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Proteomic analysis of excretory/secretory products released by *Teladorsagia circumcincta* larvae early post-infection

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SUMMARY

Teladorsagia circumcincta *is an important parasitic nematode of domestic small ruminants. Drug resistance in this species is common so alternative methods of control are required. As animals develop immunity to* T. circumcincta, *vaccination is a valid option. Little is known about the antigens that play a role in stimulating immunity at this host/parasite interface. As responses generated between 1 and 5 dpi are known to affect development of these nematodes in their gastric niche, we focused on proteins released during the early stages of infection. To identify molecules potentially involved in immunity, we undertook a proteomics analysis of proteins released from larvae harvested at 1-, 3- and 5-days post-infection (dpi). This analysis produced peptide sequence data that was used to search information available in* T. circumcincta *expressed sequence tag (EST) databases and enabled identification of a number of excretory/secretory (ES) proteins. Immunoblots were performed to assess the relative molecular weight of ES antigens that were targets of local IgA responses in mucus from sheep rendered immune to infection. ELISA was performed to assess antigen-specific mucus IgA levels in individual sheep. These experiments provided preliminary evidence that the proteins identified in the larval secretome were subject to these antibody responses.*

Keywords *excretory/secretory antigens, IgA responses,* Teladorsagia circumcincta*, proteomics*

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INTRODUCTION

Teladorsagia circumcincta (syn. *Ostertagia circumcincta*) is a parasitic nematode that resides in the abomasum of domestic small ruminants. Endemic in temperate regions, this pathogenic parasite is a major cause of economic loss and suffering in sheep and goats. Currently, *T. circumcincta* is controlled using repeated anthelmintic treatments, however, drug resistance is relatively common and now acts as a major constraint on small ruminant farming in several areas of the world (1). *Teladorsagia circumcincta* isolates that are resistant to all three available drug classes have been identified and, on farms where these are present, no practical options for nematode control exist (2,3). An obvious alternative for control is to induce immunity *via* vaccination. Sheep that are continuously exposed to *T. circumcincta* infective larvae develop resistance to re-infection and experimental challenge of such animals with third stage larvae (L3) indicates that rapid expulsion of incoming parasites occurs in the first 48–72 h of infection (4,5). Thus far, most research has focused on immune effector responses to *T. circumcincta* (6–9), however, the nature of the antigens associated with the stimulation of these responses is unknown. This is particularly the case for antigens expressed by *T. circumcincta* during the early stages of infection. This gap in knowledge exists despite the fact that antigens derived from these stages are thought to play an important role in the induction and maintenance of host protective immunity (4,10).

Nematode excretory/secretory (ES) products have been identified as important sources of protective antigens in other ruminant species (11). The potential of *T. circumcincta* ES proteins as vaccine candidates is currently under investigation. In the work presented here, we detail an analysis of the proteins potentially released by *T. circumcincta* larvae during the first 5 days of infection. One of the main technological hurdles to identifying proteins derived from such early larval stages is the low quantity of ES products that are obtainable from these parasites. To circumvent this, we have undertaken a sensitive proteomics analysis of ES products

from larvae harvested at 1-, 3- and 5-days post-infection (dpi). To investigate those antigens that may be involved in the induction of host immune responses, ES products from each stage were also subjected to immunoblotting to assess the specificity of mucus IgA responses in immune vs. non-immune sheep. This strategy was chosen as previous studies have indicated that local IgA responses are important in the prevention of establishment and growth of *T. circumcincta* in the abomasum (10,12). It is anticipated that antigens identified by this type of analysis could be incorporated into subunit vaccines for the control of Teladorsagiosis in future.

MATERIALS AND METHODS

Production of ES products from *Teladorsagia circumcincta* larvae

To provide larvae for production of ES material, helminth-free lambs (< 6 months-old) were infected orally with 150 000 *T. circumcincta* infective L3. Mucosal stage parasites were harvested at 1-, 3- and 5-dpi following methods detailed previously for the recovery of 7 day-old fourth stage larvae (L4: 13). Harvested parasites were washed and cultured in RPMI-based medium following previously published methods (14). The nematodes were cultured at the following concentrations: 1-dpi L3 approximately 100 per mL, 3-dpi L3/L4 approximately 600 per mL and 5-dpi L4 approximately 1000 per mL. Culture fluids were obtained at 24 and 48 h by centrifugation to pellet the parasites and the supernatants were passed through 0·2 μM sterile filters. Protease inhibitors (Roche Protease Inhibitor Cocktail) were added and the ES products were concentrated 10- to 20-fold at 4°C using Amicon Ultra-15 and Ultra-4 filter devices (Millipore). Protein concentrations of ES batches were determined using the Pierce BCA protein assay with BSA standards and 50–100 μL aliquots stored at –70°C.

Proteomic analysis of ES products derived from larvae harvested at 1-, 3- and 5-dpi

