hard to explain, but does indicate the heterogeneity of response to infection.

1.5 Control Strategies

Currently, control of hookworm disease relies heavily on anthelmintic drugs, predominantly benzimidazoles. Control programmes are aimed at reducing the worm burden, thus the transmission potential in an attempt to alleviate the morbidity associated with infection, rather than infection *per se*. (WHO, Prevention and control of intestinal parasitic infections, 1987). Albendazole is the treatment of choice for hookworm infection, and a single dose is sufficient to treat hookworm infection with a cure rate of 57-95% and an egg reduction rate of between 92 and 100% (Albonico *et al.*, 1999). Thus, clearly, regular anthelmintic treatment would result in lowering worm burdens and help prevent disease, but to whom should treatment be directed? The bias in worm burden distribution in the population might suggest that treatment should be aimed at the people carrying the heaviest infections, but identifying this group may prove difficult. It has been suggested that, in terms of practicality, cost effectiveness and ease of administration, a programme of school-based treatment is simplest and most effective at treating disease associated with STH (Savioli *et al.*, 2002). On this basis, the WHO put forward a resolution to see 75% of children receive regular anthelmintic treatment by 2010. However, the epidemiology of hookworm is distinct from that of the other STH, in that infection increases with age, leading some researchers to suggest that a school based treatment programme may have a more limited benefit than treatment of disease in adults attributable to hookworm (Chan *et al.*, 1997).

Despite this, regular chemotherapy can clearly reduce morbidity. In Nepal a regular deworming programme, providing 2 albendazole treatments a year to 95% of school children, has reduced anaemia by 77% since 2001 (WHO, 2005). Antenatal treatment with anthelmintics in Nepal was also found to significantly reduce maternal anaemia and improve infant survival (Christian *et al.*, 2004). These results confirm work undertaken in Tanzania that found serum ferritin and haemoglobin levels were significantly improved in children by treatment against hookworm (Bhargava *et al.*, 2003) and work in Zanzibar that demonstrated a regime of three doses per year of anthelmintic in school children to significantly

improve iron status and decrease the chances of IDA occurring (Stoltzfus *et al.*, 1998).

The importance of regular deworming treatment is highlighted by the incidence of reinfection following treatment; a study in Tanzania reported a return to pre-treatment levels of hookworm infection just 4-12 months following treatment (Albonico *et al.*, 1995). Quinnell *et al.*, (1993) found that in Papua New Guinea, levels of hookworm prevalence had returned to those pre-treatment 2 years beforehand and a geometric mean of worm burden had returned to 58% of the pre-treatment level.

A worry of large scale nematode control programmes based on chemotherapy is the emergence of drug resistant worm populations (Albonico *et al.*, 1999). This is already a massive problem with nematodes of veterinary importance (including *H. contortus*, discussed below), and there are reports of drug failure likely due to resistance in hookworms (Albonico *et al.*, 2003; de Clerq *et al.*, 1997). A problem with detecting drug resistance in hookworms is the variability in cure rates of chemotherapy, the difficulty in determining drug efficacy, and the lack of a standardised technique (Geerts and Gryseels, 2000). Recently, workers have looked to the veterinary research field to adapt established anthelmintic resistance assays for use with hookworms, and this may allow future drug resistance screening (Albonico *et al.*, 2005; Kotze *et al.*, 2005).

Improvement of diet, and iron supplementation may also contribute to reducing morbidity. Work looking at the effect of iron supplementation with or without helminth chemotherapy in adult females (with a high prevalence of hookworm, as well as concomitant STH infections) demonstrated iron supplementation alone to significantly increase haemoglobin values; though, when administered in combination with anthelmintic, haemoglobin values further improved (Gilgen and Mascie-Taylor, 2001).

That improved sanitation is an important factor in any human helminth control programme is well recognised (Albonico *et al.*, 1999); however, the impact this has is often slow to develop, may not make a measurable impact for decades and

the reduction may be greater on other STH than it is on hookworm incidence (Esrey *et al.*, 1991). These authors report only a 4% decline in hookworm infection prevalence as a result of latrine use. Use of footwear is also likely to have an effect upon infection prevalence; a Tanzanian study linked compulsory shoe wearing by school children to a lower hookworm incidence compared to children not attending school (Killewo *et al.*, 1991). Whilst an improvement in the sanitary conditions is undoubtedly of long term benefit, currently the only strategy available for effective short to medium-term disease control is chemotherapy (Brooker *et al.*, 2004).

Like human hookworm, control of GI nematodes in sheep is almost exclusively based on use of anthelmintics, there being 3 main broad spectrum drugs available: the benzimidazoles, the macrocyclic lactones and levamisole. Control is highly effective and results in an almost complete cure of the existing nematode infection. However, drug resistance is a major, and ever increasing, problem, with resistance to all classes of drug available, and a high prevalence of multi-drug resistant worms (reviewed recently by Kaplan, 2004). Reports indicating the seriousness of the situation in South America (Echevarria *et al.*, 1996; Eddi *et al.*, 1996; Maciel *et al.*, 1996; Nari *et al.*, 1996), Australia (Love *et al.*, 2003) and South Africa (Van Wyk *et al.*, 1999) suggest that *H. contortus* strains now resistant to all three types of anthelmintic in use actually threaten the viability of sheep farming in these regions.

In the UK, although not on the scale in tropical areas, anthelmintic resistance in *H. contortus* is prevalent (Jackson and Coop, 2000). Multi drug resistance is a big problem in *T. circumcincta* (the more prevalent sheep GI species in the UK) and recently triple resistant isolates have been identified (Bartley *et al.*, 2004). The problems highlighted have resulted in a concerted effort to seek alternative control strategies and research into vaccine development against the GI nematodes of ruminants, particularly *H. contortus*.

As well as the programme of chemotherapy for school children, in 2005 the WHO recommendations for STH control also stated that research into alternative control such as vaccines is considered essential. The combination of knowledge

gained from the veterinary field and the HHV1 is the subject of the following section.

1.6 Rationale of Vaccination

It would certainly seem, from the immune responses associated with hookworm infection in man, that acquisition of solid natural immunity does not follow infection. How therefore, can one expect vaccination to be a feasible strategy? Early studies focusing on stimulating immunity in dogs to *A. caninum* found that administration of live L3, either orally or subcutaneously, repeatedly in small doses elicited an artificial immunity (McCoy, 1931). The immunity was not sterile, rather it was reflected by a much reduced worm burden compared with non immune controls. Control of disease was antibody mediated (Otto, 1940) and was primarily directed at antigens secreted by L3 (Sheldon and Groover, 1942). This knowledge led to research in the veterinary field to design a vaccine against *A. caninum* for use in dogs. Irradiated L3 were shown to elicit good levels of protection, providing the damaged L3 remained viable (Miller, 1965) and able to secrete the antigens required to induce immunity (Hotez *et al.*, 2003). This established the basis for a commercial vaccine using attenuated L3 which was marketed in the USA in the 1970s. Ultimately the vaccine was a commercial failure due to the high costs involved in production, the short shelf life and an inability of clinicians and pet owners to appreciate the value of a vaccine that, whilst not resulting in sterile immunity, was still of benefit in lowering environmental contamination and in preventing disease (Miller, 1978). However, the vaccine did demonstrate proof of concept, and confirmed that the larval stages contain or release protective antigens (irradiated larvae do not develop into adults). The immunity stimulated by the murine vaccination with irradiated *N. americanus* has suggested a localised Th2 response with an increase in IL-4 and levels of IgG1, analogous to the situation described in human hookworm infection (Girod *et al.*, 2003).

Immunoepidemiological evidence that indicates a group of individuals in endemic hookworm regions develop a Th2 humoral immune response to a specific secreted antigen that results in a lower intensity of infection indicates that individuals can develop a natural immunity that could be exploited through vaccination (Brooker *et al.*, 2004). Very recent work has linked specific anti-L3

antigen IgE titre with protection against hookworm in endemic areas of Brazil and China (Bethony *et al.*, 2005). Thus there are several lines of evidence that development of a human hookworm vaccine is feasible.

Discussed in detail later, hookworms and *H. contortus* share a similar battery of proteins and enzymes that align the brush border of the alimentary canal, and may be involved in digestion. Over the past two decades, several antigen complexes have been isolated from the gut of *H. contortus* and these have been found to consistently elicit high levels of protection against homologous challenge in vaccine trials (Knox and Smith, 2001) proving vaccination against *H. contortus* a feasible strategy. *H. contortus* also has homologues of some of the hookworm secreted antigens, and protein mixtures containing these have also elicited high levels of protection (Schallig *et al.*, 1997).

1.7 Potentially Protective Antigens

There are two major classes of proteins that are suitable vaccine targets: Excretory-secretory (ES) products, that are essential for parasite survival (for example for extra corporeal digestion), and somatic proteins that are accessible to host Ig (Knox, 2000). Of the latter, much research has been focused on the "hidden" antigens of the nematode gut, termed as such as they are not naturally exposed to the host immune system. However, Ig can be directed against such antigens and is ingested with the blood meal, a concept proven by the development of a successful vaccine against a gut antigen of the cattle tick *Boophilus microplus* (Willadsen, 1995). Antibodies directed at gut proteins essential for worm survival may inhibit protein function, and protection may be due to direct antibody inhibition of proteinases (Knox *et al.*, 1999). There is also evidence that antigens secreted by hookworm infective larvae can elicit immunity. Therefore the HHVI has selected hookworm antigens as lead candidates for vaccine testing on the basis of either the link with attenuated larval vaccines, or because they are orthologues of the major *H. contortus* vaccine candidates (Hotez *et al.*, 2003). Protective antigens, and those deemed to have potential are discussed in the next section.

1.7.1 Gut-expressed antigens

Several, protective gut-expressed antigens have been isolated from *H. contortus* (Munn, 1997). H11 (a 110 kDa integral membrane glycoprotein) is one such antigen, purified from contortin-enriched preperations from adult *H. contortus* (Smith *et al.*, 1993). H11 shows aminopeptidase activities and H11 isotypes are encoded by at least 3 genes, exhibiting homology to those of mammalian microsomal aminopeptidases. Enzyme activity is localised to the intestinal brush border and can be inhibited *in vitro* by antisera raised against H11 (Smith *et al.*, 1997). The level of this enzyme inhibition correlates with protection (Munn *et al.*, 1997). In its native and purified form, H11 is the most efficient nematode immunogen, in protective terms, isolated to date (Knox and Smith, 2001). Intriguingly, a homologue of H11 in hookworms has not been identified to date.

The *H. contortus* galactose containing glycoprotein complex H-gal-GP, isolated from the gut lumen, reduced worm burdens by 72% and mean faecal egg counts (FEC) by up to 93% when used in a sheep protection trial against homologous challenge (Smith *et al.*, 1994). The complex is localised to the surface of the gut cells and has been implicated in blood feeding. Enzyme linked immunosorbent assay (ELISA) data implicates antibody-mediated protection (Smith *et al.*, 1999). H-gal-GP has been shown to contain an aspartyl protease (Longbottom *et al.*, 1997) and at least 4 metalloendopeptideases termed MEP1, 2, 3 and 4 (Redmond *et al.*, 1997; Smith *et al.*, 1999) as well as thrombospondin (Skuce *et al.*, 2001), cystatin, (Newlands *et al.*, 2001) and galectin (Newlands *et al.*, 1999) homologues. Recently Jones and Hotez (2002) have identified and characterised a 98 kDa MEP from *A. caninum* (AcMEP-1). Expression of the AcMEP-1 is restricted to the adult stage and activity is localised to the gut surface as in *H. contortus*. The elucidated amino acid sequence shows homology to the *H. contortus* MEP-1 component of H-gal-GP (66% similarity and 50% identity) thus making it a possible vaccine target for *A. caninum* protection studies. It is likely that MEPs are involved in part of a proteolytic cascade of haemoglobin digestion, so antibody directed against them might inhibit parasite feeding (Williamson *et al.*, 2003). Moreover, Culley *et al.* (2000) have demonstrated hookworm MEPs which specifically cleave eotaxin, a potent eosinophil chemokine. Discussed in **Section 1.4.2.1**, eosinophils are believed to play a role in hookworm immunity, thus AcMEP may have an immunoregulatory function.

Aspartyl protease (APR) activity capable of digestion of skin macromolecules and haemoglobin has been reported in hookworms (Brown *et al.*, 1999; Williamson *et al.*, 2002; Williamson *et al.*, 2003) and at least two distinct aspartyl proteases have been isolated from hookworms: APR-1, a cathepsin D like protease and APR-2 (Harrop *et al.*, 1996; Williamson *et al.*, 2003). A homologue of aspartyl protease of H-gal-GP, the *N. americanus* aspartyl protease Na-APR-2 can cleave human haemoglobin and serum proteins in a host-specific manner. It is more similar to a distinct nematode specific family of aspartic proteases than to Cathepsin D like proteases, represented in hookworms by the APR-1s (Harrop *et al.*, 1996), but, like APR-1, is localised to the gut surface and excretory glands (though was not detected in adult ES). As mentioned, both

proteases can cleave haemoglobin and serum proteins, and it has been suggested that the enzymes may both play roles in the proteolytic cascade of haemoglobin degradation; APR-1 an upstream protease exposing other cleavage sites and APR-2 (and the host of other haemoglobinase enzymes) degrading the exposed cleavage sites (Williamson *et al.*, 2003). Vaccination of dogs with a recombinant Ac-APR-1 resulted in a significant shift in adult worms from the normal site of predilection, the small intestine, to the colon; the significance of this in terms of potential protection is not understood (Hotez *et al.*, 2002). Recently, vaccination of dogs with Ac-APR-1 led to a significantly reduced worm burden and FEC and, most interestingly, vaccinated dogs were protected against blood loss and did not suffer anaemia (Loukas *et al.*, 2005). This evidence combined with protective capabilities of H-gal-GP certainly suggests these proteases are potential vaccine targets.

H. contortus and *A. caninum* both contain cathepsin B-like cysteine proteases (Tort *et al.*, 1999). Cox *et al.* (1990) identified a 35 kDa cysteine protease in *H. contortus* water soluble extracts that showed anticoagulant qualities and degraded fibrinogen. Immunisation with this protease conferred marginal protection in lambs. A cysteine protease-enriched fraction of detergent soluble *H. contortus* extracts, Thiol Sepharose Binding Protein (TSBP; purified by passage through a Thiol-Sepharose affinity column), proved protective in immunised lambs (Knox *et al.*, 1999). The proteins were localised to the gut surface and the microvillar surface of worms recovered from vaccinated lambs was coated in sheep immunoglobulin. TSBP consists of a 60 kDa glutamate dehydrogenase and cysteine proteases encoded by three distinct genes, cDNAs of which have been designated hmcp1, 4 and 6 (Skuce *et al.*, 1999). Further fractionation of TSBP has determined that the protection partitions to a protease-rich subfraction and is likely attributable to cysteine proteases (Knox *et al.*, 2005). Using cystatin, a cysteine protease inhibitor, to specifically purify the cysteine proteases in TSBP, Redmond and Knox (2004) demonstrated the cysteine proteases to confer a substantial level of protection. Moreover, in a vaccine trial using recombinant hmcp 1, 4 and 6 expressed as GST-fusion proteins, sheep showed a highly significant 38% reduction in worm burden. Two cysteine proteases have been cloned from *A. caninum* AcCP-1 and AcCP-2

(Harrop *et al.,* 1995). Vaccination of dogs with the latter, likely to be a gut expressed protease, resulted in reduced fecundity and a decrease in worm size corresponding to specific anti AcCP-2 antibodies binding to the worm gut and interfering with protease function (Loukas *et al.* 2004).

1.7.2 Excretory secretory antigens

Molecules released by the parasite *in vivo* or *in vitro* culture are referred to as ES products. They may be derived from the parasite surface, from specialised secretory glands or as by-products of digestion and represent antigen exposed to the host (Knox, 2000). ES products are often released in a stage specific manner. For example, an increase in the quantity of protein released by *H. contortus* L3 *in vitro* culture (as detected by gel electrophoresis and Coomassie staining) is associated with the onset of feeding (Gamble and Mansfield, 1996). *H. contortus* ES products include proteolytic enzymes probably involved in digestion of the blood meal (Knox and Jones, 1990). ES proteins usually display a simpler antigenic composition compared with somatic antigens and elicit a strong immune response, thus implying a possible protective value (Schallig *et al.,* 1994).

1.7.2.1 Adult ES

Schallig *et al.* (1994) showed ES products of adult *H. contortus* to provoke strong cellular and humoral immune responses in Texel sheep and to elicit an immunity characterised by a reduction in FEC of 32% and of worm burden by 63%. In the same study, sera from primary infected and challenged sheep used to screen immunoblots of adult ES strongly identified a 24 kDa protein and a 15 kDa protein, respectively. Later protection studies with the 15 kDa and 24 kDa ES proteins demonstrated a 72% reduction in FEC compared with control groups (Schallig *et al.,* 1997a). These workers also found an increased mastocytosis to abomasal tissue and a primarily Th2 response to the ES antigen, leading them to postulate that ES antigens mimic natural immunity to *H. contortus*. Interestingly, when looking at correlations in protection, animal age and IgE and IgG1 level, it would appear that protection is age related, but that when this is taken into consideration, it correlates with IgE antibody (Kooyman *et al.,* 2000). Cloning

and characterisation of these proteins demonstrated a 50% homology between the 24 kDa ES (Hc24) and a 24 kDa protein secreted by *A. caninum* L3, *Ancylostoma* secreted protein 2 (ASP-2) (see section 1.7.2.2). ASP-2 is expressed with the onset of feeding and restricted to parasitic life stages suggesting a role in the transition to parasitism (Hawdon *et al.*, 1999). Adult hookworm-derived ASPs also show homology with Hc24: two adult ASPs (ASP-3 and ASP-5) exhibit 25% and 32% homology with Hc24, respectively (Hotez *et al.*, 2003). More recently, trials of combined recombinant 15 kDa and 24 kDa ES with or without a glycan-rich insect cell extract gave a reduction in sheep worm burden of 55% and 65% respectively, though these results were not reproducible and protection was not apparent in young lambs (Vervelde *et al.*, 2002).

The most abundant protein secreted by *A. caninum* adults is a 16 kDa tissue inhibitor of metalloproteases (TIMP), mRNA expression for which is restricted to the adult stage (Zhan *et al.*, 2002). TIMP shows 33% identity and 50% similarity with the N-terminal of human tissue inhibitor of metalloprotease 2, and has homologues in *C. elegans*, although its function is unknown. Interestingly, an investigation into the humoral response of dogs vaccinated with irradiated *A. caninum* showed that a dominant antibody response was elicited against a second TIMP protein (TIMP-2) (Boag *et al.*, 2003). Vaccination with recombinant *A. caninum* TIMP had no effect on overall worm burden, but it did have a marked effect on the habitat of the adult worms; a proportion of worms appeared to migrate through the small intestine to the colon suggesting a genuine effect (Hotez *et al.*, 2002). No homologue has been identified in the available adult *H. contortus* gene sequence database.

A number of other proteins are secreted by adult hookworms including a neutrophil inhibitor factor (NIF), which exhibits some homology with Ac-ASP-3. Vaccination of hamsters with an *A. ceylanicum* homologue of the AcNIF resulted in a significant reduction in fecundity of worms following homologous challenge (Ali *et al.*, 2001). Secretion by adult *A. caninum* of a fatty acid and retinol binding protein has also been demonstrated (Basavaraju *et al.*, 2003); inhibition of which might be detrimental to the worm indicating it as a possible vaccine candidate. Adult hookworms secrete a family of anticoagulant peptides

(Cappello *et al.*, 1995; Stassens *et al.*, 1996), of which one (*A. caninum* anticoagulant protein, AcAP5) has shown slight promise in trials as a protective vaccine candidate (Hotez *et al.*, 2002). Moreover, purified IgG specific for AcAP5 has been demonstrated to neutralise the anticoagulant nature of the protein *in vitro*, therefore antibody targeting *in vivo* might prevent hookworm feeding (Harrison *et al.*, 2001).

In addition, adult hookworms have also been shown to secrete an aspartyl protease inhibitor (Delaney *et al.*, 2005), a novel immunoreactive protein (AcES-2; Bungiro *et al.*, 2004), acetylcholinesterases (Pritchard *et al.*, 1991), a haemolysin (Don *et al.*, 2004), a platelet inhibitor (Del Valle *et al.*, 2003) and proteins that bind selectively to natural killer cells (Hsieh *et al.*, 2004). Inhibition of some of these proteins, potentially essential for the parasite's survival, might be feasible by antibody, indicating a possibility as vaccine candidates.

1.7.2.2 Larval ES

It has been demonstrated that larval infective stages (L3) of both hookworm and *H. contortus* contain or release proteins capable of protection against further infection; vaccination using irradiated larvae of both *A. caninum* and *H. contortus* will protect against homologous challenge (Miller, 1965; Smith and Angus, 1980). Developmentally arrested L3 larvae of both hookworm and *H. contortus* activate to resume development shortly after entry to the host. This can be achieved *in vitro* by culture of *A. caninum* with a low molecular weight fraction of dog serum to stimulate feeding (Hawdon and Schad, 1990). In the case of *H. contortus*, exsheathing in CO_2 and culture in media results in the onset of feeding after 48-72 hours (Gamble and Mansfield, 1996).

On resumption of development, *A. caninum* L3 release several molecules into culture medium, the most abundant of which is a 42 kDa secreted protein termed ASP-1. A cDNA found to encode ASP-1 exhibits homology to the Antigen5/ Antigen3 (Ag5/Ag3) family of proteins from hymenoptera venom, a family of cysteine-rich secreted proteins (CRISPs) and pathogenesis-related proteins in

plants (Hawdon *et al.*, 1996). When antibodies to recombinant ASP-1 were used to probe the kinetics of expression, it was found that ASP-1 is released within 30 min of activation *in vitro*, far earlier then the onset of feeding (6-8 hours post activation) (Hawdon *et al.*, 1996). It was also shown that the serum stimulus essential for activation, was required constantly for ASP-1 release, but required only once to initiate feeding. This implied that ASP-1 had a function apart from feeding, leading Hawdon and co-workers to suggest an important role in transition to parasitism and infection. They found a 30% homology to the adult ES protein NIF mentioned above, pointing to a possible immunomodulatory role. Alternatively, on account of ASP-1's homology to Ag5 of the wasp *Vespula squamosa* they suggest its function is one of an allergen to elicit an immediate type hypersensitivity response and a Th2 cytokine profile (Hawdon *et al.*, 1996). Vaccination with a recombinant ASP-1 resulted in protection in mice against challenge infections manifested in a reduction in lung hookworm burdens and a reduction in hookworms in muscle (worms do not develop beyond L3 in the mouse). This decrease in worm burden was associated with an increase in all classes of Ig (Gosh and Hotez, 1999), leading the authors to conclude that burden reduction is antibody dependent, and may operate by blocking L3 entry to the lungs. Further weight is added to this hypothesis by their observation that ASP-1 may be an amphidial protein in a plant nematode (*Meloidigyne incognita*) and that antibodies against this could alter certain amphidial functions, such as chemosensation.

A second *A. caninum* L3 ASP, ASP-2, is approximately half the size of ASP-1, and exhibits 54% identity and 68% similarity and aligns with the C-terminal half of ASP-1 (Hawdon *et al.*, 1999). Thus, there are two types of ASP molecules in *A. caninum*: a single domain type, represented by ASP-2 and a double domain type, represented by ASP-1, both belonging to the same family (Hawdon *et al.*, 1999). Since their discovery in *A. caninum*, representatives of both the single and double domain ASPs have been discovered in the human hookworm species (Bin *et al.*, 1999; Zhan *et al.*, 2003) Like *asp*-1, *asp*-2 mRNA is synthesised in all life stages of the parasite, but its gene product is only released by activated L3. ASP-2 is represented in many species of nematode: as already mentioned, ASP-2 has a homologue in adult *H. contortus* in the form of Hc24, it also has

homologues in the filarid nematodes [an ASP-2 homologue in *Brugia malayi* has been demonstrated immunogenic (Murray *et al.*, 2001)] and in at least 17 genes in *C. elegans* (Bin *et al.*, 1999). The wide distribution of ASP-2 among nematode species suggests it serves an important function in development and physiology (Hawdon *et al.*, 1999) thus making it a prime vaccine target. Vaccine trials have demonstrated a protective function of ASP-2; vaccination with recombinant *A. ceylanicum* ASP-2 lead to 30% reduced worm burden, and to a 16% reduction in adult worm length and was associated with a high specific antibody titre (Goud *et al.*, 2004). This protection was repeatable in a second trial, again accompanied by high specific antibody titres, and enhanced by concomitant vaccination with another hookworm protein (Mendez *et al.*, 2005). Studies have also indicated that there is a relationship between human anti-ASP-2 antibody titre and reduced infection intensity in hookworm endemic regions (Hotez *et al.*, 2005). Based on this evidence, a *Pichia*-expressed recombinant *N. americanus* ASP-2 (Na-ASP-2) is now ready for use in human Phase 1 clinical trials (Goud *et al.*, 2005).

Although ASP-1 has only 1 homologue identified in *C. elegans*, suggesting there are fewer copies of this gene in the nematode genome than ASP-2 (Bin *et al.*, 1999), there is a single domain ASP-1 homologue in *H. contortus*: Hc40 (Hawdon *et al.*, 1999). This 40 kDa homologue, purified from a *H. contortus* cDNA library immunoscreened with a polyclonal serum against gut proteins (Rehman and Jasmer, 1998), is almost identical to a protein purified from *H. contortus* adults that was demonstrated to confer protection against homologous challenge in a guinea pig model (Sharp *et al.*, 1996). A single domain ASP like protein, a homologue of the N-terminal of ASP-1 as opposed to an ASP-2 homologue, has also been isolated from *H. contortus*, in this case from ES of cultured L3. This was shown protective in guinea pigs vaccinated with the native protein (80% reduction in worm burden) and with a baculovirus-expressed version (45% reduction) compared to controls (Sharp and Wagland, 1998). However, the guinea pig is a non permissive host model and no published sheep vaccine trials have been carried out. Two ASP homologues have been described in *O. ostertagi* and, like the *H. contortus* L3 ES ASP-1, both are single domain ASPs but show homology to the N-terminal part of the *A. caninum* ASP-1 as

opposed to the ASP-2; several other single domain ASPs have been found in the EST dataset (Geldhof *et al.*, 2003). Of significance is that *O. ostertagi* ASP homologues are the most abundant proteins in a protective fraction from adult *O. ostertagi* ES (Geldhof *et al.*, 2003).

Both *A. caninum* and *H. contortus* release metalloproteases (MTP) developmentally expressed in activated larval stages (Hawdon *et al.*, 1995; Gamble *et al.*, 1989; 1996). On gassing with CO_2, *H. contortus* L3 release a 44 kDa zinc MTP which mediates ecdysis *in vitro* (Gamble *et al.*, 1989). The protease acts on an area 20 μm from the anterior tip of the nematode, and degrades the refractile ring allowing the L3 to escape its sheath (Gamble *et al.*, 1989). Secretion of another, likely distinct, *H. contortus* MTP occurs coincident with the L3 to L4 moult and the onset of feeding *in vitro* (Gamble *et al.*, 1996). The authors postulate that although this 46 kDa metalloprotease may play a role in the L3 to L4 moult, it is not the enzyme's exclusive function, as it is continuously released after the moult has taken place (Gamble *et al.*, 1996). It may have a function in feeding or prevention of blood clotting.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of cDNA from *A. caninum* larval stages found cDNA encoding the *A. caninum* (Ac) MTP confined to the L3 stage. The full length cDNA encodes a protein with a calculated molecular weight of 61 kDa and shows significant homology to members of a family of zinc MTPs called the astacins (Zahn *et al.*, 2002). Homology was found with a group of astacin-like proteases in *C. elegans*, and astacin-like MTPs have since been identified in *O. ostertagi* (De Maere *et al.*, 2005) and *Strongyloides stercoralis* (Gomez Gallego *et al.*, 2005). MTP-like sequences are found in expressed sequence tag (EST) databases of *N. americanus*, *T. circumcincta* and *A. ceylanicum* indicating the ubiquitous nature of these proteases in nematodes, though no homologue has, as yet, been identified in the *H. contortus* EST dataset. Antibodies to recombinant AcMTP recognised a protein band of 47.5 kDa in activated L3 ES, most probably a form following removal of the pro-enzyme indicating that L3s release a processed form of MTP during *in vitro* activation (Zhan *et al.*, 2002). The function of AcMTP is

unknown, but it may have a role in regulating development (Zhan *et al.*, 2002), or it may facilitate skin penetration and/or tissue migration (Williamson *et al.*, 2006). Whatever the function, its stage specificity and secretion during L3 activation suggest a critical role in the infective process.

Regardless of the specific roles of these developmentally regulated astacin-like MTPs, possible *in vivo* interference of function may make them good vaccine targets, although the *H. contortus* exsheathing MTP is probably not expressed out with the cuticle, or for long enough to target it with antibody (Gamble *et al.*, 1989). At least two protection trials have been carried out using the recombinant AcMTP. In the first, utilising the dog *A. caninum* model, no difference in worm burden between *Escherichia coli*-expressed MTP-vaccinated and control animals. However, a high specific AcMTP antibody was developed and there was a significant inverse correlation between antigen specific IgE titre and worm burden (Hotez *et al.*, 2003). More recently, vaccinating hamsters with *Pichia*-expressed *A. ceylanicum* MTP resulted in a reduction in worm burden of 28%; when administered in a cocktail vaccine alongside ASP-2, the MTP significantly improved overall protection as measured by worm burden and FEC (Mendez *et al.*, 2005). Thus, the larval MTP is a promising vaccine candidate.

Infective *N. americanus* L3 have been shown to secrete aspartyl proteases with an activity similar to that of the adult APRs discussed above (Brown *et al.*, 1999). This protease activity was demonstrated to degrade skin macromolecules. Furthermore incubation of L3 with protease inhibitors found Pepstatin A, an apartyl protease inhibitor, the only class of inhibitor to significantly inhibit larval skin penetration (Brown *et al.*, 1999). In addition, antibody against APR-2 was shown to significantly inhibit L3 skin penetration. Though likely to be a result of cross reactivity with a larval APR (no APR-2 mRNA is detectable in the L3), this immune intervention with this protease might have a protective function.

Identified by immunoscreening cDNA libraries with serum from hookworm patients in China, *A. caninum* L3 secrete a protein with homology to the retinol binding-like proteins of *C. elegans* (Hawdon *et al.*, Society of Protozoologists, 53rd Annual Meeting, June 25-28, 2000). The 144 amino acid larval protein

shows homology to the transthyretin-like proteins (AcTTR), which are a nematode specific family with at least 52 representatives in *C. elegans,* and a faint homology to the transthyretin-related proteins, a highly conserved gene family represented in plants, bacteria and animals (Eneqvist *et al.,* 2003). Though the function of AcTTR is unknown, it may scavenge host retinol; vaccination of dogs with an *E. coli*-expressed AcTTR lead to a significant inverse relationship between specific IgE and worm burden (Hotez *et al.,* 2003). DNA homologues of TTR are abundant in the L4 and adult *H. contortus* EST dataset and are present at lower levels in the L3 dataset (DP Knox, personal communication). A study of the immunogenicity of adult *H. contortus* ES found a TTR-like protein to be the most immunogenic protein identified, suggesting it as a possible vaccine candidate (Yatsuda *et al.,* 2003).

1.8 Vaccine Requirements

Vaccination against GI nematodes, even with the most highly effective vaccine candidates discovered to date, is unlikely to produce sterile immunity. However, this may not necessarily be a problem. Mathematical models of vaccination against GI nematodes of sheep (for example, *H. contortus*) suggest that conventional antigen vaccines with an efficacy as low as 60% in only 80% of the flock will still produce substantial control and benefits (Barnes *et al.*, 1995). This model suggests that a higher efficacy might be required for hidden antigen vaccines, but these vaccines would still be as good at worm control as standard control strategies if levels of protection of 80% were achieved in 80% or more of the flock (Barnes *et al.*, 1995).

In the case of hookworm vaccine design, the aim of a vaccine would be to reduce the worm burden, and thus pathology associated with disease, rather than hookworm infection *per se*, thereby diminishing the reliance on anthelmintics (Brooker *et al.*, 2004). The efficacy required for this in man is, as yet, unquantified and awaits the outcome of clinical trials (Goud *et al.*, 2005). However, it is worth pointing out that recent vaccine trials in hamsters showed that vaccination with two recombinant *A. ceylanicum* larval antigens not only reduced worm burden and egg output, but also increased the haemoglobin values and the weight of the hamsters significantly, thus reducing disease (Mendez *et al.*, 2005). Epidemiological studies on the feasibility of vaccination against schistosomiasis have indicated that a 75% reduction in egg count in 75% of the infected population could lead to control within 10 years (Chan *et al.*, 1997).

It is ultimately assumed that a vaccine against hookworm will comprise a cocktail of protective antigens from the larval stage and those aligning the gut of the adult worm and likely involved in blood meal digestion (Brooker *et al.*, 2004). Certainly, it would seem that, by combining antigens that singularly promote modest protection, levels of protection can be increased (Mendez *et al.*, 2005).

Thus, further work is required to both discover novel antigens and to assess the levels of protection conferred by established antigens. The nature of the hookworm antigen discovery and testing programme is such that it is not feasible to assess protection levels of human hookworm antigens in the natural host, and thus reliance on adapted rodent and canine model systems is required. Due to the low levels of natural antigen produced *in vitro* by hookworms, it is also deemed unfeasible to trial the protective qualities of natural antigens, instead relying on recombinant antigens produced in a number of systems. Several studies point to the reduced efficacy of bacterial recombinant antigens compared to their natural counterparts because of loss of enzymatic activity or conformation and glycosylation (Knox and Smith, 2001). This can be circumvented to some extent by using eukaryotic expression systems, such as yeast or insect cells. However, eliciting the high titres of antibody potentially required for protection, as indicated in trials using both hidden antigens and ASP homologues, using such systems is difficult (Hotez *et al.*, 2003). Certainly, the levels of protection established with the major hookworm vaccine candidates are still significantly below the levels achievable using natural antigens in the veterinary field (Hotez *et al.*, 2003. HHVI 3rd Annual Report). In the veterinary field, protection trials against GI nematodes, and *H. contortus* specifically, have established high levels of protection using the natural antigen in the natural host/parasite system, confirming their efficacy as vaccine candidates, prior to attempts to recreate this immunity using recombinant antigens.

1.9 Overall Objective and Aims of the Study

Highlighted above, the adult hookworms share a similar niche in humans as *H. contortus* does in sheep. Furthermore, both nematodes are blood feeders and cause an anaemia-related disease directly attributable to the haematophagous adults. Of the already established and efficacious larval and adult vaccine candidates in *H. contortus*, several have homologues in the hookworms. Some of these hookworm antigens have been established as modestly protective in recombinant form; however, unlike their counterparts in *H. contortus*, have not been trialed in native form.

The overall objective of this body of work is to assess the usefulness of *H. contortus* as a "model" for human hookworm vaccine discovery and development and in the process discover novel *H. contortus* vaccine candidates. More specifically the aims are:

i. To identify homologues of lead larval *A. caninum* vaccine candidates in *H. contortus*.
ii. To study their biology, stage specific expression, site of expression and suitability as vaccine candidates.
iii. To attempt to purify hookworm homologues from *H. contortus* L3 ES
iv. To evaluate the protective properties of L3 ES and homologues therein in sheep vaccine trials.

Chapter 2

Materials and Methods

All chemicals and reagents were purchased from Sigma, UK or Fisher Scientific, UK, unless otherwise stated. Standard solution and buffer recipes are listed in Appendix 1. Centrifuges used were a bench top CR422 and an A14 microfuge (both from Jouan, France).

2.1 General Parasitology

2.1.1 Infective Larvae

The Moredun Ovine Susceptible (MOSI) isolate of *H. contortus* was used throughout this study. This is a worm isolate which retains full susceptibility to the three major classes of anthelmintic in current general use.

H. contortus L3 were obtained by coproculture of faeces collected from monospecifically infected sheep and larvae were stored at 4°C in distilled water, as described by Seaton *et al.* (1989). Larvae used throughout all the experiments were less than three months old and were kindly provided by D. Bartley (Moredun Research Institute).

2.1.2 Exsheathing L3

L3s were exsheathed by a method similar to that described by Gamble *et al.* (1996). In brief, following centrifugation at 770xg in for 5 min, the L3 pellet was resuspended in Earle's Balanced Salt Solution (EBSS, appendix 1) at a concentration of 20,000 L3/ml in a 500 ml conical flask. Prior to exsheathment, L3s were incubated at 39°C for 1 h in a waterbath (W14 Grant, UK); this increased the speed and synchronicity of larval exsheathment. Using narrow rubber piping attached to a regulator on a CO_2 cylinder, CO_2 was bubbled vigorously through the solution containing the L3s for 10 min, whilst maintaining a temperature of 39°C. Immediately following CO_2 gassing, the flask was sealed using nescofilm and incubated at 39°C with shaking at 150 rpm. Following incubation for 3 h, an aliquot of 100 µl was inspected by light microscopy at x20 magnification and greater than 90% of L3s had exsheathed at this timepoint.

2.1.3 Culture of L3 for ES

Following exsheathment, L3 were centrifuged for 5 min at 770xg, the supernatant was collected, termed the exsheathing fluid (EF) and this was stored at -20°C. L3s were axenised from cast sheaths by Baermanisation (active migration through lint free filter paper immersed in EBSS with 0.2 mg/ml penicillin/streptomycin, 200 µg/ml gentamycin, and 25 µg/ml nystatin in a funnel with a run off tap), allowing larvae to move through the paper and to be concentrated by gravity. Following incubation at 37°C for 12 h, L3 were collected and resuspended in fresh pre-warmed (37°C) EBSS + antibiotics as above in 225 cm^2 tissue culture flasks (Co-Star, USA) at a concentration of 25,000 L3/ml, in a maximum volume of 50 ml. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 for up to 120 h. L3 were sedimented by centrifugation at 770xg at 24 h intervals and the supernatant removed. This was designated excretory/ secretory product (ES) and was filtered through 0.2 µm pore filter (Millipore, UK) before being concentrated 50-fold by ultrafiltration using Amicon YM-10 membranes (Amicon, UK) and stored at -80°C until required. L3 were resuspended in fresh media as above and transferred to new tissue culture flasks. Viability of L3 was examined visually (x20 light microscopy) at 24 h intervals and a 20 µl aliquot of culture supernatant was plated onto Luria-Bertani (LB, appendix 1) agar plates and incubated overnight at 37°C to monitor bacterial contamination.

2.2 General Protein Techniques

2.2.1 L3 protein extracts

Protein extracts were made in 3 steps as described in the following paragraphs.

Step 1: 1 ml of ice cold S1 buffer (0.186 g ethylenediaminetetra-acetic acid (EDTA) dissolved in 500 ml Phosphate buffered saline (PBS), pH 7.4 + 1 mM phenylmethylsulphonylfluoride (PMSF) final concentration (fc)) was added to 0.5 g L3 in a 1.5 ml eppendorf and mixed. The worm suspension was transferred to a 2 ml ribolysing vial containing glass beads (Hybaid, UK) and the vial was filled with S1 buffer prior to ribolysing at 6.5 speed for 20 s in the ribolyser (Bio101/Thermosavant Fastprep 120). The vial was removed and cooled on ice for 10 min. This was repeated three times. Disrupted worms were centrifuged at 13,000xg in a bench top centrifuge at 4°C and the supernatant was removed; this was the S1 (water soluble) protein extract.

Step 2: The ribolysed L3 pellet was resuspended in 1 ml of S2 buffer (S1+ 0.1% Tween 20) and chilled on ice for 10 min prior to ribolysing as before for 5 s. The suspension was chilled on ice for a further 45 min then L3 pelleted and supernatant removed as in step 1 and this was designated the S2 extract, containing the membrane-associated proteins.

Step 3: This was carried out as in step 2 except the L3 pellet was resuspended in S3 buffer (2.5% v/v Triton X-100 in PBS + 1 mM PMSF) prior to ribolysing. Following centrifugation, the L3 pellet was discarded and the supernatant designated S3, containing the membrane-bound proteins.

All extracts were stored at -80°C until required.

Adult *H. contortus* extracts, prepared as above, were kindly provided by M. Oliver, Moredun Research Institute.

2.2.2 Protein concentration estimation

Protein concentration was estimated using the Pierce method BCA assay, and the procedure was carried out as described in the manufacturer's instructions (Pierce and Warriner, UK). Samples and bovine serum albumin (BSA) (Sigma, UK) standards were incubated in the BCA working reagent for 1 hour at 37°C allowing a colorimetric reaction to occur (based on the principal that a colour change in the BCA is brought about by the reduction of copper ions). The optical density (OD) at 562 nm was measured and the protein concentration of the sample (in triplicate) determined through comparison with a standard curve of the know BSA standards.

2.2.3 Protein visualisation by polyacrylamide gel electrophoresis

Proteins in parasite extracts and ES were visualised by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1977) using the Mini-protean cell system (BioRad, UK). Protein separation was carried out in 10% or 15% polyacrylamide gels (appendix 1) or in 4-15% gradient gels (Biorad, UK). Approximately 10 µg of protein sample was mixed 1:1 with 2X reducing or non-reducing sample buffer (appendix 1), boiled for 5 min, and applied to the gel. Following electrophoresis at 150 volts (V) for 60 min in 1X Laemmli electrode buffer (appendix 1), gels were stained with Coomassie blue (appendix 1) for 15 min and destained 4 x 15 min in the same solvent. Protein bands and molecular weight marker (Mark 12 pre-stained markers, Invitrogen, UK) bands were then visible. Silver nitrate staining was used when a more sensitive visualisation method was required. Following electrophoresis, gels were fixed in 50% (v/v) Methanol / 10% (v/v) Acetic acid for 10 min, then 5% (v/v) Methanol / 7% (v/v) Acetic acid for 10 min and finally 10% (v/v) Gluteraldehyde for 10 min before washing 4X 15 min in distilled water (dH_2O). Gels were then incubated in 5 µg/ml dithiothreitol (DTT) in dH_2O for 10 min prior to transfer into 0.1% (w/v) silver nitrate in dH_2O for 10 min. Gels were washed briefly in

dH_2O prior to addition of developer (3% (w/v) Sodium carbonate in dH_2O plus 50 µl /100 ml 37% (v/v) formaldehyde. Gels were incubated in developer until protein bands became visible, approximately 3 min. The reaction was stopped by the addition of 5 ml per 100 ml developer of 2.3 M Citric acid. The molecular weight of proteins was approximated visually by comparison to the molecular weight standards or by measuring the distance in mm from the top of the gel to each standard of known molecular weight and plotting the molecular weight against the distance migrated. The distance migrated by the protein of interest was then measured and the molecular weight against distance plot used to estimate an approximate molecular weight for the band of interest.

