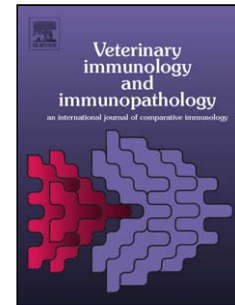


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4

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Abstract

The development of eosinophilia is a characteristic feature of helminth infection, although the exact nature of the interaction between eosinophils and parasites remains to be fully defined. Previously, it has been reported that *Haemonchus contortus* and other nematodes produce eosinophil-specific chemoattractants. This paper describes studies aimed at isolating and identifying the factor(s) responsible. Initial studies showed that soluble extracts of infective larvae (L3) of *H. contortus* provoked a chemokinetic, rather than chemotactic, response in ovine bone marrow eosinophils *in vitro*. This activity was inhibited by lactose to a markedly greater extent than sucrose suggesting a galectin-like identity. Lactose affinity chromatography of soluble *H. contortus* extracts resulted in the isolation a specific bound fraction which retained biological activity. SDS-PAGE gel electrophoresis indicated a single Coomassie-stained band at between 31 and 41kDa. Subsequent, mass spectrometric analysis confirmed that the bound fraction contained a mixture of nematode galectins. The results confirm that *H. contortus* larvae produce several galectin-like proteins, at least one of which demonstrates eosinophil chemokinetic activity *in vitro*. The possibility of the parasite-derived factor mimicking the mammalian galectin-9, a known eosinophil chemokine, is discussed.

Keywords

Eosinophils, nematodes, galectins, cell migration, ovine

Introduction

The development of systemic and local tissue eosinophilia is characteristic of the host immune response towards helminth infection (Sanderson, 1992; Jones, 1993). However, opinion is divided on the role of eosinophils during infection, in terms of both their protective effect (Behm and Ovington, 2000; Meeusen and Balic, 2000; Klion and Nutman, 2004) and their ability to mediate inflammation (Lee and Lee, 2005; Jacobsen et al., 2007). Evidence for a protective role derives mainly from *in vitro* studies on parasite killing in the presence of eosinophils or their granular components (Butterworth and Thorne, 1993; Butterworth, 1984; Rainbird et al., 1998). As yet, there are few confirmatory data from *in vivo* infections to support such an effector role. More recent studies, using transgenic and/or IL-5 knockout rodent models to alter eosinophil production and recruitment, have yielded mixed and often contradictory results with respect to disease establishment and outcome (Dent et al. 1997; Behm and Ovington, 2000; Meeusen and Balic, 2000; Klion and Nutman, 2004; Galioto et al., 2006). **Ablation of systemic and/or tissue eosinophilic responses can be associated with reduced, enhanced or unaffected severity of infection generally depending on the particular parasite and experimental situation being studied.**

In atopic conditions, the development of pathology is strongly linked to eosinophils and their products (Walsh, 1999). There is also some evidence suggesting that eosinophils may contribute to pathogenesis during parasitic infection (Hall et al., 1998; Nickdel et al., 2001). Moreover, it has previously been shown that a number of ovine parasitic gastrointestinal nematodes produce a factor(s) that promote eosinophil migration *in vitro* (Wildblood et al., 2005). This raises the possibility that helminths may actively promote eosinophil recruitment and activation, and utilize resulting tissue damage to aid their survival within the host. **For example, eosinophil-mediated mucosal damage, such as that seen in the asthmatic lung, could result in impaired absorption and, therefore, an enhanced nutrient supply for developing worms, and/or could provide a favourable microenvironment for their establishment.** The aim of this study was to attempt to characterize and identify eosinophil chemoattractant(s) produced by the infective third stage larvae (L3) of *Haemonchus contortus*.