For 1-dimensional (1-D) gel separation, ES products from 1-, 3- and 5-dpi were fractionated by SDS-PAGE under reducing conditions. Each sample (approximately 10 μg) was mixed with 10 μL SDS-PAGE sample buffer (0·05 M Tris, pH 6·8, containing 5% (w/v) SDS, 20% (v/v) glycerol, 0·01% (w/v) bromophenol blue and 10 mM DTT), boiled for 5 min before loading onto 10% gels with a 3% stacking gel. After protein separation, gels were stained with colloidal Coomassie Blue (SimplyBlue™ SafeStain, Invitrogen), destained in water and the image of each track captured. Mass spectrometry analysis was performed at the Moredun Research Institute's Proteomics Facility <http://www.mri.sari.ac.uk/fgu-functional-genomics-services.asp>. Each gel track was sliced horizontally into 27 equal gel slices of approximately 2·5 mm each and individual slices were finely chopped (approximately 1 mm^3), transferred to clean 0·5 mL Eppendorf tubes and processed using standard in-gel reduction, alkylation and trypsinolysis steps (15). Digest supernatants of 20 μL final volume were transferred to HPLC sample vials and stored at 4°C until required for liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis. Liquid chromatography was performed using an Ultimate 3000 nano-HPLC system (Dionex) comprising a WPS-3000 well-plate micro autosampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump and an SRD-3600 solvent rack controlled by Chromeleon chromatography software (Dionex: http://www1.dionex.com). Samples of 4 μL were applied to the column by direct injection. Peptides were eluted by the application of a 15-min linear gradient from 8% to 45% solvent B (80% acetonitrile, 0·1% formic acid) and directed through a 3-nL UV detector flow cell. LC was interfaced directly with a 3-D high capacity ion trap mass spectrometer (Esquire HCTplus™, Bruker Daltonics) utilizing a low-volume (50 μL/min maximum) stainless steel nebuliser (Agilent, catalogue number G1946–20260) and ESI. MS/MS analysis was performed as previously described (16). A peak list file was generated from the resultant data and submitted to a local database server using the MASCOT search engine for protein database searching against NCBInr <http://www.ncbi.nlm.nih.gov/> and Nembase <http://www.nematodes.org/nematodeESTs/nembase.html> databases. The MS data was also searched against a dataset of 833 sequences derived from two *T. circumcincta* cDNA libraries (one for exsheathed L3 and one for 7 day-old L4) generated by suppressive subtractive hybridization (17). The modifications used in these searches were a global modification of carbamidomethyl (C) and a variable modification of oxidation (M). The tolerances used were; for MS data, 1·5 Da, and for MS/MS data, 0·5 Da. Matches achieving a significant molecular weight search (MOWSE) score were considered significant if two peptides matched for each protein, each of which had to contain an unbroken *b* or *y* ion series of a minimum of four amino acid residues. The other criterion considered in assigning a positive identification for each protein was a concordance between the calculated theoretical molecular mass value of the protein and the observed position of the peptide on 1-D gel electrophoresis.

Infection protocol for the production of mucus for immunoblot analysis and ELISA

To provide material for comparison of IgA responses amongst immune and non-immune sheep, abomasal mucus was obtained from seven previously infected adult ewes

(Group 1) and from eight primary-infected (5 month-old) lambs (Group 2). The ewes had been naturally infected by grazing mixed-species nematode-infected pastures before the study. Subsequently, in an attempt to render them immune to *T. circumcincta*, the ewes were trickle-infected with 2000 L3, three times a week for 10 weeks. Faecal egg counts (FEC) were performed on samples taken from each ewe weekly and from each lamb at the start, middle and end of the trickle infection phase. FEC were performed using the modified McMaster technique (18). All animals were administered orally with 50 000 L3 3 days after the final trickle infection. Three days after challenge, all sheep were necropsied and their lumenal and mucosal *T. circumcincta* burdens (adult and larval parasites) enumerated following standard techniques (19). At necropsy, abomasal mucus was obtained from a single quarter of each stomach by wiping the mucosal surface with sterile gauze (8 ply, 10×10 cm) soaked in 3 mL sterile PBS. The gauze was placed into a 20-mL syringe and the mucus/PBS mix squeezed out in a sterile 25 mL universal tube. The samples were centrifuged at 1912 g for 5 min and supernatants aliquoted and stored at –20°C.

ELISA: mucus IgA levels to ES products from parasites harvested at 3-dpi

To measure antigen-specific mucus IgA levels in individual sheep, an ELISA was performed using ES products from parasites harvested at 3 dpi. Microtitre plates were coated overnight at 4°C with 50 μL ES products at 5 μg/mL in 50 mM bicarbonate buffer, pH 9·6. The plates were washed six times with wash buffer (PBS, 0·05% v/v Tween-20), then blocked with 5% soya milk powder in TTBS, pH 7·4, for 1 h at room temperature. After washing, 50 μL abomasal mucus (diluted 1 : 4 in TTBS) from individual ewes or lambs, were added and incubated for 1 h at room temperature. The wells were re-washed and 50 μL mouse anti-bovine/ovine IgA monoclonal antibody (MCA628, Serotec: at 1 : 250 in TTBS) added for 1 h at room temperature. After a further wash, 50 μL horseradish peroxidase-conjugated polyclonal rabbit anti-mouse IgG (P0260, DakoCytomation) at 1 : 1000 were added for a further 1 h at room temperature. After a final wash, 50 μL *o*-phenylenediamine dihydrochloride substrate (Sigma) were added to each well. After 15 min in the dark, the colour reaction was stopped by addition of 25 μL 2·5 M sulphuric acid and the OD values read at 490 nm. Each test sample was assayed in duplicate.

Immunoblot analysis of mucus IgA responses to larval ES products

To examine for regions of IgA-reactivity in 1-, 3- and 5-dpi larval ES products, proteins were separated by 1-D SDS-PAGE under reducing conditions on 4–15% gradient polyacrylamide gels (BioRad), and were transferred electrophoretically onto Immobilon P (Millipore, UK). The membranes were blocked in 0·5% (v/v) Tween 20 in Tris-Buffered Saline (TTBS), pH 7·4, containing 10% soya milk powder at 4°C overnight, then incubated at room temperature for 2 h with abomasal mucus samples (diluted 1 : 4 in TTBS) from individual ewes or mucus pooled from the lambs. Membranes were washed three times for 5 min in TTBS and incubated with mouse anti-bovine/ovine IgA monoclonal antibody (MCA628, Serotec) at 1 : 250 in TTBS for 1 h at room temperature then re-washed. Next, the blots were incubated for 1 h at room temperature in alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (A4312, Sigma) at 1 : 250 in TTBS. Following three washes in TTBS, filters were incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma Fast BCIP/NBT). Antigen membrane tracks were stained with Coomassie blue, de-stained in the solvent with extra methanol and the image of each track captured.

Statistical analysis

The median worm burdens of Groups 1 and 2 sheep were compared using a Mann–Whitney *U*-test. The relationships between immune responses and worm burdens were investigated graphically and, where appropriate, the strength of the relationship was summarized using Pearson's product moment correlation coefficient. The statistical significance of this coefficient was determined using a randomization test using StatXact version 6·0. All other analyses were carried out using Genstat (10th Edition).