2.2.4 Western Blotting

Proteins separated by SDS-PAGE were transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, UK) using a semi-dry graphite blotter (Milliblot Graphite electroblotter 1; Millipore, UK) at a constant power of 50 mA for 45 min. Transfer set up consisted of pre-wetting the PDF membrane in methanol prior to equilibration for 5 min in anode buffer 2 (appendix 1). A sandwich was created with 1 piece of Filter paper soaked in anode buffer 1 (appendix 1) on the anode. A second piece of filter paper soaked in anode buffer 2, laid on top, followed by the equilibrated PDF membrane. The SDS-PAGE gel, having been equilibrated in anode buffer 2 for 5 min was then laid on the membrane. Finally a last piece of filter paper, soaked in cathode buffer (appendix 1) completed the transfer sandwich. Following protein transfer the membrane was incubated overnight in a blocking solution (5% (w/v) Non-fat Dried Milk Powder, Marvel, UK; or 5% (w/v) Globulin Free Bovine Serum Albumin (Sigma, UK)) in Tris Buffered Saline (TBS, appendix 1) + 0.05% (v/v) Tween 20 (TBST) at 4°C.

2.2.5 Antibody detection of transferred proteins

Following overnight blocking, membranes were washed in TBST for 5 min prior to incubation of the primary anti-sera, diluted in TBST, for 2 h at room

temperature (RT). Membranes were washed 4 x 5 min in TBST before incubation in the secondary, horse-radish peroxidase (HRP) labelled anti-sera, diluted in TBST, for 1 hour at RT. Following 4 x 5 min washes in TBST, 3, 3' diaminobenzidine (DAB) substrate (One tablet set dissolved in 15 ml water; Sigma Fast DAB, UK) was added for 5-10 min until the proteins recognised by the anti-sera were visible. The reaction was terminated by washing with tap water.

When a more sensitive detection system of antibody binding was required, the Enhanced Chemiluminescence (ECL) plus system was employed (Amersham Pharmacia Biotech, UK). The protocol was as described by the manufacturer. Upon exposure to HRP, a chemi-luminescence reaction occurs and the luminescence can be detected by exposure to X-ray film. Exposure times were varied, depending on signal strength, between 5 s and 60 s.

2.2.6 Periodate Treatment

To oxidise cross-reacting carbohydrate epitopes from glycoproteins, thus allowing visualization of specific anti-protein reacting antibody following Western blotting, Periodate treatment was carried out. Following Western blotting (**Section 2.2.4**), membranes were washed 2X 20 min in 50 ml 50 mM sodium acetate pH 4.5. Membranes were removed and incubated for 1 hour in the dark in freshly prepared 50 mM sodium Periodate/ 50 mM sodium acetate pH 4.5 prior to washing 2X 10 min in 50 mM sodium acetate pH 4.5 and 2X 10 min in TBST. The membranes were then incubated in 50 ml freshly prepared 50 mM sodium borohydride in TBST followed by a final 3X 10 min washes in TBST. Membranes were then probed as described (**Section 2.2.4**).

2.2.7 Substrate Gel analysis of protease activity

Samples were fractionated under non-reducing conditions on 0.1% (w/v) gelatin-substrate SDS-PAGE gel slabs (10%). Approximately 10 μg of sample was

mixed with an equal volume of non-reducing sample buffer before application to gel as before and electrophoresis at 150V for 90 min in ice-cold 1X Laemmli buffer. Following electrophoresis, gels were washed 4 x 15 min in 2.5% (v/v) Triton X-100 to remove SDS. The gels were sliced and incubated overnight in a phosphate or acetate pH buffer (appendix 1) alone or in the presence of an appropriate protease inhibitor. After incubation, gel slices were stained with Coomassie blue. On destaining, regions of proteolysis appeared clear against the blue background of the gel.

Inhibitor studies were carried out by pre-incubating approximately 10 μg of sample in non-reducing sample buffer with the appropriate protease inhibitor(s) for 2 h on ice prior to application to the substrate gel as before. Following electrophoresis, gels were washed and sliced as above before incubation overnight in pH 8 buffer (appendix 1) with the appropriate protease inhibitor. Inhibitor concentrations during the pre-incubation and incubation were as follows: 1,10 Phenanthroline -1 mM; E-64 (L-trans-epoxysuccinyl-leucyl-amido-(4-guanidino)butane) -10 μM; AEBSF (4-2-Aminoethyl)benzenesulphonyl fluoride) - 2 mM; Pepstatin - 10 μM; EDTA (Ethylenediaminetetra-acetic acid) - 4 μM .

2.2.8 Metalloprotease substrate assay

This assay is based on the premise that when a metalloprotease cleaves a specific substrate, a fragment released is further cleaved by leucine amino peptidase (LAP, Sigma, UK) to release 4-methoxy-2-napthylamine (4-MNA), which on excitation causes fluorescence. This activity can then be measured.

For a standard curve, a dilution series from 8 μM to 0.125 μM of 4-MNA was made up in triplicate and fluorescence was measured at λ425 nm following excitation at λ340 nm using a luminescence spectrometer (LS50B, Perkin Elmer, UK).

Two µl of substrate, 50 mM Glu-Ala-Ala-Phe-4-MNA (Sigma, UK), was diluted in 88 µl 1 M Phosphate Buffer pH 7 (appendix 1), to which 10 µl (5 µg) of protein sample was added. Samples were set up in triplicate, as were blank PBS negative controls, and these were incubated at 37°C for 2 h. Seventeen µl of LAP was added to a solution of 1 µM phosphoramidon (Sigma, UK) in 1X PBS. Thirty µl of the LAP/ phosphoramidon solution was added to the substrate and mixed and incubated for a further hour at 37°C. Following this incubation, the fluorescence released by the free 4-MNA was measured as before.

2.2.9 Lentil lectin affinity chromatography

Nematodes are rich in glycosylated proteins; these proteins are usually expressed on surface of cells or are excreted. Lentil lectin binds to certain carbohydrate moieties on glycosylated proteins; therefore, lentil lectin affinity chromatography was employed to purify the lentil lectin binding proteins from the crude mixture in whole *H. contortus* L3 ES.

A 3 ml chromatography column (Pharmacia, UK) was prepared by filling with Lectin Wash Buffer (appendix 1) plus 0.5% (v/v) Triton X-100 (LWBT). This was then packed with 3 ml of lentil lectin bound agarose slurry (Vector Labs, UK). The column tubing was connected to an LKB pump (Pharmacia, UK) and flow inducer (Watson-Marlow, UK) and LWBT was run through it at 11 ml per hour for 3 h at 4°C. Two and a half mg of pooled ES protein at a concentration of 130 µg/µl was diluted 1:1 with LWBT + $MnCl_2$ and $CaCl_2$ at 5 μM and 50 μM fc, respectively ($LWBT^{++}$), and the sample was run over the column at a rate of 7 ml per hour for 8 h at 4°C, and the unbound sample was collected. The column was rinsed with $LWBT^{++}$ for 3 h until no more protein was eluted, when the protein readout, measured using a Pharmacia LKB Ultraviolet (UV) Optical Unit, returned to its baseline. The rinse was added to the unbound sample before being concentrated by ultrafiltration using Amicon YM-10 membranes, and these were stored at -80°C until required (the unbound fraction).

Elution buffer (0.2 M α-mannopyranoside in LWB + 0.5% (w/v) CHAPS) was run through the column until the column was full (approximately 30 min at 6 ml/h) and the bound protein was allowed to elute overnight at 4°C. Following the incubation, the eluate was run out of the column until the protein readout returned to the baseline. This fraction, termed the bound fraction, was concentrated and stored at -80°C until required.

2.3 General Molecular Biology Techniques

2.3.1 Total RNA extraction

Total RNA was isolated from exsheathed L3 (ExL3) or L4 *H. contortus* using TRIzol Reagent (Invitrogen, UK), according to the manufacturer's instructions. Worms, which had been snap frozen in liquid nitrogen (LN_2), were ground up to a powder in a pestle and mortar under LN_2. The LN_2 was allowed to evaporate and 2 ml of TRIzol per 100 mg worms was added and allowed to freeze onto the powdered L3. Grinding continued until the mix was fully liquid, transferred to 1.5 ml eppendorfs and allowed to stand for 5 min. 0.2 ml chloroform, per ml TRIzol originally used, was added prior to mixing thoroughly and standing for 3 min at RT. Phases were separated by centrifugation at 12,000xg for 15 min at 4°C and the upper aqueous phase containing the RNA was removed. RNA was precipitated by adding 0.5 ml isopropanol per ml TRIzol originally used, incubating at RT for 15 min, centrifuging at 12,000xg for 10 min at 4°C and removing the supernatant. RNA was washed with 75% (v/v) ethanol before being pelleted by centrifugation at 7,000xg for 5 min at 4°C. The RNA pellet was briefly air dried and resuspended in 100 µl RNase-free water. RNA was stored at -80°C until required.

2.3.2 Reverse transcription (cDNA preparation)

First strand cDNA synthesis was carried out using the BD SMART RACE cDNA amplification kit (BD Clontech, UK), according to the manufacturer's instructions. This kit is based on the switching mechanism at 5' end of RNA transcript (SMART) technology that allows generation of full length cDNAs by reverse transcription. The BD Powerscript Reverse Transcriptase, upon reaching

the end of the RNA template, adds 3-5 dC residues to the 3' end of the new 1st strand cDNA. The BD SMART oligo contains a poly dG region that anneals to the dC at the 3' end and serves as an extended template for reverse transcriptase thus allowing complete 5' synthesis. Standard 1st strand synthesis, reverse transcriptase used with a poly T primer that anneals to the poly A tail, results in cDNA ready for 3' rapid amplification of cDNA end (RACE) PCR. Thus two separate cDNAs were prepared, a 5' RACE ready cDNA and a 3' RACE ready cDNA. Briefly, 1 μg of total RNA was used as the template for both the 3' and 5' cDNA synthesis. For 3' cDNA synthesis 3' poly (dT) primer was added; for 5' reaction 5' primer (the modified oligo (dT)) and the SMART IIA oligo were added. Reactions were incubated at 70°C for 2 min before cooling on ice. First strand buffer, DTT, deoxyribonuleotide triphosphates (dNTP) and the BD Powerscript reverse transcriptase were added and the reactions incubated at 42°C for 90 min. cDNA was diluted in Tris- EDTA (TE) buffer (BD Clontech, UK) and concentration of the transcribed cDNA was determined by spectrophotometry.

2.3.3 Concentration estimation of RNA and DNA

Concentration of RNA and DNA was determined by spectrophotometry using the OD 260:280 ratio as determined using a GeneQuant Pro spectrophotometer (GeneQuant, UK).

2.3.4 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) amplification of DNA was carried out using a Perkin Elmer 2400 GeneAmp thermocycler. Roche UK Taq polymerase was used as standard and reaction mixes were prepared according to the manufacturer's instruction: 10X buffer, 0.4mM dNTPs (Roche, UK), 1 μM Primer 1, 1 μM Primer 2, 1.75 units Taq polymerase (Roche, UK) and 50-500 ng DNA template in a total volume of 50 μl. A standard reaction consisted of 4 min at 94°C to denature the DNA; 25-35 cycles of 30 s at 94°C to denature, 30 s at 50°C-72°C for primer annealing, 30 s per 500 bases at 72°C for extension; and a

final extension of 7 min at 72°C. PCR using a Taq polymerase results in a single dA at the 3' end of the DNA which can be exploited for cloning purposes, as describe in **Section 2.3.6.** All oligonucleotides (primers) were purchased from MWG, Germany.

2.3.5 Agarose gel electrophoresis of nucleic acids

PCR products, RNA and DNA samples were analysed by conventional agarose gel electrophoresis. 0.8% (w/v) agarose gels were prepared in Tris-acetate-EDTA (TAE) electrophoresis buffer (appendix 1) containing 0.5 µg/ml ethidium bromide (Promega, UK). Agarose gels were run in TAE buffer at 80 V for 45 min. Five µl of DNA was diluted 1:1 with DNA loading buffer (appendix 1). DNA fragments were visualised by UV illumination using an AlphaImager (GRI AlphaInnotech, UK) and product sizes were estimated in comparison to a DNA ladder molecular weight marker (Roche, UK).

2.3.6 Purification of PCR products

PCR products were purified using the Geneclean II kit (Q-bio gene, UK) according to the manufacturer's instructions. Briefly, PCR products were excised from agarose gels and the gel piece was dissolved in NaI at 45°C. Following this, DNA was allowed to bind to 10 µl of glassmilk (Q-bio gene, UK) before washing with the manufacturer's wash and ethanol. DNA was then eluted from the glassmilk in 10 µl water, the glassmilk pelleted at 13,000xg for 30 s and the DNA in water removed and stored at -20°C until required.

2.3.7 Ligation of PCR products into pGEM-T

Purified PCR products were cloned into pGEM-T, (Promega, UK), a propagation vector plasmid of *E. coli* with 3' dT overhangs, allowing ligation of PCR products. Purified PCR products were ligated into pGEM-T overnight at 4°C at a 3:1 ratio of insert: vector, according to manufacturer's instructions. Insert DNA was incubated with T4 DNA ligase buffer, 50 ng of vector, insert DNA, and 1 U/µl T4 DNA ligase in a total reaction volume of 10 µl. The following formula

was used to estimate the amount of insert DNA required for the ligation reaction: ng insert required = [(ng vector/ vector size (kb))] x insert size (kb) x ratio of insert:vector. Ligations were incubated overnight at 4°C.

2.3.8 Transformation of competent cells

E. coli competent cells (JM109 cells for vector propagation or BL21 codon plus cells for expression; Stratagene, UK) were transformed with plasmid DNA, according to the manufacturer's instructions. Briefly, 50 µl of cells were mixed on ice with 50ng of plasmid/insert ligation mix for 20 min. Cells were heat-shocked at 42°C for 45 s and then transferred to ice for a further 2 min. Five hundred µl of SOC medium (appendix 1) was added to the cells before incubating at 37°C in a shaking incubator (Infors AG, UK) for 90 min. Following this, 100 µl of transformed cells were plated out on LB agar plates containing ampicillin (50 µg/ml fc) (LB-Amp) and incubated overnight at 37°C.

In cases where competent cells were the JM109 strain and the plasmid was pGEM-T, blue/white colony selection was used to identify those colonies that contained plasmid with an insert. In this case, LB agar plates also contained X-gal (Bioline, UK; 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside; 80 µg/ml) and IPTG (isopropyl-1-thio-β-D-galactopyranoside; 0.5 mM). X-gal is a lactose analogue and IPTG is an inducer of the *lac* operon. The pGEM-T multiple cloning site (MCS) is in the α-peptide region of the β-galactosidase gene and protein expression is under control of the *lac* operon. Thus, when DNA is inserted into the MCS, the α-peptide is disrupted. Colonies containing plasmid with no insert synthesise β-galactosidase normally, which breaks down X-gal to produce a blue colony. Colonies containing plasmids with an insert synthesise an inactive form of the enzyme and remain white in colour.

Following overnight incubation, colonies were picked to identify insert-positive plasmid colonies. White colonies were assumed to be positive for recombinant plasmids when pGEM-T was used for cloning purposes, as previously mentioned. If blue/white selection was not possible 20 colonies were picked aseptically using pipette tips but, prior to amplification, colonies on tips were

touched to the meniscus of 100 µl of sterile water. 1 µl of this water was used as a template in a PCR reaction to identify positive recombinants (termed Colony PCR).

Colonies were amplified by inoculation into 10 ml LB broth containing ampicillin (50 µg/ml) and incubated overnight in a shaking incubator at 37°C.

2.3.9 Purification of plasmid DNA

Following cell transformant amplification, 1-5 ml of the cell culture was centrifuged at 770xg for 5 min and the supernatant was removed. The Wizard Plus *SV* Miniprep kit (Promega, UK) was used for plasmid purification and the protocol was followed as described in the manufacturer's instructions. Briefly, the cell pellet was resuspended in 50 mM Tris-HCl, pH7.5; 10 mM EDTA; 100 µg/ml RNase A prior to bacterial lysing by addition of an alkaline lysis buffer (0.2 M NaOH; 1% (w/v) SDS). Addition of alkaline protease was added to inactivate the endonucleases released upon cell lysis and an acidic neutralisation solution (4.09 M guanidine hydrochloride; 0.759 M potassium acetate; 2.12 M glacial acetic acid) added. Bacterial lysate was centrifuged at 13,000xg for 10 min and the clear supernatant containing the DNA was applied to the Wizard *SV* mini-column to which plasmid DNA binds and lysate is forced through upon centrifugation at 13,000xg for 1 min. Plasmid DNA bound to the column was washed twice with the wash solution (162.8 mM potassium acetate; 22.6 mM Tris-HCl; 0.109 mM EDTA in 95% (v/v) ethanol). Plasmid DNA was eluted in 50 µl sterile RNase-DNase free-water (Promega, UK) and stored at -20°C.

2.3.10 Sequencing of plasmid inserts

All sequencing reactions were carried out by the Moredun Research Institute Functional Genomics Unit using a GE Healthcare MegaBACE 500 48-capillary sequencer using Sanger-Coulson dideoxy chain terminator reactions. When inserts were cloned into pGEM-T, the SP6 and T7 primer sites flanking the MCS were used for sequencing reactions. In all other plasmid vectors, insert gene

specific primers were used. For sequencing of novel genes, a minimum of three independent sequencing reactions were carried out.

Sequencing results were analysed by Chromas software (Version 1.45) and DNA Star software packages to allow construction of contiguous sequences. Web based software and databases available from EBI (http://www.ebi.ac.uk) and NCBI (http://www.ncbi.nlm.nih.gov/) were used for homology analysis searches (using the basic local alignment search tool (BLAST). ClustalW and EMBOSS programmes were used for protein and nucleotide alignments programmes and protein motif searches were carried out using Signal-P and Prosite websites. Phylogenetic analyses were carried out using the MEGA version 3.1 software (www.megasoftware.net).

2.3.11 Restriction enzyme digests of DNA

Restriction enzyme digests were carried out according to manufacturer's instructions (Roche, UK). Ten U enzyme per µg DNA was used in each reaction and the appropriate buffer was included (Roche, UK). Enzymes were incubated with DNA at the appropriate temperature for 4 h, or until agarose gel electrophoresis indicated the DNA was fully digested.

2.3.12 Sub-cloning into a bacterial expression vector

Prior to recombinant protein expression, genes were sub-cloned from pGEM-T into an expression vector (either pET-22b (Novagen, UK) or pGEX-6P3 (Amersham Biosciences, UK)). pGEM-T plasmids containing the gene insert were subjected to restriction enzyme digestion in order to cut the insert out of pGEM-T. The appropriate expression vector was also digested under the same conditions with the same enzymes. Following digestion, all reactions were subject to agarose gel electrophoresis and the appropriate bands on the gel excised and purified. The gene insert was ligated into the expression vector following purification, and JM109 cells were transformed and plated out on LB-Amp agar plates and incubated overnight at 37°C.

2.3.13 Expression of recombinant protein

Following confirmation of the insert in the recombinant plasmid by colony PCR and that it was inserted in the correct reading frame, recombinant plasmids were propagated overnight in 10 ml LB-Amp broths. Plasmids were purified as described in **Section 2.3.9** and cell transformations (**Section 2.3.8**) were carried out using the *E. coli* strain BL21 codon plus (Stratagene, UK). This is a competent cell line designed for protein expression, and containing an excess of those codons not normally used by *E. coli* to circumvent codon usage problems. Transformants were plated out on LB-Amp agar, incubated overnight at 37°C and confirmation of the insert DNA in the recombinant plasmids was sought by colony PCR.

Positive recombinant colonies were picked and propagated overnight in 10 ml LB-Amp in a shaking incubator at 37°C. Following this, 0.5 ml of the overnight culture was used to spike fresh 10 ml LB-Amp broth which was then incubated on a shaking incubator at 37°C for 2 h, until the OD_{600} reached 0.6-1, as measured on a Mass Spectrometer (DU 650, Beckman, UK). One ml of culture was removed and centrifuged at 12,000xg for 5 min. The supernatant and cell pellet were stored at -20°C, protein extracts of this were the non-induced expression control. The remaining culture was induced by addition of IPTG to a final concentration of 1 mM and incubated at 37°C in a shaking incubator for a further 2 hours. Cells were pelleted by centrifugation at 770xg for 10 min and supernatant and pellets stored at -20°C.

Insoluble and soluble protein fractions were prepared from the cell pellets. These were resuspended in 50 mM Tris pH 8.5/ 2 mM EDTA in one tenth of the original volume. Lysozyme and Triton-X-100 were added to final concentrations of 100 µg/ml and 0.1% (v/v), respectively. Following incubation at RT for 15 min, $MgCl_2$ (8 mM f/c), DNase (10 µg/ml f/c) and RNase (10 µg/ml f/c) were added. The lysed cell suspension was incubated for a further 15 min, to allow full nucleic acid degradation, and then centrifuged at 13,000xg for 10 min. The supernatant constituted the soluble proteins and the pellet, resuspended in 50 mM Tris pH 8.5/ 2 mM EDTA, was the insoluble protein fraction. Protein fractions

were analysed by SDS-PAGE electrophoresis and the success of recombinant protein expression determined by comparison to empty vector and non-induced recombinant vector controls. The size of the putative recombinant protein was estimated by comparison with molecule weight standard markers (Invitrogen, UK). Confirmation of 6X histidine (6 His) tagged protein expression was sought by Western blotting and screening with an anti-6 His monoclonal antibody (Sigma, UK).

2.3.14 Purification of recombinant protein

Large format standard SDS-PAGE electrophoresis was used to separate the proteins. Blue G dye (Serva, Germany) was used in the 1X Laemmli buffer in the cathode buffer reservoir to stain proteins. A constant voltage of 50 V was applied for 12 h. The band corresponding to the recombinant protein was excised from the gel with a scalpel blade and finely chopped up. Electroelution was carried out using the AE-3590 Max Yield electroelution apparatus (Atto, USA), as described in the manufacturer's instructions. Finely chopped gel peices containing protein was placed in the cathode cup of the electroelution chamber on a semi-permeable dialysis membrane. The bottom of the cup of the electroelution chamber on the anode side was also sealed with dialysis membrane. The electroelution chamber was placed in the electroelution tank, which was filled with 0.1X Laemmli buffer, creating a seal between the cathode and anode sides of the tank. The cathode cup was filled with 0.1X Laemmli and the anode cup filled with 0.05X Laemmli buffer, until the buffer flowed, without disturbing the contents, into the cathode cup. A constant current of 50 V was passed across the apparatus for 10 hours or until all the protein had been eluted from the gel pieces and carried across to the anode. The protein in the anode cup was collected by pipette and concentrated across YM-10 filters (Amicon, UK) and stored at -20°C.

2.3.15 High molecular weight genomic DNA preperation

Approximately 0.5 g of adult *H. contortus* (a kind gift from Dr D. Redmond, Moredun Research Institute) were ground up under LN_2 and the powder

resuspended in 4.5 ml 25 mM EDTA/ 75 mM NaCl pH 8. To this, 0.5 ml 10% (w/v) SDS and 0.5 ml 10 mg/ml stock Proteinase K were added and the solution was mixed very gently and incubated overnight at 55°C. The lysate was transferred to a tissue culture flask (Co-Star, USA) and 55 ml of phenol in 0.1 M Tris pH 9 were added and the solution incubated overnight at 4°C with gentle agitation to mix the phases. Phases were separated by centrifugation at 770xg for 10 min and the aqueous phase containing the DNA was transferred to dialysis tubing using a wide bore pipette to prevent shearing. The DNA solution was dialysed against TE buffer overnight at 4°C (with 2 changes of buffer) to remove the SDS. DNA was transferred to a 50 ml Falcon tube (BD Biosciences, UK) and stored at 4°C. DNA concentration was measured (**Section 2.3.3**) at 74 ng/ul and agarose gel visualisation demonstrated it of a high quality and molecular weight.

2.3.16 Southern Blotting

Digoxygenin-dUTP (DIG) labelling of a 600 base pair fragment of the target gene was carried out using the DIG high prime DNA labelling and detection starter kit II (Roche, Germany). The target gene was excised from the pET22b plasmid, into which had previously been cloned, by a double restriction enzyme digest (**Section 2.3.11**). One µg of DNA was made up to a final volume of 16 µl in sterile water and denatured by boiling for 10 min and cooling rapidly in ice. DIG labelling was carried out overnight according to the manufacturer's instructions; the DNA is random prime labelled with DIG-dUTP by the DIG-High Prime (containing dNTPs, a labelling Klenow enzyme and reaction buffer). The DIG-dUTP is alkali labile, so the reaction was stopped the following day by addition of 2 µl 4 M lithium chloride, 2.5 µl 0.2 M EDTA in 70 µl 100% ethanol. The solution was incubated at -20°C for 2 h, centrifuged (12,000xg for 10 min) to pellet the precipitated DNA and the pellet washed in 70% ethanol, allowed to dry and resuspended in TE buffer. This was the DIG-labelled DNA probe.

High molecular weight *H. contortus* genomic DNA was digested completely using one of *Eco*RI, *Bam*HI, *Hind*III or *Not*I restriction enzymes (Roche,

Germany) (**Section 2.3.11**). Thirty µl of each digest, in DNA sample buffer, was applied to a 0.8% (w/v) agarose gel, alongside 1 µl of unlabelled DNA probe (a positive control). Southern blotting was carried out by capillary transfer method onto a positively charged nylon membrane, in 0.4 M sodium hydroxide, as described by the manufacturer (Roche, UK). Pre-hybridization, hybridization and washing was carried out according to the manufacturer (DIG high prime DNA labelling and detection starter kit II; Roche, UK). Prehybridization, hybridization and wash conditions were as follows: 42°C for 2 h in the DIG Easy Hyb Buffer (prehybridization); hybridization was carried out with approximately 100 ng of probe in 10 ml DIG Easy Hyb buffer overnight at 42°C. For stringency washing, blots were washed with 2X Sodium citrate (SSC, 0.3 M NaCl; 30 mM sodium citrate, pH 7) buffer + 0.1% (w/v) SDS for 5 min followed by 2 X 15 min washes in 0.5X SSC (75 mM NaCl; 7.5 mM sodium citrate, pH 7) + 0.1% (w/v) SDS at 68°C. Chemiluminescence detection by exposure to X-ray film for 10 min to 2 hours was carried out according to the manufacturer.

2.3.17 Matrix Associated Laser Desorption Ionisation- Time of Flight Mass Spectrometry (MALDI-TOF M/S)

Protein samples were separated by SDS-PAGE as before (**Section 2.2.3**) under reducing conditions and gels were fixed in 40% (w/v) methanol; 10% (w/v) acetic acid in dH_2O for 30 min prior to staining for 2 h in Colloidal Coomassie Blue stain and destaining in 25% (w/v) methanol overnight. Bands of interest were excised using a sterile scalpel blade. Reductive alkylation using DTT and iodoacetamide followed by overnight digestion with Trypsin (porcine, Promega, UK) and MALDI-TOF analyses (Voyager De-Pro MALDI-ToF) were carried out by the Moredun Research Institute Functional Genomics Unit. Output was evaluated using the EXPASY search tool. An in house MASCOT database was created with the suspected protein sequences and this was searched with the MALDI-TOF output to ascertain whether the excised and digested protein was a match.

2.4 Cloning of *H. contortus* Transthyretin-like protein

2.4.1 Identification of a TTR homologue in *H. contortus*

The *H. contortus* EST dataset (available on the Nembase website: http://nema.cap.ed.ac.uk/nematodeESTs/nembase.html) was searched using the tBLASTn search tool (a BLAST algorithm that allows a nucleotide database to be searched with an amino acid sequence by carrying out a conceptual six reading frame translation of the nucleotide database) with the amino acid sequence of the *A. caninum* TTR-like protein (AcTTR) that was undergoing vaccine testing by the HWVI (P. Hotez, personal communication). Several *H. contortus* ESTs showed homology to the AcTTR; the EST that was of highest homology (HcTTR) came from a library constructed by Dr D. Redmond at Moredun Research Institute from *H. contortus* egg cDNA (Hc_egg_21G10_SKPL). The clone containing the sequence was available in a λ uni-Zap XR phage vector (Stratagene, UK) and kindly provided by Dr C. Whitton at the University of Edinburgh (GenBank Accession number: CA994587).

2.4.2 *In vivo* excision and plasmid rescue of the HcTTR clone

Serial dilutions from 1:10 to 1:1,000,000 of phage stock were made in SM buffer (Appendix I). XL1 strain *E. coli* cells (Stratagene, UK) were grown overnight at 37°C in 10 mM $MgSO_4$ to an OD_{600} of 0.5. Ten µl of each dilution of phage stock was added to 200 µl of cells and incubated at 37°C for 15 min. Three ml of NZY (appendix 1) top agar, melted and cooled to 48°C, was added to 200 µl of cells/ phage, mixed and immediately poured onto LB agar plates and incubated overnight at 37°C. Single plaques were cored from the $1:10^6$ plate and inoculated into 500 µl of SM buffer + 20 µl chloroform and stocks were stored at 4°C. To plasmid rescue the phage insert, the ExAssist Interference resistant helper phage kit (Stratagene, UK) was used. This allows *in vivo* excision of the insert in the λ uni-Zap XR phage vector into a pBluescript phage plasmid (phagemid), allowing plasmid purification. Briefly, 250 µl phage stock was combined with 200 µl XL1

cells (OD_{600} of 1 in 10 mM $MgSO_4$) and ExAssist helper phage (Stratagene, UK) in a 10 ml polypropylene tube at 37°C for 15 min prior to addition of 3 ml LB broth and incubation at 37°C for a further 3 h. The suspension was then heated to 65°C for 20 min, and cell debris pelleted by centrifugation at 1000xg for 15 min. The supernatant was removed and stored at 4°C; this contained the excised phagemid. For plasmid DNA preparation, 1 µl of the excised phagemid stock was added to 200 µl of SOLR *E. coli* cells (Stratgene, UK) (an overnight stock grown in LB broth supplemented with 10 mM $MgSO_4$, 0.2% (v/v) maltose and 50 µg/ml Kanamycin at OD_{600} of 1), incubated at 37°C for 15 min then plated out on LB-Amp and incubated overnight at 37°C.

2.4.3 Cloning, sub-cloning and expression of HcTTR

Colonies were picked and propagated and plasmid DNA prepared as described in **Section 2.3.9**. PCR confirmation of an insert of the predicted size was carried out using the vector primers T3 and T7, and plasmid sequenced as described in **Section 2.3.10**. Primers were designed and primer sequences adapted to include appropriate restriction enzyme sites to allow the cloning into either pET or pGEX *E. coli* expression vectors (**Table 2.4.1**) as described in **Sections 2.3.7** and **2.3.12**. PCR amplification with the appropriate primer pairs (see **Table 2.4.2**) were carried out using the annealing temperature (Tm) as indicated. All reactions were carried out under the conditions described in **Section 2.3.4** with 30 cycles of 30 X 30 s denaturing, annealing and extension. Products were purified, ligated into pGEM-T and sub-cloned into the appropriate expression vector as described in **sections 2.3.6- 2.3.12**.

Table 2.4.1: PCR primers for the alternative TTR cloning and expression strategies, restriction enzyme sites shown in bold.

TTR amplicon and primer name	Expression Vector	Restriction Enzyme site	Primer Sequence (5′ to 3′)
Full length. Forward (TTR-1)	pET 22b	*Eco* RI	ccg **aat tcg** atg aaa gcg gtc att ctt ctt ttg
Full length. Reverse (TTR-2)	pET 22b	*Xho* I	**agc tcg agg** tca tca tag tca tca tct aaa aag ta
Full length. Forward (TTR-3)	pET 22b no peLB leader	*Nde* I	ta **cat atg** atg aaa gcg gtc att ctt ctt
No Signal Peptide. Forward (TTR-4)	pET 22b no peLB leader	*Nde* I	t**a cat atg** atg agg gat caa tcc ata gct
pGEX-6P3 Forward (TTR-5)	pGEX-6P3	*Eco* RI	ccg **aat tcg** atg aaa gcg gtc att ctt ctt ttg
pGEX-6P3 Reverse (TTR-6)	pGEX-6P3	*Xho* I	agc **tcg agt** cag tca tca tag tca tca tct aaa aag ta

Table 2.4.2: TTR primer pairs and PCR conditions for amplification.

Primers	Tm (°C)	Ultimate Expression Vector
TTR-1 / TTR-2	55	pET-22b
TTR-3 / TTR-2	55	pET-22b (no peLB leader sequence)
TTR-4 / TTR-2	55	pET-22b (TTR without signal peptide. No peLB leader sequence)
TTR-5 / TTR-6	55	pGEX-6P3

Following sub-cloning into the appropriate expression vector, all TTR inserts were confirmed by sequencing to be in the correct reading frame as the plasmid

vector prior to expression attempts (**Section 2.3.13**). Expression and recombinant protein purification were as described in **Section 2.3.13 –2.3.14**.

2.5 Cloning of *H. contortus* Metalloprotease

2.5.1 Identification, cloning and expression of Metalloprotease fragment

Degenerate primers designed to the highly conserved astacin like region of the known nematode astacin-like metalloprotease (MTP) sequence, based on a clustal alignment of the *A. caninum* and *O. ostertagi* MTP sequences (See **Figure 2.5.1**), were kindly provided by Dr A. Nisbet, Moredun Research Institute.

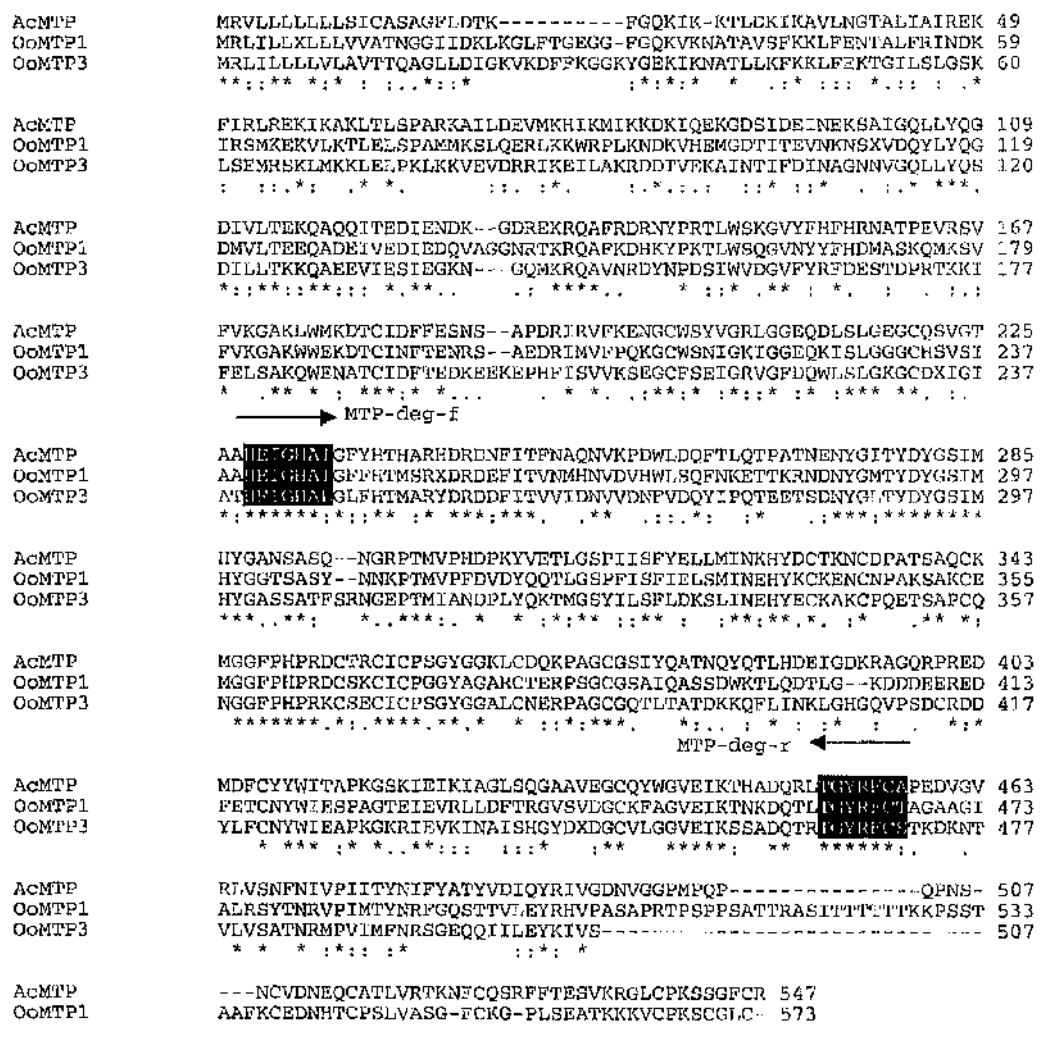


Figure 2.5.1: A clustal W alignment of the AcMTP and OoMTPs 1 and 3 (for which full sequence is available). Degenerate primers were designed to the highly conserved regions highlighted in black, the forward degenerate primer was termed MTP-deg-f; the reverse termed MTP-deg-r.

Degenerate PCR was carried out using primers MTP-deg-f and MTP-deg-r (see **Figure 2.5.1** and **Table 2.5.1**) on *H. contortus* ExL3 cDNA, (from a stock of Moredun Research Institute cDNA prepared by M. Oliver) under the following amplification conditions: 35 cycles of 30 s denaturing, 30 s annealing (Tm 52°C), 1 min extension and the expected product size was 650- 750 base pairs (bp). Following visualization of the PCR product by agarose gel electrophoresis, the approximately 700 bp product was purified, ligated into pGEM-T and sequenced

as described in **Sections 2.3.6-2.3.10.** Sequence analysis demonstrated the product to be a novel 687 bp MTP fragment and allowed design of gene specific primers (gsp) for cloning purposes and for sequence extension at the 5' and 3' ends. Primers are shown in **Table 2.5.1.**

Table 2.5.1: Primer designs for discovery, cloning and expression of HcMTP gene. Restriction enzyme sites are shown in bold.

Primer pair	Sequence (5′ to 3′)	Tm °C	Purpose of primers/ amplicon
Mtp-deg-f Mtp-deg-r	gct gc(g/a) ca(t/c) ga(g/a) at(t/a) gg(c/t) ca gc(a/g) cag aa(c/t) ctg tag cc(a/g) gt	52	To attempt to amplify a novel mtp from *H. contortus* larval cDNA
Mtp-f-1 Mtp-r-1	gaa ctc ggc cac gct ctt cga ttt gat ttc gac tcc	52	GSP to clone fragment into pGEM-T for sequence confirmation
Mtp-f-2 Mtp-r-2	ggt gct acg ggt gct tct cat aac aag aaa cct aca tgg gtg agg gaa tcc acc att ctc gca ctg gtg aga	>72 >72	To use with the SMART RACE (BD Clontech, UK) pimers for 5' and 3' end sequence.
Mtp-*Nde* Mtp-*Xho*	ata **cat atg** gaa ctc ggc cac gct ctt ac **ctc gag** cga ttt gat ttc gac tcc	55	To clone into pET for expression of recombinant protein
Mtp-F-*Nde* Mtp-R-*Xho*	gat tag **tac ata tgg** gct tac ttg aaa aag caa aa gat tag **cgc tcg agt** tcg gca att ttg tact cc ag	55	To clone into pET and express full length MTP gene (without signal sequence)
Mtp-F-whole-*Nde*	ta **cat atg** agg ctc act ata cta ctg	55	To clone into pET and express full length MTP gene

Using gsps mtp-f-1 and mtp-r-1, the novel mtp sequence was amplified by PCR from L3 and L4 cDNA (**Section 2.3.1- 2.3.2**) as described (**Section 2.3.4**) under

the following amplification conditions: 30 cycles of 30s denaturing, 30s annealing (Tm= 52°C), 1 min extension. The products were purified, ligated into pGEM-T and sequenced for sequence confirmation (see **Sections 2.3.6-2.3.10**). The MTP fragment PCR amplified from L4 cDNA using primers mtp-F-*Nde* and mtp-R-*Xho*, was cloned into the pET 22b expression vector and expressed as described in **sections 2.3.5- 2.3.13**. The recombinant protein was purified (**section 2.3.14**) and stored at -20°C.

2.5.2 Identification of full length HcMTP by extension of 3' and 5' ends using RACE PCR

Utilizing the RACE ready L4 cDNA (see section 2.3.2), extension of the MTP fragment at the 3' and 5' end was attempted using gene specific primers and the universal primer (UPM) (that anneals to the 3' or 5' adapted ends of the RACE ready cDNA) provided by the kit manufacturer (BD Biosciences, UK). The primers MTP-F-2 and MTP-R-2 were designed such that the products of the reactions would give products which overlapped by 200 bp. Mtp-f-2 was used in conjunction with the UPM and 3' RACE ready cDNA; mtp-r-2 in conjunction with the UPM and 5' RACE ready cDNA. Reactions were essentially carried out according to the kit protocol (SMART RACE cDNA amplification kit, BD Biosciences, UK). A PCR mastermix was prepared (10X BD Advantage 2 PCR buffer; 50X BD Advantage 2 polymerase mix; 0.2 mM dNTP mix in sterile water. Reactions were set up as in table 2.5.2.

Table 2.5.2: Recipe for 3' and 5' RACE PCR reactions.

5' RACE reaction	3' RACE reaction
2.5 µl 5' RACE ready cDNA	2.5 µl 3' RACE ready cDNA
5 µl UPM	5 µl UPM
1 µl mtp-r-2	1 µl mtp-f-2
41.5 µl Mastermix	41.5 µl Mastermix

PCR conditions were as follows:

5 cycles (94°C 30 s; 72°C 3 min)

5 cycles (94°C 30 s; 70°C 30 s, 72°C 3 min)

30 cycles (94°C 30 s; 68°C 30 s, 72°C 3 min)

A schematic diagram of the RACE PCR reaction is shown in **Figure 2.5.2**.