Materials and Methods

Reagents

Recombinant murine (rm) interleukin-5 (IL-5) and rm eotaxin (Eo) were obtained from Sigma Ltd, UK. **RmIL-5 and rmEo were prepared as 5µg/ml and 20µg/ml stocks in 0.1% bovine serum albumin in PBS according to the manufacturer's instructions and then, for use as positive controls** in the cell migration assay, both were diluted to **a final concentration of 100ng/ml** in 10% Iscove's Modified Dulbecco's Medium (IMDM; (Sigma Ltd, UK) supplemented with 10% heat inactivated foetal bovine serum (FBS; (Sigma Ltd, UK)), 0.05mM 2-mercaptoethanol (Sigma Ltd, UK), 100 IU/ml penicillin, 100µg/ml streptomycin (Gibco BRL, UK) and 10 IU/ml preservative free heparin (Sigma Ltd, UK)). β-Lactose (Lac) was obtained from Sigma Ltd, UK and sucrose (Suc) from Fisher Scientific Ltd, UK. **Both disaccharides were prepared as 1M stock solutions in PBS and then diluted, as required, in IMDM for use in the inhibition experiments.**

Gastrointestinal nematodes

Worm donor animals

Lambs aged 3-6 months were challenged orally with approximately 5000 *H. contortus* infective third stage larvae (L3). Animals were then left for 21 days to allow the larvae to develop into egg producing adults.

Preparation of L3

Larvae were isolated from the faeces of donor animals, by standard parasitological techniques. Briefly, faeces containing eggs was cultured at 22°C for 10 days to allow development to the L3 stage. Faecal cultures were soaked with water and filtered at 4°C. Filtered sediments were warmed with water to allow larvae to migrate through the filter for collection. L3 were stored at 4°C in saline until required.

Preparation of L3 extract

Approximately 2×10^5 L3 were centrifuged at $12000 \times g$ for 10 minutes, and the supernatant discarded. The worm pellet was resuspended in 1ml ice cold phosphate buffered saline (PBS), and transferred to a ribolyzing tube (Matrix D, Fisher Life Sciences, UK) with an additional 1ml PBS. Worms were ribolyzed for 20 seconds using a Fast Prep FP120 (Thermo Electron Corporation, UK) and then cooled on ice for 10 minutes before the ribolyzing procedure was repeated. This process was continued until the worms were completely fragmented. The worms were left on ice for 30 minutes before being spun at $14000g$ for 15 minutes. The resulting supernatant was collected and designated S1 (whole worm soluble extract).

Total protein measurement

Total protein concentrations were measured using a commercial kit (BCA reagent, Pierce UK Ltd) in accordance with the manufacturer's instructions.

Bone marrow donor animals

A variety of conventional crossbred lambs, under 1-year-old, of both sexes, were used as bone marrow donors.

Collection and preparation of ovine bone marrow cells

Bone marrow cells were isolated from the sternum at **necropsy**, and prepared as previously described (Haig et al., 1988). Briefly, bone marrow suspensions were filtered through a double layer of lens tissue (Whatman, UK). Filtered cells were washed in Hanks' balanced salt solution (HBSS; Life Technologies, UK), supplemented with 2% heat inactivated FBS, heparin, penicillin and streptomycin (as detailed above), and 0.04% sodium bicarbonate (Fisher Scientific Ltd, UK). After resuspension, a cell fraction with a density of $<1.077g/ml$ was obtained by centrifugation over Lymphoprep (Nycomed, UK) at $1000 \times g$ for 30 min at room temperature. Then, after further washing in HBSS, cells were resuspended in 10% IMDM and, for use in the chemotaxis assay, adjusted to contain 5×10^6 viable cells/ml. Differential eosinophil counts were performed

using freshly prepared Carpentier's stain containing 2% of 2% eosin-Y (Sigma Ltd, UK) and 3% of 37% formaldehyde saturated with calcium carbonate. Cells were diluted 1:10 and the percentage of eosinophils (routinely 15-25%) was determined by light microscopy.

Eosinophil migration

Eosinophil migratory activity was estimated using modified blind-well Boyden chambers (Neuroprobe, USA). Briefly, the lower compartment was filled with either a control or test sample diluted in 10% IMDM, and covered with an 8µm pore size nitrocellulose membrane (Sartorius, Germany). Approximately 1×10^6 bone marrow cells were added to the upper compartment, and then the chambers were incubated for 3 hours at 37°C, 5% CO₂. **Note: for the sugar inhibition experiments both the controls and test samples, and the cell suspensions were diluted, immediately before use, in IMDM containing lactose or sucrose to give the final assay concentrations required.**

Following incubation filters were removed, washed in PBS and fixed overnight in 70% ethanol (Fisher Scientific Ltd, UK). Prior to mounting on microscope slides, fixed filters were differentially stained with Quick-Diff staining kit (Gamidor, UK), dehydrated and rendered transparent by serial treatment with ethanol, n-propanol (Rathburn Chemicals Ltd, UK), n-propanol:xylene, and xylene (Fisher Scientific Ltd, UK). Stained cells from five randomly chosen views were counted by light microscopy (x40) at a depth of 50µm from the upper surface (cell side) of the filter.