RESULTS

Protein profile of ES products from larvae harvested at 1-, 3- and 5-dpi

Peptide sequence data from individual gel slices that provided significant MOWSE scores *and* matches of four consecutive *b* or *y* ions against two separate peptides are shown in Table 1. Using these criteria, one protein from 1-dpi ES products (gel slice 13, Figure 1a) gave a significant match. This match was observed against a *T. circumcincta* expressed sequence tag (EST) that has significant sequence identity to an astacin (AST)-like metalloprotease from *Ostertagia ostertagi*. The observed size of the *T. circumcincta* ES protein was concordant with enzymically active versions of other nematode AST metalloproteases (27–29). A protein identified in gel slices 18–20 (with significant MOWSE scores of 57, 84 (indicated in grey type in Figure 1a) and 72, respectively) provided peptide sequence data that matched

Table 1 *Teladorsagia circumcincta* larval ES proteins identified by LC-MS/MS. A match was considered significant when the number of matched peptides with four consecutive *b* or *y* ions was = or > 2 and the observed molecular mass of the protein was equivalent to the calculated theoretical value of the matched protein

Gel slice	Observed approximate MW (kDa)	MOWSE score	*T. circumcincta* EST accession number	Possible function: inferred by data on closest homologue in public databases (nematode species: relevant NCBI accession number)
ES 1-dpi				
13	40–45	117	*CB036707*	Astacin-like metalloprotease (*O. ostertagi: CAJ43810*)
ES 3-dpi				
14	38–42	129	*CB036707*	Astacin-like metalloprotease (*O. ostertagi: CAJ43810*)
19	27–30	365	*CB037400*	Cathepsin F (*T. circumcincta: ABA01328* [14])
22	22–23	182	*CB038581*	Unknown (*C. elegans* hypothetical protein described as Onchocerca related antigen family homologue [20]*: AAC02599*)
24	18–20	115	*CB038075*	Unknown (*H. contortus* 15 kDa antigen: *AAC47713* [21])
27	14–16	70	*AM744117*	Unknown (no homologues identified in database: *CB0389078*)
ES 5-dpi				
8	70–75	76	*AM744035*	Aspartyl proteinase (*C. elegans* hypothetical protein: *AAO25990*)
9	65–70	172	*AM743962*	ASP-like protein (*A. caninum* ASP-4 homologue:*AAO63576* [22])
11	50–55	106	*AM744030*	Apyrase (*C. elegans* hypothetical protein: *AAA68725*)
14	38–42	140	*BM052081*	Astacin-like metalloprotease (*H. contortus: CAJ43810*)
16	35–38	219	*CB036294*	ASP-like protein (*O. ostertagi* ASP-2 homologue: *CAD56659* [23])
17	32–35	248	*CB036585*	ASP-like protein (*O. ostertagi* ASP-1 homologue:*CAD23183* [24])
19	27–30	478	*CB037400*	Cathepsin F (*T. circumcincta* cathepsin F: *ABA01328* [14])
20	25–27	239	*CB037328*	ASP-like protein (*O. ostertagi* ASP-3 homologue: *CAO00416* [25])
22	22–25	149	*CB036229*	ASP-like protein (*O. ostertagi* ASP-3 homologue: *CAO00416* [25])
24	18–20	157	*CB036253*	Unknown (*T. colubriformis* 30 kDa antigenic glycoprotein: *O97391* [26])
24	18–20	112	*CB038075*	Unknown (*H. contortus* 15 kDa ES antigen: *AAC47713* [21])
27	14–16	189	*CB043406*	Unknown (*O. ostertagi* 20 kDa ES antigen: *CAC44259* [24])

For each sequence that provided a significant match, only the gel slice containing the highest MOWSE value for that protein is indicated. The accession number of the highest hit identified in the *T. circumcincta* Nembase EST database or *T. circumcincta* L4-specific database is shown. The possible function of each identified protein (where applicable) is inferred from the known function of its closest homologue identified in *T. circumcincta*, or other nematode species. Where relevant, the associated reference for each characterized protein is indicated in parenthesis.

only a single peptide across four consecutive *b* or *y* ions. This protein was identified as cathepsin F (Tci-CF-1). This enzyme has been identified previously as the most abundant protein in ES products derived from *T. circumcincta* L4 obtained at 7 dpi (14). The cathepsin was identified here at a MW concordant with that estimated for the pro-peptide version of Tc-CF-1. The most intensely staining bands in 1-dpi ES products (obvious in slices 8 and 9, at approximately 60–70 kDa), were identified as sheep albumin. This was despite vigorous washing of the nematodes before culture.

More protein bands were visualized in ES harvested from L3/L4 obtained at 3 dpi (Figure 1b). Five proteins gave identities which produced significant matches under the set criteria (Table 1). Again, data derived from a number of other slices gave significant MOWSE scores against *T. circumcincta* EST sequences but only covered four consecutive *b* or *y* ions on a single peptide (data not shown). Most of these (gel slices 4, 8–10 and 24) showed identity to nematode activation secreted proteins (ASPs). One set of data (gel slice 6), matched to a *T. circumcincta* L4 sequence homologous to an angiotensin converting enzyme-like protein from *Caenorhabditis elegans* (accession number: *Q18581*) shown to have a role in moulting (30). A number of proteins (gel slices 5–8) were identified as heat shock proteins (HSP), only one of which (slice 5) matched the criteria noted above. The data showed homology to HSP-1 from *C. elegans* (Accession number: NP_503068) but has not been included in Table 1 and Figure 1 as the MS data produced a higher score against mammalian HSP when the peptide masses were searched against the NCBInr database.