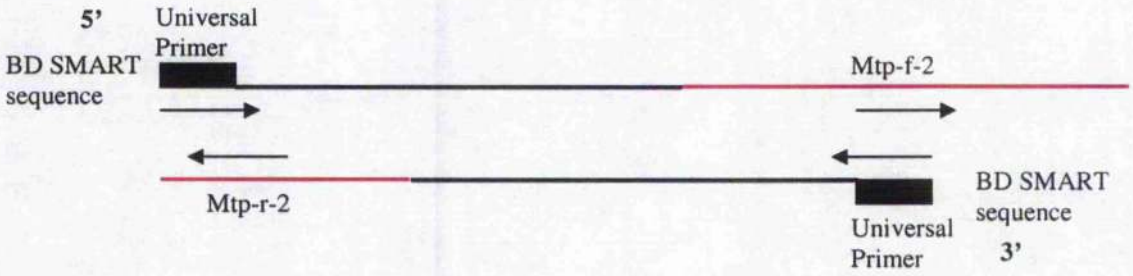


Figure 2.5.2: A schematic diagram of the primers used for RACE PCR extension of the 5' and 3' ends of the amplified HcMTP fragment. GSPs designed to the confirmed sequence of the HcMTP fragment (shown in red) were used in conjunction with the BD UPMs, that anneal to the BD SMART sequence added to the 5' and 3' ends of the RACE ready cDNA (black shaded box). Mtp-r-2 was used with the universal primer mix and 5' RACE ready cDNA to extend the 5' end; Mtp-f-2 was used in conjunction with the universal primer mix and 3' RACE ready cDNA to extend the 3' end.

PCR products from the 5' and 3' reactions were purified, ligated into pGEM-T and sequenced (**sections 2.3.6-2.3.10**). Following sequence analysis and confirmation of a 5' start codon followed by a putative signal sequence and 3' stop codon followed by a poly dA tail, gene specific primers were designed to amplify the full length novel MTP coding sequence (see **Table 2.5.1**) to allow cloning into pGEM-T for sequencing confirmation and to allow expression of the full length HcMTP (without signal sequence) in the pET 22b expression vector.

2.6 Cloning of *H. contortus Ancylostoma* secreted protein (HcASP-1)

2.6.1 PCR amplification of cloning of HcASP

To PCR amplify the single domain *H. contortus* larval ASP homologue (HcASP-1), primers were designed to the 5' and 3' ends of the published sequence (Sharp and Wagland, 1998). There was no start codon at the 5' end, so primers were designed to either the beginning of the available sequence or to the nucleotide base after the putative cleavage site of the signal peptide. PCR primers, adapted to include an appropriate restriction enzyme cleavage site, were designed to allow sub-cloning into an expression vector as before. Primers used are shown in **Table 2.6.1**.

Table 2.6.1: Primer designs for cloning and expression of HcASP-1 gene. Restriction enzyme sites are shown in bold sequence.

HcASP amplicon (and primer name)	Expression vector	Restriction enzyme site	Primer sequence (5′ to 3′)
Published Full length (ASP-1 –forward)	pGEX-6P3	*Bam HI*	ctg **gat cct** tcg tac taa cga ttc tg
Published Full length (ASP-2-reverse)	pGEX-6P3	*Xho* I	cgc **tcg agg** tta ata taa gtt tgt ctt
Published Full length (ASP-3-forward)	pET 22b	*Nde* I	tac **ata tgt** tcg tac taa cga ttc tg
Published Full length (ASP-4-reverse)	pET 22b	*Xho* I	cgc **tcg aga** tat aag ttt gtc ttt tt
No signal peptide (ASP-5- forward)	pET 22b	*Nde* I	tac **ata tga** cga aaa att gcg aga ctt cag aa
Published Full length (ASP-6- forward)	pIVEX2.3d (Roche, UK)	*Nco* I	atc atg t**cc atg** ggc ttc gta cta acg att ctg
Published Full length (ASP-7 –reverse)	pIVEX2.3d (Roche, UK)	*Sma* I	agt ata **ccc cgg g**ag ata taa gtt tgt ctt ttt
ASP-5' RACE	n/a	n/a	tgc ata ttt ctc agc caa gca gga gta

Primers were used in the combinations described in **Table 2.6.2.** Reactions were carried out under the conditions described in **Section 2.3.4** with an amplification cycle of 30 X 30 s denaturing, 30 s annealing and 1 min extension. Products were purified, ligated into pGEM-T and sub-cloned into the appropriate expression vector as described in **Section 2.3.7**. Following sub-cloning into the appropriate expression vector, all ASP inserts were confirmed to be in frame with the plasmid vector prior to expression attempts (**Section 2.3.13**).

Table 2.6.2: Primer pairs, Tm and expression vectors for cloning and expression of HcASP-1 gene.

Primers	Tm (°C)	Ultimate Expression Vector
ASP-3 / ASP-4	55	pET-22b (without peLB leader sequence)
ASP-5 / ASP-4	55	pET-22b (no signal sequence, without peLB leader sequence)
ASP-1 / ASP-2	55	pGEX-6P3
ASP-6 / ASP-7	55	pIVEX 2.3d

2.6.2 Expression of recombinant HcASP-1

Once in-frame cloning of the HcASP-1, with or without signal sequence, was confirmed by sequence analysis, attempts at recombinant protein expression in pET 22b and pGEX-6P3 were carried out as described in **section 2.3.13.**

To attempt to express HcASP-1 without the putative signal sequence in the pGEX system, it was cloned into pGEX-6P1 (a similar plasmid to pGEX-6P3, but with a frameshift in the MCS). Following PCR amplification by primers ASP-3 and ASP-4 and cloning of the full length HcL3ASP-1 product into pGEM-T, the restriction enzymes *Eco*RI and *Xho*I were used to cut the insert out of pGEM-T and ligate into the MCS of pGEX-6P1 (**Sections 2.3.11** and **2.3.12**). *Eco*RI cuts the HcASP-1 sequence just after the putative cleavage site of the signal peptide, thus allowing the sub-cloning of the gene without the signal sequence. Attempts at expression of recombinant protein were carried out as previously described.

2.6.3 Expression of HcASP-1 using an *in vitro* cell free translation system

Following cloning the HcASP-1 into the pIVEX 2.3d expression system (Roche, UK) and sequence confirmation that the insert was in frame, plasmid was propagated and purified (**Sections 2.3.8** and **2.3.9**) and 10 μg of pure plasmid

was used for protein expression in the Rapid Translation System *E. coli* HY kit (Roche, UK) using the RTS 500 Instrument (Roche, UK) according to the manufacturers instructions. Protein was analysed by SDS-PAGE electrophoresis (**Section 2.2.3**).

2.6.4 Extension of 5' HcASP-1 end by RACE PCR

A gsp (ASP-5'-RACE, **Table 2.6.1**) was designed to a region of the published HcASP sequence approximately 230 bp from the start of the published sequence to attempt amplification of the 5' end with start codon by RACE PCR. The BD Biosciences SMART RACE cDNA amplification kit was used and the protocol and PCR conditions were identical to those described in **section 2.5.2**. The PCR product was purified, ligated into pGEM-T, and sequenced (**sections 2.3.6-2.3.10**).

2.7 Immunohistochemistry on worm sections

2.7.1 Raising antibodies to recombinant protein in rabbits

Fifteen-30 μg of electroeluted, purified recombinant protein, diluted in 300 μl of sterile PBS containing 50 μg of Quil A (Brenntag Biosector, Denmark) adjuvant, was injected subcutaneously into a rabbit on 3 occasions, 3 weeks apart. Five to 10 ml blood was collected by venepuncture of the marginal ear vein immediately prior to the first vaccination, centrifuged at 770xg for 10 min and sera removed (pre-vaccinate sera) and stored at -20°C. Two weeks following the 3rd vaccination rabbits were terminally anaesthetised with Halothane and Oxygen and blood was collected by cardiac puncture and sera collected as before (vaccinate sera) and stored at -20°C.

2.7.2 Preparation of L3 sections

Exsheathed *H. contortus* L3s were fixed in zinc salts fixative (ZSF; 0.5% (w/v) Zinc acetate, 0.5% (w/v) $ZnCl_2$, 0.05% (w/v) Calcium acetate, 0.1 M Tris, pH 7-7.4), 4% (v/v) paraformaldehyde (in PBS) or Modified Bouin's (2.5% glacial acetic acid; 5% (v/v) formaldehyde (37%); 92.5% (v/v) saturated aqueous picric acid) solution for 24-28 h at 4°C. L3s were pelleted by centrifugation (10,000xg), mixed in 10% (w/v) gelatin which solidified to a soft malleable pellet, and then fixed in 70% (v/v) ethanol for a further 48 hours at 4°C. Following dehydration using graded alcohols, samples were embedded in paraffin wax at 56°C. Sections were cut as required for immunohistochemical analysis.

2.7.3 Antibody labelling of worm sections

H. contortus L3 or adult (a kind gift from M. Oliver, prepared as above) sections (5 μm; on Superfrost slides; Merck, UK) were de-waxed in xylene and dehydrated in a series of alcohols (100%, 75% (v/v) and 50% (v/v) ethanol). Non-specific binding was blocked by incubation in 5% horse serum in PBS

(PBS/5% HS) overnight at 4°C. All antibody incubation and wash steps were carried out five times in PBS/5% HS. Primary antiserum (both pre-vaccinate (negative control) and vaccinate sera) was prepared at a range of dilutions (1:100-1:2000) and slides incubated in the appropriate serum overnight at 4°C. Secondary serum control slides were incubated in PBS/5% HS. Slides were washed by gentle agitation in PBS/5% HS for 4 x 15 min. Slides were incubated with a secondary fluorescein isothiocyanate (FITC) labelled sheep anti-rabbit IgG polyclonal sera (Sigma, UK) diluted at 1:500 in PBS/5% HS for 3 h at RT and washed as before. Sections were mounted in Citifluor (Chemical Labs, UK) under a coverslip and incubated at 4°C in the dark for 1 h. Adult sections were examined under a fluorescent microscope (Axiovert 25) at x100 and photographed using a digital camera. Larval sections were examined using an Olympus BX50 fluorescent microscope at x1000 and photographed onto Kodak Ektachrome tungsten 320T film. Images were cropped in Microsoft Powerpoint.

2.8 Sheep vaccine trial

2.8.1 Vaccination and challenge strategy

A protection trial was conducted using 11-month-old Suffolk-Greyface cross lambs, assigned into groups of seven balanced for sex and weight. All animals, housed in loose boxes and fed on hay, concentrate and water *ad libitum*, received 3 immunisations at 3 weekly intervals and were challenged with 5000 *Haemonchus* L3s one week after the final immunisation. Antigens were administered with 5 mg of Quil A in PBS in a total volume of 2 ml, 1 ml being given intramuscularly into each hind leg. The control group received adjuvant alone. The unbound (**Section 2.2.9**) group received 35 µg of the non-binding ES fraction and the bound (**Section 2.2.9**) group received 5 µg of the bound and eluted ES fraction. Blood samples were taken at weekly intervals by jugular venepuncture and sera was prepared as described (**Section 2.4.1**) and stored at -20°C until required. Pre-vaccinate sera were defined as sera taken from blood samples collected immediately prior to the first vaccination, vaccinate sera as that collected 1 week post final vaccination.

2.8.2 Parasitological parameters

FEC were carried out 3 times a week starting 17 days post-infection until *post mortem* 36 days post infection. Methods for FEC and worm enumeration were as described in Smith and Smith (1993). Briefly, for worm counts, animals were stunned using a captive bolt gun and exsanguinated. At necropsy the abomasa were collected, opened along the lesser curvature and contents washed into a bucket. A saline digest was made of the abomasum, following which the abomasum was rinsed thoroughly to ensure all worms were washed out. Male and female worms were recovered from a subsample of 2% of the total abomasal wash volume for counting and worm measurements. FEC were carried out by adding 10 ml of water per gram of faeces and emulsifying, removing faecal matter by straining through a sieve and taking a 10 ml subsample for counting. The remaining faecal matter in the sub sample, containing the worm eggs, was pelleted by centrifuging at 770xg for 2 min and then mixed in a saturated salt solution. Upon repeat centrifuging, the eggs floated in the salt solution and the

top 2 ml was poured into a polystyrene spectrophotometer cuvette (Sigma, UK) and the number of eggs determined by visual inspection and enumeration under light microscopy (x20 magnification).

2.8.3 Worm measurements

Worm measurements were made as described in Redmond and Knox (2004). Male and female adult worms recovered from individual animals in each group were pooled into their respective groups. Worm length was measure for a random sample of 50 worms (male or female) from each pool. Worms were photographed individually under 10X light microscopy and the length determined using the semi-automated Image-Pro Express (Media Cybernetics, UK) software programme.

2.8.4 Egg hatch assay

Following individual animal egg counts, eggs were emptied from the cuvette and washed in a 38 µm sieve with tap water for 1 min to remove any excess salt solution. Eggs were resuspended in 2 ml dH_2O in a Petri dish and allowed to develop at room temperature for 3 days. Following 3 days of incubation, the culture was fixed by addition of 10 µl helminthological iodine and mixed by pipetting. One hundred µl of the 2 ml culture was removed and streaked onto a microscope slide. The number of unhatched eggs and L1 larvae were enumerated under x20 light microscopy and the percentage of eggs hatched worked out as follows: eggs unhatched/ L1 larve X 100. This assay was performed on eggs collected on days 20, 22, 29 and 36 post infection.

2.8.5 Detection of circulating total IgG levels in vaccinated animals by enzyme linked immunosorbent assay

An ELISA method was used for the detection of systemic total IgG and IgE antibody in sera in response to vaccination. IgG ELISA assays were carried out by Kay Hall (Moredun Research Institute). Microlon (high binding) 96 well ELISA plates (Greiner/Bio-one, UK) were coated with 5 µg/ml of pooled L3 ES in 50 µl of bicarbonate buffer pH 9.6 (appendix 1) per well and incubated overnight at 4°C. Plates were then washed 6 times with TBST and non-specific

binding blocked by incubation with 200 µl per well in 10% (w/v) non fat dried milk in 50 mM bicarbonate buffer pH 9.6 overnight at 4°C. Primary antibody samples (vaccinate sheep sera from individual animals) were made up in a dilution series ranging from 1:100 to 1:102,400 in TBST for IgG ELISA or 1:20 to 1:20480 for IgE and 50 µl was added per well. A 1:1280 for IgG or 1:20 for IgE dilution of pre-vaccinate control sera was also made and 50 µl was added per well. Following incubation at RT for 2 h, plates were washed as before 6 times in TBST. Secondary HRP-labelled goat anti-sheep IgG antibody or mouse anti-sheep IgE (Sigma, UK) was diluted 1:2000 in TBST, 50 µl added per well and plates were incubated at RT for 2 h. Following a further 6 washes in TBST, the HRP-labelled secondary antibody was detected by the addition of 100 µl per well of Sigma Fast-O-phenylenediamine dihydrochloride (OPD, Sigma, UK) and colour allowed to develop (approximately 10 min) and the reaction stopped by addition of 50 µl per well of 2.5 M sulphuric acid before reading the OD_{492} of each well using a microtitre plate reader (Labsystems iEMS Reader MF, UK). IgG and IgE levels were calculated as a titre against the IgG levels in the dilution of control sera.

2.9 RNA interference

2.9.1 Production of double stranded RNA

To produce double stranded RNA, gene specific DNA was cloned into pGEM-T, then subcloned into the vector L4440 (kindly provided by J. Ahringer, University of Cambridge) (**section 2.3.12**) using restriction enzymes that flank the MCS, and cells transformed and plasmid propagated as described in **sections 2.3.8-2.3.9**. Each side of the MCS of L4440 is flanked by a T7 promoter and gene specific double stranded RNA (dsRNA) can be produced by transcription with a T7 polymerase. dsRNA was prepared using the T7 Ribomax RNAi kit (Promega, UK), according to the manufacturer's instructions. Briefly, plasmids were linearised by digestion with one of *Xba*I and *Xho*I (on opposite sides of the insert). Separate reactions were set up using 1 μg of the linearised DNA, the T7 polymerase mix (Promega, UK) and 2X buffer (Promega, UK) and incubated for 30 min at 37°C thus allowing transcription of single stranded RNA (ssRNA). Following ssRNA transcription in both directions, both reactions were mixed incubated at 70°C for 10 min and then allowed to slowly cool to RT to allow annealing. The resulting dsRNA was purifed, ethanol precipitated and resolved in sterile water according to the manufacturer's protocol.

2.9.2 dsRNA production of *C. elegans* genes

Genes highlighted through homology to *H. contortus* proteins as potential candidates for RNAi loss of function studies in *C. elegans* were cloned from mixed stage *C. elegans* cDNA (prepared previously by methods similar to those described for *H. contortus* cDNA production **Section 2.3.1- 2.3.2**) as described in **Section 2.9.1**. The genes cloned were a transthyretin-like protein (Genebank Accession number Q18878, CeTTR) identified as the closest homologue to the HcTTR (**Section 2.4**) and two members of the nematode astacin-like family (NAS) of *C. elegans* (*nas*-31 and *nas*-32) that were homologues of the HcMTP (**Section 2.5**). Primers were designed to PCR amplify the whole CeTTR sequence and to unique regions approximately 200 bp in length of *nas*-31 and *nas*-32 of the published sequence. dsRNA was produced as in **Section 2.9.1**. The Primers that were used are described in **Table 2.9.1**.

Table 2.9.1: Design of primers for dsRNA production of *C. elegans* target genes.

Gene	Primers	Sequence	Tm(°C)	Product Size (bp)
CeTTR	CeTTR_f	atg cag act tta ctg tta gct	58	471 base pairs
	CeTTR_r	cta gaa gct atc ttc ctc att		
nas-31	nas-31_f	cta tcc tgg aag ccc gcg atg	56	208 base pairs
	nas-31_r	tct gca tta tat tga tgc aag		
nas-32	nas-32_f	att tga ata aca acc aaa tcg	54	212 base pairs
	nas-32_r	tca att cga att tca agc atg		

2.9.3 RNA interference of *C. elegans*

C. elegans were maintained on nematode growth medium (NGM) agar under standard conditions (as described by Sulston and Hodgkin (1988)) and RNAi soaking carried out essentially as decribed by Tarbara *et al.* (1998). Twenty L4 worms were soaked in 1 ng of target dsRNA in PBS + 1 µl lipofectin (Gibco-BRL, UK) in a total volume of 11 µl for 12 hours. Worms were re-plated on to NGM agar (seeded with a lawn of *E. coli* strain OP50) and grown overnight. The worm phenotype was inspected visually and compared with worms incubated in PBS and lipofectin alone (negative control) and worms soaked in RME-2 dsRNA (a positive control, RNAi with which leads to a phenotype of reduced progeny; a kind gift from Dr C. Britton, University of Glasgow). Fifteen adult worms were picked for RT-PCR analysis to establish that RNAi had resulted in a reduction in the mRNA expression of the targeted gene.

2.9.4 One-step Reverse Transcription-PCR

Total RNA was prepared from 15 *C. elegans* adult worms that had undergone RNAi. Worms were added to 200 µl of lysis buffer (10 mM Tris-HCl pH 7.5; 0.5% (w/v) SDS; 5% (v/v) β mercaptoethanol; 10 mM EDTA) and stored at -80°C for 10 min. Following this, 0.5 mg/ml Proteinase K was added and the solution mixed then incubated at 55°C for 1 h. To this, 100 µl of Total RNA reagent (AB Gene, UK) was added, the solution mixed and incubated on ice for 5 min. Zero point two ml chloroform was added; the solution mixed and incubated on ice for 5 min prior to centrifuging at 12,000xg for 15 min. The upper phase, containing the RNA, was collected to which an equal volume of isopropanol was added followed by 10 min incubation on ice. Following precipitation, RNA was pelleted by centrifugation at 12,000xg for 10 min, the RNA was washed with 75% (v/v) ethanol, and the RNA pellet dissolved in 50 µl sterile water. To ascertain that target gene expression had been reduced, a one step RT-PCR was carried out using the Superscript One Step RT-PCR with platinum *Taq* kit (Invitrogen, UK) and appropriate gene specific primers, according to the manufacturer's instructions with an amplification of 29 cycles. This system allows the combining of cDNA synthesis and PCR in a single reaction. One step RT-PCR was also carried out on negative control worm RNA using gene specific primers to the experiment's target gene to act as a PCR control. A second control one step RT-PCR using gene specific primers to amplify a known expressed *C. elegans* gene from RNA prepared from the RNAi treated worms confirmed RNA quality.

2.10 Direct challenge assay

To investigate the ability of specific antisera directed against *H. contortus* proteins to inhibit larval penetration of the gut mucosa, a direct challenge assay was carried out. The protocol was based on that described by Jackson *et al.* (2004). The abomasum from a worm free sheep was removed, cut along the lesser curvature and the contents gently rinsed off with physiological saline (0.85% w/v). Folds were removed, cut into 2 cm^2 sections and placed into wells of a 6 well plate (Corning, UK). Hank's medium (Sigma, UK) supplemented with 20 mM Hepes (Boehringer Mannheim, Germany) pH 7.6, pre-warmed to 39°C, was added to surround, but not submerge the explant. An isolation chamber was provided by the barrel of a 5 ml syringe and this was placed on the centre of the explant firmly to create a seal. A challenge dose of 2,500 exsheathed *H. contortus* L3 in 0.5 ml of PBS or sera, which were pre-incubated with appropriate sera (see **Table 2.10),** was added to the isolation chambers. Plates were transferred to an incubator (Stuart Scientific S160) and maintained at 39°C in an oxygen rich environment for 3 hours. All samples and controls were set up in triplicate.

Following incubation, isolation cylinders were removed and rinsed into a 50 ml Falcon tube (Wash 1: worms that were not associated with the tissue). The explant was removed and washed vigorously by plunging 30 times in to a 50 ml Falcon tube containing 25 ml of saline, this was termed Wash 2: worms that were associated with, but had not penetrated the tissue. The explant was transferred to a third 50 ml falcon tube containing 1% (w/v) Pepsin (1:2500 (w/v) Porcine pepsin, Sigma, UK) and 1% (v/v) HCl. Tissue was allowed to digest thoroughly overnight at 37°C. This third tube, termed the digest, contained worms that had penetrated the gastric tissue. Thus, there were 3 tubes for each piece of abomasal tissue, and each of these was fixed with 2 ml of helminthological iodine (250 g Potassium iodide; 50 g resublimed iodine dissolved in 500 ml distilled water) and the final volume was adjusted to 50 ml with distilled water. The number of larvae present in a 2% aliquot of each was counted using a 9 cm Petri dish with a 1 cm grid drawn on it under x100 light microscopy.

The percentage of larvae that had penetrated the tissue was worked out by dividing the number of larvae in the digest by the total number of recovered larvae and multiplying by 100.

Table 2.10: Sera used to pre-incubate exsheathed *H. contortus* L3 for the direct challenge experiment.

Experimental Group	**Antibody used for pre-incubation**	**Antibody Dilution Factor**
Positive Control	None (PBS)	Not applicable
Unbound	Pooled Unbound sheep vaccinate sera	Neat
Bound	Pooled Bound sheep vaccinate sera	Neat
Pre-vaccinate sheep	Pooled sheep pre-vaccinate sera	Neat
Metalloprotease	Rabbit anti rMTP vaccinate sera	Neat
Pre-vaccinate rabbit	Rabbit pre-vaccinate sera	Neat

Chapter 3

H. contortus larval somatic and excretory/ secretory proteins

3.1 Introduction

Third stage larvae of both hookworm species and *H. contortus* contain and/ or release proteins that can induce protective immunity (Miller, 1965; Smith and Angus, 1980). As discussed in **Chapter 1**, developmentally arrested L3s reactivate upon entry to the host as a result of certain stimuli. This can be mimicked *in vitro* by culturing *A. caninum* L3 at 37°C with host serum (Hawdon and Schad, 1990), and by exsheathing *H. contortus* L3 with CO_2 and subsequently culturing at 37°C (Gamble and Mansfield, 1996). Upon activation and culture, *A. caninum* release a variety of proteins into the culture fluid, some of which are promising vaccine candidates (discussed in **section 1.7.3.2**).

Following a period of "dormancy" on the pasture and upon ingestion by the ruminant host, L3 *H. contortus* exsheath in the rumen and develop in the abomasum to L4 stage larvae within approximately 72 hours. This is reported to be replicated *in vitro* and during culture L3 release proteins (ES products) (Gamble *et al.*, 1996). Proteases are released during exsheathing, one of which, a metalloprotease, digests a refractory ring on the sheath that allows the L3 to escape from the 2nd stage cuticle or sheath (Gamble *et al.*, 1989). Proteins have also been reported to be released concomitantly with the molt from the ExL3 to the L4 (Gamble *et al.*, 1996) including a protease of metallo-nature and a number of unidentified proteins. Evidence from the analogous situation in hookworm would suggest that some of these *H. contortus* proteins may be potential vaccine candidates; in particular those proteins that are homologous to the already identified L3 ES hookworm candidate antigens.

Studies carried out by Sharp and Wagland (1998) demonstrated that *H. contortus* L3 ES could be fractionated by lentil lectin affinity chromatography and that the resulting lectin binding fraction was highly enriched for a 40 kDa protein. Subsequent analysis showed that this 40 kDa protein corresponded to single domain *A. caninum* ASP-1 homologue.

This chapter describes work that was undertaken to harvest the ES proteins of L3 *H. contortus* and to define the protease content as well as to seek homologues of the lead hookworm larval vaccine candidates. In addition, it was the intent to obtain sufficient ES protein to enable its fractionation with selected sub-fractions evaluated in a vaccine trial in **Chapter 4**. Lentil lectin binding was evaluated here because this had been used with success previously (Sharp and Wagland, 1998) to enrich for the *H. contortus* ASP-1 homologue.

3.2 Results

3.2.1 Characterisation of *H. contortus* L3 proteins and proteases

To investigate the proteins released by *H. contortus*, L3 were exsheathed and cultured and ES collected as described in **Section 2.1.3.** *H. contortus* ExL3 extract (prepared as described in **Section 2.2.1**), exsheathing fluid (EF) and ES products were analysed by SDS-PAGE electrophoresis. The profile of proteins released by L3 following exsheathing and during culture was compared to that of the soluble (S1), membrane associated (S2) or membrane bound (S3) somatic extracts (**Figure 3.2.1**). The profile of the protein extracts appeared distinct to and more complex than that of the ES. The profile of the S1, S2 and S3 were also distinct to one another, the S1 appearing most complex.

The protein profile of EF was quite distinct to that of both ES and extracts with a prominent band at approximately 100 kDa and a doublet running at approximately 45 - 50 kDa. These proteins may correspond to the previously described major exsheathing protein at 98 kDa (Gamble *et al.*, 1989a) and a 44 kDa metalloprotease thought to mediate ecdysis (Gamble *et al.*, 1989a).

Parasites released ES proteins into the culture media and the protein profile increased in both concentration and complexity as culture time progressed as judged by SDS-PAGE (**Figure 3.2.1**).

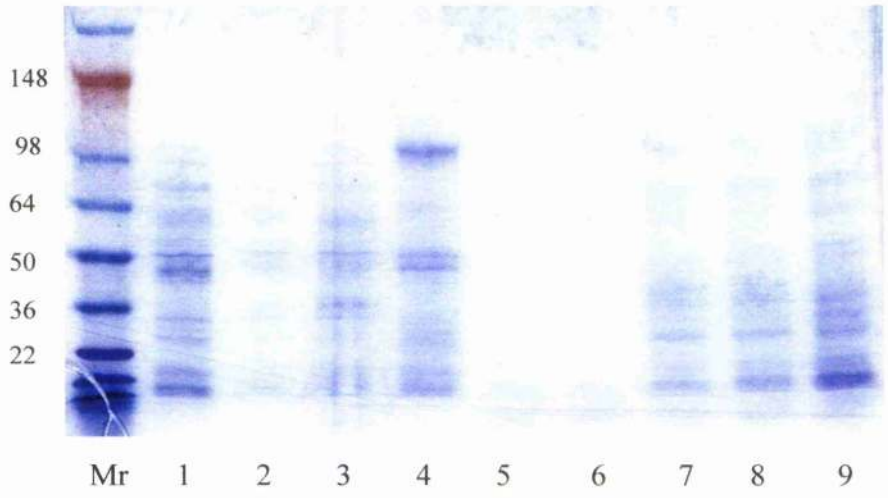


Figure 3.2.1: Coomassie stained SDS PAGE Gel of *H. contortus* L3 extracts, EF and ES products. The gel profile is representative of all the cultures carried out.
Mr- Molecular weight markers (kDa); Extracts: **1** - Soluble (S1); **2** - Membrane associated (S2); **3** - Membrane bound (S3); **4** - Exsheathing fluid; **5** - 24hrES, **6** - 48hrES, **7** -72hrES, **8** -96hrES, **9** - 120hrES.

On average, 10 million L3 released 250 µg protein in the first 24 hours of the culture period with a total of approximately 2.5 mg released during the 120 hour culture period, although it is worth noting that the quantity of protein released did vary between cultures therefore several very large (10 million L3) cultures were required to produce a manageable quantity of protein. Concentration of protein from the cultures resulted in a slight loss of protein and a further compounding factor was that early cultures were carried out in EBSS with a phenol red dye which required dialysing out prior to concentration measurement resulting in a further loss of protein. In total, 5 cultures (amounting to 50 million L3) were required to produce a total of 4 mg of concentrated ES protein.

Greater than 95% of larvae remained viable, as evaluated by visual inspection, up to 120 hours in culture. Following this period the viability decreased. Very few or none depending on the culture, of the worms developed into L4 larvae. This was in contrast to reports by Gamble *et al.* (1996).

Assessment of protease activity in protein extracts, EF and ES was carried out as described in **Section 2.2.6**. Exsheathed L3 extracts were made as described in **Section 2.2.1** however protease inhibitor (PMSF) was not added. Exsheathing fluid contained several regions of protease activity, as indicated by digestion of gelatin in substrate gel SDS-PAGE (**Figure 3.2.2**). The most active proteolysis occurred in a region of 100–140 kDa, with other prominent bands visible at approximately 80 kDa and 50 kDa (**Figure 3.2.2**). A band of proteolysis was also apparent, upon visual inspection of the gel, at approximately 37 kDa that is clearer in **Figure 3.2.5** ES contained protease activity that appeared following 72 hours in culture, and this activity was more intense at 96 and 120 hours in culture. The protease activity appeared to be limited to a band of activity that migrated with a weight of approximately 40 kDa (**Figure 3.2.2**) and was most active at a pH of 8 (**Figure 3.2.3**). Protease activity was not visible in any of the somatic extracts (**Figure 3.2.2**).

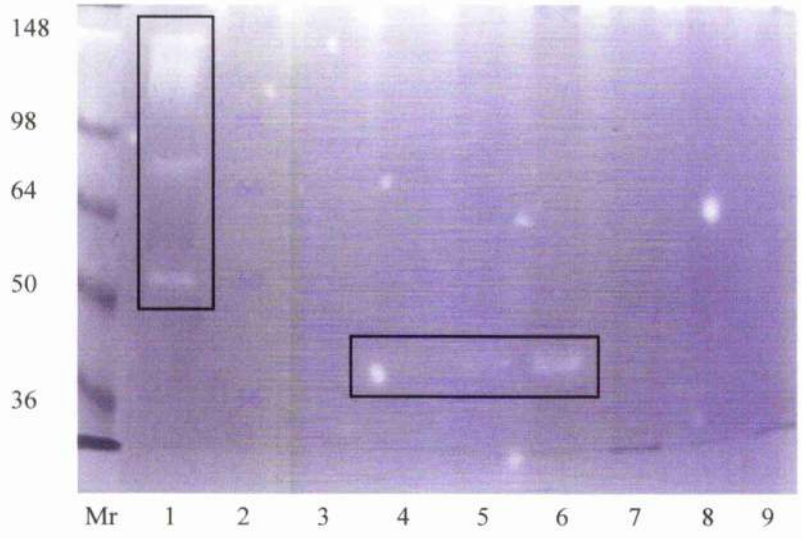


Figure 3.2.2: EF and ES Substrate Gel. 0.1% w/v Gelatin Substrate, 10% SDS PAGE gel showing protease activity in *H. contortus* larval EF, ES collected at 24 hour intervals during culture and larval extracts. Regions of proteolysis detectable in EF and 72, 96 and 120 hour ES are boxed.
1 - Exsheathing fluid; **2** - 24hrES; **3** - 48hrES; **4** -72hrES; **5** -96hrES; **6** - 120hrES; **7** - S1; **8** - S2; **9** - S3.

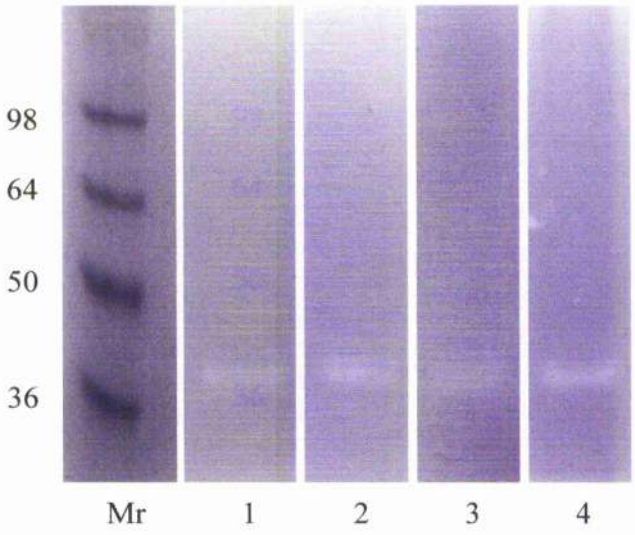


Figure 3.2.3: ES Substrate gel at various pHs. 0.1% gelatin substrate, 10% SDS PAGE gel showing protease activity in 120 hour *H. contortus* larval having been incubated at various pH.
1 – pH 5; **2** – pH 6; **3**– pH 7; **4** – pH 8

To confirm that the protease activity observed in ES could be ascribed a metalloprotease nature, inhibition studies involving pre-incubation with a range of specific protease inhibitors were carried out as described in **Section 2.2.6**. The range of inhibitors used combine to inhibit all four classes of protease: 1,10 Phenanthroline and EDTA are both metalloprotease inhibitors, E-64 is a cysteine protease inhibitor, AEBSF inhibits serine proteases and Pepstatin is an aspartyl protease inhibitor. The only inhibitor that reduced activity was 1, 10 Phenanthroline, (**Figure 3.2.4a**). A combination of 1, 10 Phenanthroline and EDTA (another metalloprotease inhibitor) completely abrogated activity (**Figure 3.2.4a**) implying the protease to be of a metallo nature. Substrate gel analysis of 96 hour ES pre-incubated in increasing concentrations of 1, 10 Phenanthroline (**Figure 3.2.4b,** pH 8) demonstrated a dose-dependant reduction in protease activity confirming that the visible protease activity in *H. contortus* L3 ES be attributable to metalloprotease activity.

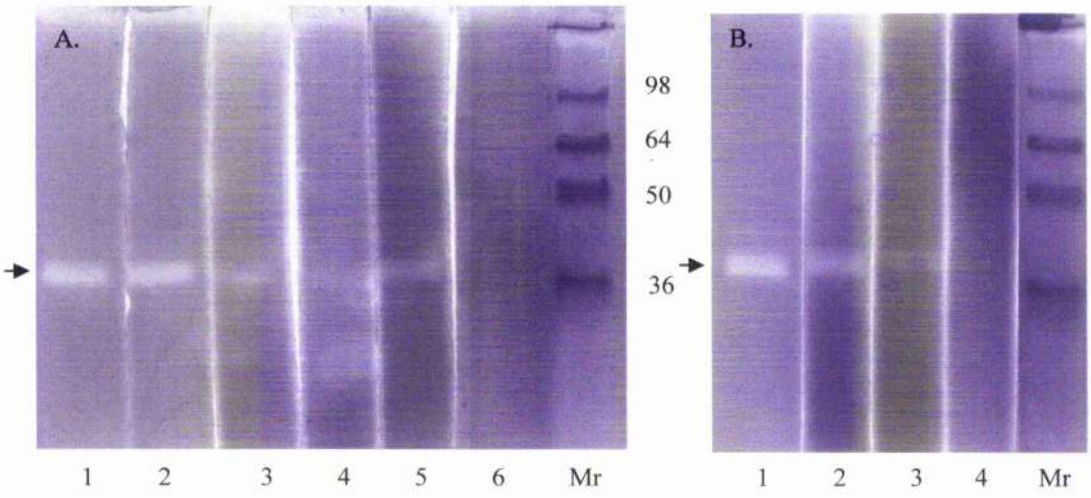


Figure 3.2.4: Substrate gel of ES with protease inhibitors. 0.1% Gelatin Substrate, 10% SDS PAGE gel showing protease activity in *H. contortus* larval ES collected after 96 hours in culture. Bands of proteolysis are marked by arrows. Panel A: 96hr ES incubated with a range of specific protease inhibitors. **1** – pH 8 buffer; **2** - AEBSF (2 mM); **3** - E-64 (10 μM); **4** - 1, 10 Phenanthroline (1 mM); **5** - Pepstatin (10 μM); **6** - 1, 10 Phenanthroline (1 mM) + EDTA (4 μM).
Panel B: 96hr ES incubated with increasing concentrations of 1, 10 Phenanthroline. **1** - pH8 buffer; **2** - 1 mM; **3** - 10 mM; **4** - 100 mM.

Similar inhibitor studies carried out with EF demonstrated 1,10 Phenanthroline to be the most effective protease inhibitor, reducing the intensity of all activity compared with the control and in particular being the only class of protease inhibitor tested to completely abrogate the band of activity at approximately 37 kDa implying this band to be attributable to metalloprotease activity (**Figure 3.2.5**). Interestingly all classes of protease inhibitor led to a slight reduction in protease activity compared with the control perhaps suggesting a range of proteases to be present in EF.

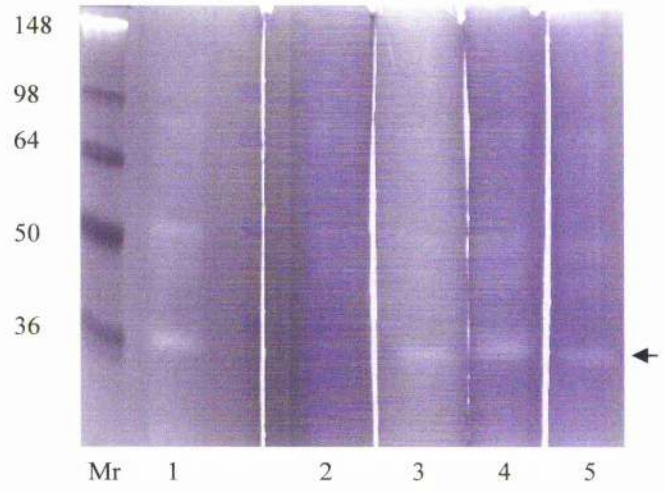


Figure 3.2.5: Substrate gel of EF with protease inhibitors. 0.1% Gelatin Substrate, 10% SDS PAGE gel showing protease activity in *H. contortus* larval EF. **1** - pH 8 buffer; **2** - 1, 10 Phenanthroline (1mM); **3** - AEBSF (2mM); **4** - E-64 (10μM); **5** - Pepstatin (10μM). The prominent band at 37 kDa

Further confirmation of metalloprotease activity in *H. contortus* L3 ES came from a metalloprotease specific assay (see **Section 2.2.7**). Metalloprotease activity was measured by the release of a 4-methoxy-2-napthylamine substrate following cleavage of an amino acid substrate between the alanine and phenylalanine residues. This was compared to a standard curve of fluorescence detectable by serial dilutions of 4-MNA (**Figure 3.2.6**). Some enzymatic activity, expressed as μM 4-MNA per μg protein per hour, was found in all ES, with the highest activity in 96 hr ES (**Table 3.2.2**). Notably, the levels of metalloprotease activity were far higher in EF and in the positive control, the metallo-endopeptidase containing H-gal-GP complex.

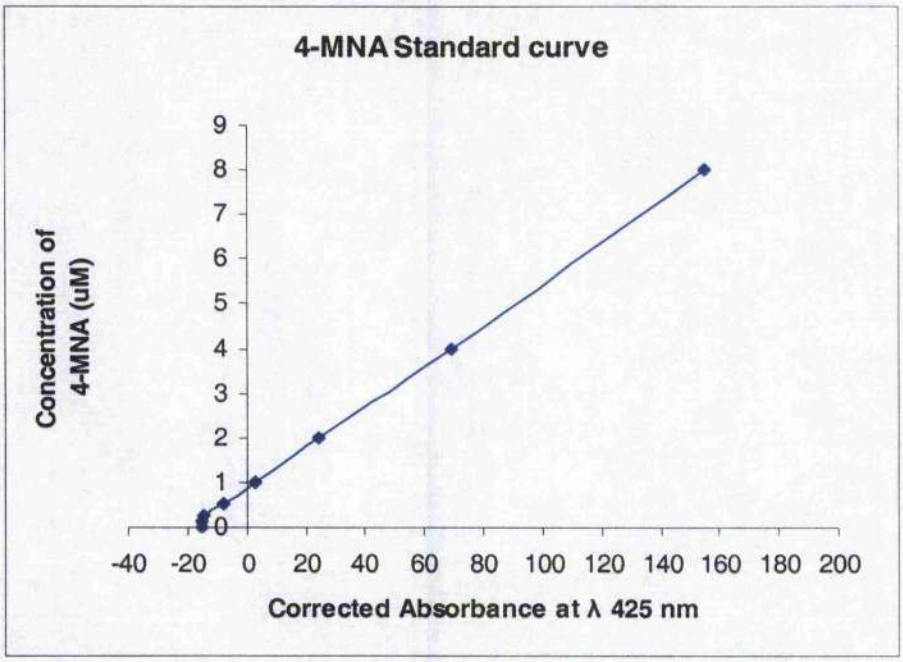


Figure 3.2.6: The standard curve showing the fluorescent absorbance detected at λ 425 nm of a diluted series (from 0.125 µM to 8 µM) of 4-MNA standards

Table 3.2.1: The mean enzymatic activity of *H. contortus* L3 ES, EF and H-gal-GP (positive control) as a measure of the 4-MNA released when a metalloprotease cleaves a specific substrate. (Fluorescence +/- SEM)

Sample	24 hr ES	48 hr ES	72 hr ES	96hr ES	EF	H-gal-GP
Fluorescence (λ 425 nm)	11.5 (4.2)	7.7 (5.45)	12 (1.28)	31 (12)	148 (1.8)	200 (9.7)
4-MNA concentration	1.36 µM	1.17 µM	1.41 µM	2.38 µM	8 µM	>8 µM
Protein quantity (µg)	15	15	15	15	15	10
Enzymatic activity (µM 4-MNA / µg protein / hour)	0.05	0.04	0.05	0.08	0.27	>0.40

3.2.2 Lentil lectin affinity chromatography of *H. contortus* L3 ES

Previous reports have indicated that Lentil lectin binds specifically to an ASP-1 homologue in *H. contortus* L3 ES (Sharp and Wagland, 1998). Therefore, attempts were made to identify this homologue by Western Blot and enrich for it by affinity chromatography. A Western blot of *H. contortus* L3 ES and somatic extracts, probed with biotinylated *Lens culinaris* lentil lectin identified several bands of reactivity in L3 ES that tended to become progressively more intense as culture time proceeded. The most intense region of reactivity was at approximately 40 kDa (**Figure 3.2.7**) and may represent the 40 kDa ASP-1 homologue. This band appeared after 48 hours in culture and was most intense in the 72 hour and 120 hour cultures, the reason for the apparent decline in abundance in the 96 hour culture are unclear. Other prominent bands reacting with the lentil lectin included those at approximately 60 kDa and a smear at approximately 90- 120 kDa. These appeared after 72 hours in culture and were most intense in the 120 hour culture. Strikingly, no prominent binding was detected in S1, S2 and S3 larval extracts produced immediately following exsheathing (**Figure 3.2.7**) and those produced from worms cultured for 96 hours (data not shown) implying the lectin binding proteins to be of a genuinely excreted/secreted nature.