Statistical analysis of cell migration data

Due to inherent biological variation in cell responsiveness from different bone marrow preparations, results are presented as the log₁₀ transformed total eosinophil counts from the five random fields of view. For statistical analysis the log₁₀ transformed counts from three **replicate** experiments per test stimulus were compared with the appropriate control by paired Student's *t*-test: *p*-values <0.05 were considered statistically significant.

Preparation of lactosyl-sepharose

Lactosyl-sepharose was prepared essentially as previously described (Levi and Teichberg, 1981). Briefly, 10ml sedimented sepharose 4B beads (Sigma Ltd, UK) were suspended in 10ml 0.5M sodium carbonate (Na_2CO_3 ; Sigma Ltd, UK) and washed several times until a pH of 11.0 was obtained. The washed beads were filtered using a Buchner funnel with 185mm thickness filter paper (7cm diameter, No 1, Whatman, UK), resuspended in 10ml 0.5M Na_2CO_3 and placed on a magnetic stirrer. 1ml divinyl-sulfone (Sigma Ltd, UK) was then added and allowed to react for 70 minutes. Beads were then re-filtered and washed in 0.5M Na_2CO_3 several times to remove unreacted divinyl-sulfone. Filtering was repeated, the beads suspended in 10ml 10% lactose in 0.5M Na_2CO_3 , and then placed on a magnetic stirrer overnight. The beads were re-filtered and the serially washed in 0.5M Na_2CO_3 , deionised H_2O (dH_2O) and finally in 4mM 2-mercaptoethanol in PBS (MePBS), pH 7.0. Beads were stored in MePBS supplemented with 0.02% sodium azide (Sigma Ltd, UK) at 4°C.

Lactose affinity separation

S1 extract, diluted to 20µg/ml total protein content with MePBS, was added to an equal volume of lactosyl-sepharose beads equilibrated with MePBS and allowed to react at room temperature on a roller for 1 hour. Beads were then allowed to sediment out before the supernatant was collected as the unbound fraction. Beads were washed 3-4 times in an equal volume of MePBS, prior to elution of the bound components in an equal volume of 10% lactose in MePBS, and allowed to react at room temperature for a further hour. The beads were then allowed to sediment again after which the supernatant was collected and designated the bound fraction.

SDS-PAGE analysis

Fractions were concentrated sufficiently to allow for visualization on a 12% SDS-PAGE gel fixed with 25% isopropanol (Sigma Ltd, UK), 10% glacial acetic acid (Fisher Scientific Ltd, UK) and stained with PAGEBlue (Fermentas Ltd, UK). Prior to running on the gel, samples were reduced in 50mM dithiothreitol (Sigma Ltd, UK) and boiled for 5 minutes. Samples were then loaded onto freshly prepared 12% SDS-PAGE mini-gels and run at 120V for 60 minutes.

Mass spectrometry

The band of interest was excised from the colloidal Coomassie blue-stained SDS-PAGE gel, destained and then subjected to in-gel tryptic digestion. Following digestion, LC-ESI-MS/MS analysis was performed using a modification of the method described by Batycka *et al.*, 2006). Briefly, HPLC was carried out using an Ultimate 3000 nano-HPLC system (Dionex, LC-Packings) comprising autosampler, 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 software. Samples (4µl) were applied to the system by direct injection and separated on a reverse phase 200µ i.d. x 5cm monolithic capillary column (LC-Packings) at a flow rate of 3µl/min. The column was maintained at constant temperature (50°C) throughout. Peptides were eluted in order of increasing hydrophobicity by the application of a 15 min linear gradient of 8%-45% acetonitrile, 0.1% formic acid, with the eluant directed through a 3nl UV detector flow cell. The LC system was coupled to a 3D high-capacity ion trap tandem mass spectrometer (Esquire HCTplusTM, Bruker Daltonics, Germany) via an API-ESI interface utilising a low volume stainless steel nebuliser (Agilent) at 15psi. ESI-MS/MS data acquisition was initiated on a contact closure signal triggered by the Chromeleon software.