A higher number of identities were obtained for proteins present in ES products from L4 obtained at 5 dpi (Figure 1c). All 27 gel slices contained data that provided significant MOWSE scores and 12 of these provided matches that met the set criteria. With the exception of the ORA-1 homologue and the *T. circumcincta* sequence that showed no significant identity to other proteins identified in 3-dpi ES products, all proteins that had been identified in the previous time points were observed in 5-dpi ES products. Significant matches to ASP molecules were identified in 16 slices: only the highest scoring matches for these proteins are summarized in Figure 1c and Table 1. Five different

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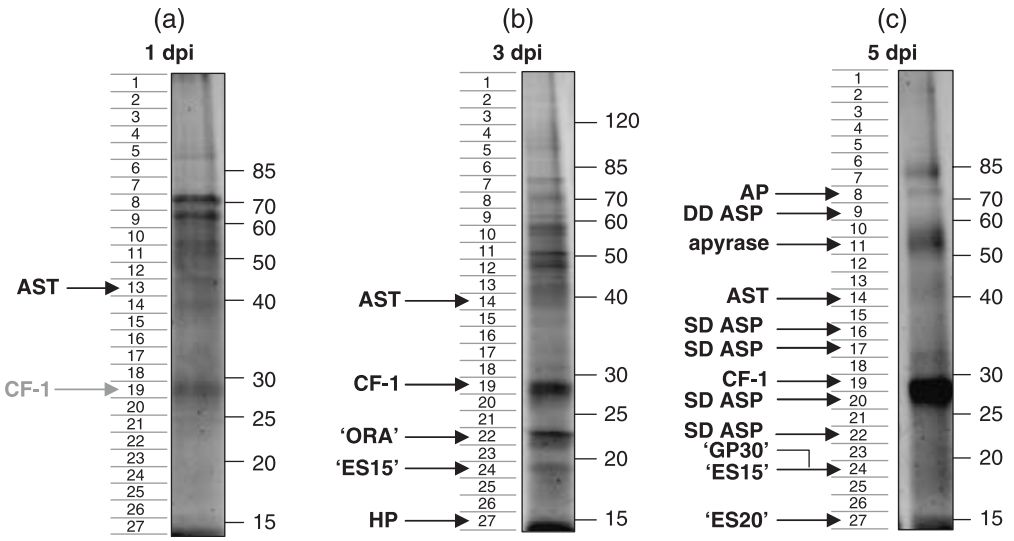

Figure 1 1-D SDS-PA gel tracks stained with colloidal Coomassie. For each track, 10 µg ES products from *T. circumcincta* harvested at 1-, 3- and 5-dpi were loaded. The estimated molecular mass is indicated on the right hand side of each track. Bars on the left of the tracks correspond to the 27 gel slices excised from each. For each slice, putative identifications (based on the presumed or true function of the most homologous protein identified in the NCBInr database) for significantly matching proteins (under the criteria described in materials and methods) are indicated on the left of each panel. In some cases, matching proteins attained these criteria over a number of gel slices. For brevity, only those slices that gave data that provided the highest matches for each protein are indicated. AST, astacin; CF-1, cathepsin F-1; ORA, *Onchocerca* related-antigen homologue; ES15, excretory/secretory 15 kDa antigen (*H. contortus*) homologue; HP, hypothetical protein; AP, aspartyl proteinase; DD-ASP, double domain activation secreted protein; SD-ASP, single domain ASP; GP30, 30 kDa glycoprotein antigen (*T. colubriformis*) homologue; ES20, excretory/secretory 20 kDa antigen (*O. ostertagi*) homologue.

ASP sequences present in *T. circumcincta* EST datasets provided significant MOWSE scores and matches of four consecutive *b* or *y* ions against two separate peptides to the MS data from 5-dpi ES products. Data from slice 9 matched to a *T. circumcincta* EST from the L4-specific dataset, the closest homologue of which is a double domain (DD)-ASP (ASP-4) from *Ancylostoma caninum* (22). The MS data from lower regions of the gel matched to single domain (SD)-ASPs. For example, data from slice 16 matched an EST cluster (Nembase cluster TDC00460) that shows most identity to an N-terminal, SD-ASP, ASP-2, from *O. ostertagi* (23). A separate N-terminal, SD-type ASP in the *T. circumcincta* dataset (Nembase cluster TDC00468) matched to data derived from gel slice 17. This cluster exhibits highest identity to another *O. ostertagi* ASP, ASP-1, which had been identified via immunoscreening (Vercauteren et 2003). The MS data in gel slice 20 matched to an ASP-encoding EST (Nembase cluster TDC01271) with identity to a C-terminal, SD-ASP, ASP-3, from *O. ostertagi* (25). Another C-terminal, SD-ASP (Nembase cluster TDC00462) was identified by matches with MS data derived from slice 22. These results indicate the complexity of the ASP gene family in *T. circumcincta*.

Three proteins, identified as potential vaccine candidates in other ruminant nematodes, are homologues of ESTs that provided significant matches under the set criteria to MS data obtained from the lower molecular weight regions of the gel. These were: *Trichostrongylus colubriformis* 'GP30' (gel slice 24: Reference (26)), *H. contortus* 'ES15' (gel slice 24: Reference (21)) and an *O. ostertagi* 20 kDa ES antigen ('ES20', slice 27: Reference (24)).

Nematode burdens and IgA reactivity to larval ES products

The *T. circumcincta* mucosal L3/L4 burdens (Table 2), most of which should be derived from the 50 000 L3 challenge three days previously, varied substantially among the trickle-infected animals in Group 1 (range: 0–12 750 L3/L4). Three sheep had larval establishment rates of approximately 20%, while in the remaining sheep; two animals had no larvae and the other two, 950 and 1300 L3/L4, respectively. The time point of necropsy chosen was too early to assess if these mucosally established larvae were stunted or arrested in their development due to the effects of immunity. Despite the variation in larval establishment among the sheep in Group 1, when the median mucosal larval burdens were compared between this group and the primary-infected lambs in Group 2, the difference was statistically significant ($P = 0{\cdot}026$). Mucus IgA levels to ES products obtained at 3 dpi were measured by ELISA. Comparatively high variability in