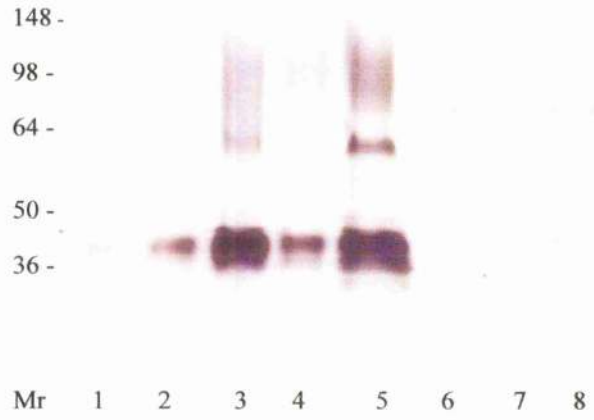


Figure 3.2.7: Western Blot of *H. contortus* larval ES proteins and somatic extracts probed with biotinylated lentil lectin.
1 - 24hrES; **2** - 48hrES; **3** - 72hrES; **4** - 96hrES; **5** -120hrES; **6** - S1; **7** - S2; **8** - S3.

To enrich for the lentil lectin binding proteins and in particular the putative 40 kDa ASP-1 homologue, affinity chromatography of *H. contortus* L3 ES was carried out. A total of 2.5 mg of pooled 24-120 hr *H. contortus* L3 ES was applied to a chromatography column containing agarose-bound lentil lectin as described in **Section 2.2.8**. The bound and eluted protein fractions were clearly enriched for the lentil lectin-binding proteins, and the non-binding fraction exhibited no reactivity with lentil lectin as judged by SDS-PAGE gel and Western blot (**Figure 3.2.8**). Seven hundred and fifty μg of unbound protein and 105 μg of bound protein were recovered following concentration (amounting to an approximately 3 fold loss of protein during the entire process). A band of protease activity, corresponding in size to the previously described metalloprotease, was visible only in the unbound fraction (**Figure 3.2.9**).

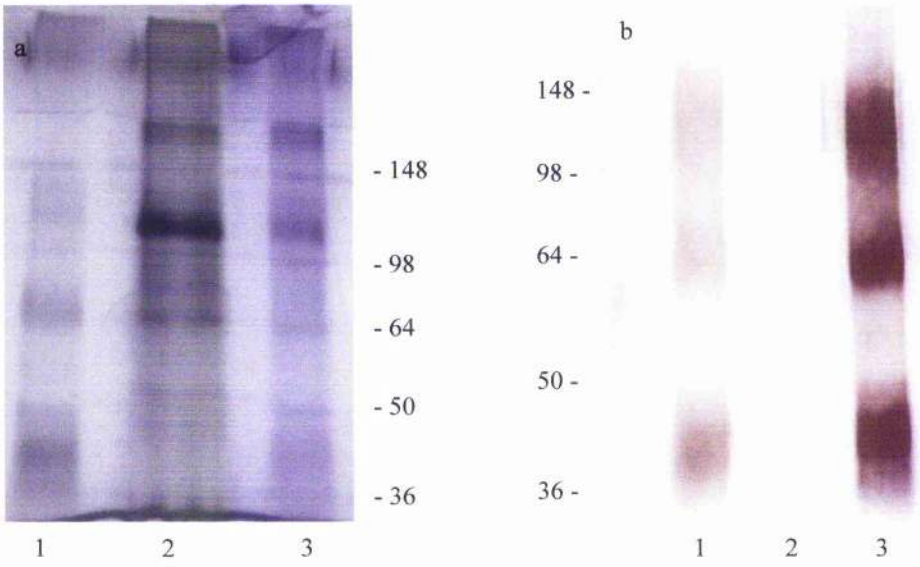


Figure 3.2.8: A 10% SDS-PAGE profile of whole ES, lentil lectin binding ES, and unbound ES (Panel **a**). Panel **b** shows a Western blot of identical samples probed with biotinylated lentil lectin. Molecular weight standards are in kDa. Panel **a**: **1** - bound and eluted ES; **2** - unbound; **3** - unfractionated ES. Panel **b**: **1** - unfractionated ES; **2** - unbound ES; **3** - bound and eluted ES.

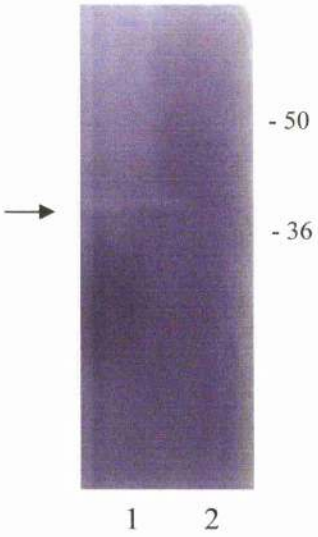


Figure 3.2.9: Substrate gel of unbound and bound fractions. 0.1% gelatin substrate, 10% SDS PAGE gel showing protease activity in *H. contortus* larval ES unbound fraction. Molecular weights indicated in kDa. Arrow points to band of proteolysis in unbound ES.
1 – unbound ES; **2** – bound and eluted ES

3.2.3 Attempts to seek *A. caninum* homologues by immunoblot

Western blots of *H. contortus* ES and somatic extracts were screened with sera raised against some of the lead larval hookworm vaccine candidates to attempt to identify potential homologues in *H. contortus* and to confirm the 40 kDa lectin binding protein as an ASP-1 homologue. Sera raised against the following *E. coli* expressed recombinant *A. caninum* proteins were kindly supplied by P. Hotez (HHVI): ASP-1, ASP-2, transthyretin-like protein (TTR) and the larval metalloprotease (MTP). Western blots were carried out with primary antibodies at a range of dilutions (from the recommended 1:4000 to 1:100) and developed using both DAB and the more sensitive ECL substrates (**Section 2.2.6**) (data not shown). Upon screening *H. contortus* whole ES, larval and adult extracts, an immune reaction was only observed using the TTR sera, which recognised a band at approximately 15 kDa, and more faintly at 60 kDa (see **Chapter 6**). Interestingly, the only sera to recognise a band in *A. caninum* L3 extract, a positive control kindly provided by P. Hotez (HHVI), was the ASP-1 sera, which very faintly labelled a band at 45 kDa (data not shown). A more accurate positive control of *A. caninum* L3 ES was unavailable. Due to the nature of the recombinant proteins, it may be that antibody raised against a potentially incorrectly folded *E. coli*-expressed protein would not cross-react with the native homologous protein.

3.3 Discussion

Resumption of development from the quiescent environmental L3 to the parasitic nematode occurs in response to entry into the host, and the environmental changes therein (Rogers and Sommerville, 1968). In the case of *H. contortus*, entry to the host is by ingestion and the larvae exsheath in the rumen in response to various factors, likely to include an increase in temperature and exposure to CO_2 and carbonic acid in the rumen prior to migration into the slightly acidic environment of the abomasum (Rogers and Sommerville, 1968). During or prior to the exsheathing process, L3 release a 44 kDa zinc-metalloprotease (Gamble *et al.* 1989a). Following exsheathment, L3 move from the rumen into the abomasum where they become closely associated with the gastric glands and develop into the blood feeding L4 by shedding their third stage cuticle (Urquhart *et al.,* 1987). Initiation of exsheathing and culturing L3 through to the moult to L4s can be replicated *in vitro* and the proteins released by L3 following resumption of development attained from culture (Gamble and Mansfield, 1996; Sharp and Wagland, 1998).

The results reported here confirm the secretion of a range of proteins (**Figure 3.2.1**), including active proteases (**Figure 3.2.2**), during the exsheathing process with secretion or excretion of proteins being apparent for up to 120 hours. As indicated by Gamble and Mansfield (1996), the quantity and complexity of proteins released into culture increases with time, proteins released following 24 or 48 hours in culture being barely detectable by Coomassie staining. After 72 hours in culture, a range of proteins was visible including an active protease with a molecular weight of approximately 40 kDa. Interestingly, the quantity of protein produced by larvae in culture in this study was 5-fold less than reported by Gamble and Mansfield (1996). The difference in ES quantity is difficult to explain as the exsheathing and culture methods were very similar, but may be due to a difference in *H. contortus* strains used (an American Beltsville strain versus the MOSI strain used in this study). There have been several reports of variation in the activity and character of adult gut-associated and ES proteases between different strains (Redmond and Windham, 2005; Karanu *et al.*, 1997); however there are no publications detailing a difference in the actual quantity of

proteins released. Throughout the culture period, worms remained viable and no physical deterioration was visible. Indeed, Gamble and Mansfield (1996) demonstrated that L3 maintained in culture for 96 hours remain infective to sheep.

The MTP (**Figure 3.2.4**) activity that appeared in ES after 72 hours (**Figure 3.2.2**) in culture is likely to be similar to that which was characterised, but as yet unidentified, by Gamble *et al.* in 1996 in that it appears at the same time point in culture has a pH optima of 8 and is of an approximately similar molecular weight (**Figure 3.2.3**). The difference in observed estimated size of the ES MTP in this report (approximately 40 kDa) and that of the previously reported ES MTP (46 kDa) is likely due to the substrate gels in this study necessitating the electrophoresis of proteins under non-reducing conditions. Because of the non-reduced nature of the protein, it may run slightly faster in comparison with the already reduced molecular standard. This had been reported for other *H. contortus* proteins (Dr G. Newlands, personal communication).

Gamble *et al.* (1996) proposed that the ES MTP is released coincidentally with the molt from the L3 to the L4. This molt corresponds with the onset of feeding of larvae in culture and the general increase in proteins released by the larvae as ES (Gamble and Mansfield, 1996). During the cultures carried out in this study, most larvae did not appear, by visual inspection, to have developed to the L4, even after 120 hours; similar findings have been reported by other groups (C. Britton, personal communication). Although no feeding assay was carried out, larvae at all culture time points were evaluated for development to the fourth stage visually by an expert in the field (D. Bartley, Moredun Research Institute). The reason for the apparent lack of development to L4 is unknown and may be due to the different strains of worm involved. However it does question the theory that secretion of significant amounts of protein and appearance of the MTP occur coincident with the third moult and transition to the fourth larval stage (Gamble *et al.*, 1996). It might be that the metalloprotease described, or other unidentified proteases, are involved in an early step in the L3 to L4 moult but that other later factors essential for completion of the moult do not occur. It

is also possible that the small numbers of worms that successfully moult to L4 are releasing the protease although this seems unlikely.

MTP activity was much higher in EF compared to the ES timepoints (**Table 3.2.1**), the levels of the latter remaining relatively constant throughout the culture period, despite the observed appearance of MTP activity after 72 hours in culture. As noted earlier, a 44kDa EF MTP has been implicated in larval exsheathment (Gamble *et al.*, 1989) and the relatively high levels of activity detected in EF compared to ES could reflect the synchronous release of enzyme induced by the exsheathing process. Alternatively, the EF and ES MTPs may be distinct enzymes as suggested by studies by Gamble *et al.* (1989) and Gamble et al, (1996) where the enzymes had slightly differing molecular weights of 44 kDa and 46 kDa in EF and ES respectively. The results described here also suggest the EF and ES MTPs to have different molecular weights (37 kDa versus 40 kDa); in combination with the temporal expression patterns this does suggest the enzymes to be distinct. This leads to the possibility that the enzymes have differing substrate specificities with the result that the EF enzyme cleaves the assay substrate used here more efficiently than its ES counterpart. This possibility could be addressed by a detailed substrate preference comparison of the purified EF and ES enzymes and, ultimately, by analysing the genes encoding them. In *C. elegans* there is a gene family of 39 astacin-like MTPs, one of which (NAS-37) has been demonstrated to be important in ecdysis, and can actually mediate refractile ring formation in *H. contortus* (Davis *et al.*, 2004). This suggests that there are likely to be a number of astacin-like MTPs with distinct functions.

No protease activity was detected by substrate PAGE analysis of L3 extract made following exsheathing, and no activity is reported in L4 extract by Gamble and Mansfield (1996). This would indicate that at least some of the proteins within the ES are genuinely secreted, the proteins being processed to yield active enzyme during secretion, and not products of larval degradation during *in vitro* culture.

Contrary to expectations, sera raised against *A. caninum* ASP-1, ASP-2 and MTP failed to cross-react with *H. contortus* larval ES, larval extract or adult extract. This meant that the *A. caninum* sera could not be used to identify and localise the homologues in *H. contortus*. Interestingly, the sera, with the exception of the ASP-1, failed to cross-react with proteins in the provided positive control *A. caninum* L3 extract. The ASP-1 sera did recognise a protein in the *A. caninum* extract, but this was very faint. This result might question the quality of the sera following trans-Atlantic transport, although a better positive control would have been *A. caninum* L3 ES. The results of the TTR labelling are discussed in **Chapter 6.**

Glycosylated nematode proteins are very immunogenic with the carbohydrate component being particularly immunogenic. Indeed it has been proposed that a substantial part of the host immune response to *H. contortus* is likely to be directed against carbohydrate epitopes on parasite antigens (Schallig and Van Leeuwen, 1996). The protective gut-associated antigen complexes isolated from *H. contortus* [TSBP (Knox *et al.*, 1999), H-Gal-GP (Smith *et al.*, 1994) and H11 (Munn *et al.*, 1997)] are heavily glycosylated with the glycan being highly immunogenic. Glycoproteins are normally found in the plasma membrane, are extracellular proteins (as in the case of the gut associated-complexes) or secreted, like those found in ES. Adult ES contain several N-linked glycoproteins that afford significant protection against homologous challenge (Schallig and Van Leeuwen, 1997).

Lectin-binding studies carried out to investigate the carbohydrate moieties in *H. contortus* extracts showed several lectins to bind to a variety of glycoproteins in both adult and L3 soluble extract (Schallig *et al.*, 1996). Lentil lectin is specific for terminal α-D-mannosyl and α-D-glucosyl residues and has been reported to specifically enrich *H. contortus* L3 ES for the 40 kDa larval ASP-1 homologue (Sharp and Wagland, 1998). In this study, lentil lectin reacted strongly with glycoproteins in the region of 40 kDa, which appeared in L3 ES after 48 hours and tended to increase in intensity as culture time progressed (**Figure 3.2.6**). This protein is in the appropriate size region to be the larval ASP-1 homologue. No such protein was detected in L3 extracts made either before or after culture,

indicating that the 40 kDa protein is of a genuinely ES nature, and is transcribed and released quickly, rather than stored by the worms prior to secretion.

Lentil lectin-affinity chromatography allows fractionation of whole ES into a lentil lectin-binding and a non-binding fraction. This clearly enriched for the protein/s at 40 kDa, but also for the other proteins (**Figure 3.2.7**). With the exception of 40 kDa proteins that might be the putative ASP-1 homologue, the identification of the proteins within the bound or unbound fractions remains unknown; although it would appear that the MTP activity partitions with the unbound fraction (**Figure 3.2.8**). Previous reports (Sharp and Wagland, 1998) suggest that lentil lectin chromatography enriches for only the 40kDa protein, however this does not seem the case in this study. This result has implications for the interpretation of vaccine trial outcomes with reference to selecting protective proteins for recombinant expression.

In summary, upon exsheathing and culture, *H. contortus* L3 release a range of proteins, including proteases, that increase in both quantity and complexity as culture time persists. Despite earlier reports to the contrary, in this study L3 larvae did not molt to the fourth stage; however, they did release into culture proteins that have been previously associated with this moult and the resumption of feeding, including a metalloprotease. Probing L3 ES with lentil lectin confirmed strong binding to a protein or proteins with a molecular weight of approximately 40 kDa. This may include the ASP-1 homologue, and lentil lectin affinity chromatography allowed partial purification and enrichment of these proteins.

Chapter 4

Sheep Protection Trial

4.1 Introduction

Vaccination with irradiated *H. contortus* and *A. caninum* larvae can achieve high levels of protection against homologous challenge (Smith and Angus, 1980; Miller, 1965). However, attempted vaccination with *H. contortus* somatic larval antigens failed to induce immunity in sheep (Smith, 1977); yet, as vaccination with irradiated larvae induces immunity, it is likely L3 and possibly L4 secrete proteins that can elicit immunity. Indeed, metabolites secreted by *H. contortus* during the molt from L3 to L4 were effective immunogens in sheep (Ozerol and Silverman, 1970) although no attempt was made to isolate the protective component(s).

Hookworm L3, on transition to parasitism, release a number of developmentally regulated proteins, several of which have been identified as vaccine candidate antigens (see **Chapter 1**). Protection trials with hookworm antigens have been carried out using recombinant proteins and some of the most promising antigens identified to date include members of the ASP family, ASP-1 and ASP-2 (Hotez *et al.*, 2003). *H. contortus* L3 release a single domain ASP-1 homologue into ES and this can be partially purified by lentil lectin chromatography and has been shown to confer protection against homologous challenge in guinea pigs (Sharp and Wagland, 1998). Several other hookworm candidate larval antigens are likely to have homologues in *H. contortus*, including the hookworm larval MTP; MTP activity in *H. contortus* ES has been reported previously (Gamble *et al.* 1997) and confirmed in **Chapter 3**. It would be of benefit to evaluate the protective potential of L3 *H. contortus* ES components in a sheep vaccine trial. Adult *H. contortus* ES components do induce protective immune responses (summarised in Knox, 2000), but similar trials with L3 ES have not been reported.

This chapter describes a sheep vaccine trial to evaluate the protective efficacy of *H. contortus* L3 ES fractions with the aim of indicating the worth of the native proteins therein as potential vaccine candidates. L3 ES were harvested and subsequently fractionated into lentil lectin-binding [potentially enriched for the

ASP-1 homologue (Sharp and Wagland, 1998)] and unbound fractions, containing MTP activity, these fractions being used as immunogens in the protection trial. The trial design and execution were described in **Chapter 2** and the peptide content of the immunogens described in **Chapter 3**.

4.2 Results

4.2.1 Serological response to vaccination

The vaccine trial was carried out as described in **Chapter 2.** Briefly 3 groups of 7 animals were vaccinated on 3 occasions 21 days apart intramuscularly with either 5 μg of the lentil-lectin binding ES fraction plus Quil A (bound group), 35 μg of the non-binding lentil lectin ES fraction plus Quil A (unbound group) or Quil A in PBS (Control group). One week following the final immunisation animals were challenged with a single dose of 5,000 L3 *H. contortus*.

To examine the serological antibody response to immunogens, sera was collected (as described in **Section 2.8.1** prior to initial vaccination and 1 week post the third and final immunisation) and used in ELISA and Western blot analyses. All sheep in both the unbound and the bound vaccine groups had a significant ($P<0.01$, data not shown) circulating IgG response to whole ES (**Table 4.2.1**). The response varied between individuals with an approximate 3-fold difference between the highest and lowest responders. The variation in IgE analysis was much more pronounced both within and between groups (**Table 4.2.2**). Animals vaccinated with the bound ES fraction showed only a very low IgE response, the group mean of which was not statistically different to the control group (data not shown). All sheep in the unbound group, except animal 2, had a significant ($P<0.01$, data not shown) circulating IgE response. Both ELISA analyses and Western blot analyses were carried out using whole ES, rather than the appropriate fraction that animals received during the vaccine trial, due to the difficulty in obtaining large amounts of protein.

As can be seen from the ELISA data (**Tables 4.2.1** and **4.2.2**), those animals vaccinated with the unbound fraction generally had a higher total IgG and IgE titre than those vaccinated with the bound fraction. Western blots of whole ES probed with pooled pre-vaccinate sera from either group showed only a very faint reaction with the ES, likely to be due to non-specific binding (**Figure 4.2.1**). With pooled sera from the unbound vaccinate group, the intensity of staining increased with ES culture time, reflecting the increasing quantity and

perhaps complexity of proteins released into culture over time (**Figure 4.2.1A**). Twenty four hour ES was strongly recognised by antiserum from the unbound vaccinate group, despite the fact that ES proteins are barely visible following staining of protein gels (**Chapter 3, Figure 3.2.1**). There was particularly pronounced recognition of a peptide at ~48 kDa, also seen in 48 hour ES. Other peptides that were prominently recognised in later time point ES included those at 33, 47, 50, ~60, 93 and ~100 kDa. Western blot analysis of whole ES probed with pooled vaccinated sera from the bound group (**Figure 4.2.1B**) demonstrated a distinct pattern of recognition compared with the unbound group sera. Again the complexity of peptides recognised increased with culture time of ES with peptides at 36, 50, ~60, ~95 and ~130 kDa being recognised most strongly by the antiserum. As seen in the lentil lectin binding profile (**Chapter 3, Figure 3.2.7**), a protein corresponding in size with the putative ASP-1 homologue, (Sharp and Wagland, 1998) at approximately 40 kDa was recognised by bound antisera in 72 hour and later time point ES cultures, however this band is not particularly prominent. Periodate treatment of blots prior to antibody probing reduced the number of bands recognised by both unbound and bound sera, indicating that a proportion of the antibody was directed against carbohydrate epitopes (**Figure 4.2.2**). Reduction of reactivity was more pronounced with sera from animals vaccinated with the bound fraction, perhaps not unexpectedly as this fraction was enriched for glycoproteins. However, this treatment revealed three peptides (~42 kDa, 60 kDa and 98 kDa) which were distinctly recognised by the bound sera although antigens of similar size were also recognised in the unbound fraction.

Table 4.2.1: Total IgG analysis from individual animals following vaccination with either the bound or unbound antigen. IgG values are expressed as the dilution factor required to obtain the mean of the group pre-immune serum values at a dilution of 1:1280. * The mean was calculated using the value of 104,200 where appropriate; the real mean would likely be higher.

Group	**Bound**	**Unbound**
Animal No.	*IgG Titre*	*IgG Titre*
1	36,272	65,021
2	69,581	91,532
3	40,433	>104,200
4	68,615	92,742
5	18,263	>104,200
6	23,581	>104,200
7	19,543	>104,200
Mean	**39,470**	**95,156***

Table 4.2.2: IgE analysis from individual animals following vaccination with either the bound or unbound antigen. IgE titres are expressed as the dilution factor required to obtain the mean of the group pre-immune serum values at a dilution of 1:20. * The mean was calculated using the value of 20,480 where appropriate; the real mean would likely be higher.

Group	**Bound**	**Unbound**
Animal No.	*IgE Titre*	*IgE Titre*
1	1227	6012
2	11	918
3	187	>20,480
4	2448	7301
5	46	>20,480
6	262	>20,480
7	192	>20,480
Mean	**624**	**13,736***

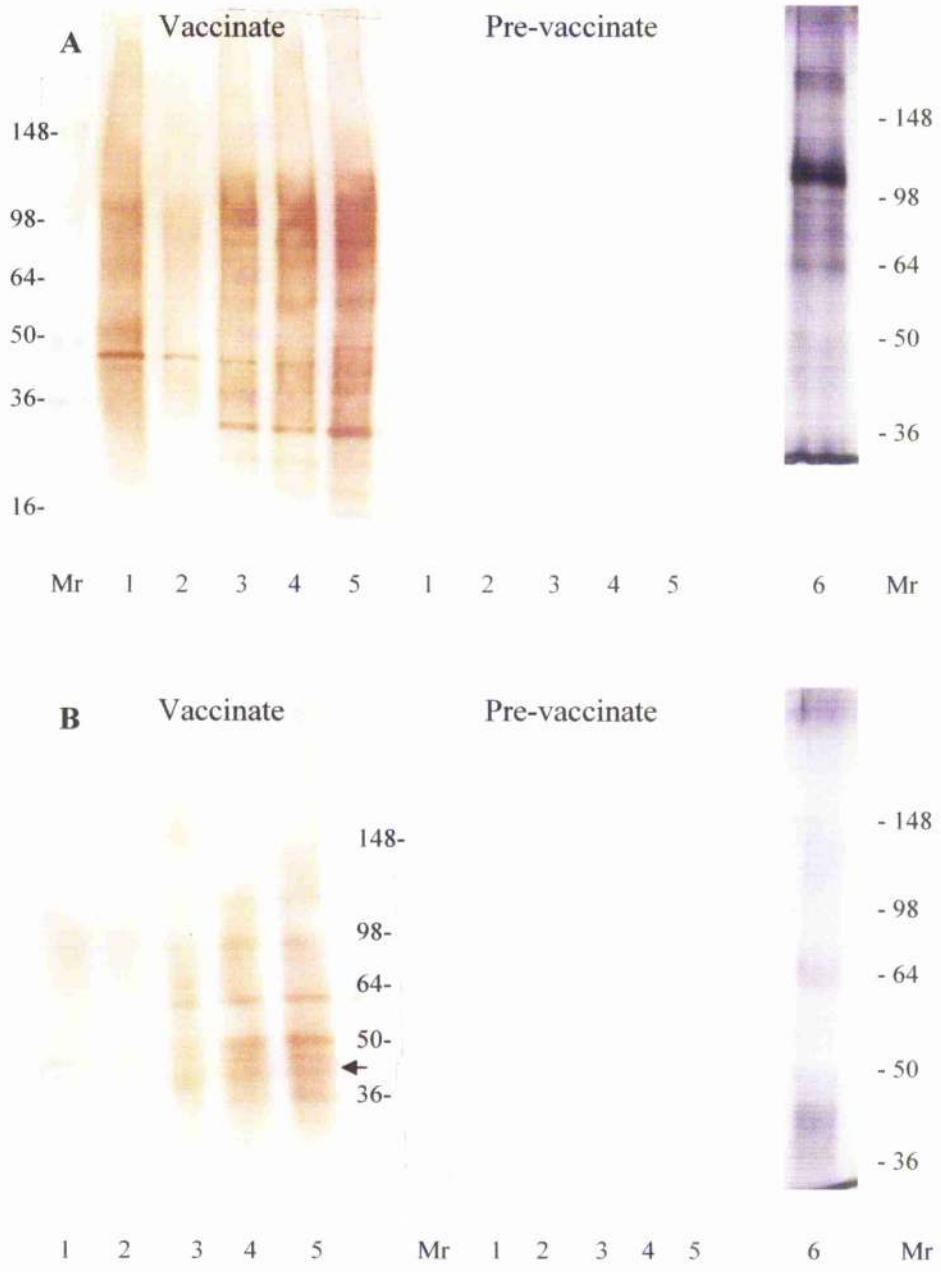


Figure 4.2.1: Western blots of whole ES probed with 1:1000 diltuion of pooled pre-vaccinate or vaccinate sera from unbound group (**panel A**); and bound group (**panel B**). An HRP labelled rabbit anti-sheep IgG secondary antibody (1:1000) detected the sheep total IgG. The arrow in **B** marks peptide at 40 kDa recognised by antibody. Lane 6 shows silver stained 10% SDS PAGE profile of the appropriate ES fraction animals were vaccinated with alongside the Mr markers appropriate for this gel (see also Figure 3.2.8).
Culture time of ES: **1** - 24 hr; **2** - 48 hr; **3** - 72 hr; **4** - 96 hr; **5** - 120 hr; **6** – ES fraction used as immunogen.

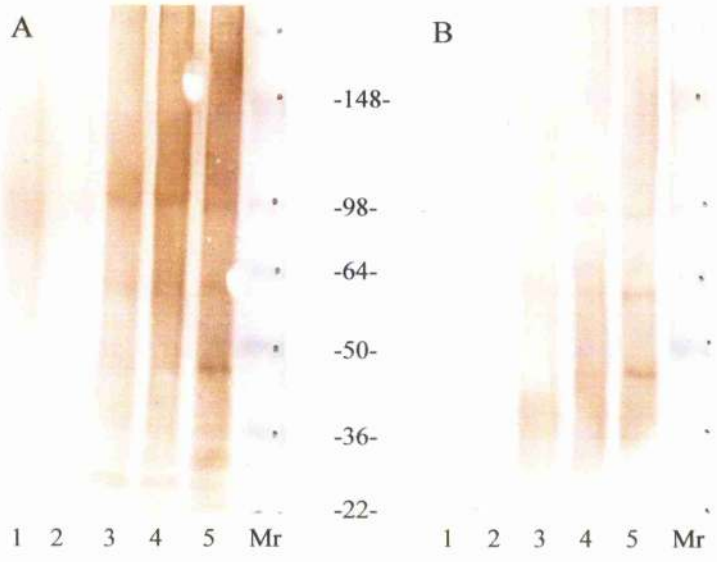


Figure 4.2.2: Periodate treated Western blots of whole ES probed with 1:1000 pooled vaccinate sera from unbound group (panel A); and bound group (panel B).
Culture time of ES: **1** - 24 hr; **2** - 48 hr; **3** - 72 hr; **4** - 96 hr; **5** - 120 hr.

4.2.2 Levels of protection

To quantify any protective affect of vaccination, individual animal FEC were made during the trial; at the end point animals were sacrificed and final adult worm burdens enumerated, male to female ratios established and a sub-sample of worms measured (as described in **Sections 2.8.2** and **2.8.3**). The FEC for the trial are plotted as group means in **Figure 4.2.3**. Although not found to be statistically significant by one way analysis of variance (*ANOVA*), there was a clear trend towards reduced egg output compared with the adjuvant alone control group in both vaccinate groups. Cumulative FEC was reduced by 10% for the bound group and 17% for the unbound group.

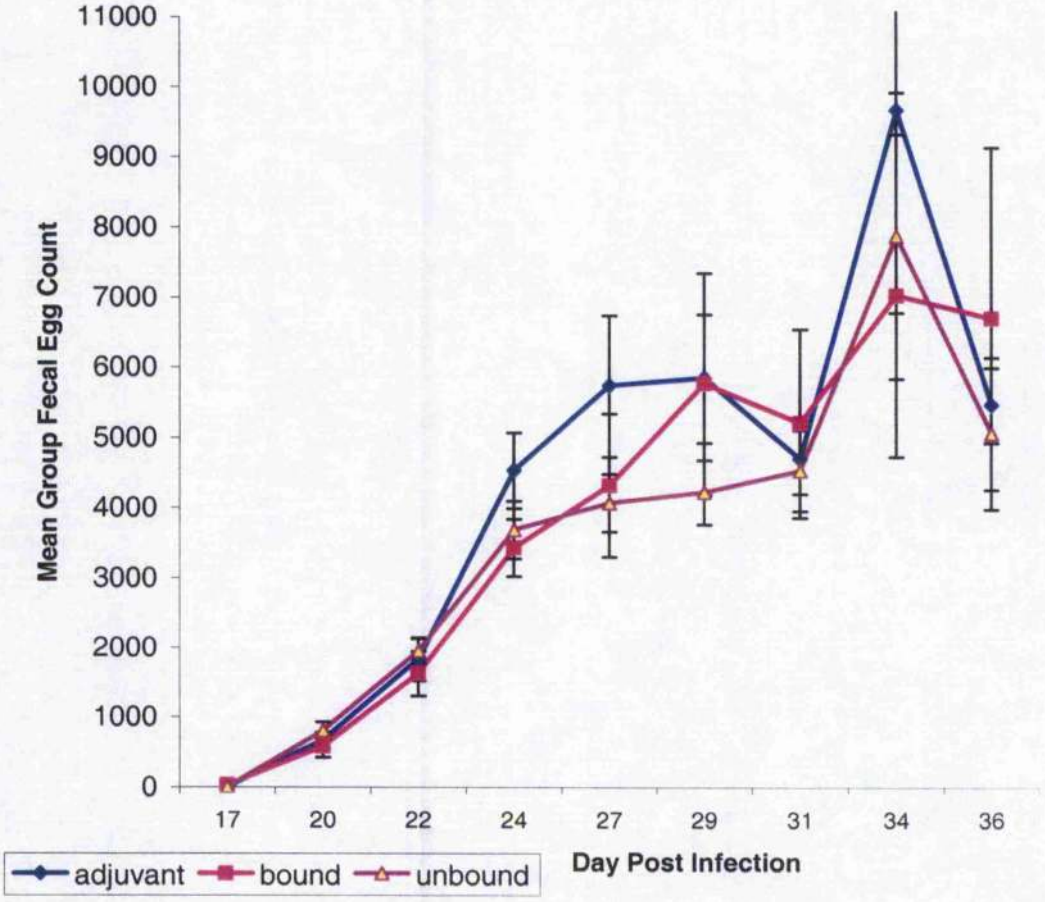


Figure 4.2.3: Mean group faecal egg counts plotted against day post infection. Standard error of mean (SEM) is shown by error bars above and below each point.

Mean cumulative egg counts and final adult worm burdens for the individual animals in each group are summarized in **Table 4.2.2**. Lambs immunised with the bound and unbound fractions had reduced group mean final worm burdens of 16% ($P<0.01$) and 12% (not found to be statistically significant by one way *ANOVA*) respectively, when compared to the control group.

In vaccinated groups the male to female ratio (m:f) did not differ significantly from that of the control (control 45:55; bound 43:57; unbound 43:57). Despite there being no effect on the length of surviving male worms in either of the groups, there was a small, yet statistically significant reduction in female worm size in the bound group (mean length of 19.89 mm SEM +/-0.18, n=50),

amounting to a decrease of 6.2%, compared with the controls (21.20 mm SEM +/-0.18, n=50 P<0.01 by one way *ANOVA*). To investigate a potential affect of vaccination on the capability of eggs to hatch successfully an egg hatch assay was carried out (**Section 2.8.4**). The viability of eggs, evaluated by egg hatch assay on days 20, 22, 29 and 36 post-challenge, was >80% at all time points with no statistical differences between the viability of eggs from control and vaccinated animals were observed (**Table 4.2.4**).

Table 4.2.3: Individual animal mean FEC and abomasal worm counts. Reductions in egg counts and worm counts are expressed as a percentage reduction compared to the control, adjuvant group. SEM is shown. One-way *ANOVA P* values are reported on cumulative group means following log_{10} transformation of data. * marks a statistical significance of $P<0.05$

Group	**Adjuvant**		**Unbound**		**Bound**	
Animal Number	*FEC*	*Adult counts*	*FEC*	*Adult counts*	*FEC*	*Adult counts*
1	4129	3800	3610	4500	2218	3800
2	3631	4550	2797	3000	3320	3050
3	2467	4450	5984	4850	1239	3800
4	3126	4350	3815	3050	8711	4350
5	5707	5700	2474	4500	6027	4700
6	3773	4100	2686	4000	2707	3750
7	7257	5350	3675	4500	2734	3650
Mean	**4299**	**4614**	**3577**	**4057**	**3851**	**3871**
S.E.M.	621	256	449	283	984	199
Reduction (%)	**n/a**	**n/a**	**17** (P=0.47)	**12** (P=0.08)	**10** (P=0.46)	**16*** (P=0.01)

Table 4.2.4: Egg viability assay. Percentage of eggs that hatched following collection from individual animals on days 20 to 36 post challenge, expressed as a group mean +/- SEM.

Group	Percentage of eggs that hatched (+/-S.E.M)			
	Day 20	**Day 22**	**Day 29**	**Day 36**
Control	88 (+/-2.6)	97 (+/-0.9)	99 (+/-0.6)	98 (+/-1.2)
Unbound	96 (+/-1.4)	98 (+/-0.7)	98 (+/-0.6)	95 (+/-1.9)
Bound	89 (+/-6.9)	96 (+/-1.4)	97 (+/-1.0)	95 (+/-2.4)

4.2.3 Direct challenge assay

To establish whether sera from vaccinated animals could inhibit larval establishment within the abomasal tissue, a direct challenge assay was carried out as described in **Section 2.10**. ExL3 were pre-incubated with PBS alone, pooled pre-vaccinate, bound or unbound vaccinate sera, and their ability to penetrate abomasal tissue *in vitro* evaluated. The results are summarised in **Table 4.2.5**. The percentage of the larval population that were tissue associated was calculated using the formula:

$$\text{Percent tissue associated L3} = \frac{\text{No. of L3 in digest}}{\text{Total No. of L3 in washes + digest}} \times 100$$

While pre-incubation with bound sera was without effect, worthy of note is that pre-incubation with unbound vaccinate sera resulted in a highly significantly lower percentage of tissue associated larvae compared to pre-vaccinate sera (mean of 64% versus 79%, P=0.001 by chi-square test on original counts).

Table 4.2.5: The worm counts in a 2% sub-sample of washes or digests (tissue associated) from abomasal tissue challenged *in vitro* with *H. contortus* ExL3 following pre-incubation with sera. Mean percentage of L3 that were tissue associated are reported with the SEM, n=3 in each sample. * marks statistically significant result compared to the pre-vaccinate control group (P=0.001 by chi-square test on count data).

Treatment	L3 in Washes	Tissue associated L3	Total L3 recovered	Mean % Tissue associated
Pre-vaccinate sera	19	68	87	
	17	47	64	**79%**
	11	58	69	(+/- 3.1)
Unbound sera	28	37	65	
	16	28	44	**64%***
	22	54	76	(+/- 4.1)
Bound sera	12	72	84	
	6	56	62	**87%**
	9	48	57	(+/-1.8)
PBS (no sera)	41	55	96	
	26	33	59	**60%**
	37	68	105	(+/- 2.7)

4.3 Discussion

This chapter described the outcome of a protection trial where lambs were immunised with L3 ES fractions prepared by lentil lectin chromatography. Antigens binding to this matrix included the 40 kDa likely ASP-1 homologue while the MTP homologue did not bind (see **Chapter 3**). The trial had two purposes: 1) to repeat the purification protocols described in Sharp and Wagland, (1998) and to evaluate the protective efficacies of the fractions in sheep (previously only guinea pig trials had been carried out) and 2) to attempt to provide an indication as the whether the ASP-1 homologue or the MTP, partitioning in the bound and unbound fractions respectively, or both may be involved in inducing protective immunity. The results provided some evidence that vaccination with either lentil lectin-binding, or non-binding fractions of *H. contortus* L3 ES can promote a modest level of protection against homologous challenge as judged by a reduction in final worm burdens and a clear trend towards reduced egg production. Vaccination with the bound fraction resulted in a small reduction in female worm length while an intriguing observation was that sera from animals vaccinated with the unbound fraction containing the MTP inhibited *in vitro* larval penetration of abomasal tissue.

There was a trend towards lower egg counts in both the bound and unbound groups amounting to an overall reduction of 10% and 17% respectively, although this was not statistically significant. Worthy of note is the pattern of reduction in egg counts in the bound group between days 24 and 29 post-challenge and to a greater extent in the unbound group between days 24 and 31. This might correspond to a delay in maturation of larvae to adult, or to a postponement of egg production by the adult female worms. A similar FEC pattern, during the equivalent time points, was observed by Redmond and Knox (2004) following vaccination with a cysteine protease fraction and the corresponding recombinant proteins. In the UK, *H. contortus* has been reported to undergo a hypobiosis at the early L4 stage, though the trigger for this is unknown. It may be speculated that some factor of an immune response, stimulated through vaccination, might have delayed the onset of development to the gravid adult stage, though this

would require extensive further investigation. A feature of this study, and many other studies investigating the protective potential of nematode extracts or ES, is the large variability between animals in the same group in terms of egg count and worm burden. This does complicate interpretation of results and often makes it difficult to define statistically significant differences, particularly with relatively crude antigen extracts (D.P. Knox, personal communication).

Both vaccinated groups showed a reduction in abomasal worm count, the reduction of 16% in the bound group being statistically significant. Also of note was the detected 6% reduction in mean female worm length in this group. As discussed in **Chapter 3**, and above, lentil lectin binding purportedly enriches *H. contortus* L3 ES for a single domain ASP-1 homologue, larval Hc40 (Sharp and Wagland, 1998). Several vaccination studies with recombinant hookworm ASP-1 and ASP-2 have been carried out in a variety of model systems. Bacterially expressed recombinant *A. caninum* ASP-1 in the murine model reduced lung worm burden by up to 57% and this protection could be passively transferred with antibody (Ghosh *et al.*, 1996; Ghosh and Hotez, 1999). However, the mouse is a non-permissive host, and the relevance of a reduction in lung worm burden to a natural infection is unknown, though the antibody mediated nature of protection does indicate ASP-1 as a potential vaccine candidate. Vaccination with *Pichia pastoris*-expressed recombinant *A. ceylanicum* ASP-1 in the hamster model (permissive) had no effect on worm burden (Goud *et al.*, 2004), though *P. pastoris*-expressed *N. americanus* ASP-1 showed a trend towards a reduction in egg counts in vaccinated dogs challenged with *A. caninum* (Hotez *et al.*, 2003 HHVI 3rd Annual Report). ASP-2 has shown slightly more promise in vaccine trials; levels of protection corresponding to a 32% reduction in worm burden and 56% reduction in egg count have been achieved using *P. pastoris*-expressed *A. ceylanicum* protein in the permissive hamster model (Mendez *et al.*, 2005). Results have been more variable in dogs, with protection using *P. pastoris*-expressed Ac-ASP-2 ranging from an 8% to 47% reduction in worm burden, with a similar inconsistency in egg counts (Hotez *et al.*, 2003 HHVI 3rd Annual Report).

Reductions in female worm lengths induced by vaccination are common observations, particularly with vaccines targeting digestive processes, for example gut associated cysteine proteases (Redmond and Knox, 2004). In this situation, it is thought female worm growth and egg production are compromised by nutrient depletion, the latter imposing a considerable metabolic burden on female worms which is not experienced by the males. A similar level of size reduction in adult worms to that found in this study has been reported in *A. ceylanicum* recovered from hamsters vaccinated with ASP-2 (Goud *et al.*, 2004). This is also similar to the significant reduction in length (10%) of female *H. contortus* induced by previous infection of sheep with *Oestrus ovis* (Terefe *et al.*, 2005). The authors explain this phenomenon by the enhanced recruitment of activated inflammatory cells.