Mass spectrometry data analysis

Raw MS/MS data was processed using DataAnalysisTM software (Bruker Daltonics) and converted into Mascot generic files (mgf). Database searches were performed using BioToolsTM software (Bruker Daltonics) and the Mascot search algorithm (Matrix Science Ltd) to mine an in-house primary sequence database (Nematode-db). **Nematode-db is a specific compilation of all available sequence data for nematode proteins, and excludes data from other species.** Mass tolerances for precursor (MS¹) and fragmentation ions (MS²) were set at 1.5Da and 0.5Da respectively. Acceptance criteria for confirmation of protein identity were at least two identified peptides per protein, and a minimum of 4 contiguous “b” and/or “y” ions for at least one peptide.

Results

Chemotaxis vs. chemokinesis

Note: In all the experiments described, **the observed** migratory responses for the positive controls were significantly ($p < 0.05$) higher than for the negative IMDM control.

Three separate **replicate** experiments **were carried out to** evaluate the comparative effects of chemoattractant gradient on the migratory responses of bone marrow-derived eosinophils to two known eosinophil-specific chemoattractants (rmEo and rmlL-5) and the soluble extract (S1) from *H. contortus* L3. **The** S1 extract significantly promoted eosinophil movement, independently of chemoattractant gradient, in a similar fashion to rmlL-5 (**Figure 1**). In contrast, for rmEo, significantly increased eosinophil migration was observed when it was present only in the lower compartment *i.e.* only when there was a positive rmEo gradient with respect to the cells.

Carbohydrate inhibition

In a further three replicate experiments **to** evaluate the effect of lactose on S1-induced eosinophil migration, **it was shown that millimolar concentrations of** lactose had a marked and dose-related inhibitory effect on migration which reached statistical significance ($p < 0.05$) at the two highest concentrations (50 and 100mM) tested (**Figure 2**). *Note:* In a previous series of experiments 100mM lactose was shown to have no effect on enhanced eosinophil migration in response to either rmEo or rmlL-5 (data not shown).

Finally in a further series of three replicate experiments **which directly** compared the effects of two similar disaccharides at the same concentration (50mM) on S1-induced eosinophil migration, **it was shown** that lactose had a significantly greater ($p < 0.01$) effect than sucrose (**Figure 3**).

Lactose affinity

Whole S1 extract was separated based on its affinity for lactose, and the resulting unbound and bound fractions were tested for their ability to induce eosinophil migration. The **results** from **three replicate** experiments indicated that the bound fraction retained the ability to induce eosinophil migration, and that this effect was concentration-

dependent (**Figure 4**). In contrast, the unbound fraction displayed virtually no promigratory activity.

SDS-PAGE and mass spectrometric analysis

Lactosyl-sepharose fractionated S1 material was concentrated sufficiently to allow visualization by colloidal Coomassie blue staining on a 12% SDS-PAGE mini-gel (Figure 5). The biologically active bound fraction contained only a single dominant band of protein corresponding to a molecular weight of between 31 and 41kDa. The material in this band was subsequently analysed by LC-ESI-MS/MS in an attempt to identify the component protein(s). The mass spectrometry data, summarized in Table 1, indicated the presence of a mixture of proteins. There were three significant matches to entries on the local nematode EST database, all of which shared significant homology with nematode galectins. *Note:* Since this database is incomplete, scores and coverage of proteins is likely to be lower than would be the case if a fully sequenced and annotated genomic database were available.

Discussion

Work previously conducted in this laboratory demonstrated that live L3 and whole adult worm extracts from *H. contortus* has an associated eosinophil chemoattractant activity (Wildblood et al., 2005). The results presented here offer insight into the nature of the active component from soluble L3 extracts.

Using an *in vitro* assay based upon the modified Boyden chamber (Boyden, 1962), it was initially shown that the factor(s) had chemokinetic, rather than chemotactic, properties (see Figure 1) since there was no requirement for a chemical gradient to induce cell migration. Previous reports concerning parasite-derived chemoattractants have implied the presence of both chemotactic and chemokinetic activities, and there have been suggestions that multiple factors may be present which differ in their mode of action (Klesius et al., 1985; Horii et al., 1986; Potter and Leid, 1986; Klesius, 1993;).