Table 2 Nematode (*T. circumcincta* adult and total larval) burdens of sheep. Group 1 consisted of seven previously field-infected ewes that were subsequently trickle-infected for 10 weeks (2000 L3 three times a week)

Sheep number	Group 1 Adult burden	Group 1 L3/L4 burden	Group 2 Adult burden	Group 2 L3/L4 burden
1	0	0	0	10 050
2	750	950	0	7 950
3	112	0	0	18 800
4	0	8 200	0	17 050
5	600	12 750	0	16 650
6	508	1 300	0	16 700
7	10 200	12 500	0	12 300
8	–	–	0	18 450
Median	508	1 300	0	16 675
Median % larval establishment	–	2·6%	–	33·3%

Three days after the final trickle infection, the ewes were challenged with 50 000 L3. Group 2 consisted of eight helminth-free lambs that received a single 50 000 L3 challenge at the same time as Group 1. Samples were harvested for nematode burden analysis three days post-challenge.

levels of antigen-specific IgA was observed in Group 1 sheep, whereas Group 2 displayed little IgA reactivity to the 3-dpi ES products (Figure 2a). The relationship between larval burdens and IgA responses to the larval ES products were investigated for animals in Group 1. These animals had both varying worm burdens and varying levels of antigen-specific IgA. Lower antigen specific IgA levels were evident in those sheep with the highest larval burdens (Figure 2b); the Pearson correlation coefficient was estimated as –0·80 ($P = 0{\cdot}046$).

Immunoblotting was performed using ES products from all time points in combination with mucus from individual sheep in Group 1 (Figure 3) and a pool of mucus derived from all lambs in Group 2. Several regions of IgA reactivity were identified using mucus derived from the infected ewes. With the exception of a band of reactivity between 35 and 40 kDa in 1-dpi ES products, no IgA reactivity was observed in the mucus obtained from the primary-infected lambs. For all three antigen time points, those animals in Group 1 that had higher larval burdens (sheep numbers 4, 5 and 7) displayed reduced reactivity to the ES antigens compared with the animals that had 0 or low larval burdens. As would be expected, there was also a degree of heterogeneity in IgA reactivity to individual antigens. Most IgA immunoreactivity was observed against antigens at estimated molecular masses of 25–40 kDa in 1-dpi ES products. IgA reactivity was more obvious to higher molecular weight components in 3-dpi ES products: clear bands of IgA reactivity were obvious at 50, 60, 100 and > 200 kDa. More IgA-reactive bands were observed in ES products from 5 dpi and, again, was most intense in those sheep with 0 or low larval burdens.

DISCUSSION

Nematode ES components from parasitic larvae are difficult to study because these developmental stages cannot be generated *in vitro* and the proteins are often released in low quantities (31). Recently, several proteomic analyses have proven the value of this type of approach to the investigation of protein identities in the nematode secretome (32–34). In the present study, we used a 1-D proteomics strategy to identify proteins released by *T. circumcincta* larvae harvested from ovine abomasa at 1-, 3- and 5-dpi. Larvae harvested at 1 dpi represent post-infective L3 that have lost their external sheaths and have just penetrated the gastric gland (35). These larval stages embody the first phase of the host/parasite interaction at this site and are important targets of the host response in immune sheep. Here, we unequivocally identified two abundant proteins secreted by these stages: an AST-like metalloproteinase and a cathepsin. The lack of significant matches obtained for the other components of 1-dpi ES products may be attributable to the amount of protein harvested (and hence the quality of the MS data obtained), the presence of contaminating host proteins (despite extensive washing of the nematodes prior to culture) and the fact that there is poor genomic coverage for *T. circumcincta*. The EST data that is currently available for the parasitic stages of this nematode is derived from transcripts expressed by later developmental stages (7 day-old L4 and adult worms) and so transcripts specific to early post-infective stages may not be represented in these EST datasets.

Tci-CF-1 has previously been identified as the most abundant protein in ES products obtained from L4 harvested at

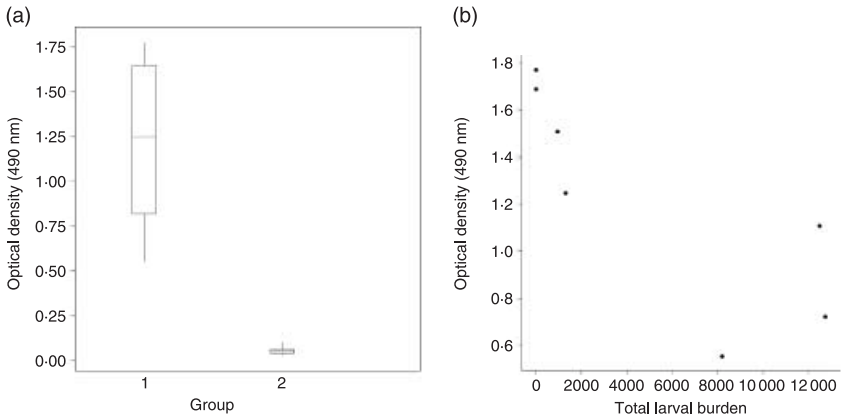

Figure 2 Mucus IgA responses to 3-dpi ES products as measured by ELISA. Each test sample was assayed in duplicate. (a) Boxplots depicting the distribution of mucus IgA responses to 3-dpi ES products in Groups 1 and 2. (b) The relationship between mucus IgA responses to 3-dpi ES products and larval nematode burdens in Group 1 ewes ($r = -0{\cdot}80$).