Pre-incubation of *H. contortus* ExL3 with neat sera from the group vaccinated with the unbound fraction significantly reduced the penetration and establishment of larvae in explanted abomasal tissue compared with L3 incubated in pre-vaccinate sera. The addition of sera generally increased the percentage of larvae becoming tissue associated, possibly reflecting activation of the larvae in coming into contact with host fluids. *In vitro* bioassays have been used to investigate the effect of mucous from immune cattle on *O. ostertagi* larval migration (Claerebout *et al.*, 1999); the authors reported that both mucous and sera from partially immune cattle contain larval migration inhibitory activity. The results reported here are the first to use an abomasal *in vitro* direct challenge assay to assess the effect of pre-incubation of larvae with sera on larval establishment. The level of reduction fell short of the levels seen when incubating *T. circumcincta* L3 with abomasal explants from immune animals; of course in this case the tissue will contain local antibody and immune cells primed by previous infection (Jackson *et al.*, 2004). Nonetheless, the level of reduction in larval establishment is similar to the level of larval migration inhibition immune sera had on *O. ostertagi* L3 in comparison to naive sera (Claerebout *et al.*, 1999). Also worth noting is that the larval establishment is reduced by unbound sera compared to not only pre-vaccinate control sera but also to bound vaccinate sera. In analogous experiments with hookworm larvae, incubation with sera from dogs vaccinated with irradiated L3 (Fujiwara *et al.*, 2005),

AcASP-2 or AcMTP (Williamson *et al.*, 2006) reduced skin penetration by up to 60%; it is noteworthy that the *H. contortus* MTP activity partitions with the unbound fraction. Perhaps, immune inhibition of the MTP prevents larval penetration of the abomasal glands?

The protection achieved was considerably lower than that reported in a similar study where the protective efficacy of similar sub-fractions of L3 ES proteins were evaluated using a guinea pig model (Sharp and Wagland, 1998). These authors reported levels of protection of up to 85% as judged by worm numbers although the statistical significance was not reported. Also, the guinea pig is an unnatural and non permissive host for *H. contortus* (worms do not develop to adults, Wagland *et al.*, 1989) and as such is far from ideal as a vaccine model. Computer modelling predicts that a vaccine which stimulates 60% protection in 80% of the flock on the basis of worm burdens would provide effective control in the field (Barnes *et al.*, 1995). Against this background, it is clear that the L3 lentil lectin affinity binding fraction tested here would be an ineffective vaccine. Future work could focus on further purifying ES components in an attempt to more closely define the proteins responsible for the protection observed here. It is possible that only trace amounts of the actual protective protein were actually injected here and that increasing this may increase the magnitude of the protective immune response. It is also possible that 2 or more protective components that might act in a synergistic manner in whole ES partition into different lectin chromatography fractions.

Animals vaccinated with bound or unbound material showed a strong IgG response with those animals vaccinated with the unbound fraction having a higher titre (at least a 2-fold higher mean titre) than those vaccinated with the bound fraction. Animals vaccinated with the unbound fraction also showed a strong IgE response whereas those vaccinated with the bound fraction did not. These results are most likely explained by the fact that animals vaccinated with the unbound fraction received 7 times the quantity of protein than those vaccinated with the bound fraction. However, previous vaccine trials using *Haemonchus* have resulted in a protective effect using less than 3 μg of antigen (Redmond and Knox, 2004); in this trial, animals vaccinated with the bound and

unbound fractions received 5 μg and 35 μg protein per immunisation respectively. Circulating antibody titres have been closely correlated with successful immunisation against *Haemonchus* using gut-expressed antigens (Smith *et al.*, 1994; Smith *et al.*, 1996). Here, there was no correlation between circulating IgG or IgE levels and egg counts or worm counts. This may simply reflect the small differences in the parasitological parameters observed when vaccinates were compared to controls as well as the small number of animals used in the trial. However, it is worth noting that vaccination studies using a Thiol Sepharose-binding fraction of adult ES (Bakker *et al.*, 2004) and a purified larval surface antigen (Jacobs *et al.*, 1999) found no correlation between circulating or mucus antibody levels and protection.

Vaccination with the adult *H. contortus* ES proteins Hc15 and Hc24 (an ASP-2 homologue) promoted good levels of protection with 70 and 99% reduction in FEC and worm count, respectively (Schallig and Van Leeuwen, 1997; Schallig *et al.*, 1997; Kooyman *et al.*, 2000). Again vaccination was accompanied with a general increase in total IgG levels but no correlation with protection was found. Of interest though is that protection with this fraction in lambs is, when age is controlled for, correlated with IgE, not IgG1 (Kooyman *et al.*, 2000). This, combined with the increase seen in abomasal eosinophil and mast cell infiltration, led the authors to suggest that vaccination with ES proteins mimics a natural immunity with the hallmarks of a Th2 response. These studies suggest that the local immune response may play an important role. The present study could be extended to analyse other circulating antibody isotypes and those found at the abomasal surface as well as examining the cellular response to vaccination. In other vaccination studies which used QuilA as adjuvant, significant local antibody and cellular responses were observed (Redmond and Knox, 2004; Geldhof *et al.*, 2002).

Western blot analysis with vaccinate sera from individual animals (data not shown) and pooled sera from each group (**Figure 4.2.1**) demonstrated that a variety of whole ES antigens were recognised following vaccination with either the bound or unbound ES fractions. The antigens that were strongly recognised with antisera were not necessarily the most prominent on a peptide profile of the

immunogen used; a greater variety of proteins were recognised by the sera than were visible on SDS-PAGE profiles of ES (compare **Figures 4.2.1** and **3.2.1**). Some parallels could be drawn between areas of highest immunogenicity when probed with the bound sera and proteins most visible in the lectin binding profile at ~60 kDa and 98 kDa (**Figure 3.2.7**), although it is notable that the highly lentil lectin reactive ~40 kDa protein was only weakly recognised by bound sera. The proteins most highly recognised by the unbound sera are not visible in the unbound immunogen, however a protein at ~33 kDa is visible in later ES protein profiles (see **Figure 3.2.1**). The bulk of the bound and unbound sera reactivity was abolished by periodate treatment (**Figure 4.2.2**), a treatment which oxidises carbohydrate epitopes (Woodward *et al.*, 1985). Clearly, the glycan components of L3 ES are highly immunogenic. Periodate treatment did reveal that a limited number of ES peptides was recognised by the pooled sera of both groups and, somewhat surprisingly, that the prominent immunogenic peptides in each fraction were similar in size (**Figure 4.2.2**). Peptides at ~40 kDa, 60 kDa and 98 kDa were recognised by sera from both groups, although the 40 kDa ASP homologue was expected to partition in the bound fraction (Sharp and Wagland, 1998). Of course these antigens could have the same molecular weight but be distinct and unrelated proteins. This could be clarified by undertaking a proteomic-based analysis of each extract. Moreover, the recognition profile of the unbound vaccinate group did differ from the bound with, for example, peptides between 22 and 31 kDa and above 98 kDa being recognised by sera from the unbound vaccinate group only.

In this study, a systemic immunisation regime, injecting the antigen intra-muscularly with the saponin adjuvant Quil A, was chosen. This protocol has been used successfully in several vaccine trials against *H. contortus* (Knox *et al.*, 1999; Redmond and Knox, 2004; Bakker *et al.*, 2004; Geldhof *et al.*, 2003) and Quil A is a commercially acceptable adjuvant (Cox and Coulter, 1997). Quil A induces both a Th1 and Th2 type response, associated with induction of circulating IgG (Cox and Coulter, 1997). As it is likely that the naturally protective response is Th2, inducing a Th2 response using an alternative adjuvant may increase protection with natural antigens (Geldhof *et al.*, 2004). It was previously found that sheep immunised with a larval surface antigen in Quil A

were not protected, however when immunised with the same antigen in a Th2 only inducing adjuvant (aluminium hydroxide) sheep were significantly protected (Jacobs *et al.*, 1999). However, Geldhof *et al.* (2004) found that protection against *O. ostertagi* in calves induced by vaccination with a subset of adult ES proteins using Quil A as adjuvant was not repeatable when antigen was delivered with aluminium hydroxide. It is quite possible that by switching adjuvant, protection levels using the bound (or unbound) fraction could be increased considerably.

A further feature of the vaccine trial was the single challenge dose of 5,000 *H. contortus* L3. Though routinely used for testing adult antigens it is perhaps not the best vaccination strategy for testing larval antigens as it may overwhelm the naive animal with a high one-off dose, though it should be pointed out that a dose in this region or even higher may be expected during the high transmission season in the tropics. A trickle infection might be more suitable as in this study (using larval antigens), antibody/cells primed by vaccination will only target developing larvae; by the time worms have developed into adults the vaccine may not work. However as a vaccine evaluation strategy this would have drawbacks; it would be very difficult to distinguish between any potentially protective effects of the vaccine as opposed to an acquirement of immunity from the trickle infection, which has been demonstrated in *H. contortus* (Emery *et al.*, 2000).

Because of the lack of availability of the native protein, ASP vaccine trials in hookworms have relied upon the use of recombinant proteins; the relatively low levels of protection may well be due to the recombinant proteins not performing as the native protein would. Trials using protein fractions enriched for ASP homologues in either *H. contortus* (Hc24, Schallig and Van Leeuwen, 1997; Schallig *et al.*, 1997; Kooyman *et al.*, 2000) or *O. ostertagi* (Geldhof *et al.*, 2003) have achieved much higher levels of protection. In the former study, the Hc 24 enriched ES fraction provided a far higher degree of protection than whole ES. In the latter, a fraction enriched for *O. ostertagi* ASPs protected calves against homologous challenge with a 60% reduction in egg count and a 16% reduction in worm burden. Interestingly, the ASP homologues identified in this fraction were

single domain, like the hookworm ASP-2, but showed homology to the N-terminal of hookworm ASP-1, reminiscent of the *H. contortus* larval Hc40 ASP-1 homologue (Geldhof *et al.*, 2003; see **Chapter 5**). Assuming the bound fraction is enriched for the L3 *H. contortus* ASP-1 homologue, the results reported here add to the evidence that native nematode fractions, enriched for ASP homologues, can confer significant protection; thus confirming the status of ASPs as lead hookworm specifically, and nematode generally, vaccine candidates. In this study, alternative adjuvant formulation and further fractionation might increase the protective efficacy of the lentil lectin-bound and unbound fractions. It is likely that the *H. contortus* larval ES also contains other homologues of hookworm vaccine candidates (for example, MTP activity has been identified, yet the protein responsible remains unknown) thus further fractionation and identification of some of these antigens would allow more evaluation of their potential efficacy.

In summary, vaccination with a non lentil lectin-binding fraction of *H. contortus* L3 ES resulted in a reduced FEC and final worm burden that, whilst not statistically significant, was lower than the adjuvant control group and pre-incubation of L3 with vaccinate sera significantly reduced larval establishment in an *in vitro* assay. The reduced egg count, significantly reduced adult worm burden and reduction in female worm length culminate to suggest that vaccination with the lectin binding fraction had a genuine, albeit modest, protective affect against homologous challenge.

Chapter 5
Further Characterisation of the *H. contortus* larval ES ASP-1 homologue.

5.1 Introduction

Members of the Ancylostoma secreted protein (ASP) family have been identified in a variety of parasitic nematodes since their discovery in *A. caninum* (Hawdon *et al.*, 1996). As described in detail in **Section 1.7.3.2**, upon activation *in vitro*, *A. caninum* L3 secrete two distinct types of ASP: a double domain type (ASP-1) with two pathogenesis-related domains and a single domain type with one pathogenesis-related domain (ASP-2), which shows homology to the C-terminal of ASP-1 (Hawdon *et al.*, 1996; 1999). Orthologues of both the double and single domain ASPs have been identified in the L3 stage of *A. duodenale*, *A. ceylanicum* and *N. americanus* (Zhan *et al.*, 1999). These sequences exhibit very high degrees of amino acid identity to the original *A. caninum* ASP-1 or ASP-2 sequences, for example the *N. americanus* ASP-1 is 97% identical to the *A. caninum* ASP-1. Despite being originally associated with the transition to parasitism, ASPs are also expressed by adult *A. caninum* worms: ASP-3 represents a homologue of the single domain protein; ASP-4, -5 and -6 represent double domain protein homologues (Zhan *et al.*, 2003). It is now recognised that ASPs are ubiquitous in many parasitic nematode species: single domain ASPs have been identified in filarial nematodes including *O. volvulus* (Tawe *et al.*, 2000) and *B. malayi* (Murray *et al.*, 2001) and in *O. ostertagi* (Geldhof *et al.*, 2003). Both single and double domain ASPs have been recognised in *H. contortus* (Rehman and Jasmer, 1998; Sharp and Wagland, 1998). The ASPs are related to the venom allergen-like proteins (VAP) of *C. elegans*.

The recent characterisation of the X-ray structure of *N. americanus* ASP-2 has indicated a structural and charge mimicry of chemotaxins, suggesting a possible immunomodulatory function (Asojo *et al.*, 2005) although, there is little experimental evidence for this. Early vaccination studies with *A. caninum* ASP-1 in the murine model suggested that antibody against recombinant ASP-1 might inhibit larval migration, indicating a chemosensory role for ASPs (Ghosh and Hotez, 1999). ASPs from *O. volvulus* can stimulate angiogenesis in the blood vessels of the cornea (Tawe *et al.*, 2000), though this is likely to be an indirect effect (Higazi *et al.*, 2003). Interestingly, *O. volvulus* ASP-1 is also a potent adjuvant, eliciting a high Ig titre of a mixed Th1/Th2 type (MacDonald *et al.*,

2005) perhaps suggesting an immunomodulatory role. Despite the function of the ASPs remaining poorly understood, they are likely to have an affect on host cells, and thus remain potential vaccine targets.

Discussed in both **Sections 1.7.3.2** and **4.3**, ASPs, both of the single and double domain type, induced protective immune responses in a number of vaccination models. *O. ostertagi* ASP-1 and ASP-2 were the most abundant proteins in a host-protective fraction of adult ES (Geldhof *et al.*, 2003); *O. volvulus* ASP-1 can induce an anti-larval protective immunity (MacDonald *et al.*, 2004). Of the hookworm species, vaccination with *A. caninum*, *A. duodenale* and *N. americanus* ASP-1 reduced worm burdens in the mouse model (Ghosh and Hotez, 1999; Liu *et al.*, 2000), and vaccination with *N. americanus* ASP-1 reduced mean FEC in dogs challenged with *A. caninum* (Hotez *et al.*, IHHVI 3rd Annual Report). *A. caninum*, *N americanus* and *A. ceylanicum* ASP-2 have all shown considerable promise as vaccine candidates (Hotez *et al.*, 2003; Goud *et al.*, 2004). The *H. contortus* ASP-2 homologue, Hc24, protects sheep against homologous challenge (Schallig *et al.*, 1997a; Vervelde *et al.*, 2002) and ES enriched for the L3 ASP-1 homologue can reduce worm burden in guinea pigs (Sharp and Wagland, 1998). Described in **Chapter 3,** vaccination of sheep with a fraction of *H. contortus* ES purportedly enriched for the larval HcASP-1 significantly reduced adult worm burden and led to a reduction in female worm length and a trend towards reduced faecal egg output. It would be of benefit to establish whether this lectin binding fraction was genuinely enriched for the larval HcASP-1 protein.

This chapter describes the work that was undertaken to ascertain the temporal pattern of expression and to obtain full length sequence of the previously described *H. contortus* larval ASP-1 homologue. Furthermore it was the aim to express the larval HcASP-1 as a recombinant protein and produce an antibody probe thus enabling localisation studies.

5.2 Results

5.2.1 Temporal expression of HcASP-1 mRNA

RT-PCR using primers ASP-3 forward and ASP-4 reverse (**Section 2.6.**1) was carried out on approximately 50ng of cDNA made from various life stages of *H. contortus* (egg, L3, ExL3, L4, day 11 immature adults, day 28 adults). A product of the appropriate size (660 bp) was amplified from ExL3, L4 and adult stage cDNA (**Figure 5.2.1**). Despite the bands being visibly brighter in the L4 and day 11 samples, a conclusion about levels of template expression cannot be drawn due to the non-quantitative nature of this PCR.

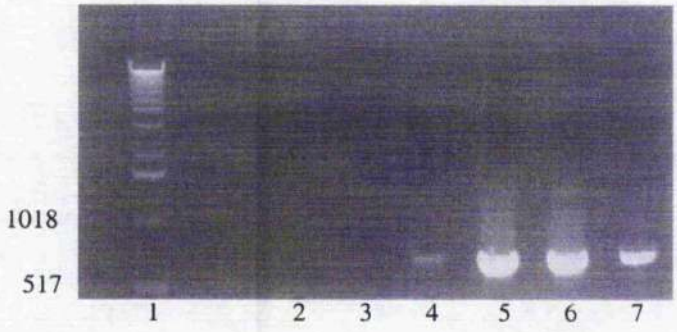


Figure 5.2.1: Expression of ASP-1 homologue in different life stages of *H. contortus*. PCR on cDNA prepared from various stages of *H. contortus* using primers designed to the larval HcASP-1 homologue.
1 – DNA ladder (bp); **2** – egg; **3** – L3; **4** – ExL3; 5 – L4; **6** – day 11 adults; **7** – day 28 adults.

5.2.2 RACE PCR to obtain full 5' sequence

The published HcASP-1 (Sharp and Wagland, 1998) sequence lacked a 5' initiation codon. The 5' end containing the ATG (Met) start codon was isolated from ExL3 cDNA by 5' RACE PCR (**Sections 2.5.2** and **2.6.4**) using a gene specific primer (ASP-5'-RACE) designed to a region of the published sequence (**Figure 5.2.2**). Three 5' end clones were sequenced and found to encode identical sequences that were in frame with and overlapped the published sequence. The full length amino acid sequence (**Figure 5.2.2**) encodes an open

reading frame (ORF) of 226 amino acids and a predicted molecular weight of 24.7 kDa with a pI of 5.02. The amino-terminal 19 amino acids are highly hydrophobic and analysis using the Signal-P software indicates this a signal peptide with a predicted cleavage site between positions S_{19} and A_{20}. The full length protein contains 4 potential *N*-linked glycosylation sites and 12 cysteine residues (**Figure 5.2.2**).

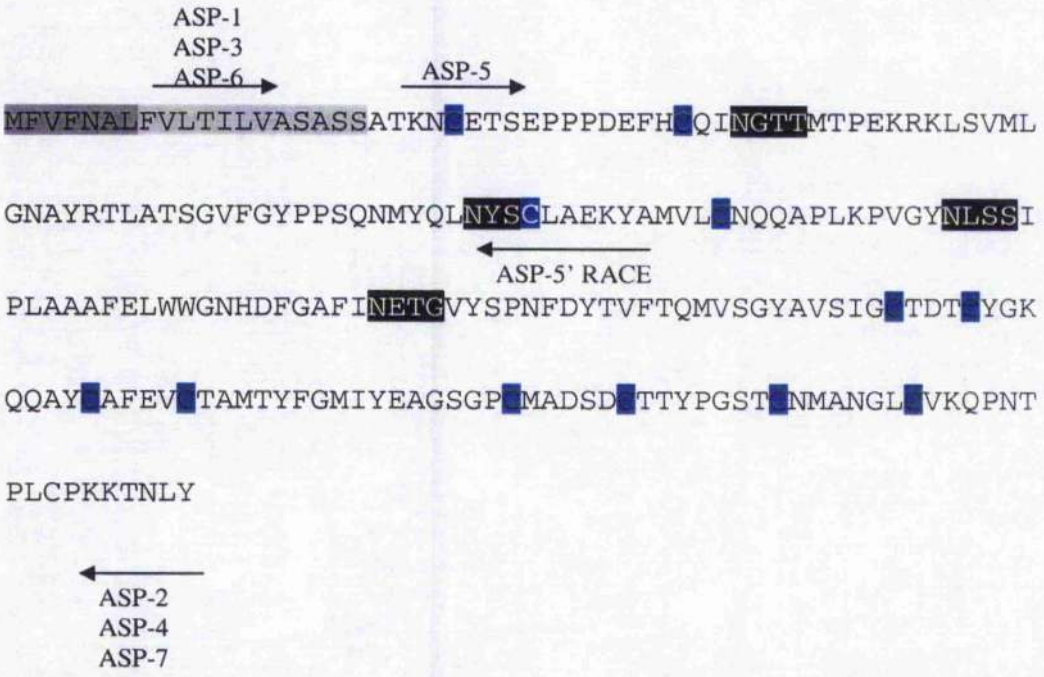


Figure 5.2.2: Amino acid sequence of the elucidated full length HcASP-1 protein. The signal peptide sequence is boxed in grey. The 7 amino acids extended by 5' RACE PCR are boxed in dark grey. Predicted *N*-linked glycosylation sites are boxed in black and cysteine residues are boxed in blue. Primer sites are marked above or below indicating the forward or reverse nature as appropriate. The primers differed slightly as they were designed for different expression systems or RACE extension as detailed in tables 2.6.1 and 2.6.2 in Chapter 2.

A BLAST search (February 2004) of the published *H. contortus* EST sequences (http://zeldia.cap.ed.ac.uk/nematodeESTs/nembase) showed 17 clusters that contained sequences that demonstrated a significant homology to the HcASP-1 protein, including a sequence matching the published 5' truncated HcASP-1 exactly, indicating a gene family (**Table 5.2.1**). Of note, all the sequences were

from adult cDNA libraries and the areas of greatest divergence were at the amino-terminal end.

Table 5.2.1: HcASP-1 like sequences from the *H. contortus* EST dataset. All ESTs are from adult cDNA libraries.

EST Cluster	Accession numbers	Identity to HcL3ASP-1	Contig Length (aa)	Closest homologue
HCC00180	A30245	100%	219	Identical to HcL3ASP-1
HCC00248	BF059782, BF060135, BF662831, BM173860, BU665925	77%	217	AAM54195 VAP-1 protein (*C. elegans*)
HCC04130	CB012540	72%	228	AAC69015 VAP-1 protein (*C. elegans*)
HCC00248	BE496784	73%	220	AAM54195 VAP-1 protein (*C. elegans*)
HCC00248	BE496761, CB014990	73%	218	AAC69015 VAP-1 protein (*C. elegans*)
HCC00548	CB015234, CB015315	72%	219	AAM54195 VAP-1 protein (*C. elegans*)
HCC01296	BF422978, BF423346, CB015186, CA868666, CA869655	70%	164	No Hit
HCC00548	CB015570	71%	216	No Hit
HCC04130	CA869697	71%	206	No Hit
HCC00248	CB016123, CA869755	71%	198	No Hit
HCC00548	BF060300, BF423191, CB015942, CB016035, CB016213	66%	225	No Hit
HCC01296	CA033884	71%	197	No Hit
HCC04130	CB015930, CA870089, CA958015	72%	185	No Hit
HCC00248	BF060342, BF422799, BF423385, CA957984	72%	187	No Hit
HCC04130	CA958199	75%	182	No Hit
HCC00248	CA959003, CB019980	72%	181	No Hit
HCC03964	CA869433	83%	116	No Hit

Multiple alignments of the full length HcASP-1 protein sequence with the *A. caninum* ASP-1 and ASP-2 sequences showed the HcASP-1 to align closer to the N-terminal of the ASP-1 protein (**Figure 5.2.3**), despite having a total length that would infer it is a single domain type ASP (like ASP-2). HcASP-1 showed 26% identity and a 39% similarity to the N-terminal of *A. caninum* ASP-1 and a 21% identity and a 35% similarity to *A. caninum* ASP-2. As is typical within the ASP family, the cysteine residues are highly conserved.

Figure 5.2.3: EMBOSS alignment showing regions of homology between the N-terminal of *A. caninum* ASP-1 and the HcASP-1 proteins. Regions of amino acid identity are shaded in dark grey; regions of high similarity scores are shaded in light grey; conserved cysteine residues are boxed in blue.

Phylogenetic analysis was carried out using the published *A. caninum*, *O. volvulus*, *B. malayi* and *O. ostertagi* ASPs and the *H. contortus* Hc24 and Hc40 and HcASP-1 proteins. A dendrogram was created using the neighbour-joining method in the MEGA version 3.1 software and is shown in **Figure 5.2.4**. There was strong support (88%) for a clade containing the *A. caninum* ASP-1 and -2, the *H. contortus* Hc24 and Hc40, and the *C. elegans* VAP-1. The HcASP-1 would appear to fit best within the clade containing the filarial ASPs (69% bootstrap support).

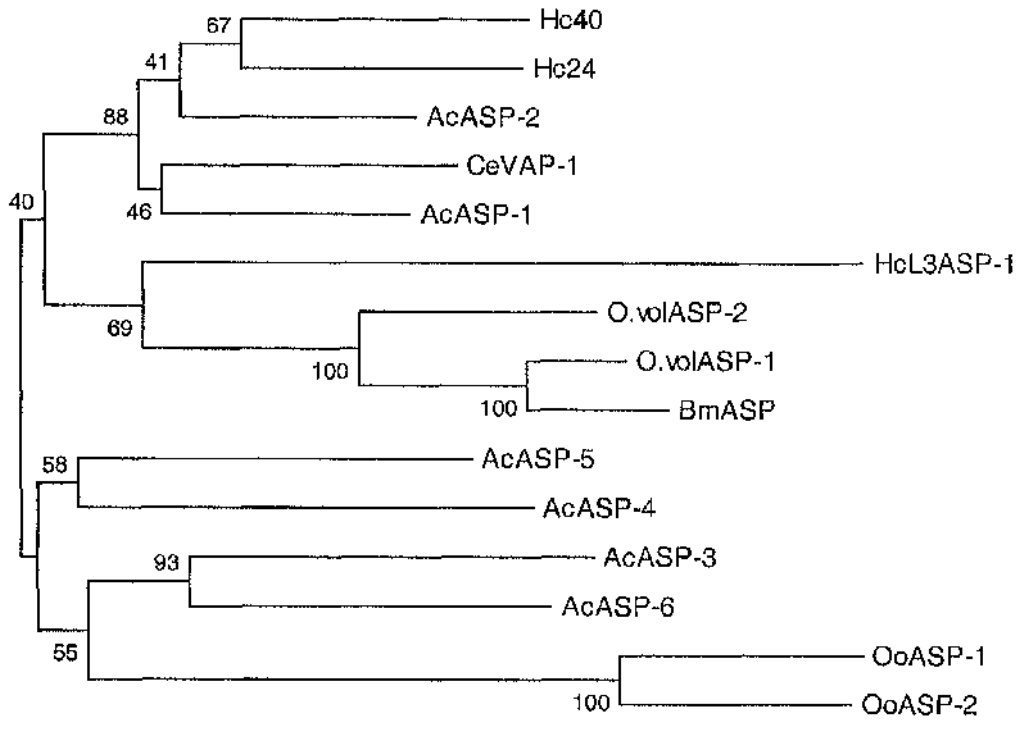


Figure 5.2.4: Dendrogram of the ASPs from *A. caninum (Ac), O. ostertagi (Oo), O. volvulus (O.vol), B. malay (Bm)i, C. elegans (Ce)* and *H. contortus*. Numbers indicate bootstrap support values for the groups distal to the labelled node. Divergence is indicated by length of line.

5.2.3 Attempted expression of HcASP-1

The published, slightly 5' truncated, form of HcASP-1 was successfully cloned into the pET 22b and the pGEX-6P expression vectors either with or without the putative signal peptide and the pIVEX2.3d vector with the signal sequence, as indicated in **Sections 2.6.1** and **2.6.2** and **Table 2.6.2**. Successful amplification of HcASP-1 using adapted primers from ExL3 cDNA, cloning into pGEM-T and subcloning into an expression vector is shown in **Figures 5.2.5** and **5.2.6** for the HcASP-1 with no signal peptide into pET22b. Sequencing of the correct insert in the expression plasmid sequence (**Figure 5.2.6**) demonstrated correct in frame cloning.

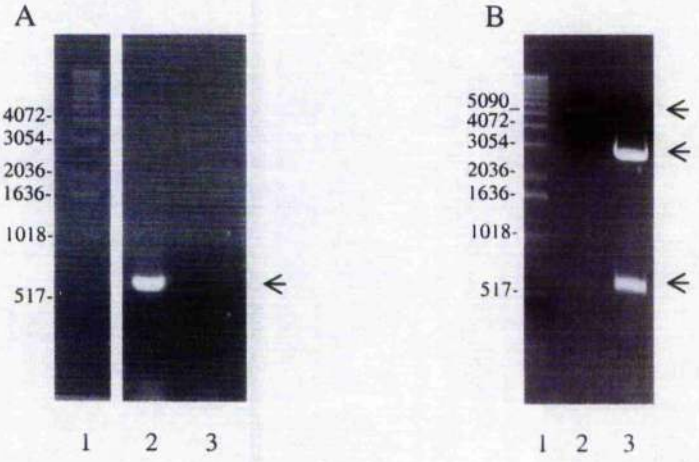


Figure 5.2.5: Cloning strategy of HcASP-1 without signal sequence into pET 22b for expression of recombinant protein.

Panel **A** shows PCR of HcASP-1 using primers ASP-5 –forward/ ASP-4-reverse from ExL3 cDNA. **1**- DNA ladder (bp); **2**- PCR on ExL3 cDNA; **3**- no template negative control. Arrow points to product of 630 bp.
Panel **B** shows restriction enzyme digests using *Nde*-I and *Xho*-I of empty pET 22b and of pGEM-T containing the HcL3ASP-1 amplified in **A**. **1**- DNA ladder (bp); **2**- linearised pET22b plasmid; **3**- linearised pGEM-T and HcL3ASP-1 (without signal peptide) cut out of plasmid following *Nde*-I /*Xho*-I double digest. Top arrow points to pET22b (5493 bp); middle arrow points to pGEM-T (3000 bp); bottom arrow points to HcL3ASP-1 (620 bp).

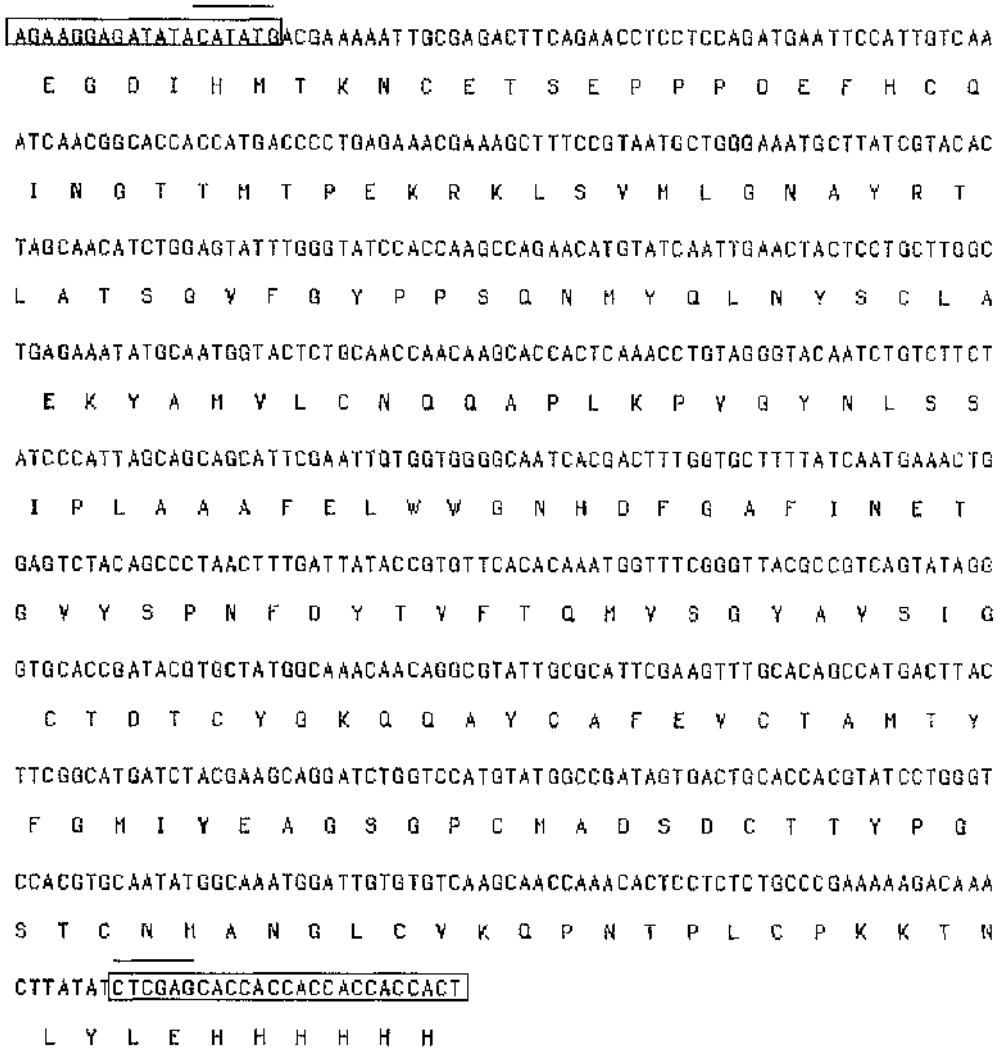


Figure 5.2.6: Nucleotide sequence and amino acid translation demonstrating successful in frame cloning of HcASP-1 without signal sequence into pET 22b for expression of recombinant protein. Boxed regions indicate plasmid vector sequence and the restriction enzyme sites used for cloning are highlighted by a black line above the sequence.

Following sequencing reactions to confirm the HcASP-1 sequence was cloned in frame within the vector in all instances, competent cells (BL21 and BL21 codon plus cells were both tried) were transformed with plasmid containing the HcASP-1 sequence and expression was attempted as described in **Section 2.3.13**. Soluble and insoluble cell fractions and supernatants were analysed by SDS-PAGE and the presence of recombinant protein sought by comparison with cell fractions made from induced cells containing the appropriate vector with no

insert, and non-induced cells. In the case of the Rapid Translation expression system, protein was analysed as before, and a band at the estimated molecular weight sought by visual inspection. In all attempts, no expression of the HcASP-1 protein was observed using any of the aforementioned systems (data not shown). Western blot analysis of cell fractions, probing with an HRP labelled anti-Histidine tag antibody also proved negative (data not shown).

5.3 Discussion

Originally identified in *A. caninum* larvae and now recognised as ubiquitous throughout the parasitic nematodes, the ASPs are promising vaccine candidates in a number of species (Hotez *et al.*, 2003). The single domain type ASP-2 is the most promising larval vaccine candidate against hookworms and as such is going into Phase 1 clinical trials (Goud *et al.*, 2005). The double domain hookworm ASP-1 has also shown promise as a vaccine candidate, and both single and double domain ASP-1 homologues in other species are hopeful vaccine candidates (Sharp *et al.*, 1992; Sharp and Wagland, 1998; Geldhof *et al.*, 2003). Here, further characterisation of the *H. contortus* L3 ASP-1 (HcASP-1) homologue, identified in a non-permissive host-protective fraction (Sharp and Wagland, 1998) and potentially enriched for in the modestly host-protective lectin-bound ES fraction described in **Chapter 4,** is provided. The HcASP-1 was cloned, full length sequence confirmed by comparison to the described sequence (Sharp and Wagland, 1998), and PCR carried out to investigate temporal expression. Bacterial expression of the recombinant protein was attempted with the aim of conducting a vaccine trial in sheep but this failed in several systems.

PCR using cDNA prepared from the different life stages of *H. contortus* demonstrated expression of the HcASP-1 mRNA transcript in the ExL3, L4 and adult stages. This concurs with the protein being expressed by the larvae following the transition to parasitism as reported by Sharp and Wagland (1998), though these workers did not investigate the possibility of the protein being expressed by the adult stages. *A. caninum* ASP-1 and ASP-2 proteins are secreted following L3 activation (Hawdon *et al.*, 1996; 1999), and protein expression is confined to the L3 stage. However, the mRNA transcript for the ASP-2 is present throughout all life stages of the parasite (Hawdon *et al.*, 1999).

The published HcASP-1 sequence lacked a 5' initiation codon. Here, the 5' end containing the ATG (Met) start codon was isolated by 5' RACE PCR. Like the other members of the ASP family, HcASP-1 contains a signal peptide, indicating it is likely to be secreted. The predicted molecular weight of the full ORF,

including the signal peptide, is 24.7 kDa; however the native protein migrates with a molecular weight of approximately 40 kDa on an SDS-PAGE gel (**Chapter 3**; Sharp and Wagland, 1998). The mature protein has 4 predicted *N*-linked glycosylation sites which, if occupied, would account for some of the difference between the predicted and observed molecular weights. The adult *A. caninum* ASP-4 and ASP-6 have 2 and 1 *N*-linked glycosylation sites respectively, and both migrate with a larger molecular weight than that predicted by sequence alone; the former with a relatively larger weight increase reflecting the 2 glycosylation sites (Zhan *et al.*, 2003). A feature of the ASP family is the high conservation of cysteine residues (Hawdon *et al.*, 1996). As the cysteine residues are involved in protein structure through disulfide bonding, it is possible that, despite the relative divergence in other parts of the sequence, a similar secondary structure is shared by the ASP-like proteins and HcASP-1. Although the function of ASPs remains unknown, the recently elucidated X-ray structure of the *N. americanus* ASP-2 led the authors to suggest a chemokine-like function, perhaps as an extracellular ligand of an as yet unidentified receptor (Asojo *et al.*, 2005). The ASPs are represented in *C. elegans* by a protein family with at least 20 members designated venom allergen-like proteins (VAPs) or testis-specific proteins, the vast majority of which are of the single domain type. Nothing is known of the function and RNAi experiments, the results of which are published on http//:www.wormbase.org, have not indicated a phenotype other than wildtype.

A search of the *H. contortus* EST dataset with the HcASP-1 sequence identified 16 contiguous sequences with a significant identity over the length of the EST indicating that the HcASP-1 is a member of a gene family. Interestingly all these sequences were identified from adult cDNA libraries inferring these genes are expressed by adult stage worms and were not present in the L3 EST dataset. However, there are some 15,000 adult ESTs compared to 1,100 from L3 and L4s. Given that the native protein has been confirmed in L3 ES (Sharp and Wagland, 1998), that transcript is detectable by RT-PCR (**Figure 5.2.1**) and that the latter assay indicates a lower abundance compared to 11 day and adult worms, it is likely that the absence of ASPs from the L3 EST dataset simply reflects low

transcript levels. The construction of the L3 EST dataset from sheathed rather than ExL3s may also provide an explanation for the HcASP-1 not being present.

The ASPs belong to a phylogenetically diverse group; analyses using representative ASPs from several nematode species including the hookworms, filarids, *C. elegans* and *O. ostertagi* imply the HcASP-1 to group in a clade with the filarid ASPs. The *H. contortus* Hc24, Hc40 and the *A. caninum* ASP-1 and -2 proteins comprise a different and distinct clade perhaps suggesting a function different to that of the HcASP-1.

Despite repeated attempts, with both the full length available sequence and a truncated version without the signal peptide, bacterial expression of the ASP-1 homologue continued to evade success. Constructs in the pET22b and pGEX vector systems were exhaustively sequenced to confirm correct in frame cloning. A final attempt at expression, using a Roche cell free rapid translation expression system, also proved unproductive. Difficulty in expressing bacterial recombinant ASPs have been reported by several workers (Zhan *et al.*, 2003; P. Geldhof, personal communication; M. E. Botazzi, personal communication). In spite of this, successful bacterial expression of several ASPs, including the HcASP-1 without the signal peptide, has been reported (Sharp and Wagland, 1998; Murray *et al.*, 2001; Hawdon *et al.*, 1999). The HcASP-1 protein was cloned into the pBTA721 vector and expressed as a fusion protein in *E. coli* (Sharp and Wagland, 1998) and the AcASP-1 and AcASP-2 were cloned and expressed using the pET vector system and BL21 cells (Hawdon *et al.*, 1996; 1999). Reasons for the failure of HcASP-1 expression in this study are unclear as the pET and BL21 system have worked for other ASP family proteins. The same system used in this study has also resulted in successful expression of other *H. contortus* genes (see **Chapters 6** and 7). Codon usage, a problem sometimes encountered when attempting to express *H. contortus* proteins (D. Redmond, personal communication), was addressed by also using a codon plus cell line (BL21 DE3 RIPL codon plus). This is a cell line that contains extra copies of the genes that encode tRNAs that are rarer in *E. coli* (arginine, leucine, isoleucine and proline) and is designed to allow expression of non-*E. coli* proteins that contain these amino acids. Zhan *et al.* (2003) found expression of the protein

without the signal peptide successful when the full length protein failed to express; this was attempted without success. ASPs are cysteine rich and recombinant production could result in aberrant folding due to the high number of disulphide bridges; however this is more likely to affect the solubility of an expressed protein, rather than prevent expression *per se*. An alternative to this might have been expression in a eukaryotic system such as the methane utilizing yeast (*P. pastoris*) system or an insect cell line, as these have both proved successful for ASP proteins (Hotez *et al.*, 2003). However, attempting expression with a eukaryotic system was deemed beyond the time constraints of this project. The lack of a recombinant HcASP-1 prohibited antibody probe production. Antiserum to ASP-1, kindly provided by Professor Peter Hotez, did not recognise any proteins in *Haemonchus* adult worm extracts or L3 ES indicating a, somewhat surprising, lack of antigenic cross-reactivity. Thus it was impossible to carry out localisation studies to confirm the presence of the HcASP-1 in the fractionated ES used in the **Chapter 4** vaccine study.

In summary, the work described in this chapter shows that HcASP-1 is more homologous to ASPs from filarial nematodes than those from hookworms and those already identified in the ES products of adult *H. contortus*. RT-PCR indicated that the transcript is present at in adult worms, L4 and exsheathed L3, suggesting that the protein product has functional significance for adults as well as larvae. Attempts to express the protein in bacteria failed and future work would focus on expression in yeast or insect cells. Another possibility would be to synthesise the gene with *E. coli* codon usage. Recombinant protein can be used to raise antibodies to permit the identification of the protein in the L3 ES fractions discussed in chapter 4 and for immunolocalisation in L3 and adult worms. The latter experiments might provide an indication of *in vivo* function. Finally, a recombinant protein could be used as an immunogen in a vaccine trial.

Chapter 6

Identification and characterisation of an *A. caninum* transthyretin-like protein homologue in *H. contortus*

6.1 Introduction

Upon activation *in vitro*, *A. caninum* L3 release several proteins likely to be important in the transition to parasitism, thus prime vaccine candidates. These include the previously discussed ASP-1 and ASP-2, a MTP and a transthyretin-like protein (TTR) (Hotez *et al.*, 2003). The latter was originally identified by screening an *A. caninum* L3 cDNA library with sera from patients naturally infected with hookworm (Hawdon *et al.*, Society of Protozoologists, 53rd Annual Meeting, June 25-28, 2000). The *A. caninum* TTR belongs to a large nematode-specific protein family, represented by at least 52 genes in *C. elegans*, that show a loose homology to mammalian transthyretin (19% amino acid identity to human TTR) and a family of transthyretin-related proteins (TRPs) represented in nematodes, plants, bacteria and animals (Eneqvist *et al.*, 2003).