Currently two potent mammalian eosinophil-specific chemokinetic factors have been described, namely IL-5 and galectin-9 (Matsushita et al., 2000). Data obtained in this

study (see Figures 2 and 3), based on preferential inhibition by lactose, suggested that the pro-migratory activity from *H. contortus* was galectin-like. Interestingly, this is not an isolated finding in that a chemotactic activity from *Ostertagia ostertagi* was also suggested to be a lectin, due to its inhibition by various saccharides (Klesius, 1993). The finding here that, following lactosyl sepharose affinity chromatography, only the bound S1 fraction retained eosinophil chemokinetic activity (see Figure 4) provided further evidence for the presence of a galectin. Following staining with Coomassie blue on an SDS-PAGE gel this bound S1 fraction was shown to contain only one prominent protein band. Subsequent mass spectrometric analysis revealed that this band comprised a mixture of nematode galectins with demonstrable homology to Hco-Gal-1, *Caenorhabditis elegans* Lec-4, Hco-Gal-3a/b, Hco-Gal-2 and Hco-Gal-m/f. This confirmed the observations from the sugar inhibition studies that the *H. contortus* eosinophil chemoattractant is likely to be a galectin.

The function of nematode galectins is not well understood, although they are believed to be important for survival and interaction with the host (Young and Meeusen, 2004). One possibility raised is that parasite-derived galectins mimic the action of host proteins which have been shown to modulate a range of immune responses including cell recruitment, activation, development and apoptosis (Leffler et al., 2004; Young and Meeusen, 2004). Of particular interest within the context of the present work is galectin-9 which has been reported to have specific effects on both eosinophil migration (Hirashima, 2000) and apoptosis (Saita et al., 2002). Expression of various *H. contortus* galectins, which may or may not be related to the observed eosinophil chemokinetic activity, has been demonstrated to occur at different levels depending on the life cycle stage (Klion and Donelson, 1994; Young and Meeusen, 2004). For example, Hco-Gal-1 is expressed by all developmental stages, although the highest levels were reported in L2 and unexsheathed L3. A second galectin was produced preferentially in the adult stage, whilst yet another was expressed maximally in unexsheathed L3, and declined in the later parasitic stages (Greenhalgh et al., 2000). Although the biological functions of these proteins are poorly understood, ligands have been isolated both from host and parasite extracts by affinity chromatography which ultimately may lead to the assignment of specific biofunctionality. Similar work on *C. elegans* galectins showed that LEC-1 (*C. elegans* galectin 1) was associated to the cuticle and pharynx (Greenhalgh et al., 2000).

340

341 Conclusions

342

343 The results presented here have established that *H. contortus* produces an, as yet,
344 unidentified galectin, or possibly mixture of galectins, which have potent chemokinetic
345 activity for ovine eosinophils *in vitro*. It is hypothesised that the parasite-derived
346 protein(s) may mimic the action of the host galectin-9, and could play an important role in
347 the development of nematode-associated, eosinophilic inflammation *in vivo*. Studies are
348 underway to clone, express and test the biological activities of recombinant galectins,
349 based on those identified by the current mass spectrometric analysis.

350

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352

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357

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Figure Captions

Figure 1: Effect of chemoattractant gradient on eosinophil migratory activity. Results from three **replicate** experiments, expressed as mean ($\pm$ S.E.M) $\log_{10}$ total eosinophil counts per 5 fields of view, are shown. Treatments were: Column 1, negative control (IMDM); Columns 2-4, rmEo (100ng/ml) in the lower, upper or both compartments respectively; Columns 5-7, rmlL-5 (100ng/ml) in the lower, upper or both compartments respectively; Columns 8-10, S1 (500ng/ml) in the lower, upper or both compartments respectively. Asterisks above columns indicate a statistically significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p \leq 0.001$) from the IMDM negative control by paired Student's *t*-test.

Figure 2: Effect of increasing concentrations of lactose on eosinophil migratory activity. Results from three **replicate** experiments, expressed as mean ($\pm$ S.E.M) $\log_{10}$ total eosinophil counts per 5 fields of view, are shown. Treatments were: Column 1, negative control (IMDM); Column 2, rmEo (100ng/ml); Column 3, rmlL-5 (100ng/ml); Columns 4-9, S1 (500ng/ml) with 0, 5, 10, 20, 50, 100mM lactose respectively. Asterisks above columns indicate a statistically significant difference (* $p < 0.05$) from S1 alone (Column 4) by paired Student's *t*-test.