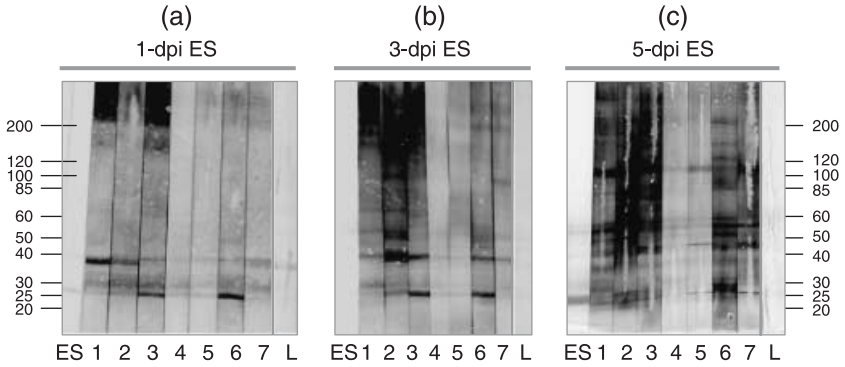

Figure 3 Immunoblots of (a) 1-, (b) 3- and (c) 5-dpi ES products probed with mucus from individual ewes in Group 1 (numbered 1–7) and a pool of mucus derived from eight lambs in Group 2 (L). A blot strip stained with Coomassie for the ES antigen from each time point is also shown (ES).

7 dpi (14): we also identified this protein in the 3- and 5-dpi ES material analysed here. Because of its ubiquity across these stages and its immunogenicity (14), this protein probably represents an important vaccine candidate for future studies. Likewise, the AST-like metalloprotease was identified in ES products from 1-day-old-parasites and from the later stages studied here. The dominance of two proteinases in the *T. circumcincta* L3/L4 secretome probably reflects the nematode's adaptation to feeding and growing after entry to the gastric gland. Indeed, these parasites grow at an impressive rate over the first five days of infection: on 1 dpi the mean length of L3 has been measured at approximately 0·68 mm compared to L4 harvested at 5-dpi, which attain an average length of approximately 3·1 mm (35). Supporting this view of the proteinases' roles in nutrition, nematode cathepsins (36) and ASTs (37) have been shown to digest host proteins. Of relevance, nematode AST-like metalloproteinases have been implicated in stimulating innate and adaptive immune responses early in infection (29).

Around 3 dpi, *T. circumcincta* undergo a moult to L4 (10). Therefore, the secretome obtained at this time point is likely to be derived from a mixed-stage larval population. At 3 dpi, five ES proteins displayed significant matches to *T. circumcincta* ESTs. Based on the comparative identity of these ESTs to sequences from other species, it was shown that, in addition to the two proteinases identified in 1-dpi ES products, three proteins, which are homologues of immunogenic secreted proteins described in other parasitic nematodes, were identified. The function of each of these secreted immunogens in other nematodes is currently not known, however, some have shown promise as vaccine candidates: for example, a recombinant version of 'ES15' from *H. contortus*, when combined with a recombinant 24 kDa ES protein, conferred significant protection in 9 month-old sheep (38).

Many proteins identified in ES products obtained at 5-dpi were classified as ASPs. These proteins are members of a group of nematode-specific molecules that exhibit homology to a large evolutionarily related, though diverse, group of secreted proteins classified as the SCP/Tpx-1/Ag5/PR-1/Sc7 family (CCD domain cl00133: SCP Super-family: 39). Proteins in this family have been identified in a wide range of organisms. In mammals, members of the SCP/Tpx-1/Ag5/PR-1/Sc7 family have been ascribed roles in male reproduction (40), while in insects they have been described as venom allergens (41). In nematodes, their true function remains to be elucidated, however, ASPs have been suggested as playing a part in the transition to parasitism (42) or as allergens

(41). Nematode ASPs have been most characterized in the canine hookworm, *Ancylostoma caninum* (42), and their potential as valid vaccine candidates has been investigated most intensively in this system (43). Three types of ASP have been described in nematodes: DD molecules that contain two similar, though distinct, domains and SD-ASPs in which the domains exhibit homology either to the C- or N-terminal domains present in DD-ASPs (25). Both DD- and C-terminal type SD-ASPs have been identified in many nematode species, whereas, N-terminal type SD-ASPs have only been described in other ruminant nematode species, *O. ostertagi* and *Cooperia punctata* (23,44). Here we identified a DD-ASP that exhibits most homology to an ASP identified in the ES products and on the cuticle of adult *A. caninum* (22). In terms of SD-ASPs, we identified both C- and N-terminal type proteins. These four SD-ASPs displayed most homology to proteins identified previously in *O. ostertagi* (25). Two contain a C-terminal type domain and two, a N-terminal type domain. Based on localization studies, two of the *O. ostertagi* ASPs (ASP-1 and -3) have been implicated as having roles in male reproduction (25), however, true functional data for these nematode proteins does not exist. Also identified in the 5-dpi ES products was an aspartyl proteinase. This is in agreement with a previous enzymic analysis of *T. circumcincta* L4 ES products (45).

Previously, ES products from 7-dpi *T. circumcincta* larvae have been subjected to MS analysis (34). Only one of the proteins identified here (Tci-CF-1) was described in the previous study. Several proteins identified in 7-dpi ES products (34) were likely to be structural or metabolic in origin and this may reflect the state of integrity of the nematodes. The immunogenicity of these 'metabolic' and 'structural' proteins was not assessed in the previous study so evidence of *in vivo* release was not obtained. The differences observed between the current study and this previous investigation of *T. circumcincta* L4 ES products are therefore likely to be due to the precise developmental stages that were examined and also to subtle differences in the culture methodology that was used.

The rationale for probing *T. circumcincta* ES antigens released early in infection with locally derived IgA was based on elegant adoptive transfer experiments where lymphocytes and IgA, transferred intravenously from the gastric lymph of immune to naive sheep, were shown to confer partial protection against challenge (10). In the previous studies, gastric lymph obtained only during the donors' lymphoblast response (between 1 and 5 dpi) transferred immunity to the recipient animals. An indication that IgA is involved in this protective response has been suggested by data analysis that showed that levels of this antibody isotype correlated negatively with *T. circumcincta* length and fecundity (7,10). Based on these observations, we measured ES-specific IgA levels in mucus from previously infected ewes and primary-infected lambs and found a significant difference between the two groups. Moreover, within Group 1, we demonstrated a significant negative correlation between antigen-specific mucus IgA levels and larval burden. The immunoblot analysis indicated that the ES products from larvae at 1-, 3- and 5-dpi are subject to these IgA responses and demonstrated the relative molecular mass of those proteins that are targets of IgA in these compartments. It should be emphasized here though, that although we identified proteins in regions of IgA reactivity, the antibody may be reacting with less abundant proteins (or carbohydrates) that comigrate at the same molecular mass as the identified proteins. For this reason, immunoblotting needs to be extended to 2-D analysis, however, this methodology requires larger amounts of input protein which would involve the use of increased numbers of sheep for antigen generation.