Transthyretin in mammals acts as a transport protein for thyroid hormones. In conjunction with retinol-binding protein it also transports retinol (Schreiber *et al.*, 1997). The TRP family are, as the name suggests, related to transthyretin (approximately 35% identical) and likely to have a similar homotetrameric structure, although the function is not well understood. RNAi loss of function studies carried out on the two TRP genes in *C. elegans* resulted in no observed phenotype (Eneqvist *et al.*, 2003). The nematode-specific TTR-like proteins are distinguished from the TRPs, by their lack of a highly conserved four amino acid motif at the C-terminal (Enequist *et al.*, 2003). Little is known about the function or structure of the nematode TTR-like proteins.

Despite the function of the AcTTR remaining unknown, it has been suggested that it might scavenge host retinol (Hotez *et al.*, 2003). Recombinant AcTTR has been evaluated in at least two protection trials, the first in the dog-*A. caninum* natural host system and the second in the hamster-*A. ceylanicum* model. In the former, antibody titre in response to vaccination was low. However, there was a significant inverse relationship between AcTTR specific IgE titre and worm burden (Hotez *et al.*, 2003). The data from the hamster model trial is not yet

available, however it has been reported that a similarly low antibody titre was elicited (Hotez *et al.*, HHVI 3rd Annual Report).

There are several TTR-like genes in the *H. contortus* ESTs represented in libraries of all life stages. A proteomic analysis of the proteins excreted/secreted by adult *H. contortus* in culture identified several TTR-like proteins. Of interest, several of these TTR-like proteins were found to be amongst the most immunogenic proteins released by adult worms as judged by Western blot using sera from sheep naturally immune to *H. contortus* (Yatsuda *et al.*, 2003).

On the basis of the reported immunogenicity of TTR-like proteins and the promise shown by the AcTTR as a vaccine candidate, a homologue of the AcTTR was sought in *H. contortus* and the biology and suitability as a potential vaccine candidate investigated. This chapter describes the identification of a homologous TTR in *H. contortus,* its expression and characterisation and studies carried out to identify the site of expression of the native protein.

6.2 Results

6.2.1 Reactivity of *H. contortus* ES and extracts with anti-recombinant *A. caninum* TTR serum

To attempt to seek a homologue of the *A. caninum* TTR-like protein in *H. contortus*, extracts and ES were probed with sera against the AcTTR, as described in **Sections 3.2.3** and **2.2.4**. Larval extracts and ES from *H. contortus* were screened by Western blot and probed with rabbit sera raised against a bacterial recombinant AcTTR (kindly provided by Professor P. Hotez). The AcTTR probe labelled a protein of approximately 16 kDa in larval soluble extract, but not in ES from any time point in culture (**Figure 6.2.1**). The predicted molecular weight of the AcTTR is 16 kDa thus the cross-labelling is likely to be of a similar protein. A second protein band at an estimated 60 kDa was also labelled, though the identity of this band remains unknown.

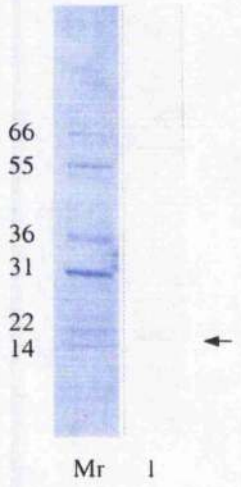


Figure 6.2.1: Western blot of *H. contortus* L3 S1 extract probed with a primary anti-recombinant AcTTR sera (1:2000 dilution) and HRP labelled secondary anti-rabbit IgG (1:1000 dilution). **1** - *H. contortus* L3 S1 extract. An arrow marks the putative HcTTR between 14 and 22 kDa in size.

6.2.2 Identification of *A. caninum* TTR homologue in *H. contortus* EST dataset

Having confirmed cross-reactivity of sera raised against the AcTTR with a band of the appropriate size in *H. contortus* extract, a bioinformatic approach was taken to investigate TTR-like proteins within the available *H. contortus* dataset. The published *H. contortus* EST dataset was searched (May 2003) with the *A. caninum* TTR amino acid sequence (kindly provided by Professor P. Hotez) as described in **Section 2.4.1**. Seventeen contiguous sequences (**Table 6.2.1**) produced significant alignments (similarity score over 150) with the AcTTR. The full length EST CA994587, clustered in HCC01741, proved the closest homologue. Full length cDNA containing the CA994587 was amplified by PCR and cloned (**Section 2.4.2** to **2.4.3**). The full length gene was designated HcTTR.

Table 6.2.1: Closest homologues to the AcTTR protein in the *H. contortus* EST dataset. The % identity over the length of the sequence and the corresponding E-value are shown.

Cluster containing contig	Accession numbers in contig sequence	% identity to AcTTR	E-value	Stage
Hcc01741	CA994587, CA994643	64	8e-48	Egg
Hcc01741	BF423360 , CB020963	62	8e-48	Adult
Hcc00775	BF186796, BM173984, BM174173, CA994790	61	2e-47	L4
Hcc00775	CA994783	61	2e-47	L4
Hcc02240	BM138829, BM138889, BM138926, BM139052, BM139325, CB012730	57	3e-42	Adult
Hcc02240	CB013103, CB015232	57	3e-42	Adult
Hcc02240	CB012455	57	3e-42	Adult
Hcc02240	CB020655	57	3e-42	Adult
Hcc00756	BF186811, BM174148	54	7e-42	L4
Hcc02251	BM138844, CB018766, CA957008, CB020778	53	9e-42	Adult
Hcc00365	BM139192, BF059980	56	1e-41	Adult
Hcc00365	CA957899, CB020811, BF060064, BF422778, BF423083	55	6e-41	Adult
Hcc02849	BM174112, BM174127	55	2e-40	L4
Hcc00365	BM138977	55	6e-40	Adult
Hcc00365	CB013024, AW670763	58	2e-39	Adult
Hcc00756	BF186771,	52	4e-39	L4
Hcc00365	CB021070, CA869898	57	2e-38	Adult

Closer examination of the HCC01741 cluster that contained the CA994587 sequence showed it to comprise of 2 contiguous sequences (Contigs), the first containing sequences BF423360 and CB020963 from an adult cDNA library; the second, CA994587 and CA994643 from an egg cDNA library. A Clustal alignment of the predicted protein sequence from Contigs 1 and 2 is shown in **Figure 6.2.2**. As can be seen, a full ORF is not available for Contig 1 but, other than the first residue, the sequences are identical at the amino acid level thus likely represent the same gene.

Figure 6.2.2: Clustal alignment of the protein sequences encoded by the 2 Contigs in cluster HCC01741. Contig 1 contains sequences BF423360 and CB020963; Contig 2 CA994587 and CA994643. Regions of identity are highlighted in dark grey; amino acids showing a high similarity score are highlighted in light grey.

The HcTTR shows a 64% identity and a 74% similarity across the whole sequence to the AcTTR and has an ORF of 152 amino acids compared with the AcTTR ORF of 144 amino acids. The predicted molecular weight of the HcTTR is 16.9 kDa. Like the AcTTR, the first 15 amino acids of HcTTR constitute a predicted signal peptide with a likely point of cleavage between A_{15} and M_{16}; the predicted molecular weight of the protein without signal peptide is 15.3 kDa. An alignment highlighting regions of homology between the AcTTR and the HcTTR is shown in **Figure 6.2.3**.

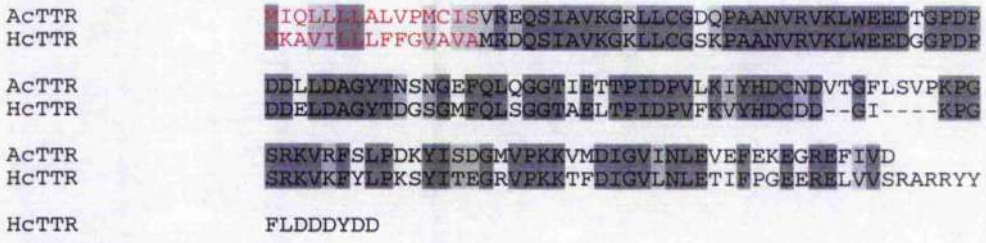


Figure 6.2.3: A Clustal alignment of the HcTTR and AcTTR protein sequences. Regions of identity are highlighted in dark grey; amino acids showing a high similarity score are highlighted in light grey. The amino acids in the predicted signal peptides are in red.

6.2.3 Expression of recombinant Hc TTR and production of antibody probe

In order to investigate whether the HcTTR protein was secreted and to allow immunolocalisation studies to be carried out, the HcTTR was expressed as a bacterial protein and rabbit antisera produced. Following subcloning of the

bacterial protein and rabbit antisera produced. Following subcloning of the CA994587 HcTTR into pBluescript, adapted primers (as described in **Section 2.4.3**) were used to amplify a product, either with or without the predicted signal sequence, for expression in *E. coli* using the pET 22b or pGEX-6P-3 plasmids. The periplasmic leader sequence (peLB) in pET vectors is provided to direct an expressed protein to the cell periplasm. Inclusion of the peLB leader has previously been reported to block expression of certain *H. contortus* proteins (D. Redmond, personal communication), thus expression attempts were made following cloning into pET both with and without this peLB sequence. However, expression of the full length protein in both pET 22b with or without the vector peLB leader sequence, and in an alternative *E. coli* expression plasmid pGEX-6P-3, failed. Following this failure, expression attempts were carried out having removed the signal peptide from HcTTR. Expression in pET 22b of the recombinant HcTTR (rHcTTR) without the signal peptide and without the peLB leader sequence resulted in expression of a His-tagged protein migrating with an estimated weight close to that of the predicted molecular weight of 16 kDa (**Figure 6.2.4**).

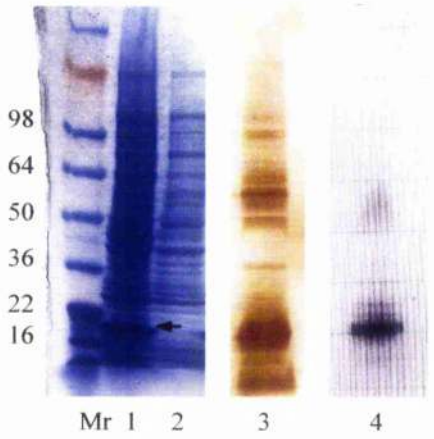


Figure 6.2.4: 4-15% SDS PAGE gel of insoluble bacterial fraction containing expressed recombinant HcTTR and the purified rHcTTR. **1** – Induced cell insoluble fraction; **2** – non induced cell insoluble fraction; **3** – Western blot of lane 2 probed with 1:500 rabbit anti-rHcTTR sera; **4** – electroeluted and purified rHcTTR. The arrow points to the rHcTTR in lane 2 between 16 and 22 kDa.

The rHcTTR was excised and subjected to a trypsin digest and MALDI-TOF analysis (**Section 2.3.17**); a comparison of the peptide mass fingerprint of the fragments with an *in silico* digest of the HcTTR amino acid sequence confirmed the expressed protein as the HcTTR (see **Appendix 2**). The rHcTTR was subsequently electroeluted and purified (**Figure 6.2.4**). An antibody probe was raised in rabbits using the purified rHcTTR (**Section 2.7.1**) and sera were confirmed to recognise a product of the appropriate size in an insoluble bacterial fraction (**Figure 6.2.4**). Pre-vaccinate rabbit sera did not react with a band at this size (not shown).

6.2.4 Temporal expression pattern of HcTTR

An investigation into the expression pattern of the HcTTR was made using PCR. RT-PCR using primers TTR-3 and TTR-2 (**Section 2.4.3**) was carried out on cDNA prepared from all life stages of *H. contortus*; a band of the appropriate size for the HcTTR appears to be expressed throughout all the life stages of the parasite (**Figure 6.2.5**). No firm conclusions regarding expression level can be drawn due to the non-quantitative nature of this PCR.

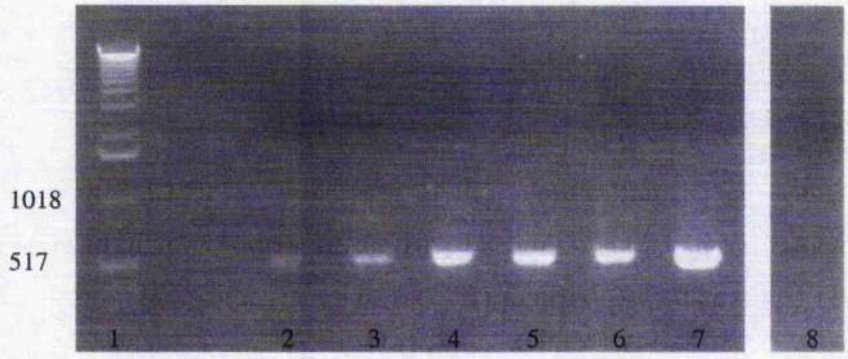


Figure 6.2.5: Expression of HcTTR in different life stages of *H. contortus*. Qualitative PCR on cDNA prepared from various stages of *H. contortus* using primers designed to the HcTTR.
1 – DNA ladder (bp); **2** – egg; **3** – L3; **4** – ExL3; 5 – L4; **6** – day 11 adults; **7** – day 28 adults; **8** – negative (no template).

Using the rHcTTR probe, the expression pattern of the native protein was sought by probing Western blots of *H. contortus* L3 extracts and ES and adult extracts.

The antibody probe reacted very faintly with a protein of the approximate predicted weight of HcTTR (~17 kDa) in soluble extract of both exsheathed L3 (data not shown) and adult worms (**Figure 6.2.6**). Antibody labelling was stronger in the adult extract and intriguingly a band migrating at approximately 60 kDa was also labelled, as detected by the AcTTR probe in L3 extract (**Figure 6.2.1**). There was no reactivity with pre-vaccinate rabbit sera in any of the extracts or ES.

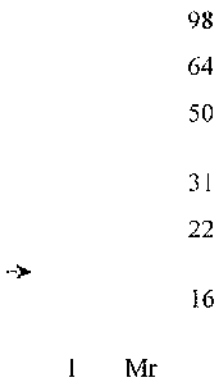


Figure 6.2.6: Western blot of adult *H. contortus* S1 extract probed with 1:500 anti rHcTTR rabbit sera. **1** – adult S1. The arrow marks the peptide at ~17 kDa

6.2.5 Immunolocalisation of native Hc TTR

To localise the native HcTTR in the nematode, immunohistochemistry was carried out using ExL3 and adult *H. contortus* sections as described in **Section 2.7**. In ExL3 sections, there was not a clear cut difference between the level of staining observed when sections were probed with rabbit anti-TTR and pre-vaccinate sera (**Figure 6.2.7**). However, the exposure time required to photograph the negative control sections was longer than that required to photograph the positive sections (45s versus 15s) leading to false positives. There was a tendency for more intense staining at focal points beneath the cuticle and foci of staining in the gut region in sections probed with the rHcTTR antibody compared to the control serum. (**Figure 6.2.7a versus 6.2.7b**). Binding

in adult sections was to the subcuticular muscle, the wall of the uterus in female worms, and to the surface of eggs within the uterus (**Figure 6.2.7**). Adult sections that were probed with pre-vaccinate rabbit sera and those probed with the secondary FITC labelled anti-rabbit IgG alone showed only background fluorescence compared with the post-vaccinated sera antibody probe.

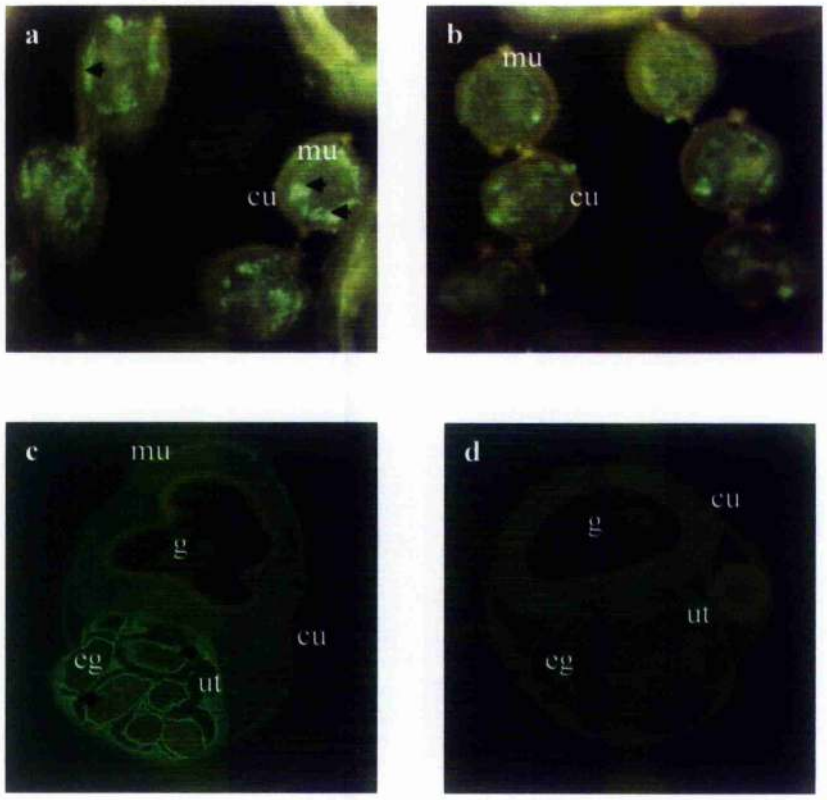


Figure 6.2.7: Immunohistochemistry of ExL3 and adult *H. contortus* transverse sections probed with anti-rHcTTR rabbit sera. Larval sections were probed with 1:500 anti-rHcTTR rabbit sera (positive) or 1:500 pre-vaccinate control sera (negative); Adult sections were probed with 1:2000 positive sera or 1:2000 negative sera **a**- ExL3 positive; **b**- ExL3 negative control; **c**- adult positive; **d**- adult negative control. Labels: g- gut; cu- cuticle; ut- uterus; eg- egg; mu- muscle.

6.2.6 RNA interference of a *C. elegans* HcTTR homologue

To investigate a possible gene function of the HcTTR, its homologue in *C. elegans* was sought and RNAi experiments were carried out to search for a phenotype that might be associated with ablation of the TTR transcript. A BLASTp search of the *C. elegans* database (Wormbase) with the HcTTR amino acid sequence identified a large gene family of TTR-like proteins within the *C. elegans* genome, the closest homologue of which was *C. elegans* TTR-like family protein, accession number CE09030 (CeTTR). CeTTR demonstrated a 68% identity at the amino acid level to HcTTR and a 61% amino acid identity to the AcTTR. Interestingly, the AcTTR showed a closer homology to a different *C. elegans* TTR-like family protein, CE06389, with an amino acid identity of 66%.

The entire CE09030 open reading frame (471 bp) was cloned from mixed stage *C. elegans* cDNA into the L4440 vector and sequenced to confirm its identity. To investigate the gene function of transthyretin-like proteins, RNAi was carried out on the CeTTR, as described in **Section 2.9**. Following three attempts at RNAi, no visible phenotype was observed; in all experiments a phenotype of reduced progeny was achieved using dsRNA to *C. elegans* rme-2 (yolk protein receptor, kindly provided by C. Britton) as a positive control, confirming success of the RNAi procedure. One-step PCR (**Section 2.9.4**) demonstrated success of the RNAi to reduce the CeTTR transcript (**Figure 6.2.8**). Amplification by PCR of CeTTR was not achieved on worms treated with RNAi of CeTTR inferring gene transcript knockdown; amplification of the CeTTR sequence was achieved from the control treated worms and amplification of a control gene, F54D5.4 (F54.2 primers were kindly supplied by P. Geldhof; Geldhof *et al.*, 2005) was successful, confirming the RNA quality. This lack of an RNAi phenotype for the CeTTR is in agreement with the published CE09030 RNAi phenotype of wild type (http://www.wormbase.org).

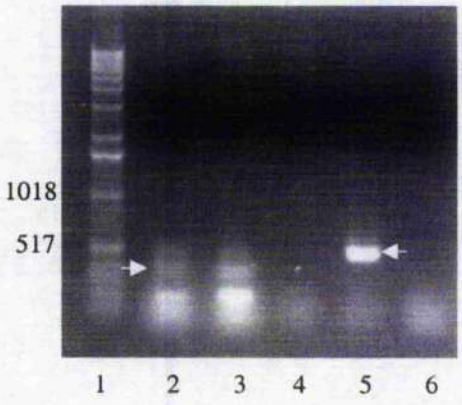


Figure 6.2.8: One step RT-PCR on RNA prepared from CeTTR RNAi treated and control worms confirming knockdown of CeTTR in RNAi treated worms but not in control worms. **1**- DNA ladder (bp); **2**- F54.2 PCR on control treated *C. elegans* RNA; **3**- F54.2 PCR on CeTTR RNAi treated *C. elegans*; **4**- TTR PCR on CeTTR RNAi treated worms; **5**- TTR PCR on control treated worms; **6**- negative TTR PCR control with no template. Arrow in lane 2 points to the 250 bp F54.2 product in lanes 2 and 3; the upper band is genomic DNA. The arrow in lane 5 points to the 471 bp CeTTR product.

6.3 Discussion

Activated L3 *A. caninum* release, amongst other characterised proteins, a transthyretin-like protein, AcTTR (Hotez *et al.*, 2003). At least one vaccine trial using a *P. pastoris*-expressed, recombinant AcTTR has confirmed its protective potential, however the antibody titres raised against AcTTR were comparatively weak (Hotez *et al.*, 2003). A significant negative correlation between AcTTR-specific IgE titre and worm burden was found. Yet, as with other hookworm antigens tested, there was an inherent difficulty reported in eliciting high antibody titres using yeast expressed proteins (Hotez *et al.*, 2003). Consequently, it is difficult to establish the potential efficacy of antigens such as the AcTTR. Identification of an AcTTR homologue in *H. contortus* would allow studies of its suitability as a vaccine candidate and, ultimately, discovery of the native protein, thus opening the door to potential vaccine efficacy studies using native protein in a natural host.

Using sera raised against the recombinant AcTTR, Western blot analysis of *H. contortus* ES and extracts identified a protein of approximately 16 kDa in adult and L3 soluble extracts but not in L3 ES. A previous study (Yatsuda *et al.*, 2003) had used proteomic analysis to show that TTRs were present in adult ES. The predicted molecular weight of the AcTTR is 16 kDa, thus the band labelled in *H. contortus* extract appears to be of the correct molecular weight to be attributed to a TTR-like protein. A second band at about 60 kDa was also labelled, however the possible identity of this is unknown. Mammalian transthyretin and the TRP proteins exist as homotetramers with a non-reduced molecular weight of about 58 kDa (Eneqvist *et al.*, 2003). It is interesting that the only other band in *H. contortus* extract recognised by AcTTR sera is of a molecular weight that would correspond to a homotetrameric version of the TTR. However, as the protein should have been completely reduced, this is deemed unlikely. This issue may be resolved by isolating the ~60 kDa peptide and subjecting it to proteomic analysis.

The AcTTR is excreted/secreted by L3 but no protein was recognised in L3 ES by antisera to AcTTR or the HcTTR described here despite the fact that the transcript is present.

To investigate the identity of the potential AcTTR homologue in *H. contortus* (HcTTR) a bioinformatic approach was taken. The option of creating a cDNA library and then screening the library with the AcTTR sera was decided too time consuming. Therefore, BLAST searches of the published *H. contortus* ESTs were carried out with the AcTTR sequence. Of the 17 contigs identified with a significant similarity score of over 150, the 2 contigs that were most homologous are likely to be the same gene identified from an adult and an egg cDNA library. Like the AcTTR, the HcTTR had a 15 amino acid signal sequence and the 2 proteins shared an identity of 64%.

Following bacterial expression of HcTTR as an insoluble protein, antibody raised against the electroeluted protein was confirmed to cross-react with the bacterially-expressed protein. The HcTTR probe recognised a protein band corresponding to the predicted molecular weight of HcTTR in L3 and adult soluble extract. Despite the protein band labelled being of a similar molecular weight to that labelled by the AcTTR probe, no conclusion can be drawn from 1D analysis as to whether the same protein is cross-reacting. Future studies immunoblotting 2D gels of *H. contortus* extract would likely be more conclusive. No HcTTR band was recognised in either L3 ES at any time point or EF suggesting that the HcTTR under investigation is perhaps not secreted, although a lack of recognition of a potentially processed and secreted form cannot be ruled out. A band at 60 kDa in L3 and adult soluble extract was recognised by the HcTTR probe in an analogous situation to the blots probed with the AcTTR probe. As discussed above, the identity of this protein remains unknown.

Further evidence of expression of HcTTR in both larval and adult life stages came from RT-PCR carried out on cDNA prepared from all life stages of *H. contortus*. Despite the product of this PCR appearing more abundant in the ExL3 to adult stages, conclusions regarding transcript abundance can not be drawn due to the non-quantitative nature of this assay.

The site of immunolocalisation of HcTTR in L3 sections was not conclusive, however it appeared that the protein localised to subcuticular cells that are most probably muscle cells. Despite the negative control L3 sections appearing to show high background fluorescence, this was actually an artefact of the increased exposure time required to photograph these sections due to the lower levels of fluorescence. Immunolocalisation in adult sections appeared more decisive, with the HcTTR localising to the subcuticular muscle tissue and to the wall of the uterus in female worms. Bright staining was also observed on the surface of eggs *in utero*. These immunolocalisation studies corroborate the RT-PCR and bioinformatic data and confirm that the mRNA is translated into a protein product in the egg, L3 and adult stages. Despite the predicted signal sequence, the sites of protein expression do not imply an excreted/secreted role for the HcTTR, although as the protein lines the uterus and egg cuticle it may well be detectable in adult ES cultures. Yatsuda *et al.*, (2003) found several TTR-like proteins in adult *H. contortus* ES, although it is worth pointing out that these proteins are distinct to the described HcTTR and therefore may have different functions and expression patterns. It is also possible that the HcTTR probe cross-reacts with other members of the TTR-like family and that the immunolocalisation observed is of several different, but related proteins.

As mentioned previously, there are 52 TTR-like genes in *C. elegans*. A brief loss of function study of the CeTTR most homologous to the HcTTR confirmed reports on Wormbase of a wildtype RNAi phenotype and as such, did not point to a possible function for the TTR-like proteins. It is interesting though that the CeTTR (CE09030) showed a 68% identity to the HcTTR, yet the AcTTR has a 64% identity to HcTTR. This might suggest that the HcTTR is not the true homologue of the AcTTR as if this were the case they would likely show a closer homology to one another than to TTR-like proteins in the more distantly related *C. elegans*. As the ESTs available do not give a full coverage of the genome, the anticipated publishing of the annotated *H. contortus* genome will allow confirmation of this.

The AcTTR and HcTTR proteins both share a 19% amino acid identity with human TTR, but their tertiary structure is not understood. Human TTR acts as a homotetramer that binds the small (21 kDa) retinol binding protein (bound itself to retinol) and prevents its loss during glomerular filtration (Monaco, 2000). The *C. elegans* TRP proteins share a similar structure to mammalian TTR, and although there is at least one TRP in *O. ostertagi* and two in *T. circumcincta* (Eneqvist *et al.*, 2003) there were none found in the *A. caninum* or *H. contortus* datasets. Fatty acid and retinol binding proteins of nematodes (FARs) are small (20 kDa) proteins that have been shown to bind retinol (Garofalo *et al.*, 2002; Basavaraju *et al.*, 2003). Studies have pointed towards a requirement of retinol for nematode survival. For example, in rodents that were retinol depleted, the microfilaria of *Litosomoides sigmodontis* showed a retarded development (Bradley *et al.*, 2001). Two FAR proteins were described from *A. caninum* that are secreted by the worm following transition to parasitism (Basavaraju *et al.*, 2003) and these were demonstrated to bind lipids and retinol. Thus it could be that the TTR-like proteins function by forming a homotetramer similar to the mammalian TTR that binds FAR proteins bound to retinol. This theory could be tested by both protein structure modelling and ligand binding studies. However, whilst this would fit with the TTR-like proteins in parasitic nematodes being secreted, it does not necessarily fit with the localisation data reported here, or with the free-living nematode *C. elegans* having such a large TTR-like gene family. If a soluble form of HcTTR were produced, it might be possible to investigate ligand binding studies in the future.

In summary, there is a TTR-like gene family in *H. contortus*, one of which shows a 64% identity to the AcTTR but, unlike the AcTTR, is not detectable in L3 ES. The HcTTR protein is localised to the musculature, the uterus wall and to the egg surface, however its function remains unknown. The sites of localisation and lack of detectable protein in L3 ES combined with the fact that there is a TTR in *C. elegans* that shows closer homology with HcTTR than AcTTR, suggests the HcTTR and the AcTTR may not necessarily be orthologous proteins. The non-ES nature of HcTTR and its localisation within the worm make it an unlikely antibody target. Thus, although possibly unsuitable as a vaccine target, HcTTR may have potential as a drug target. The HcTTR antibody probe does bind to the

egg therefore it would be very interesting to investigate whether bound antibody might have deleterious effect on egg hatching. The potential as a vaccine candidate could be investigated by carrying out sheep protection trials using the recombinant protein, or affinity purified native protein.

Chapter 7

Larval Astacin-like Metalloprotease

7.1 Introduction

Astacin-like MTPs have been reported from several parasitic nematodes and comprise a 39 member gene-family in *C. elegans* (Mohrlen *et al.*, 2003). Those discovered in parasitic nematodes are generally associated with the transition to parasitism and are developmentally regulated (Zhan *et al.*, 2002; Lun *et al.*, 2003; De Maere *et al.*, 2005; Gomez Gallego *et al.*, 2005). The suggested roles for the parasitic MTPs include moulting, feeding, tissue penetration and immunomodulation, thus making them prime targets for intervention by vaccination to control infection.

MTP activity was described in *A. caninum* L3 ES and its release demonstrated to be coincident with activation (Hawdon *et al.*, 1995). More recently, Zhan *et al.*, (2002) isolated a full length cDNA encoding a member of the astacin-like MTP family from *A. caninum* (AcMTP). The gene was identified by screening cDNA libraries with sera from hookworm infected patients and was cloned and expressed in *E. coli* (Zhan *et al.*, 2002). Importantly, recognition by sera from infected patients indicates that the protease is released *in vivo*. AcMTP antiserum was used to screen Western blots of activated L3 ES and recognised a protein of a similar molecular weight to the MTP activity described in ES suggesting the *Acmtp* may code for this protease. There are homologues of AcMTP in the ESTs from *N. americanus* as well as at least another 3 similar genes in the *A. caninum* dataset (Zhan *et al.*, 2002). Since then an almost identical MTP gene has been identified in *A. ceylanicum* (Hotez *et al.*, 2003 HHVI 3rd annual report).

Like the AcMTP, the astacin-like MTPs identified in *O. ostertagi* are strongly recognised by serum from immune animals (De Maere *et al.*, 2002). Four MTPs have been found in *O. ostertagi* and these are developmentally expressed, the majority of expression of all but one limited to the L3 or L4 parasitic stage (De Maere *et al.*, 2005). Other parasitic nematode species from which astacin-like MTPs have been described include *Trichinella spiralis* (Lun *et al.*, 2003), *Strongyloides stercoralis* (Gomez Gallego *et al.*, 2005) and *Trichostrongylus vitrinus* (A. Nisbet, personal communication).

Vaccine trials have been carried out using recombinant AcMTP in dogs and, although the levels of protection varied, there was a significant inverse relationship between worm burden and specific anti-AcMTP IgG2 (Hotez *et al.*, 2003). Moreover, the dog with the highest specific antibody titre showed a decrease in worm burden of 50% compared to the controls (Hotez *et al.*, 2003). In addition, sera from dogs vaccinated with AcMTP significantly reduced the number of activated larvae that could penetrate the skin (A. Loukas, personal communication).

MTP activity has been described in *H. contortus* L3 EF and in ExL3 ES (see Gamble *et al.*, 1989a; Gamble *et al.*, 1996 and **Chapter 3**). Although no MTP gene has been reported, it was recently demonstrated that NAS-37 (the NAS protein that mediates ecdysis in *C. elegans*) can specifically lead to refractile ring formations in the L3 sheath and mediate exsheathment of *H. contortus* (Davis *et al.*, 2004). This evidence would suggest that there are likely to be astacin-like MTPs in *H. contortus*. MTP activity was also demonstrated in the unbound fraction used in the sheep vaccine trial described in **Chapter 4**, sera from which reduced larval abomasal penetration *in vitro*.

As with many of the hookworm vaccine candidates, levels of protection using a bacterially-expressed AcMTP have been highly variable. Assessment of the native protein in a natural host/ parasite model might allow better indication as to the true protective efficacy of nematode astacin-like MTPs. It would therefore be of benefit to both the HHVI and to the development of a vaccine against *H. contortus* to identify homologue(s) of the AcMTP in *H. contortus*, thus opening the door for future vaccine trials. The work in this chapter describes the isolation and characterisation of a homologue of the AcMTP from *H. contortus* and presents data on its immunolocalisation.

7.2 Results

7.2.1 Degenerate PCR identification of a novel astacin-like metalloprotease transcript in *H. contortus*

MTP activity has been identified in *H. contortus* EF and ES, however no astacin-like MTP sequences have been identified to date. A BLAST search (April 2004) of the *H. contortus* EST dataset with the AcMTP sequence revealed no homologues, though this could be due to the fact that the datasets are heavily biased towards adult stage ESTs. Therefore, a degenerate PCR approach was taken to attempt to identify astacin-like genes in *H. contortus*. Degenerate primers were designed as described in **Section 2.5.1** and were a kind gift from A. Nisbet. PCR was carried out using primers MTP-deg-f and MTP-deg-r (**Section 2.5.1**) on *H. contortus* ExL3 cDNA and resulted in the amplification of a band at approximately 700 bp (**Figure 7.2.1**).

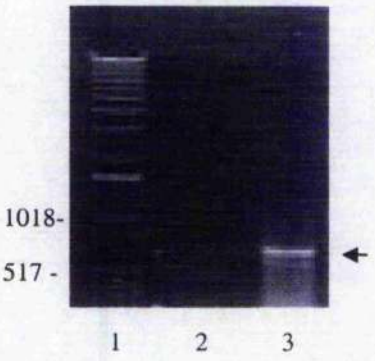
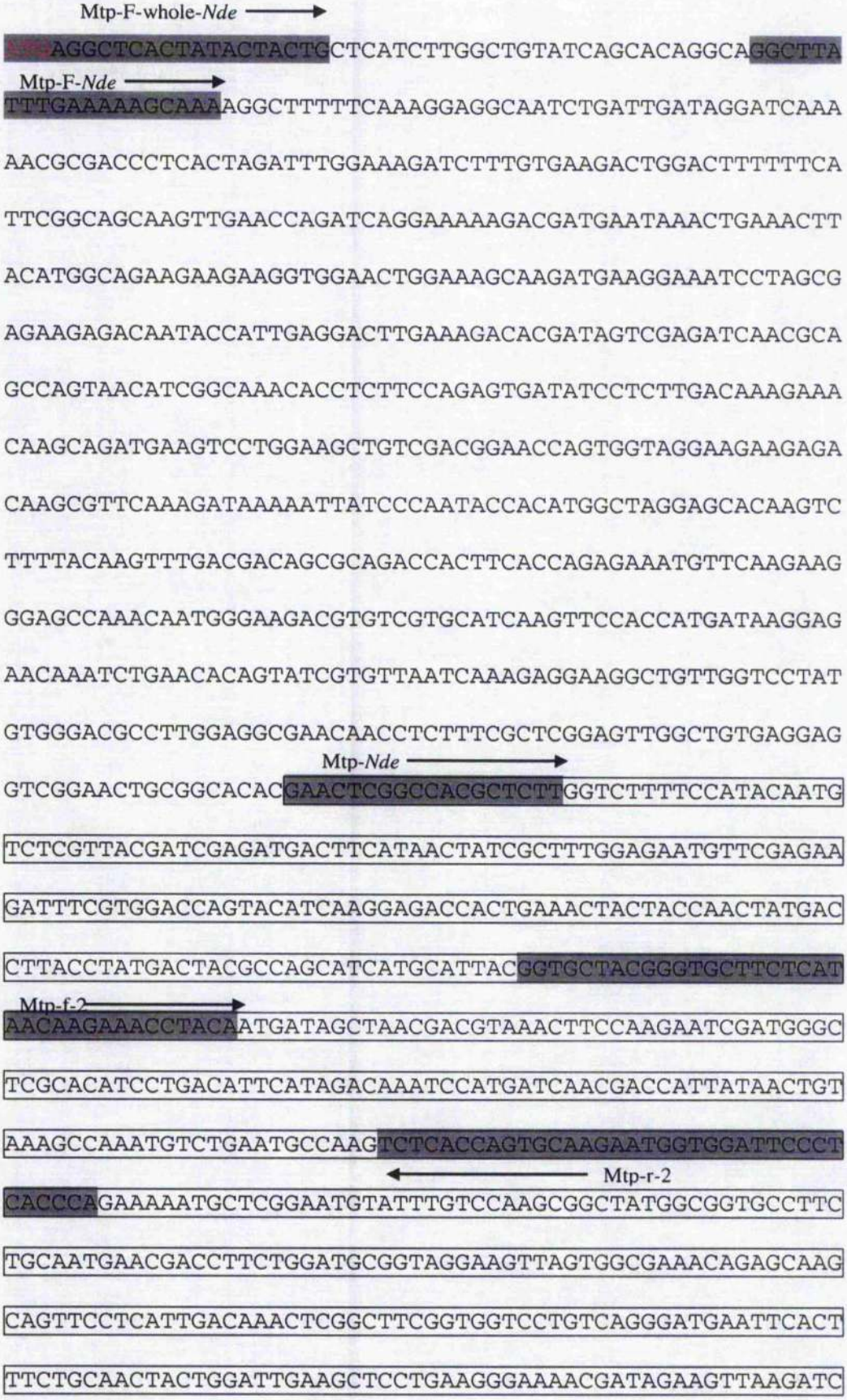


Figure 7.2.1: Degenerate PCR on *H. contortus* ExL3 cDNA using primers designed to regions of homology with the published nematode astacin-like protein sequences. **1** - DNA ladder (bp); **2** - negative control (no template); **3** - ExL3 cDNA. Arrow marks product at approximately 700 bp that was excised and cloned for sequencing.

Following PCR, the product of approximately 700 bp was excised and cloned into pGEM-T (**Section 2.5.1**) and sequenced. Six sequencing reactions of three individual clones resulted in near identical 680 bp sequences (sequencing reactions of other clones resulted in vector only sequence being identified). An amino acid translation of the contiguous nucleotide sequence from the 6 sequencing reactions was used in a Clustal alignment with the AcMTP and the *O.*

ostertagi MTP sequences (used in the design of the degenerate primers). The alignment confirmed the cloning of a novel astacin-like MTP fragment from *H. contortus* (HcMTP) and indicated that the full length MTP was likely to be in the region of 500-600 amino acids. Re-amplification of the HcMTP fragment from freshly synthesised 3' or 5' RACE ready ExL3 cDNA using gene-specific primers was not successful although a product of 640 bp (using primers MTP-f-1 and MTP-r-1) was amplified from L4 3' and 5' RACE ready cDNA. This sequence of this product was identical to the original sequence derived from ExL3 cDNA.

To obtain full length sequence, 3' and 5' RACE PCR was carried out as described in **Section 2.5.2**. 3' and 5' RACE PCR resulted in the cloning of a full length gene sequence that included an ATG start codon and a TAG stop codon with a total length of 1509 bases (**Figure 7.2.2**).


Mtp-F-whole-*Nde*
ATGAGGCTCACTATACTACTGCTCATCTTGGCTGTATCAGCACAGGCAGGCTTA
Mtp-F-*Nde*
TTTGAAAAAGCAAAAGGCTTTTTCAAAGGAGGCAATCTGATTGATAGGATCAAA
AACGCGACCCTCACTAGATTTGGAAAGATCTTTGTGAAGACTGGACTTTTTTCA
TTCGGCAGCAAGTTGAACCAGATCAGGAAAAAGACGATGAATAAACTGAAACTT
ACATGGCAGAAGAAGAAGGTGGAACTGGAAAGCAAGATGAAGGAAATCCTAGCG
AGAAGAGACAATACCATTGAGGACTTGAAAGACACGATAGTCGAGATCAACGCA
GCCAGTAACATCGGCAAACACCTCTTCCAGAGTGATATCCTCTTGACAAAGAAA
CAAGCAGATGAAGTCCTGGAAGCTGTCGACGGAACCAGTGGTAGGAAGAAGAGA
CAAGCGTTCAAAGATAAAAATTATCCCAATACCACATGGCTAGGAGCACAAGTC
TTTTACAAGTTTGACGACAGCGCAGACCACTTCACCAGAGAAATGTTCAAGAAG
GGAGCCAAACAATGGGAAGACGTGTCGTGCATCAAGTTCCACCATGATAAGGAG
AACAAATCTGAACACAGTATCGTGTTAATCAAAGAGGAAGGCTGTTGGTCCTAT
GTGGGACGCCTTGGAGGCGAACAACCTCTTTCGCTCGGAGTTGGCTGTGAGGAG
Mtp-*Nde*
GTCGGAACTGCGGCACACGAACTCGGCCACGCTCTTGGTCTTTTCCATACAATG
TCTCGTTACGATCGAGATGACTTCATAACTATCGCTTTGGAGAATGTTCGAGAA
GATTTCGTGGACCAGTACATCAAGGAGACCACTGAAACTACTACCAACTATGAC
CTTACCTATGACTACGCCAGCATCATGCATTACGGTGCTACGGGTGCTTCTCAT
Mtp-f-2
AACAAGAAACCTACAATGATAGCTAACGACGTAAACTTCCAAGAATCGATGGGC
TCGCACATCCTGACATTCATAGACAAATCCATGATCAACGACCATTATAACTGT
AAAGCCAAATGTCTGAATGCCAAGTCTCACCAGTGCAAGAATGGTGGATTCCCT
Mtp-r-2
CACCCAGAAAAATGCTCGGAATGTATTTGTCCAAGCGGCTATGGCGGTGCCTTC
TGCAATGAACGACCTTCTGGATGCGGTAGGAAGTTAGTGGCGAAACAGAGCAAG
CAGTTCCTCATTGACAAACTCGGCTTCGGTGGTCCTGTCAGGGATGAATTCACT
TTCTGCAACTACTGGATTGAAGCTCCTGAAGGGAAAACGATAGAAGTTAAGATC

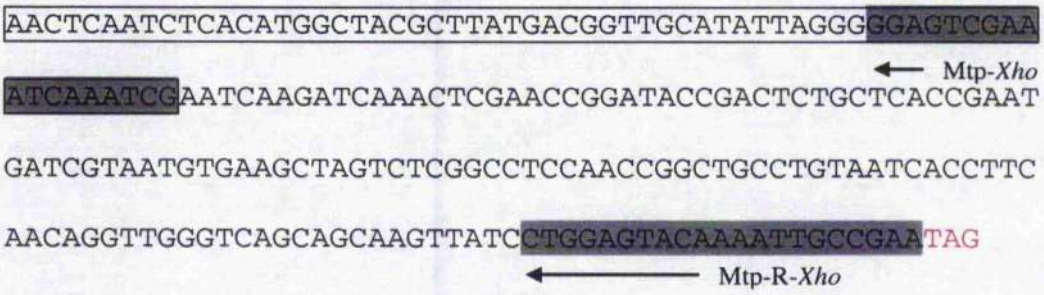


Figure 7.2.2: Nucleotide sequence of novel astacin-like MTP amplified by degenerate PCR from *H. contortus* ExL3 cDNA and extended by RACE PCR from L4 cDNA. Primer sites for specific cloning, expression and 3' and 5' extension are shown above or below sequence (forward and reverse respectively) and shaded in grey. Boxed region was cloned into pET and expressed. (Accession number AM159505). Start (ATG) and stop (TAG) codons are in red.