Figure 3: Differential effect of equivalent concentrations (50mM) of lactose (Lac) and sucrose (Suc) on S1-induced eosinophil migration. Results from three **replicate** experiments, expressed as mean ($\pm$ S.E.M) $\log_{10}$ total eosinophil counts per 5 fields of view, are shown. **Treatments were: Column 1, negative control (IMDM); Column 2, S1 (500ng/ml); Column 3, S1 (500ng/ml) with 50mM sucrose; Columns 4-9, S1 (500ng/ml) with 50mM lactose.** Common letters above the columns indicate a statistically significant (a, $p < 0.005$, b, $p < 0.01$, c, $p < 0.005$) difference between those columns.

Figure 4: Effect of bound and unbound S1 fractions following affinity separation upon eosinophil migratory activity. Results from three **replicate** experiments, expressed as mean ($\pm$ S.E.M) $\log_{10}$ total eosinophil counts per 5 fields of view, are shown. Treatments were: Column 1, negative control (IMDM); Column 2, rmEo (100ng/ml); Column 3, rmlL-

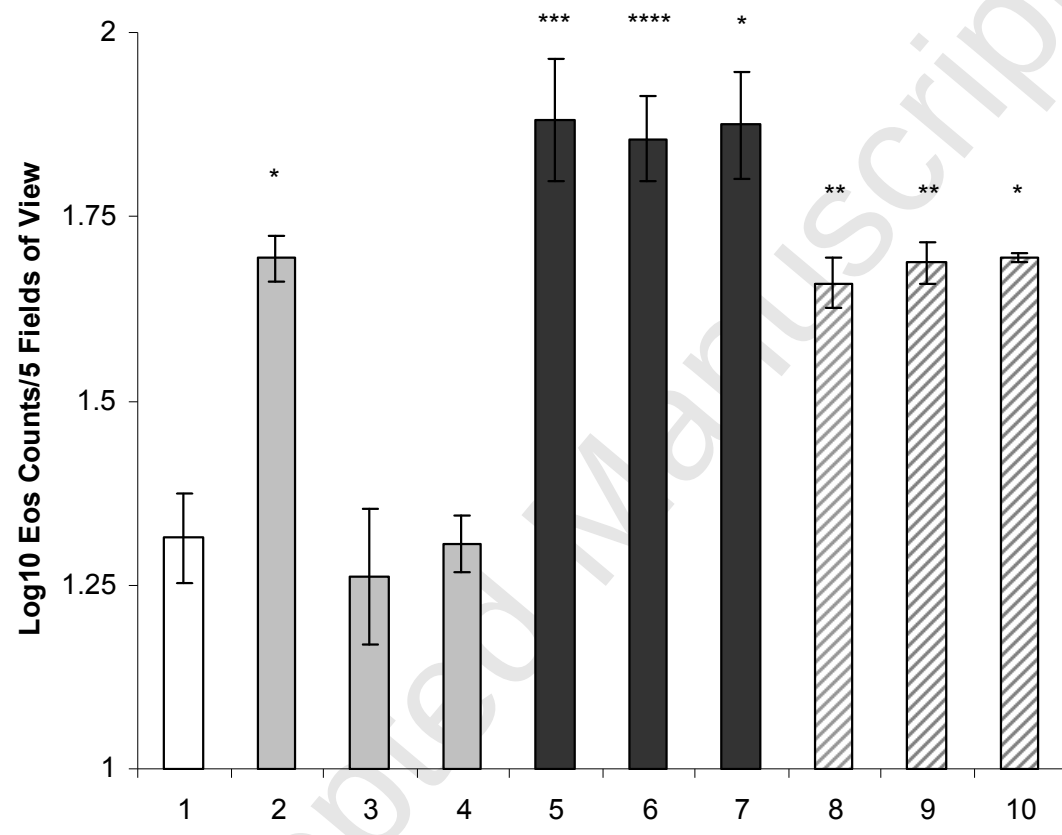
5 (100ng/ml); Column 4, Unfractionated S1 (500ng/ml); Column 5, Bound S1 fraction (equivalent to 500ng/ml unfractionated S1); Column 6, Bound fraction (equivalent to approximately 1µg/ml unfractionated S1); Column 7, Unbound fraction (equivalent to approximately 500ng/ml unfractionated S1); Column 8, Unbound fraction (equivalent to approximately 1µg/ml unfractionated S1). Asterisks above columns indicate a statistically significant difference (* $p < 0.05$, *** $p < 0.005$) from negative control IMDM (Column 1) by paired Student's *t*-test.