Immune responses directed against a number of nematode antigens are likely to be required for protection against challenge. This has been confirmed by the lack of success in nematode immunization trials that have tested only a single recombinant antigen (46). The data presented here indicates the dynamicity of the *T. circumcincta* secretome, even over the relatively short time frame of 96 h, and is relevant because previous nematode immunization studies have often utilized antigens identified during a single time point. The results obtained here therefore represent the first step to identifying those proteins that may be incorporated into a vaccine for immunization against this important disease of sheep.

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REFERENCES

1 Kaplan RM. Drug resistance in nematodes of veterinary importance: a status report. *Trends Parasitol* 2004; **20**: 477–481.
2 Waghorn TS, Leathwick DM, Rhodes AP, *et al*. Prevalence of anthelmintic resistance on sheep farms in New Zealand. *N Z Vet J* 2006; **54**: 271–277.
3 Sargison ND, Jackson F, Bartley DJ, Wilson DJ, Stenhouse LJ & Penny CD. Observations on the emergence of multiple

anthelmintic resistance in sheep flocks in the south-east of Scotland. *Vet Parasitol* 2007; **145**: 65–76.
4 Seaton DS, Jackson F, Smith WD & Angus KW. Development of immunity to incoming radiolabelled larvae in lambs continuously infected with *Ostertagia circumcincta*. *Res Vet Sci* 1989; **46**: 241–246.
5 Macaldowie C, Jackson F, Huntley J & Mackellar A. A comparison of larval development and mucosal mast cell responses in worm-naive goat yearlings, kids and lambs undergoing primary and secondary challenge with *Teladorsagia circumcincta*. *Vet Parasitol* 2003; **114**: 1–13.
6 Balic A, Bowles VM, Liu YS & Meeusen EN. Local immune responses in sensitized sheep following challenge infection with *Teladorsagia circumcincta*. *Parasite Immunol* 2003; **25**: 375–381.
7 Stear MJ, Bairden K, Innocent GT, Mitchell S, Strain S & Bishop SC. The relationship between IgA activity against 4th-stage larvae and density-dependent effects on the number of 4th-stage larvae of *Teladorsagia circumcincta* in naturally infected sheep. *Parasitology* 2004; **129**: 363–369.
8 Craig NM, Miller HR, Smith WD & Knight PA. Cytokine expression in naive and previously infected lambs after challenge with *Teladorsagia circumcincta*. *Vet Immunol Immunopathol* 2007; **120**: 47–54.
9 Halliday AM, Routledge CM, Smith SK, Matthews JB & Smith WD. Parasite loss and inhibited development of *Teladorsagia circumcincta* in relation to the kinetics of the local IgA response in sheep. *Parasite Immunol* 2007; **29**: 425–434.
10 Smith WD, Jackson F, Jackson E & Williams J. Local immunity and *Ostertagia circumcincta*: changes in the gastric lymph of immune sheep after a challenge infection. *J Comp Pathol* 1983; **93**: 479–488.
11 Meyvis Y, Geldhof P, Gevaert K, Timmerman E, Vercruysse J & Claerebout E. Vaccination against *Ostertagia ostertagi* with sub-fractions of the protective ES-thiol fraction. *Vet Parasitol* 2007; **149**: 239–245.
12 Henderson NG & Stear MJ. Eosinophil and IgA responses in sheep infected with *Teladorsagia circumcincta*. *Vet Immunol Immunopathol* 2006; **112**: 62–66.
13 Knox DP & Jones DG. Studies on the presence and release of proteolytic enzymes (proteinases) in gastro-intestinal nematodes of ruminants. *Int J Parasitol* 1990; **20**: 243–249.
14 Redmond DL, Smith SK, Halliday A, *et al*. An immunogenic cathepsin F secreted by the parasitic stages of *Teladorsagia circumcincta*. *Int J Parasitol* 2006; **36**: 277–286.
15 Shevchenko A, Wilm M, Vorm O & Mann M. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Anal Chem* 1996; **68**: 850–858.
16 Batycka M, Inglis NF, Cook K, *et al*. Ultra-fast MS/MS scanning combined with monolithic column LC increases throughput in proteomic analysis. *Rapid Comm Mass Spec* 2006; **20**: 2074–2080.
17 Nisbet AJ, Redmond DL, Matthews JB, *et al*. Stage-specific gene expression in *Teladorsagia circumcincta* (Nematoda: Strongylida) infective larvae and early parasitic stages. *Int J Parasitol* 2008; **38**: 829–838.
18 Christie M & Jackson F. Specific identification of strongyle eggs in small samples of sheep faeces. *Res Vet Sci* 1982; **32**: 113–117.
19 Jackson F & Christie MG. Observations on the egg output resulting from continuous low level infections with *Ostertagia circumcincta* in lambs. *Res Vet Sci* 1979; **27**: 244–245.
20 Braun G, McKechnie NM, Connor V, *et al*. Immunological crossreactivity between a cloned antigen of *Onchocerca volvulus* and a component of the retinal pigment epithelium. *J Exp Med* 1991; **174**: 169–177.
21 Schallig HD, van Leeuwen MA, Verstrepen BE & Cornelissen AW. Molecular characterization and expression of two putative protective excretory secretory proteins of *Haemonchus contortus*. *Mol Biochem Parasitol* 1997; **88**: 203–213.
22 Zhan B, Liu Y, Badamchian M, *et al*. Molecular characterisation of the *Ancylostoma*-secreted protein family from the adult stage of *Ancylostoma caninum*. *Int J Parasitol* 2003; **33**: 897–907.
23 Geldhof P, Vercauteren I, Gevaert K, *et al*. Activation-associated secreted proteins are the most abundant antigens in a host protective fraction from *Ostertagia ostertagi*. *Mol Biochem Parasitol* 2003; **128**: 111–114.
24 Vercauteren I, Geldhof P, Peelaers I, Claerebout E, Berx G & Vercruysse J. Identification of excretory-secretory products of larval and adult *Ostertagia ostertagi* by immunoscreening of cDNA libraries. *Mol Biochem Parasitol* 2003; **126**: 201–208.
25 Visser A, Van Zeveren AM, Meyvis Y, *et al*. Gender-enriched transcription of activation associated secreted proteins in *Ostertagia ostertagi*. *Int J Parasitol* 2008; **38**: 455–465.
26 Savin KW, Dopheide TA, Frenkel MJ, Wagland BM, Grant WN & Ward CW. Characterization, cloning and host-protective activity of a 30-kilodalton glycoprotein secreted by the parasitic stages of *Trichostrongylus colubriformis*. *Mol Biochem Parasitol* 1990; **41**: 167–176.
27 Zhan B, Hotez PJ, Wang Y & Hawdon JM. A developmentally regulated metalloprotease secreted by host-stimulated *Ancylostoma caninum* third-stage infective larvae is a member of the astacin family of proteases. *Mol Biochem Parasitol* 2002; **120**: 291–296.
28 Lun HM, Mak CH & Ko RC. Characterization and cloning of metallo-proteinase in the excretory/secretory products of the infective-stage larva of *Trichinella spiralis*. *Parasitol Res* 2003; **90**: 27–37.
29 Borchert N, Becker-Pauly C, Wagner A, Fischer P, Stocker W & Brattig NW. Identification and characterization of onchoastacin, an astacin-like metalloproteinase from the filaria *Onchocerca volvulus*. *Microbes Infect* 2007; **9**: 498–506.
30 Brooks DR, Appleford PJ, Murray L & Isaac RE. An essential role in molting and morphogenesis of *Caenorhabditis elegans* for ACN-1, a novel member of the angiotensin-converting enzyme family that lacks a metallopeptidase active site. *J Biol Chem* 2003; **278**: 52340–52346.
31 Robinson MW, Greig R, Beattie KA, Lamont DJ & Connolly B. Comparative analysis of the excretory-secretory proteome of the muscle larva of *Trichinella pseudospiralis* and *Trichinella spiralis*. *Int J Parasitol* 2007; **37**: 139–148.
32 Matthews JB, Davidson AJ & Beynon RJ. The application of mass spectrometry to identify immunogenic components of excretory/secretory products from adult *Dictyocaulus viviparus*. *Parasitology* 2004; **128**(Suppl.): S43–S47.
33 Robinson MW & Connolly B. Proteomic analysis of the excretory-secretory proteins of the *Trichinella spiralis* L1 larva, a nematode parasite of skeletal muscle. *Proteomics* 2005; **5**: 4525–4532.
34 Craig H, Wastling JM & Knox DP. A preliminary proteomic survey of the in vitro excretory/secretory products of fourth-stage larval and adult *Teladorsagia circumcincta*. *Parasitology* 2006; **132**: 535–543.
35 Denham DA. The development of *Ostertagia circumcincta* in lambs. *J Helminthol* 1969; **43**: 299–310.
36 Williamson AL, Brindley PJ & Loukas A. Hookworm cathepsin D aspartic proteases: contributing roles in the host-specific