The full length cDNA (submitted to EBI, Accession number AM159505) codes a 502 aa open reading frame (**Figure 7.2.3**). The first 16 aa represent a predicted signal sequence with a putative site of cleavage between Ala_{16} and Gly_{17}. The protein contains a 127 amino acid putative pro-peptide with a likely site of cleavage between Arg_{142} and Lys_{143} (ProP 1.0). A search of the protein structure databases (Prosite) showed the catalytic region of the protein contains the characteristic metzincin domains including the zinc-binding region HELGHALGLFH (Prosite PDOC00129) and the Met-turn (SIMHY) (**Figure 7.2.3**). Furthermore, the extended astacin-family specific motif, HEXXHXXGFXHEXXRXDR is found in HcMTP and the HcMTP shares all but 2 of the 18 residues with this motif (the HcMTP extended motif is HELGHALGLFHTMSRYDR). Homology modelling of the 3D protein structure was carried out using the SwissModel software (http://swissmodel.expasy.org//SWISS-MODEL.html) and the Deepview Swiss-pdbViewer version 3.7. A comparison of the catalytic region (Tyr_{152} – Ser_{305}) of HcMTP with the crystal structure of crayfish astacin (pdb 1QJ1) predicts that the HcMTP will have a similar structure (**Figure 7.2.4**). The model demonstrates how the side chains of the three Histidine residues from the zinc binding domain and the glutamic acid side chain from the Met turn form the zinc-binding site. The catalytic region is followed by a 34 aa epidermal growth factor-like domain

containing 6 conserved cysteine residues (EGF, Prosite PDOC00021) and a CUB (*C*lr/s complement-like, sea urchin protein, *U*egf and *B*MP-1-like, (Bork and Beckman, 1993)) domain, both of which are characteristic of the nematode astacin-like MTPs identified to date (**Figure 7.2.5**). The full 502 aa ORF has a predicted molecular weight of 56.5 kDa and contains 3 putative *N*-linked glycosylation sites (N-X-S/T-X) at Asn_{37}, Asn_{154} and Asn_{199}. Cleavage of the pro-peptide would result in a protein with a predicted molecular weight of 41 kDa (not including the impact of possible glycosylation).

MRLTILLLILAVSAQAGLFEKAKGFFKGGNLIDRIKNATLTRFGKIFVKTGLFS
FGSKLNQIRKKTMNKLKLTWQKKKVELESKMKEILARRDNTIEDLKDTIVEINA
ASNIGKHLFQSDILLTKKQADEVLEAVDGTSGRKKRQAFKDKNYPNTTWLGAQV
FYKFDDSADHFTREMFKKGAKQWEDVSCIKFHHDKENKSEHSIVLIKEEGCWSY
VGRLGGEQPLSLGVGCEEVGTAAHEIGHALGFWHTMSRYDRDDFITIALENVRE
DFVDQYIKETTETTTNYDLTYDYASIMHYGATGASHNKKPTMIANDVNFQESMG
SHILTFIDKSMINDHYNCKAKCLNAKSHQCKNGGFPHPEKCSECICPSGYGGAF
CNERPSGCGRKLVAKQSKQFLIDKLGFGGPVRDEFTFCNYWIEAPEGKTIEVKI
NSISHGYAYDGCILGGVEIKSNQDQTRTGYRLCSPNDRNVKLVSASNRLPVITF
NRLGQQQVILEYKIAE

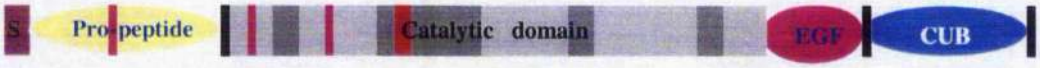


Figure 7.2.3: Amino acid sequence and schematic diagram of full length *H. contortus* MTP. Putative signal peptide (S) is shown in purple. Putative pro-peptide is shaded in yellow. Predicted *N*-linked glycosylation sites are shown in pink. The catalytic domain is shaded in light grey and the astacin-like domains are shaded in dark grey. The zinc-binding site is highlighted in red and the Met-turn motif is boxed. The EGF domain is highlighted in pink and the CUB domain is highlighted in blue.

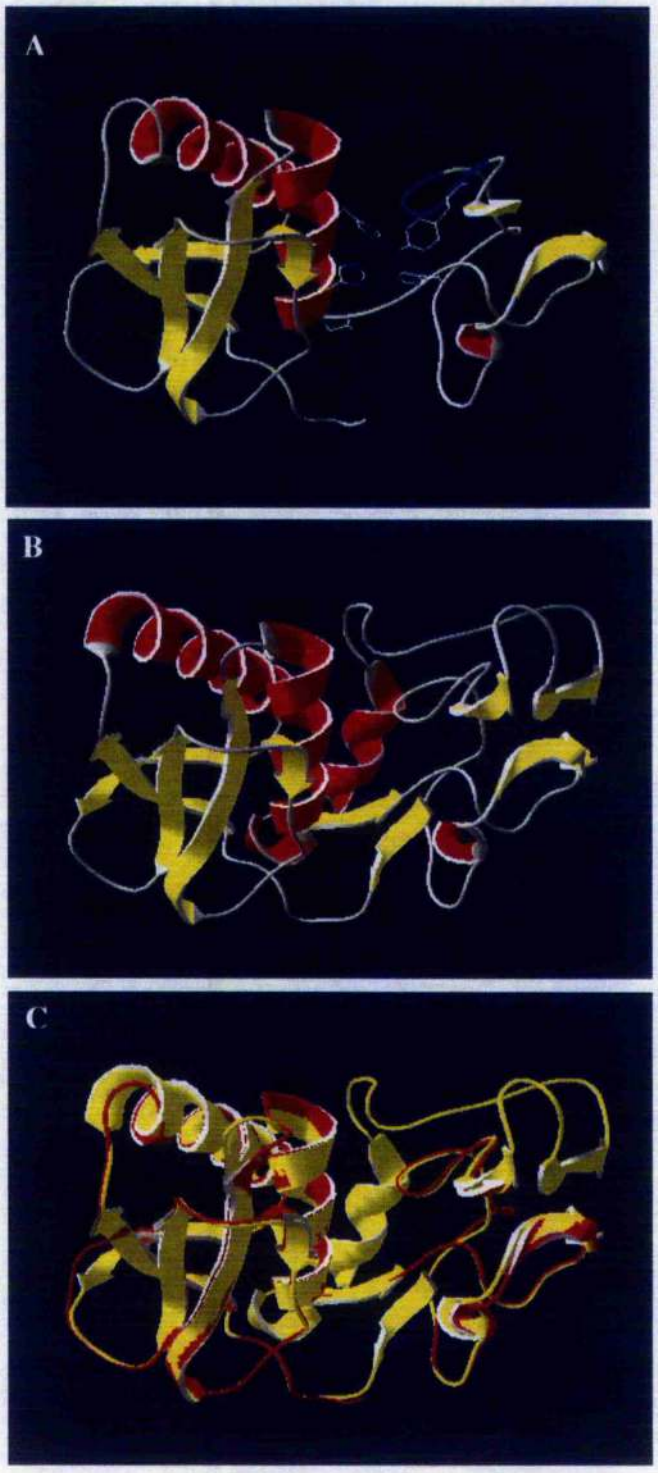


Figure 7.2.4: Molecular model of the catalytic domain of HcMTP based on the crystal structure of Crayfish Astacin (PDB 1QJ1). Beta sheets are shown in yellow and alpha-helices shown in red. **A** shows the ribbon structure of amino acids Tyr_{152} to Ser_{305} of HcMTP based on homology modelling to Astacin. The sidechains of the three His in the zinc binding domain and the Glu in the MET turn are highlighted; these make up the zinc ligation site. Amino acids in the MET turn are highlighted in blue. **B** shows the ribbon structure of Astacin (PDB 1QJ1). **C** shows an overlay of HcMTP (red) superimposed onto Astacin (yellow) and highlights the similar structure and folding.

7.2.2 Homology of HcMTP with other nematode astacin-like metalloproteases

Astacin-like MTPs have been reported in several parasitic nematode species, and constitute a 39 member (NAS proteins) family in *C. elegans* (Mohreln *et al.*, 2003) therefore the relationship of the HcMTP to MTPs from other species was investigated. A WuBlast search of the NCBI database with the determined HcMTP aa sequence revealed closest homology to MTPs I and III (61% and 46% identity, respectively) from the cattle stomach worm *O. ostertagi* and a ClustalW alignment highlighting the regions of identity to these and the AcMTP (46% identity) is shown in **Figure 7.2.5**. Of the NAS proteins from *C. elegans*, NAS-31 and NAS-32 show the closest homology to HcMTP with an amino acid identity of 42% and 34%, respectively. A phylogenetic tree was produced using the MEGA 3.1 programme and neighbour-joining method with the published astacin-like MTPs from other parasitic nematodes and NAS-31, -32 and -37 from *C. elegans* (**Figure 7.2.6**). The protein sequences for the *T. vitrinus* MTPs were kindly provided by Dr A. Nisbet. HcMTP is situated in a group with *O. ostertagi* MTP3 and NAS-31 but was in a distinct group from AcMTP. A distinctive feature of the tree is that the MTPs from *O. ostertagi*, for which the most sequences are available, do not cluster together suggesting that there may be distinct functions attributable to certain groups of the protease.

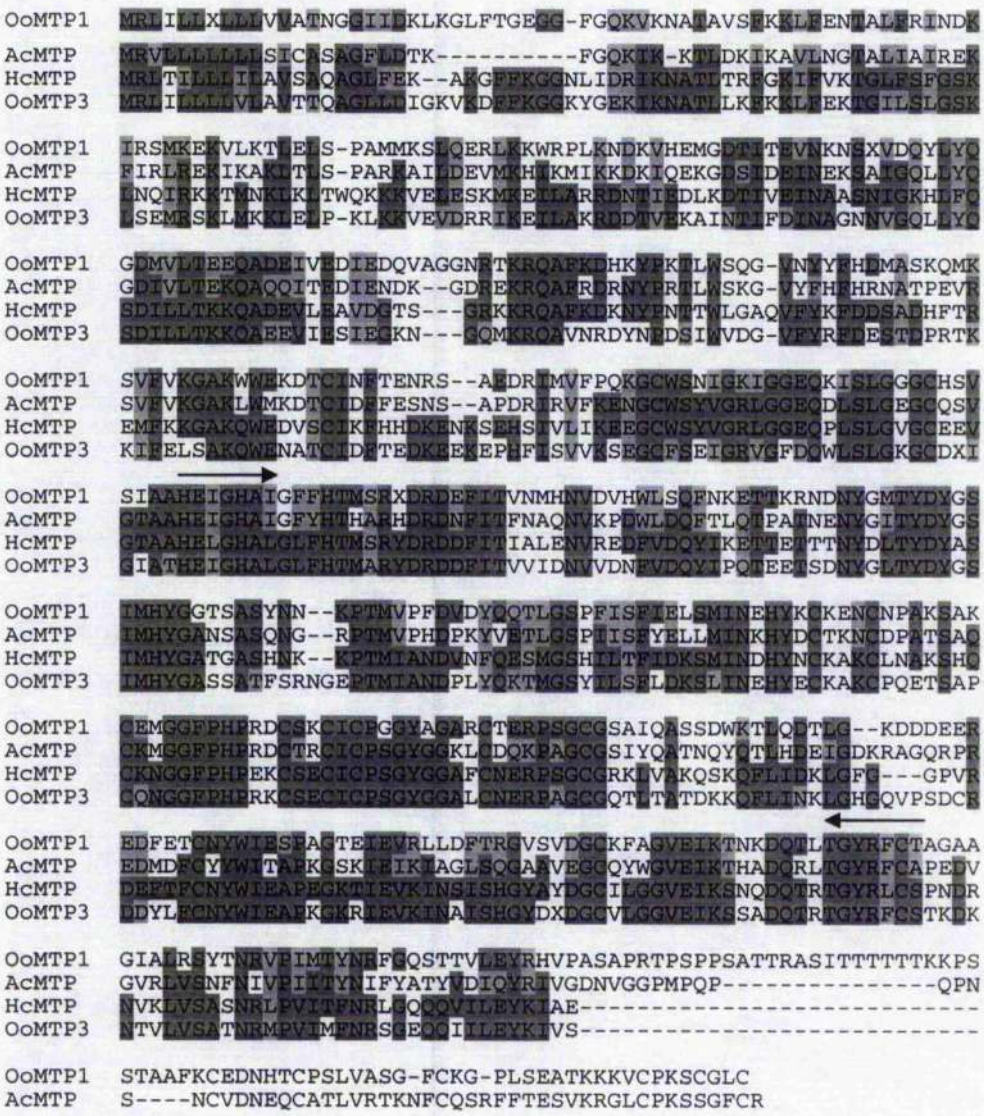


Figure 7.2.5: ClustalW alignment of a protein translation of the *hc-mtp* nucleotide sequence amplified from *H. contortus* cDNA with the amino acid sequences of *A. caninum* MTP and the *O. ostertagi* MTPs 1 and 3. Regions of amino acid identity to the HcMTP are shaded in dark grey; regions of similarity are shaded in light grey. Regions to which the original degenerate primers were designed are highlighted with black arrows (see **Chapter 2, Figure 2.5.1**)

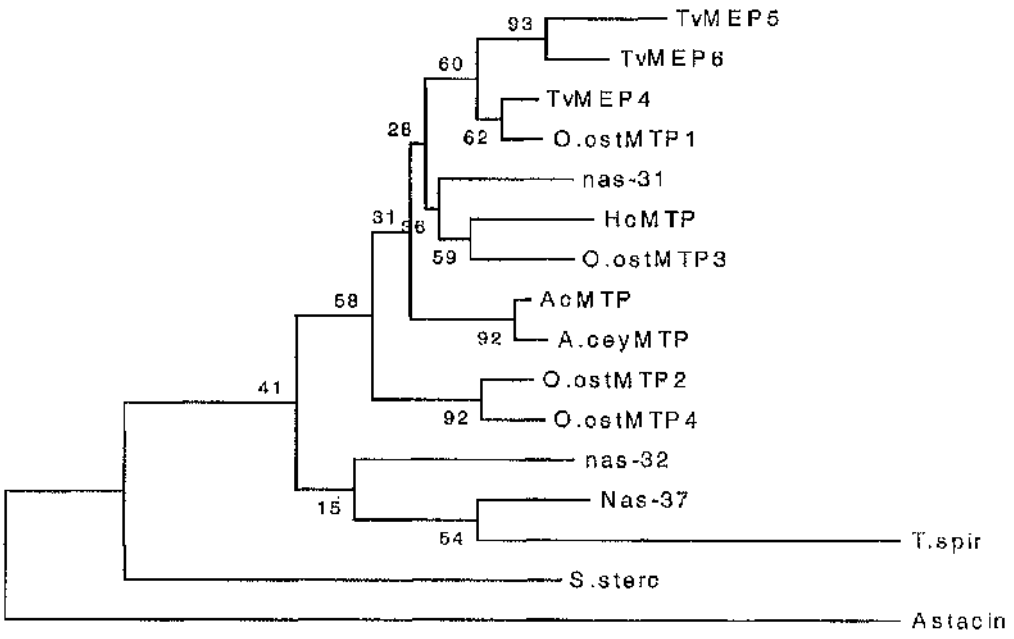


Figure 7.2.6: Phylogenetic tree of the published parasitic nematode astacin-like metalloprotease sequences and three NAS family proteins from *C. elegans*. Crayfish astacin is included for comparative purposes. Tv- *T. vitrinus*; O.ost- *O. ostertagi*, Hc- *H. contortus*, Ac- *A. caninum*, A.cey- *A. ceylanicum*, T.spir- *T. spiralis*, S.sterc- *S. stercoralis*. Line length indicates divergence.

7.2.3 Expression of recombinant HcMTP

To provide an antibody probe to investigate expression of the native protein, the 210 aa HcMTP fragment (boxed region in **Figure 7.2.2**) was cloned into pET using primers MTP-*Nde* and MTP-*Xho* and expressed in *E. coli* as an insoluble recombinant protein (rHcMTPf) with an estimated molecular weight of 24 kDa (**Figure 7.2.7**). The protein was expressed abundantly within the induced bacterial insoluble fraction and was also visible at a lower level in the insoluble fraction from non-induced bacteria. The vector pET 22b has been previously demonstrated a "leaky" vector (G. Ball, personal communication), thus this is likely to represent low level bacterial expression prior to induction. The rHcMTPf was excised and subjected to a trypsin digest and MALDI-TOF analysis (**Section 2.3.17**); a comparison of the peptide mass fingerprint of the

fragments with an *in silico* digest of the HcMTP fragment aa sequence confirmed the expressed protein as rHcMTPf (see **Appendix 2.2**). The expressed fragment was purified by electroelution and used to raise antibody in rabbits. The vaccinated rabbit sera recognised the recombinant HcMTPf in *E. coli* insoluble extract, and cross-reacted with a band at 38 kDa (**Figure 7.2.7**). Pre-vaccinate sera also cross-reacted with a bacterial protein at 38 kDa in the extract, but showed only a very weak recognition of the recombinant HcMTPf, likely to be attributable to non-specific binding.

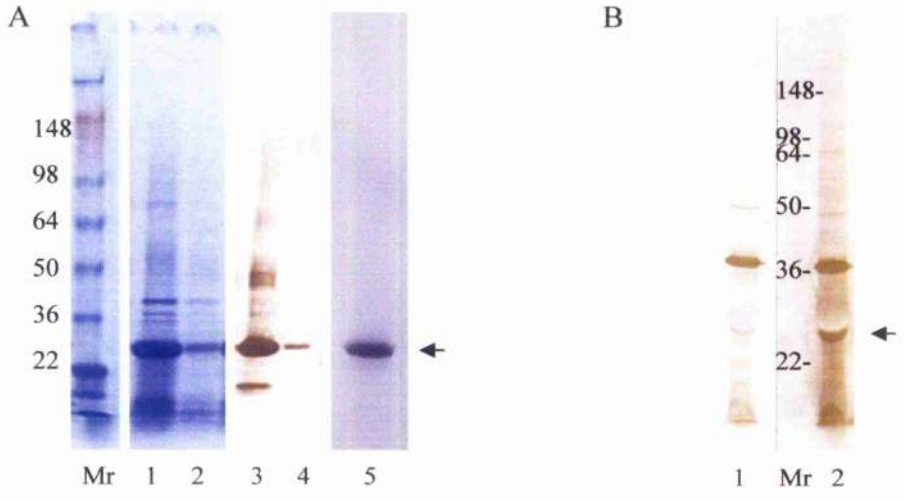


Figure 7.2.7: SDS PAGE gel of recombinant HcMTPf expressed as an insoluble bacterial protein. An anti-His(6) antibody probe recognises the expressed rHcMTPf protein in a Western blot. The rHcMTPf protein was purified by electroelution and used to raise antisera in rabbits. **Panel A**: **1**- Insoluble fraction from induced bacteria; **2**- insoluble fraction from non-induced bacteria; **3**- Western blot of Lane 1 probed with anti-His antibody; **4**- Western blot of Lane 2 probed with anti-His antibody; **5**- electroeluted rHcMTPf. An arrow marks the rHcMTPf protein **Panel B**: Western blot of insoluble protein fraction from *E.* coli expressing rHcMTPf. **1**- probed with pre-vaccinate rabbit sera; **2**- probed with rabbit anti-rHcMTPf sera. A protein of the appropriate size to be rHcMTPf is recognised by the sera (arrow).

The full length MTP without signal peptide was cloned into pET/ *E. coli* (rHcMTP). Upon induction, three proteins were expressed, the largest with a molecular weight of approximately 55 kDa (corresponding to the predicted

molecular weight of the expressed His-tagged HcMTP without signal sequence, of 55.7 kDa) and a protein doublet migrating with an estimated molecular weight of 49 and 45 kDa (data not shown). All three bands were cleanly excised and subjected to trypsin digest and MALDI-TOF analysis. Comparisons with an *in silico* digest of the full length amino acid sequence confirmed that all three represented the HcMTP. An insight into possible processing or truncation could not be gained. However, it is interesting that a peptide fragment matching position 43 in the expressed rHcMTP (without signal peptide) was identified in the 55 kDa band and not in either of the lower molecular weight bands perhaps indicating they may have been slightly truncated at the *N*-terminal (**Appendix 2.3**).

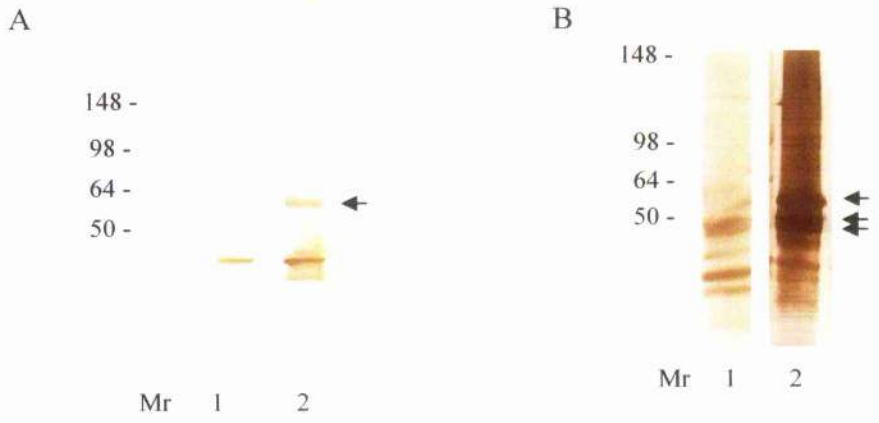


Figure 7.2.8: Western blots of insoluble bacterial fractions expressing full length rHcMTP probed with either rabbit anti-rHcMTP antisera, diluted at 1:1000 (**A**) or sera from *H. contortus* immune sheep, diluted at 1:500 (**B**). Panel **A**: **1**- pre-vaccinate rabbit sera; **2**- anti-rHcMTP rabbit sera. The arrow marks the 55 kDa rHcMTP. Panel **B**: **1**- pre-immune sheep sera; **2**- *H. contortus* immune sheep sera. Arrows mark the three expressed forms of rHcMTP

When an insoluble fraction from bacteria expressing the full length rHcMTP was probed with rHcMTPf antiserum, a band, likely to correspond to the 55 kDa rHcMTP, was recognised at 55 kDa (**Figure 7.2.8**). Pre-vaccinate rabbit serum

only recognised a band at 38 kDa, likely to be a result of non-specific binding. Sera from a sheep immune to *H. contortus* reacted strongly with the three bands corresponding to the expressed forms of rHcMTP, sera from naïve sheep showed background reactivity likely to be the result of non-specific binding and did not recognise the 55 kDa band.

7.2.4 Southern blot using the *hc-mtp* gene fragment

To investigate whether the *hc-mtp* was a member of a gene family, Southern blots were carried out on high molecular weight *H. contortus* genomic DNA digested completely using one of *Eco*RI, *Bam*HI, *Hind*III or *Not*I restriction enzymes. The blots and hybridisation conditions were as described in **Section 2.3.16.** The probe used was the fragment that was cloned into pET for expression cut out by restriction enzyme digest and DIG-labelled. Despite several attempts, the blots remained very smeary and could not be interpreted.

7.2.5 Immunolocalisation of HcMTP in worm extracts and sections

The site of protein expression within the ExL3 was investigated by immunohistochemistry studies using the rHcMTPf antiserum. This analysis indicated that the protein localises to the lateral hypodermal cords, adjacent to the lateral alae or possibly to the excretory/secretory system (**Figure 7.2.9**). Neither pre-vaccinate rabbit serum (the negative control) nor the secondary FITC labelled anti-rabbit IgG serum (a background control) labelled any structure within the ExL3 sections. Background fluorescence was only detected following a prolonged exposure (45s) time compared to the test sections. This was necessary as control sections were not visible when photographed at the same exposure time (15s) as control sections due to the lack of fluorescence.

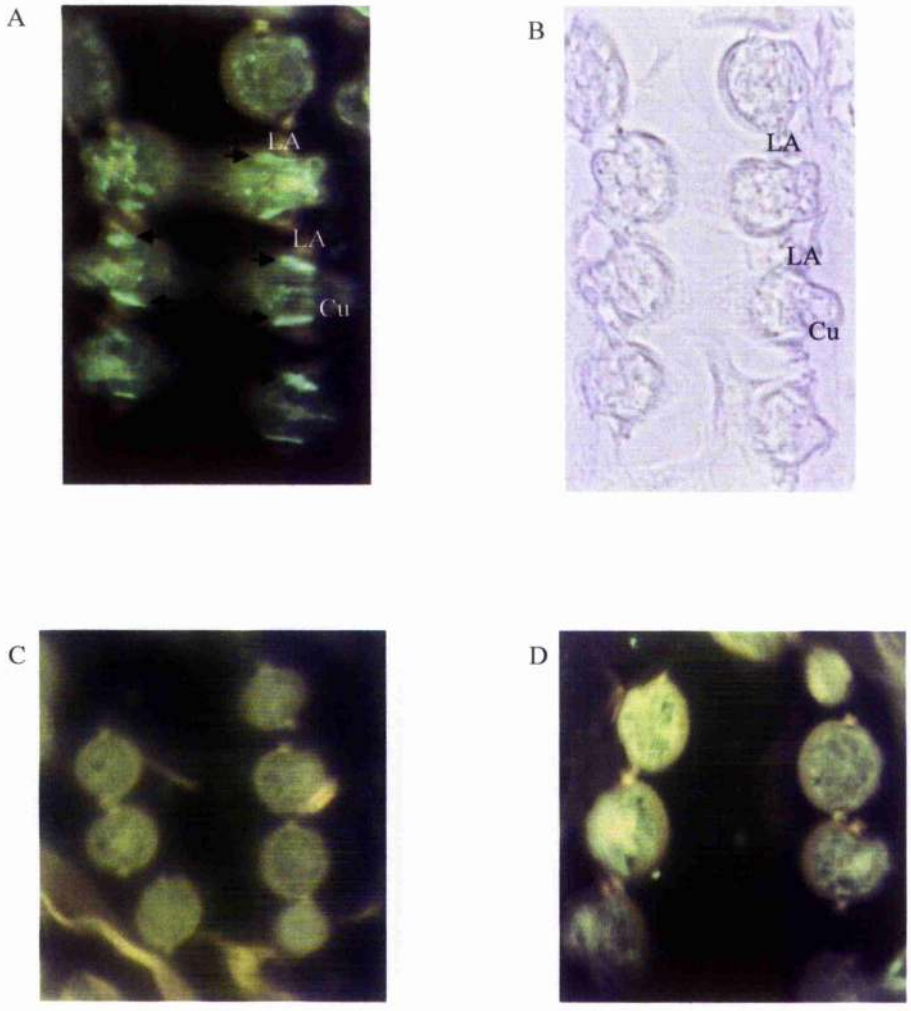


Figure 7.2.9: Immunohistochemical labelling of *H. contortus* ExL3 transverse section probed with rabbit rHcMTPf antiserum. Sections were probed with 1:200 anti-rHcMTPf rabbit sera (positive) or 1:200 pre-vaccinate control sera (negative) or 1:50 FITC labelled anti-rabbit IgG (background control). **A**- rHcMTPf antiserum; **B**- light microscopy of panel A; **C**- background control; **D**- negative control. *Cu*- cuticle; *LA*- lateral alae. Arrows mark the site of immunolocalisation in Panel **A** labelling the lateral hypodermal cords.

To determine the expression and excreted/secreted nature of the HcMTP identified, Western blots were carried out on ExL3 and adult extracts and L3 EF and ES using sera raised against the rHcMTPf as a probe. The rHcMTPf antiserum recognised a band of an estimated 47 kDa in ExL3 soluble extract

(**Figure 7.2.10**). The antiserum failed to recognise any bands in any other ExL3 or adult extract, EF or ES (data not shown).

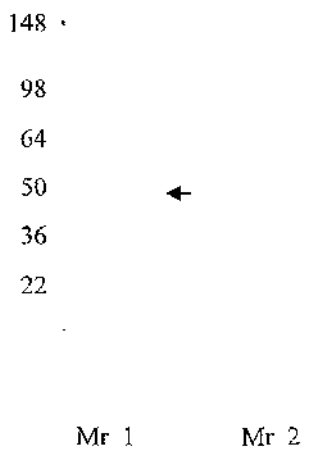


Figure 7.2.10: Western blot of reduced *H. contortus* ExL3 soluble extract probed with rabbit sera raised against rHcMTPf. **1**- anti-rHcMTPf sera; **2**- pre-vaccinate rabbit sera. Arrow marks the protein band reacting with the sera in Lane 1.

An insoluble bacterial fraction containing the full length recombinant rHcMTP was probed with sera from animals vaccinated with the unbound ES fraction (**Chapter 4**), to which MTP activity partitioned (**Figure 7.2.11**). Two bands of the appropriate size to be the expressed rHcMTP were differentially recognised by vaccinate sera compared with pre-vaccinate sera. These bands were also absent from insoluble fraction made from cells containing an empty pET vector. However a general recognition of several *E. coli* proteins by both vaccinate and pre-vaccinate sera rendered the blot difficult to interpret.

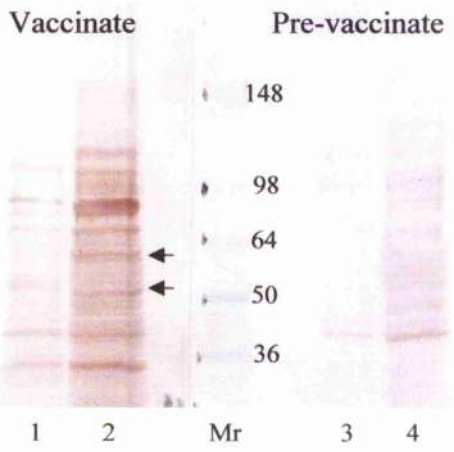


Figure 7.2.11: Western blot of reduced insoluble bacterial fraction expressing either empty pET vector (negative control) or full length recombinant HcMTP. Blots were probed with a 1:1000 dilution of sera from sheep vaccinated with the unbound ES fraction or pre-vaccinate (control) sera. **1**- Empty pET; **2**- rHcMTP; **3**- Empty pET; **4**- rHcMTP.

An investigation of the stage specific expression of HcMTP was carried out using cDNA prepared from all life stages of *H. contortus*. Transcript was detected from L4, day 11 (immature) adult and day 22 (young but mature) adult cDNA. HcMTP was also detected in egg, ExL3 and day 28 adults but at far lower levels (**Figure 7.2.12**). Despite the non-quantitative nature of the PCR, it is suggestive of increased expression of HcMTP in the L4 and early adult stages when compared with the HcTTR stage specific RT-PCR analysis of identical template cDNA run under the same amplification conditions (**Chapter 6, figure 6.2.5**).

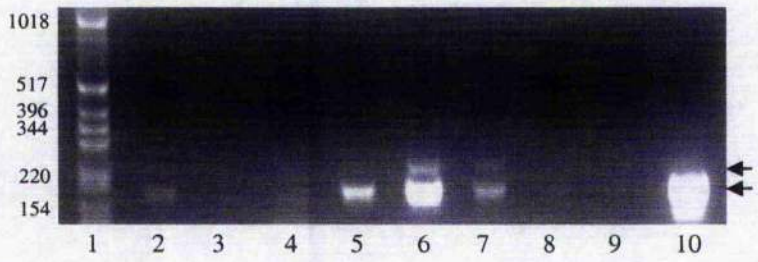


Figure 7.2.12: RT-PCR of HcMTP on cDNA prepared from all life stages of *H. contortus*. Primers (Mtp-f-2 and Mtp-r-2) produce a 200 bp product marker by lower arrow. Upper arrow marks probable genomic product. **1**- Molecular weight marker (bp); **2**- egg cDNA; **3**- L3 cDNA; **4**- ExL3 cDNA; **5**- L4 cDNA; **6**- day 11 cDNA; **7**- day 22 cDNA; **8**- day 28 cDNA; **9**- negative control (no template); **10**- positive control (full length HcMTP cloned into pET).

7.2.6 RNA interference of nematode-astacin genes in *C. elegans*

The possible function of the astacin-like MTP was investigated by conducting RNAi experiments astacin-like genes in *C. elegans* that showed closest homology to HcMTP, *nas*-31 and *nas*-32. RNAi was carried out using a soaking method. Successful knockdown of mRNA transcript was achieved by RNAi in experiments targeting *nas*-31 and *nas*-32 individually and in combination (**Figure 7.2.13**). The F54D5.3 gene (Geldhof *et al.*, 2005) was used as a control gene to check the uniformity of RNA preparations using F54 primers, kindly provided by Dr P. Geldhof. Amplification of F54D5.3 was successful on all RNA prepared from treated and control worms, confirming success of the one-step PCR. It would suggest however, that the RNA prepared from worms that underwent combinatorial RNA was of a slightly lower concentration (**Figure 7.2.13** B). No visible phenotype was observed when either *nas*-31 or *nas*-32 was targeted individually or in combination.

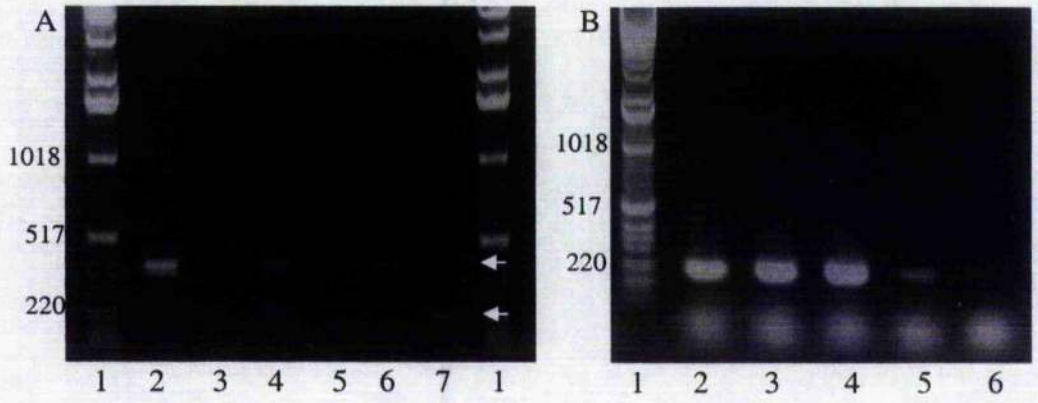


Figure 7.2.13: One-step RT-PCR on *C. elegans* that have undergone RNAi for *nas*-31 and/or *nas*-32. The gene targeted by RNAi (A) and the PCR positive control gene (B) were amplified by RT-PCR to ascertain that both RNAi and RT-PCR had been successful. Panel A: **1**- DNA ladder; **2**- *nas*-31 RNAi, *nas*-31 PCR; **3**- *nas*-32 RNAi, *nas*-32 PCR; **4**- Control, nas-31 PCR; **5**- *nas*-31 and *nas*-32 combinatorial RNAi, *nas*-32 PCR; **6**- *nas*-31 and *nas*-32 combinatorial RNAi, *nas*-31 PCR; **7**- Control, *nas*-32 PCR. Panel B, control PCR using F54 primers: **1**- DNA ladder; **2**- Control cDNA; **3**- *nas-31* RNAi; **4**- *nas-32* RNAi; **5**- *nas*-31 and *nas*-32 combinatorial RNAi; **6**- negative control (no template).
In Panel A, upper arrow marks the genomic DNA product for *nas-32*; lower arrow marks the cDNA product for both *nas*-31 and *nas*-32 visible only in the control worm cDNA.

7.2.7 Direct challenge assay

AcMTP antiserum has been demonstrated to inhibit larval penetration of the skin by *A. caninum*, suggesting the AcMTP may play a role in invasion of host tissue (A. Loukas, personal communication). The ability of rHcMTPf antiserum to prevent *in vitro* establishment of *H. contortus* ExL3 in abomasal tissue was examined as described previously (**Sections 2.10** and **4.2.3**). Pre-incubation with rHcMTPf antiserum actually increased the percentage of tissue associated L3 (83% +/- 4.4) compared to those pre-incubated with pre-vaccinate rabbit control serum (64% +/- 7.5).

7.3 Discussion

On transition to parasitism, *A. caninum* infective larvae secrete an astacin-like MTP enzyme (Hawdon *et al.*, 1995; Zhan *et al.*, 2002) and specific antiserum can inhibit tissue penetration by infective larvae *in vitro* implying a role in skin penetration. The recombinant AcMTP has shown potential as a vaccine candidate (Hotez *et al.*, 2003). Since then, astacin-like MTPs have been isolated from the infective larval stages of several parasitic nematodes (Lun *et al.*, 2003; De Maere *et al.*, 2005; Gomez Gallego *et al.*, 2005). *H. contortus* larvae release a MTP that mediates exsheathment and a likely distinct MTP into ES after 72 hours *in vitro* culture (Gamble *et al.*, 1989a; Gamble *et al.*, 1996; see **Chapter 3**). Despite characterisation of the protease activity, no sequence data has been forthcoming, and astacin-like genes are notably absent from the *H. contortus* EST dataset. Considering the potential of MTPs as vaccine targets, it would be of benefit to identity homologues of the AcMTP in *H. contortus*. Here, the isolation and characterisation of a novel *H. contortus* MTP belonging to the astacin family is described.

An initial BLAST search (April 2004) of the *H. contortus* available ESTs with the AcMTP sequence resulted in no significant hits, however the EST dataset is incomplete and is heavily biased towards adult stage sequences (15,000 adult compared to 1100 larval ESTs). Therefore transcripts expressed predominantly by larvae may not be represented. Degenerate PCR followed by RACE extension of the 3' and 5' ends resulted in the amplification and cloning of a novel gene, HcMTP.

The astacin family of MTPs were recognized during the 1990s following the characterisation of the eponymous crayfish (*Astacus astacus*) protease, astacin (Titani *et al.*, 1987). Members of this diverse family include a sea urchin developmental protein (SpAN; Reynolds *et al.*, 1992), the human bone morphogenetic protein (BMP-1; Wozney *et al.*, 1988), the mammalian and *Drosophila* tolloids (HuTLD; Takahara *et al.*, 1994 and DrTLD; Shimmel *et al.*, 1991), the mammalian meprins (Bond and Beynon, 1995), a 39 strong gene family in *C. elegans* (Mohrlen *et al.*, 2003) and several MTPs identified in

parasitic nematodes. On account of the uniformity of the zinc-binding site amongst the astacin-like MTPs, a group of snake venom metalloproteases, the serralysins and the matrix MTPs, Bode *et al.* (1993) suggested they be grouped into a common family called the metzincins. The astacin-family belongs to the M12A subfamily of the metzincin superfamily (www.merops.sanger.ac.uk/). Metzincins are characterised by a catalytic domain including the HEXXHXXGXXH motif (which includes the zinc-binding site) and Met turn (SXMHY) 50 or so residues downstream (Bond and Beynon, 1995). Members of the astacin-like family have a conserved extended motif of HEXXHXXGFXHEXXRXDR (Stocker *et al.*, 1995). HcMTP shares 15 of the 18 residues with this conserved astacin-specific extended motif, however does not have the Glutamic acid residue following the third Histidine that is a feature of astacins (Hooper *et al.*, 1994). HcMTP exhibits a conserved Met turn a further 38 amino acids downstream (**Figures 7.2.3**).

The structure of astacin has been solved by X-ray crystallography and shown to consist of an *N*-terminal and *C*-terminal domain with a deep cleft in between the two domains (Gomis-Ruth *et al.*, 1993) as seen in **Figure 7.2.4B.** The zinc ion, which is essential for activity, is located at the bottom of this cleft and is thought to be penta-coordinated through the three histidine residues and glutamic acid residue of the zinc binding motif and the tyrosine residue from the Met turn; the Met turn directing the residue towards the binding site (Stocker *et al.*, 1993). Despite crayfish astacin itself only consisting of a signal peptide, pro-peptide and the catalytic domain, most other members of the astacin-family show several C-terminal domains following the catalytic region, normally containing one or more EGF and CUB domains (Bond and Beynon, 1995).

A molecular model of the catalytic region of HcMTP, based on the structure of astacin (Gomis-Ruth *et al.*, 1993), highlights the similar structure and folding of the two enzymes (**Figure 7.2.4A** and **C**). Based on this model, it is likely that HcMTP also binds the zinc atom with the imidazole sidechains of the three histidine residues (His_{240}, His_{244} and His_{250}) and a water molecule from the glutamic acid (Glu 243) of the zinc binding site, with the Met turn positioning an oxygen from a tyrosine (Tyr_{299}) to complete the 5 ligands (Bond and Beynon,

1995). HcMTP shares all the characteristics, with the exception of the glutamic acid following the third histidine, to be classified as a member of the astacin-family of MTPs.

The *C*-terminal region of HcMTP contains an EGF domain followed by a CUB domain. Both of theses domains are common in the *C*-terminal of astacin family proteins though are both notably absent from astacin itself (Bond and Beynon, 1995). These *C*-terminal domains are shared by the astacins described to date from other parasitic nematodes (Zhan *et al.*, 2002; Lun *et al.*, 2003; De Maere *et al.*, 2005; Gomez Gallego *et al.*, 2005) and from the *C. elegans* astacin-like proteases from subgroup III, IV and V (Mohrlen *et al.*, 2003). It has been suggested that these are non catalytic domains that play a role in protein-substrate (or protein-protein) interactions (Bond and Beynon, 1995). EGF domains are present in single copies in the parasitic nematode sequences determined to date (Zhan *et al.*, 2002; Lun *et al.*, 2003; De Maere *et al.*, 2005; Gomez Gallego *et al.*, 2005) and exist as single or multiple copies in several astacin family proteases and other secreted proteases more generally (Bond and Beynon, 1995). CUB domains have been suggested to form anti-parallel β-barrels similar to those in immunoglobulins and are features of diverse members of the astacin-family MTP including the tolloid proteins, BMP-1 and subgroup III, IV and V NAS proteins from *C. elegans* (Mohrlen *et al.*, 2003) as well as non-MTP proteins such as the complement subcomponents Cls/Clr (Bork and Beckmann, 1993).