degradation of serum proteins and skin macromolecules. *Parasitology* 2003; **126**: 179–185.
37 Feng J, Zhan B, Liu Y, *et al*. Molecular cloning and characterization of Ac-MTP-2, an astacin-like metalloprotease released by adult *Ancylostoma caninum. Mol Biochem Parasitol* 2007; **152**: 132–138.
38 Vervelde L, Van Leeuwen MA, Kruidenier M, *et al*. Protection studies with recombinant excretory/secretory proteins of *Haemonchus contortus. Parasite Immunol* 2002; **24**: 189–201.
39 Marchler-Bauer A, Anderson JB, Derbyshire MK, *et al*. CDD: a conserved domain database for interactive domain family analysis. *Nucleic Acids Res* 2007; **35**: D237–D240.
40 Krätzschmar J, Haendler B, Eberspaecher U, Roosterman D, Donner P & Schleuning WD. The human cysteine-rich secretory protein (CRISP) family. Primary structure and tissue distribution of CRISP-1, CRISP-2 and CRISP-3. *Eur J Biochem* 1996; **236**: 827–836.
41 Lu G, Villalba M, Coscia MR, Hoffman DR & King TP. Sequence analysis and antigenic cross-reactivity of a venom allergen, antigen 5, from hornets, wasps, and yellow jackets. *J Immunol* 1993; **150**: 2823–2830.
42 Hawdon JM, Jones BF, Hoffman DR & Hotez PJ. Cloning and characterization of *Ancylostoma*-secreted protein. A novel protein associated with the transition to parasitism by infective hookworm larvae. *J Biol Chem* 1996; **271**: 6672–6678.
43 Ghosh K & Hotez PJ. Antibody-dependent reductions in mouse hookworm burden after vaccination with *Ancylostoma caninum* secreted protein 1. *J Infect Dis* 1999; **180**: 1674–1681.
44 Yatsuda AP, Eysker M, Vieira-Bressan MC & De Vries E. A family of activation associated secreted protein (ASP) homologues of *Cooperia punctata*. *Res Vet Sci* 2002; **73**: 297–306.
45 Young CJ, McKeand JB & Knox DP. Proteinases released in vitro by the parasitic stages of *Teladorsagia circumcincta*, an ovine abomasal nematode. *Parasitology* 1995; **110**: 465–471.
46 Hein WR & Harrison GB. Vaccines against veterinary helminths. *Vet Parasitol* 2005; **132**: 217–222.