The closest homologue of HcMTP was *O. ostertagi* MTP III (OoMTP III) which showed a 61% amino acid identity and was of a similar length. Both OoMTP I and AcMTP shared a 46% homology with HcMTP and had extended *C*- termini, the significance of which is unknown (**Figure 7.2.5**). Like the OoMTPs I and III and AcMTP, but unlike other astacin-family members, the residue immediately following the third histidine in the zinc-binding site is a threonine not a glutamic acid; this has lead some authors to suggest a reclassification of astacin-family into two distinct families (De Maere *et al.*, 2005). The closest homologues of HcMTP in *C. elegans* were NAS-31 and -32 (42% and 34% amino acid identity respectively). Phylogenetic analysis confirmed OoMTP III and the *C. elegans*

NAS-31 as the closest relatives of HcMTP (**Figure 7.2.6**). An interesting feature of the phylogenetic analysis was that MTPs from the same species do not necessarily group together suggesting that they may have evolved distinct functions. Further evidence for different functions comes from the temporally regulated nature of the *O. ostertagi* MTP family; OoMTP I being expressed in the adult stage, OoMTPs II and IV in the L3 and L4 and OoMTP III expressed by the L3 and the adult (De Maere *et al.*, 2005). Of note, the *T. spiralis* MTP clusters with NAS-37 perhaps suggestive of a role in moulting rather than in digestion as implied by Lun *et al* (2003).

The catalytic domain of HcMTP was expressed as an insoluble bacterial recombinant protein and the reduced protein was used to raise a specific antiserum (**Figure 7.2.7**). Expression of the full length HcMTP (without the signal peptide), also as an insoluble bacterial recombinant, resulted in expression of three proteins with molecular weights of 55 kDa, 49 kDa and 45 kDa. MALDI-TOF analysis confirmed that all three proteins represented HcMTP but this analysis did not allow an insight into possible truncation to be gained. Like the full length expression of HcMTP described here, bacterial expression of AcMTP also resulted in a triplet of proteins being expressed, one of which corresponded to the predicted size of the pro-enzyme; the authors suggested the other bands were likely to be processed or partially processed forms (Zhan *et* al., 2002; Hotez *et al.*, 2003). Following vaccination with recombinant AcMTP, sera from dogs recognised a triplet of closely migrating bands in activated *A. caninum* L3 ES corresponding to the pro-enzyme, the processed from of enzyme and a band migrating with a weight of between the two (Hotez *et al.*, 2003). This might correspond to a partially processed form, or to a related MTP.

Immunolocalisation studies focusing on the larval stage identified foci of antibody recognition within a strip subcuticular to the lateral alae that extend from within 14 μm of the anterior end of the worm to the end of the tail (Lichtenfels *et al.*, 1990) (**Figure 7.2.9**). The identity of the labelled region is difficult to categorise for certain, however it may be the secretory/excretory system or the hypodermal chords (I. Miskelly, personal communication). Interestingly, this focus of localisation is somewhat similar to that of the *A.*

caninum MTP (Williamson *et al.*, 2006). These authors describe the AcMTP as localising to secretory channels to the L3 cuticle; however, this work was carried out by electron microscopy so a direct comparison is difficult to make. Considering the immunoreactivity of the recombinant HcMTP, it is likely that HcMTP is at least accessible to antibody or secreted, despite the lack of detection of the native protein in ES. It may be stored in the foci immunolabelled prior to secretion/excretion. An alternative theory is that the protein is stored prior to its involvement in the moult from the L3 to L4. Nematode astacins have been implicated in ecdysis, (Hishida *et al.*, 1996; Davis *et al.*, 2004); AcMTP, OoMTP III and IV and strongylastin are associated with transition to parasitism therefore have been attributed roles such as larval penetration. However they may also be involved in the L3 to L4 moult which occurs soon after the parasitic transition (Zhan *et al.*, 2002; De Mare *et al.*, 2005; Gomez Gallego *et al.*, 2005).

RT-PCR analysis in 1st strand cDNA prepared from all the life stages of *H. contortus* resulted in HcMTP transcript detection in the L4 and immature adult (day 11) and young adult (day 22) stages (**Figure 7.2.12**). Transcript was also detected in egg and in ExL3 cDNA. A low level of transcript in ExL3 might explain why, despite original identification of the HcMTP by degenerate PCR in ExL3 cDNA, RACE PCR and extension was only achieved from RACE-ready L4 cDNA, not the RACE-ready ExL3 cDNA. Moreover, a protein of the correct size was detected in ExL3 S1 extracts, but not in adult extracts (**Figure 7.2.10**). Like AcMTP, despite mRNA transcript being detectable in the adult stage, the protein product was only detected in the larvae (Zhan *et al.*, 2002). Detection of HcMTP transcript in egg cDNA is interesting, as astacin-like proteins in *C. elegans* have been implicated in embryonic development and hatching (Hishida *et al.*, 1996).

Sera from sheep immune to *H. contortus* infection reacted strongly with the rHcMTP implying that in natural infection HcMTP is recognised by the immune system (**Figure 7.2.8B**). Both the *A. caninum* and the *O. ostertagi* MTPs were demonstrated antigenic proteins providing a basis for identification as lead vaccine candidates (Zhan *et al.*, 2002; De Maere *et al.*, 2002). The AcMTP is secreted by activated L3 although it is not known whether the OoMTPs are

secreted. Clearly to be a viable vaccine candidate a protein must be accessible to antibody (or cellular) attack; immune recognition of the MTPs in these species and of rHcMTP confirms this. The rHcMTPf antiserum recognised a single protein band only in the soluble extract of ExL3 *H. contortus*. The band had an estimated molecular weight similar to that of the MTP activity previously reported in *H. contortus* ES (**Chapter 3** and Gamble *et al.*, 1996) and to the predicted molecular weight of the processed form of HcMTP (47 kDa versus 41 kDa, the disparity likely due to the two *N*-linked glycosylation sites in the processed enzyme). It is surprising that the rHcMTPf antiserum does not recognise the MTP confirmed in L3 ES (**Chapter 3**). It is possible that this is due to a distinct and as yet unidentified MTP being responsible for the observed protease activity, a possibility that could be investigated by 2D gel analysis and mass spectrometry analysis. Two bands of an appropriate size to be recombinantly expressed full length forms of HcMTP were recognised when an insoluble bacterial fraction expressing rHcMTP was probed with sera from sheep that were vaccinated with the unbound ES fraction (to which MTP activity partitioned, **Chapter 4**) (**Figure 7.2.11**). Bands at this size were not recognised by pre-vaccinate sera, nor was there recognition by vaccinate sera of bands in an empty vector control fraction. This might suggest that the MTP in the unbound ES is encoded for by the HcMTP or a homologous protein, however recognition by both vaccinate and pre-vaccinate sera of *E. coli* proteins makes interpretation difficult.

The role played by astacin-like MTPs has only been well studied in *C. elegans*. However, the function of NAS-31 is unknown. In fact of the 39 NAS genes in *C. elegans* the functions of only a few are understood (Mohrlen *et al.*, 2003). NAS-34 (also known as HCH-1) is involved in embryonic hatching and ecdysis (Hishida *et al.*, 1996) and NAS-37, as discussed in **Section 7.1**, is involved in moulting and ecdysis (Davis *et al.*, 2004). The Genome wide RNAi analysis (results of which are published on www.wormbase.org) that has targeted the *nas* genes, describes an obvious visible phenotype for only 3 of the 39 genes: *nas*-9, -11 and -37 (Mohrlen *et al.*, 2003). However, mutants for NAS-34 (HCH-1) and NAS-35 show a defective embryonic hatching and a dumpy phenotype respectively, but RNAi results in no phenotype. Reasons for this could be

incomplete gene-silencing by RNAi (transcript knockdown was not investigated) or that the NAS proteins exhibit a high degree of functional redundancy (Mohrlen *et al.*, 2003). Recent work has shown that combinatorial RNAi can be used to silence the products of two or more related genes thereby circumventing to a degree the problem posed by functional redundancy (Geldhof *et al.*, 2006). Here, RNAi of *nas*-31 and *nas*-32 resulted in a knockdown of transcript as measured by RT-PCR. RNAi of neither gene alone gave a phenotype (in agreement with the published RNAi phenotype on Wormbase) and combinatorial RNAi resulted in no detectable phenotype; feeding and reproduction appeared as in the control worms. NAS-31 and NAS-32 are the only two members of subgroup IV (groups based on structural similarity, Mohrlen *et al.*, 2003) thus it might be that this sub-type of astacin-like MTP is not essential for survival and development or that another subtype of NAS protein can provide a degree of compensation. Recent work in our lab has demonstrated that effective RNAi of an entire class of proven *H. contortus* vaccine candidates in *C. elegans* had no phenotypic effect on worms (Geldhof *et al.*, 2006 and unpublished results). Thus, care is needed in the interpretation of such RNAi experiments.

The closest *C. elegans* homologues to HcMTP are NAS-31 and NAS-32 therefore, it seems unlikely that HcMTP plays a role in exsheathing as NAS-37 has been determined to be an exsheathing protease orthologue (Davis *et al.*, 2004), although there may be more than one metalloprotease involved in exsheathing. This observation, coupled with the number of astacin-like MTPs in *C. elegans* and those identified so far in parasitic nematodes (for example, the three in *T. vitrinus* and 4 in *O. ostertagi*) suggest that HcMTP is likely to be a member of a protein family. Confirmation of a gene family was attempted by Southern blot analysis however the probe failed to hybridize clearly to the digested genomic DNA on several attempts. The failure of Southern blots using *H. contortus* genomic DNA is something that has been reported by several other co-workers (D. Redmond, personal communication). Although not complete, the *H. contortus* genome is searchable but has not been annotated. A BLAST of the current genome with the HcMTP sequence indicated there is at least one other similar gene, although the number of potential MTPs is difficult to conclude.

Vaccination experiments using the recombinantly expressed OoMTP I in cattle did not result in protection. However, the antigen was expressed in a highly truncated form and antiserum from vaccinated animals did not recognise native proteins in *O. ostertagi* ES or extract suggesting the recombinant protein was aberrantly folded compared to the native antigen (De Maere *et al.*, 2005). The AcMTP however, has shown considerable promise as a vaccine candidate; protection was correlated with AcMTP-specific IgG2 (Hotez *et al.*, 2003). Moreover when the *A. ceylanicum* MTP was used as an immunogen in combination with the lead larval hookworm vaccine candidate (ASP-2) in the hamster-*A. ceylanicum* challenge model, it increased the levels of protection compared with either immunogen alone confirming the MTP's status as a hookworm vaccine candidate (Mendez *et al.*, 2005). That pre-incubation of infective hookworm larvae with sera against AcMTP reduced larval skin penetration *in vitro* added further evidence to the MTP as a vaccine candidate (Williamson *et al.*, 2006). Pre-incubation of *H. contortus* ExL3 with HcMTP rabbit antiserum did not reduce abomasal establishment *in vitro* compared with those larvae exposed to pre-vaccinate serum. However, incubation with sera from sheep vaccinated with the Unbound ES fraction (see **Chapter 4, Table 4.2.4**) did result in a reduction in tissue associated larvae; metalloprotease activity partitioned with this ES fraction.

The *H. contortus* MTP described here is a homologue of the *A. caninum* MTP therefore represents a potential larval vaccine candidate against homologous challenge. The full length recombinant HcMTP is recognised by sera from naturally immune sheep thus the native protein is likely to be available for targeting by immune cells. Full length recombinant HcMTP is awaiting trial in a sheep protection experiment. It may be possible to use antibody affinity chromatography to purify enough of the native protein to allow a trial using the native antigen in the natural host, thus assessing the efficacy of the HcMTP specifically and the nematode MTPs, including the hookworm MTPs more generally.

Chapter 8

General Discussion

The recognition of hookworm infection as a leading cause of global morbidity has led to the establishment of a major initiative to develop a vaccine against human hookworms (Hotez *et al.*, 2003). Despite an apparent absence in humans of the acquirement of natural immunity to hookworms, several lines of evidence point towards the feasibility of vaccination (Loukas *et al.*, 2005). Vaccination with irradiated *A. caninum* larvae could induce high levels of protection (albeit not sterile) and this protection could be transferred passively in serum (Miller, 1965; Miller, 1967). In regions of endemic hookworm infection, levels of IgE specific for a larval ES antigen (ASP-2) are significantly associated with lower hookworm infection (Bethony *et al.*, 2006). An alternative line of evidence comes from the veterinary field. Like hookworms, L4 and adult *H. contortus* are voracious blood feeders and research towards vaccine development against this parasite has identified several highly protective protein complexes from the worm gut proving vaccination against *H. contortus* is a feasible strategy (Knox and Smith, 2001). Efficacious protective antigens have also been identified in *H. contortus* ES (Schallig *et al.*, 1997). Of these already well established vaccine candidates, several have homologues in the hookworms. Lead candidate hookworm antigens have thus been selected on account of either their association with the attenuated larval vaccine, or because they are homologues of the major *H. contortus* vaccine candidates (Hotez *et al.*, 2003).

Using these selection criteria, several hookworm antigens, from both larval and adult stages have been identified as potential vaccine candidates and expressed as recombinant proteins for vaccine testing (Hotez *et al.*, 2003). Some of these antigens have been established as modestly protective in recombinant form, but due to the difficulties involved in obtaining sufficient quantity of native protein, have not been tested as native antigens. In comparison, higher levels of protection against homologous challenge have been achieved using the corresponding native *H. contortus* antigen in sheep trials. The inference here is that the native hookworm antigens may be highly protective but that this level of protection cannot be obtained with recombinants tested to date due to, perhaps, inappropriate post-translational processing. It is quite feasible to obtain sufficient biomass of the different *H. contortus* life cycle stages to purify the homologues of putative hookworm vaccine targets and to evaluate their efficacy

in vaccine trials in sheep using infection regimes mimicking natural exposure. This fact and the convergence of vaccine targets in hookworms and *H. contortus* render the latter a very useful model to aid vaccine development against the former.

Upon activation by incubation with host serum, *A. caninum* L3 begin to secrete proteins that are thought to be essential for the transition to parasitism, host entry and adaptation to the new host environment. Notably, activated *A. caninum* L3 release several well characterised proteins including AcASP-1, AcASP-2 and AcMTP, therefore it is likely that homologues of some or all of these are represented within *H. contortus* L3 ES. In an analogous situation, *H. contortus* L3s can be exsheathed by re-creating *in vitro* the likely stimuli that larvae are exposed to upon host infection. Exposure to 37°C and to CO_2 results in the release of a number of proteins including a MTP that mediates exsheathment (Chapter 3; Gamble *et al.*, 1989). *In vitro* culture of L3 following exsheathment resulted in the secretion of a range of ES proteins, the concentration and complexity of which increased with time in culture (Chapter 3). These included a MTP with a molecular weight of 40 kDa, as estimated by non-reducing gelatin-substrate SDS-PAGE, similar to that described by Gamble *et al.* (1996) and likely distinct from the EF MTP. A single domain homologue of ASP-1 has been previously identified in *H. contortus* L3 ES (Sharp and Wagland, 1998), the full coding sequence of which is described in Chapter 5 (HcASP-1), and shows a 26% identity at the amino acid level to the *N*-terminal domain of AcASP-1. In this study, production of a recombinant HcASP-1 proved impossible, however the elucidation of the full length sequence confirmed the HcASP-1 as a single domain type ASP demonstrating a higher homology to the *N*-terminal domain of the double domain ASP-1 than to ASP2, similar to those described from *O. ostertagi* (Geldhof *et al.*, 2003).

H. contortus L3 ES can be fractionated by lentil lectin affinity chromatography with the binding fraction purportedly enriched for the larval HcASP-1 (Sharp and Wagland, 1998). These authors infer that this enriched fraction contained little other contaminating protein, however in this study it was clear from Western blots, and SDS PAGE examination of the bound and subsequently eluted protein

fraction, that L3 ES contained lentil lectin binding proteins other than the 40 kDa protein previously identified as the ASP-1 homologue (Chapter 3). The presence of contaminating proteins within a crude fractionate complicates the interpretation of vaccine trials, particularly as in a previous study the authors implied the HcASP-1 protein to be the protective component of lectin bound ES in vaccine trials using a guinea pig model (Sharp and Wagland, 1998).

A sheep protection trial aimed at evaluating the protective potential of *H. contortus* larval ES proteins was carried out. ES was fractionated into a lentil lectin binding fraction and a non-binding fraction, the former purportedly enriched for the larval HcASP-1 (Sharp and Wagland, 1998). The trend towards lower FEC, statistically significant reduction in worm numbers and reduction in the length of female adult worms suggest that the lectin-binding fraction can confer a modest degree of protection in systemically immunised sheep. Assuming the bound L3 ES fraction described in this study did contain the larval HcASP-1, the modest protection reported here provides further evidence for the protective potential of ASPs confirming their status as lead hookworm specifically, and nematode generally, vaccine candidates.

The levels of protection reported here fall short of the 85% reduction in worm burden reported in the guinea pig trials (Sharp and Wagland, 1998) and of those reported using ASP enriched adult *H. contortus* ES fractions (Hc24, Schallig and Van Leeuwen, 1997; Schallig *et al.*, 1997; Kooyman *et al.*, 2000) and *O. ostertagi* fractions (Geldhof *et al.*, 2003). However it is possible that only trace amounts of protective protein were actually injected here; future work could focus on the identification and further purification of the protein(s) responsible for the protection reported here. Clearly a first step would be confirmation of the presence of the larval HcASP-1 either by an antibody probe, by using sera from vaccinated sheep to screen *H. contortus* cDNA libraries or through a proteomic approach. Both the latter two approaches would also likely yield information on other potentially protective proteins within the bound L3 ES fraction. Expression of the HcASP-1 in an alternative expression system would also allow exploration of its protective potential in a vaccine trial.

Hookworm studies have revealed that IgE specific for *N. americanus* ASP-2 reduces the intensity of infection in man (Bethony *et al.*, 2005) and anti-NaASP-1 IgE also correlates with a reduction in worm burden in dog protection studies (Hotez *et al.*, 2003). It may also be relevant that protection in lambs vaccinated with an adult *H. contortus* ES fraction enriched for an ASP-2 homologue correlated with IgE levels (Kooyman *et al.*, 2000). In this study, IgE titres were not significantly increased in the group that received the bound L3 ES fraction in comparison with the adjuvant control group. It might be that an increase in the specific IgE titre could improve protection levels, although a recent vaccine study, trialing microparticles as a delivery system for hookworm calreticulin as a potential vaccine against *N. americanus,* questioned the protective effect of specific IgE (Winter *et al.*, 2005).

Vaccination with a lentil lectin non-binding fraction of L3 ES resulted in a trend towards lower FEC and reduced worm burdens in vaccinated lambs compared to controls. Notably, MTP activity partitioned in this fraction (**Chapter 3**) and sera from sheep vaccinated with the unbound ES fraction inhibited the percentage of larval establishment in an *in vitro* abomasal penetration assay (**Chapter 3**). Pre-incubation of *A. caninum* L3 with AcMTP specific sera can inhibit *in vitro* tissue penetration suggesting that the AcMTP may play a role in larval migration (Williamson *et al.*, 2006). Sera against the MTP in the *H contortus* unbound fraction may have had an effect on the establishment of L3, analogous to the situation in hookworm. MTPs have also been implicated in establishment of *O. ostertagi* larvae; L3s are non-feeding yet L3 metalloprotease activity could digest mucin, perhaps suggesting a role in penetration of the abomasal glands (Geldhof *et al.*, 2000). The preliminary work described here requires further investigation; confirmation that a MTP might play a role in establishment could be sought by pre-incubating exsheathed L3 in a range of specific protease inhibitors prior to application to explanted abomasal tissue.

Another consideration is the nature of the vaccine-induced immune response desired to give maximum efficacy. Adult *H. contortus* imbibe host blood and, therefore, circulating antibody. Hence, antigens expressed on the surface of the worm intestine can be readily targeted. The L3s penetrate the abomasal glands,

and are thus in intimate contact with potential local immune effectors (antibody and cells) but not necessarily exposed to systemic immune effectors. In the trial described in **Chapter 4**, the vaccine was administered systemically using Quil A as adjuvant, thus favouring high systemic antibody titres. Perhaps, protection would be enhanced by delivering antigen directly to the GI lymphoid system. Adult *H. contortus* ES induces a strong IgE response during natural infection, levels of which correlate negatively with worm burden, and this may be required for the full expression of protective immunity (Kooyman *et al.*, 1997). In the trial described here, systemic IgE levels in sheep vaccinated with the lectin-bound L3 ES fraction were not significantly higher in vaccinates compared to control animals, although those animals vaccinated with the non-binding fraction did have a significant specific IgE response.

Despite only eliciting a modest degree of protection, the vaccination trials reported here do confirm the usefulness of targeting the larval stage worms as a vaccination strategy. The reduction in female worm size following vaccination with the lectin bound fraction indicates that an immune response against the larval worm may result in a detrimental effect carried through to adulthood. Hookworm trials in the hamster model also indicated that vaccination with larval antigens (ASP-2) could lead to a measurable reduction in adult worm size [10% compared with worms from control animals (Goud *et al.*, 2004)]. Recently, Bethony *et al.* (2005) reported that vaccination of dogs with ASP-2 resulted in a 69% reduction in FEC yet only a 26% reduction in adult worm burden. Given this large reduction in fecundity of worms, an immune response directed towards an antigen of the L3 must have an attenuating effect on adult worms. These findings are of significance as a successful hookworm vaccine is likely to comprise of a cocktail of larval and adult antigens (Hotez *et al.*, 2003). Should the immune response against larval antigens not only prevent some worms developing to adult but also have a deleterious effect on those worms that do reach maturity, targeting an adult antigen may result in a higher efficacy against these "weakened" adults. This principal would also apply to *H. contortus* vaccination. It would be interesting to investigate whether combining larval antigens with the recombinant cysteine proteases, [the best recombinant immunogens described in *H. contortus* to date, vaccination with which achieved

a 38% reduction in adult worm burden (Redmond and Knox, 2005)], could increase the levels of protection.

A. caninum L3 secrete a transthyretin-like protein, specific IgE titre against which showed a slight negative correlation with worm burden in a dog vaccine trial (Hotez *et al.*, 2003). However, only low antibody titres were elicited to the recombinant AcTTR, thus a more appropriate indication of its prospect as a vaccine candidate might come from a trial using native protein, potentially by identifying its homologue in *H. contortus*. A bioinformatics approach allowed the identification of the *H. contortus* TTR that showed highest homology to the AcTTR (Chapter 6). The HcTTR was expressed throughout all life stages, but unlike the AcTTR did not appear to be secreted. Western blots identified the protein in soluble extract from L3 and adult worms, but not in ES. This result strongly suggests that the HcTTR was not in either of the previously discussed ES fractions evaluated in the protection trial. Immunolocalisation studies showed the native HcTTR to be strongly expressed in the lining of the uterus and on the eggs *in utero* as well as in the sub cuticular muscle tissue.

The function of the nematode TTR-like family of proteins is not well understood and a brief loss of function study in *C. elegans* demonstrated RNAi of a homologous TTR-like protein to have effect on worm phenotype. Some authors have suggested that they might play a retinol scavenging role (Hotez *et al.*, 2003). As discussed at length in **Chapter 5**, several nematodes, including hookworm, have been shown to secrete retinol binding proteins. It might be suggested that the TTR like proteins act much like mammalian transthyretin to stabilise retinol bound to the retinol-binding proteins. The site of localisation of the HcTTR neither confirms nor dismisses this theory. Parasitic nematodes are likely to require retinol for a variety of demands; indeed model systems have demonstrated retarded development of nematodes in retinol depleted hosts (see review by Bradley *et al.*, 2001). Retinol is required for vertebrate and invertebrate embryo development (Maden, 2001) and as such may be required for nematode embryogenesis, hence the localisation of HcTTR to the egg and uterus. In filarial nematodes, lipid-binding proteins have been localised to the egg and developing embryo (Michalski *et al.*, 2002). Of course this does not

explain how retinol is initially scavenged from the host. Future work should concentrate on expression of TTR-like proteins in a soluble form to investigate ligand binding and to assess whether, like mammalian transthyretin, they form homotetramers that can interact with retinol binding proteins.

The potential of the HcTTR as a vaccine candidate would at first seem limited, as the site of expression is unlikely to be available to antibody. However, related TTR-like proteins have been detected in adult *H. contortus* ES (Yatsuda *et al.*, 2003) and have been shown to be immunoreactive. These proteins may play a different role and be expressed at other sites accordingly, or they may have been detected in culture because of release with eggs. Because of the intense expression of HcTTR on the egg, any immune interference (such as antibody binding) to the egg might inhibit or prevent successful hatching. It would, therefore, be of interest to investigate the effect of antibody on the development of eggs *in vitro*. The HcTTR may also represent a novel drug target. Although the HcTTR may be of value to intervention research in *H. contortus*, its lack of secretion suggests it plays a different role to that of the AcTTR identified as a potential vaccine candidate, thus the findings here may be of limited usefulness in its evaluation as a hookworm immunogen.

The identification of a novel astacin-like MTP from *H. contortus* cDNA (HcMTP, **Chapter 7**) provides further evidence of the ubiquitous nature of these proteins in parasitic nematodes. *A. caninum* L3 secrete an astacin-like MTP that is likely to play a role in skin penetration and degradation of skin macromolecules (Williamson *et al.*, 2006). The AcMTP has shown promise as a vaccine candidate (Hotez *et al.*,2003) and vaccination with the homologue in *A. ceylanicum* increased levels of protection against homologous challenge in combination with ASP-2 when compared with vaccination using ASP-2 alone (Mendez *et al.*, 2005). MTP activity has been described in *H. contortus* larval ES (Gamble *et al.*, 1996), the presence of which is confirmed in this study. Interestingly the former study associated protease release with the L3 to L4 moult *in vitro*, ascribing it a potential role within this moult; in this study most L3 did not moult to L4, even after 120 hours in culture, similar to findings described by other groups (C. Britton, personal communication). The MTP may

be involved in moulting and be released by the few worms that did develop to L4. Or it may play a role in moulting but other proteins essential for the completion of the L3 to L4 moult may not have been appropriately expressed, thus moulting could not be completed. An alternative explanation is that the metalloprotease activity is not involved in moulting but, like the AcMTP, functions in larval establishment within the host.

Sera raised against a fragment of the HcMTP localised the native protein to exsheathed L3 soluble extract, but interestingly did not react with any proteins within the larval ES. This may suggest that the HcMTP cloned and characterised in this study is not responsible for the MTP activity described in L3 ES, something proteomic analysis could substantiate. An alternative explanation might be that the native protein within the ES was at a far lower and more dilute concentration, thus antibody binding was not detected by Western blot. The full length recombinant HcMTP was recognised by sera from naturally immune sheep, suggesting secretion of the native protein, and was weakly recognised by sera from sheep vaccinated with the unbound L3 ES fraction. In other related parasitic nematodes (*O. ostertagi* for example, De Maere *et al.*, 2005) and in *C. elegans* (Mohrlen *et al.*, 2003) astacin-like MTPs form a gene family and this may be the case in *H. contortus*. A search of the unannotated available *H. contortus* genome suggests there is at least one, and potentially more, other related but distinct astacin-like MTPs in *H. contortus*. Should this gene(s) prove to be coding, the protein product(s) may account for the activity in either EF (Gamble *et al.*, 1989) or ES.

The HcMTP described in this study was immunolocalised within exsheathed L3 sections. Foci of localisation labelled either the lateral hypodermal chords, or possibly the excretory/secretory system. Very recent studies have localised AcMTP to secretory granules in the oesophagus and to channels that connect the oesophagus to the cuticle (Williamson *et al.*, 2006). These studies were conducted using electron microscopy, therefore it is difficult to compare them directly with the results described here. However, the focus of localisation of HcMTP within the subcuticular region might suggest a similar site of localisation. Williamson *et al.* (2006) postulate that secreted proteins involved in

the invasion and larval establishment processes in hookworm are packaged in eosphageal granules prior to secretion across the cuticle via secretory channels. This may also prove the case in *H. contortus*. Like the hookworm MTP, the HcMTP represents a potential larval vaccine candidate. Recombinant HcMTP is recognised by sera from naturally immune sheep and the full length recombinant HcMTP is awaiting vaccine trial. To gain a real insight into the protective potential of the HcMTP, it may be possible to purify enough native protein, using a high avidity antibody, to carry out a protection trial using the native antigen.

In summary, this body of work has demonstrated that vaccination of sheep with a non lentil lectin-binding fraction of *H. contortus* L3 ES resulted in a reduced FEC and final worm burden that, whilst not statistically significant, was lower than the adjuvant control group. Pre-incubation of L3 with vaccinate sera significantly reduced larval establishment in an *in vitro* assay. MTP activity partitioned with this fraction and by analogy to studies in hookworm, sera directed against the MTP may be responsible for the reduction in *in vitro* abomasal establishment. Vaccination with the lectin-binding fraction (purportedly containing the larval HcASP-1) had a modest protective affect against *H. contortus* challenge. Homologues of the hookworm larval TTR and MTP vaccine candidates were identified and characterised in *H. contortus* and represent novel potential vaccine and/or drug targets.

Future work should concentrate on identification of homologues of other hookworm vaccine candidates in *H. contortus*. For example several anticoagulant serine protease inhibitors are secreted by adult *A. caninum* (see Stanssens *et al.*, 1996), *in vitro* neutralization of which can be achieved by specific antibody (Harrison *et al.*, 2001). At least two homologous proteins have recently been identified in *H. contortus* (Clark and Knox, unpublished results). Identification of the native proteins would allow examination of protective potential in sheep vaccine trials.

A proteomic approach using 1D SDS-PAGE (thus requiring less protein than needed for 2D analysis) followed by analysis through tryptic digest and Tandem MS/MS was successful in identifying proteins in *T. circumcincta* L4 and adult

ES (Craig *et al.*, 2006). This approach could be applied to both the unbound and bound fraction of L3 ES described here. Future work should focus on the identification of the proteins within the lectin binding and non-binding fractions; further purification of the proteins responsible for protection may lead to an increase in vaccine efficacy.

As a model for hookworm vaccine development, verification of the HcASP-1 in the lectin bound ES fraction as a target for protective immune responses would confirm the ASPs as lead larval vaccine candidates. The ability of sera from sheep vaccinated with the unbound ES, containing a MTP, to reduce *H. contortus* larval establishment in abomasal tissue may indicate that this, in part, contributes to vaccine-induced protective immunity and supports the exploration of the hookworm MTPs as lead vaccine candidates. Purification of the natural HcMTP would also allow native protein vaccine trials. In terms of the usefulness of *H. contortus* as a model for hookworm larval vaccine development, the limited concentration of protein available from ES culture will hamper progress in carrying out native protein vaccination trials. This paradigm has however led to the discovery of novel potential larval vaccine candidates against *H. contortus* and may lead to discovery of novel adult vaccine candidates, allowing their trial as native antigens and providing evidence of their potential in hookworm vaccine design.

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Appendices

Appendix 1 Common Buffers and Reagents

Reagent	Recipe
Acetate pH buffer (pH range 4-7)	0.1 M Sodium Acetate; adjusted to required pH by addition of glacial acetic acid
Acrylamide Gels: 10% Resolving Gel	3.3 ml 30% Acrylamide; 2.5 ml 4X Separating buffer; 4.2 ml dH_2O; 0.01% w/v Sodium Dodecyl Sulphate (SDS); 50 µl 10 % w/v ammonium persulphate; 10 µl TEMED.
Acrylamide Gels: 3% Stacking Gel	1 ml 30% Acrylamide; 5 ml 2X stacking buffer; 4 ml dH_2O; 0.01% w/v SDS; 100 µl 10 % w/v ammonium persulphate; 10 µl TEMED
Anode buffer 1	0.3 M Tris in dH_2O
Anode buffer 2	25 mM Tris in dH_2O
Anode buffer 3	25 mM Tris-Glycine pH 9.4 in dH_2O
Bicarbonate Buffer pH 9.6	0.25 mM Na_2CO_3 adjusted to pH 9.6 with 0.25 mM $NaHCO_3$
Coomassie Blue Stain	0.5% v/v Coomassie blue in 10% v/v methanol; 30% v/v acetic acid in dH_2O
DNA Loading buffer	30% v/v glycerol, 0.25% v/v Bromophenol blue, 0.05% v/v xylene cyanol in dH_2O
Earles Balanced Salt Solution (EBSS)	25 mM $NaHCO_3$; 5 mM KCl; 0.1 M NaCl; 1 mM NaH_2PO_4; 0.1% w/v glucose pH 7.2
Laemmli electrophoresis buffer	0.25 M Tris; 2 M Glycine; 1% w/v SDS in dH_2O and pH to 8.3 with HCl. Diluted 1 : 10 to give 1X Laemmli
Lectin Wash Buffer (LWB)	0.5 M NaCl; 0.01 M Tris; 0.05% w/v

	NaN_3 in dH_2O. Adjust pH 7.4 with 1 M HCl
Luria-Bertani Agar (LB agar)	1% w/v Bacto-tryptone; 0.5% w/v Bacto yeast extract; 1% w/v NaCl; 1.5% w/v agar
Luria-Bertani Broth (LB broth)	1% w/v Bacto-tryptone; 0.5% w/v Bacto yeast extract; 1% w/v NaCl;
NZY top agar	85 mM NaCl; 10 mM $MgSO_4$; 0.5% w/v Bacto-yeast extract; 1% w/v casein hydrolysate; 0.7% w/v agarose pH 7.5 in dH_2O
Phosphate buffer (Sorenson's) (pH range 7-9)	0.1 M K_2HPO_3 adjusted to required pH with 0.1 M KH_2PO_4 in dH_2O
Phosphate buffered saline (PBS)	0.1 M Phosphate buffer pH 7.5 with 0.9% w/v NaCl in dH_2O
Protein Sample Buffer (Reducing)	0.125 M Tris-HCl pH6.8; 20% v/v glycerol; 0.01% w/v SDS; 0.02% v/v bromophenol blue; 0.4 ml 2-β-mercaptoethanol
Protein Sample Buffer (Non-reducing)	0.125 M Tris-HCl pH6.8; 20% v/v glycerol; 0.01% w/v SDS; 0.02% v/v bromophenol blue
Separating buffer (4 X)	1.5 M Tris-HCl pH 8.8
SM buffer	0.1 M NaCl; 0.01 $MgSO_4$; 0.05 M Tris-HCl pH 7.5; 0.01 % w/v Gelatin
SOC Medium	2% w/v Bacto-tryptone, 0.5% w/v Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
Stacking buffer (2 X)	0.5 M Tris-HCl pH 6.8
TAE Agarose gel electrophoresis buffer	40 mM Tris-acetate, pH 8.0; 1 mM EDTA in dH_2O
Tris-EDTA buffer	10 mM Tris-HCl (pH 7.4); 1 mM

	EDTA (pH8.0)
Tris Buffered Saline (TBS)	0.5 M Tris-HCl pH 8.8; 1.5 M NaCl adjusted to pH 7 with HCl in dH_2O. Dilute 1: 10 to give 1X TBS
Tris Buffered Saline plus Tween 20 (TBST)	1X TBS + 0.05% Tween 20

Appendix 2.1 MASCOT Output for HcTTR (Section 6.2.3)

Match to: **HcTTR** Score: **71** Expect: **1.6e-07**
HcTTR

Nominal mass (M_r): **16892**; Calculated pI value: **4.87**
NCBI BLAST search of HcTTR against nr
Unformatted sequence string for pasting into other applications

Variable modifications: Carbamidomethyl (C),Oxidation (M),Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **28**
Number of mass values matched: **7**
Sequence Coverage: **41%**

Matched peptides shown in **Bold Red**

```
     1 MKAVILLLFF GVAVAMRDQS IAVKGKLLCG SKPAANVRVK LWEEDGGPDP
    51 DDELDAGYTD GSGMFQLSGG TAELTPIDPV FKVYHDCDDG IKPGSRKVKF
   101 YLPKSYITEG RVPKKTFDIG VLNLETIFPG EERELVVSRA RRYYFLDDDY
   151 DD
```

Appendix 2.2 MASCOT Output for rHcMTP fragment (Section 7.2.3)

Match to: **MTP** Score: **76** Expect: **5.5e-08**
MTP

Nominal mass (M_r): **22402**; Calculated pI value: **6.30**
NCBI BLAST search of MTP against nr
Unformatted sequence string for pasting into other applications

Variable modifications: Carbamidomethyl (C),Oxidation (M),Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **26**
Number of mass values matched: **9**
Sequence Coverage: **68%**

Matched peptides shown in **Bold Red**

```
     1 MEIGHALGLF HTMSRYDRDD FITIALENVR EDFVDQYIKE TTETTTNYDL
    51 TYDYASIMHY GATGASHNKK PTMIANDVNF QESMGSHILT FIDKSMINDH
   101 YNCKAKCLNA KSHQCKNGGF PHPEKCSECI CPSGYGGAFC NERPSGCGRK
   151 LVAKQSKQFL IDKLGFGGPV RDEFTFCNYW IEAPEGKTIE RKINSISHG
```

Appendix 2.3 MASCOT Output for rHcMTP (Section 7.2.3)

55 kDa rHcMTP

Match to: **hcMTP** Score: **155** Expect: **9.5e-16**
hcMTP

Nominal mass (M_r): **55874**; Calculated pI value: **8.64**
NCBI BLAST search of hcMTP against nr
Unformatted sequence string for pasting into other applications

Variable modifications: Carbamidomethyl (C),Oxidation (M),Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **41**
Number of mass values matched: **20**
Sequence Coverage: **44%**

Matched peptides shown in **Bold Red**

1 MGLFEKAKGF FKGGNLIDRI KNATLTRFGK IFVKTGLFSF GSK**LNQIR**KK
51 TMNKLKLTWQ KKKVELESKM KEILARRDNT IEDLKDTIVE INAASNIGK**H**
101 **LFQSDILLTK** K**QADEVLEAV DGTSGRK**KRQ AFKDK**NYPNT TWLGAQVFYK**
151 **FDDSADHFTR** EMFKKGAK**QW EDVSCIK**FHH DKENKSEHSI VLIK**EEGCWS**
201 **YVGR**LGGEQP LSLGVGCEEV GTAAHELGHA LGLFHTMSR**Y DRDDFITIAL**
251 **ENVREDFVDQ YIKETTETTT NYDLTYDYAS IMHYGATGAS HNKKPTMIAN**
301 **DVNFQESMGS HILTFIDK**SM INDHYNCKAK CLNAKSHQCK NGGFPHPEKC
351 SECICPSGYG GAFCNERPSG CGRKLVAKQS KQFLIDK**LGF GGPVRDEFTF**
401 **CNYWIEAPEG K**TIEVK**INSI SHGYAYDGCI LGGVEIK**SNQ DQTRTGYRLC
451 SPNDRNVKLV SASNR**LPVIT FNRLGQQQVI LEYK**IAELEH HTHH

49 kDa rHcMTP

Match to: **hcMTP** Score: **213** Expect: **1.5e-21**
hcMTP

Nominal mass (M_r): **55874**; Calculated pI value: **8.64**
NCBI BLAST search of hcMTP against nr
Unformatted sequence string for pasting into other applications

Variable modifications: Carbamidomethyl (C),Oxidation (M),Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **30**
Number of mass values matched: **20**
Sequence Coverage: **49%**

Matched peptides shown in **Bold Red**

```
  1 MGLFEKAKGF FKGGNLIDRI KNATLTRFGK IFVKTGLFSF GSKLNQIRKK
 51 TMNKLKLTWQ KKKVELESKM KEILARRDNT IEDLKDTIVE INAASNIGKH
101 LFQSDILLTK KQADEVLEAV DGTSGRKKRQ AFKDKNYPNT TWLGAQVFYK
151 FDDSADHFTR EMFKKGAKQW EDVSCIKFHH DKENKSEHSI VLIKEEGCWS
201 YVGRLGGEQP LSLGVGCEEV GTAAHELGHA LGLFHTMSRY DRDDFITIAL
251 ENVREDFVDQ YIKETTETTT NYDLTYDYAS IMHYGATGAS HNKKPTMIAN
301 DVNFQESMGS HILTFIDKSM INDHYNCKAK CLNAKSHQCK NGGFPHPEKC
351 SECICPSGYG GAFCNERPSG CGRKLVAKQS KQFLIDKLGF GGPVRDEFTF
401 CNYWIEAPEG KTIEVKINSI SHGYAYDGCI LGGVEIKSNQ DQTRTGYRLC
451 SPNDRNVKLV SASNRLPVIT FNRLGQQQVI LEYKIAELEH HTHH
```

45 kDa rHcMTP

Match to: **hcMTP** Score: **167** Expect: **8e-17**
hcMTP

Nominal mass (M_r): **55874**; Calculated pI value: **8.64**
NCBI BLAST search of hcMTP against nr
Unformatted sequence string for pasting into other applications

Variable modifications: Carbamidomethyl (C),Oxidation (M),Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **28**
Number of mass values matched: **17**
Sequence Coverage: **39%**

Matched peptides shown in **Bold Red**

```
  1 MGLFEKAKGF FKGGNLIDRI KNATLTRFGK IFVKTGLFSF GSKLNQIRKK
 51 TMNKLKLTWQ KKKVELESKM KEILARRDNT IEDLKDTIVE INAASNIGKH
101 LFQSDILLTK KQADEVLEAV DGTSGRKKRQ AFKDKNYPNT TWLGAQVFYK
151 FDDSADHFTR EMFKKGAKQW EDVSCIKFHH DKENKSEHSI VLIKEEGCWS
201 YVGRLGGEQP LSLGVGCEEV GTAAHELGHA LGLFHTMSRY DRDDFITIAL
251 ENVREDFVDQ YIKETTETTT NYDLTYDYAS IMHYGATGAS HNKKPTMIAN
301 DVNFQESMGS HILTFIDKSM INDHYNCKAK CLNAKSHQCK NGGFPHPEKC
```

351 SECICPSGYG GAFCNERPSG CGRKLVAKQS KQFLIDK**LGF GGPVRDEFTF**
401 **CNYWIEAPEG KTIEVKINSI SHGYAYDGCI LGGVEIK**SNQ DQTRTGYRLC
451 SPNDRNVKLV SASNR**LPVIT FNRLGQQQVI LEYK**IAELEH HTHH