Figure 5: Colloidal Coomassie blue-stained 12% SDS-PAGE mini-gel of unfractionated and fractionated soluble (S1) larval *H. contortus* extract. M, molecular weight markers; S1, unfractionated S1 extract; U, unbound S1 fraction; B, bound S1 fraction.

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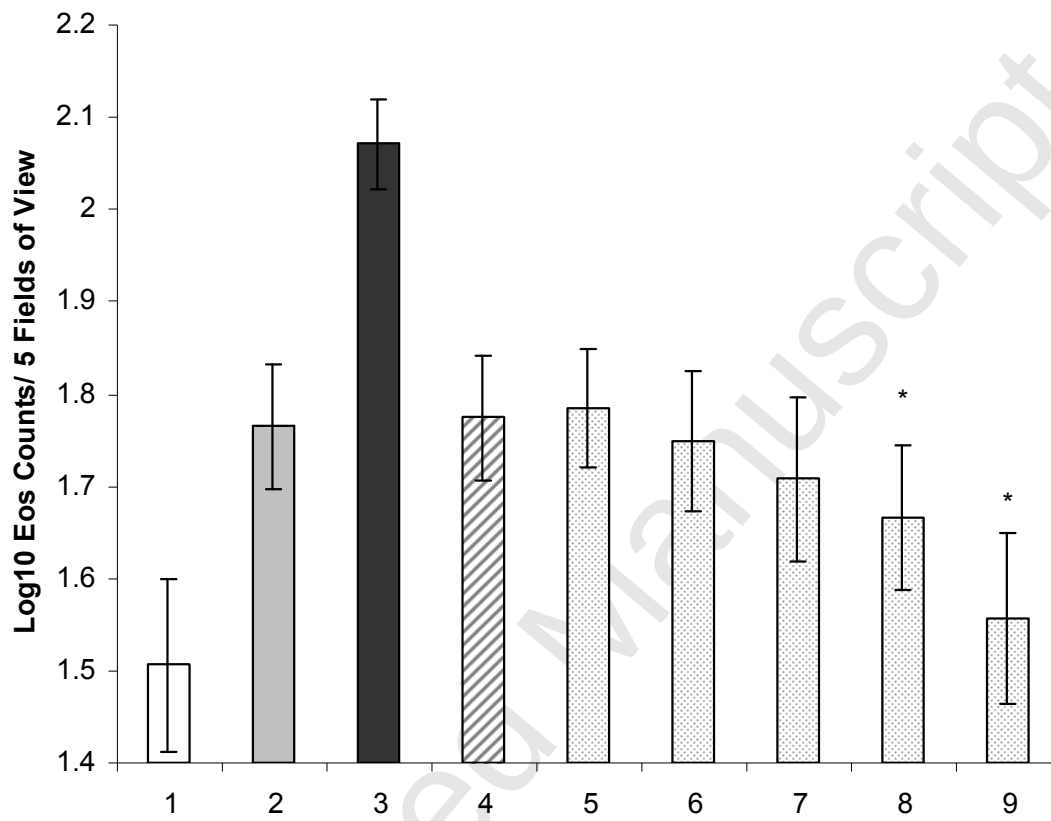
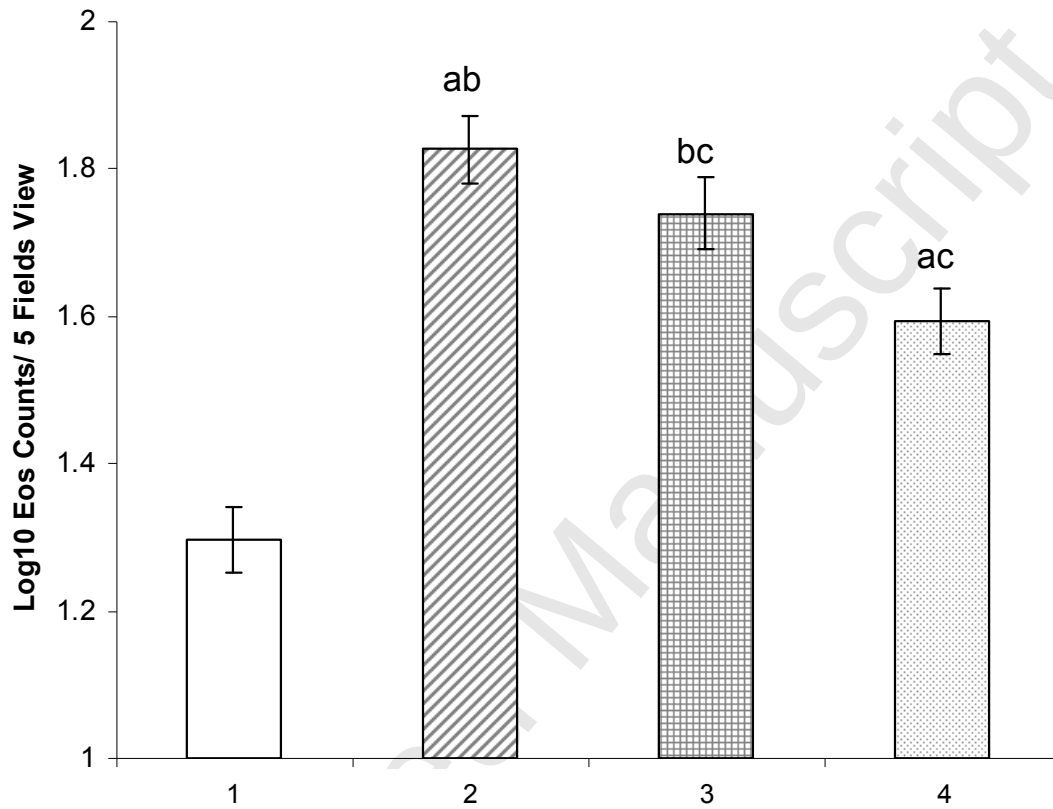
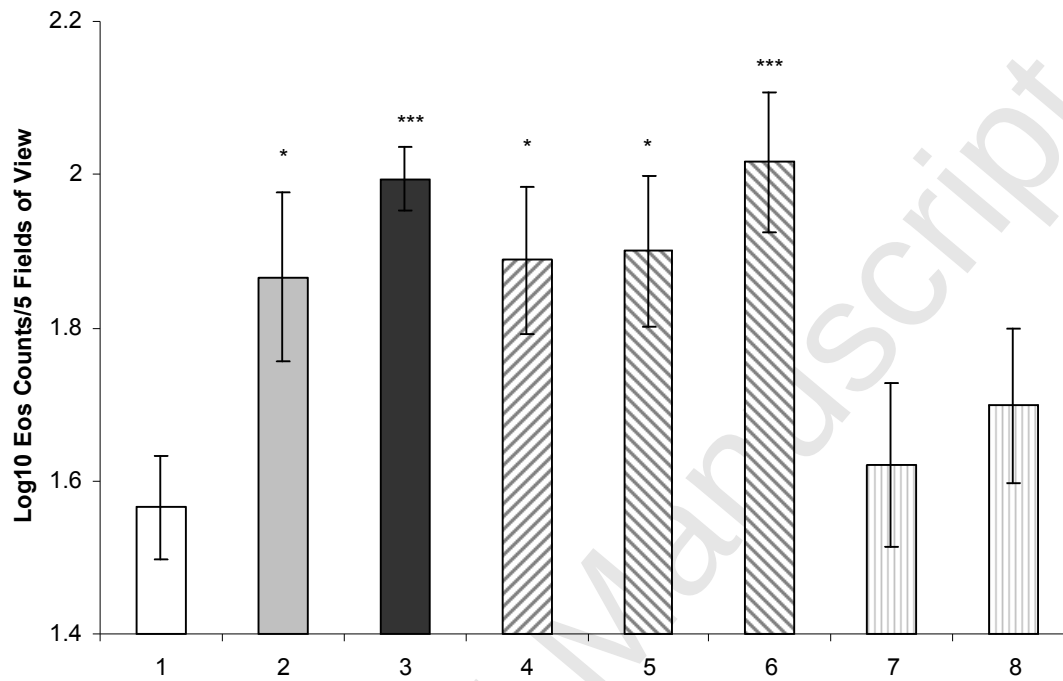
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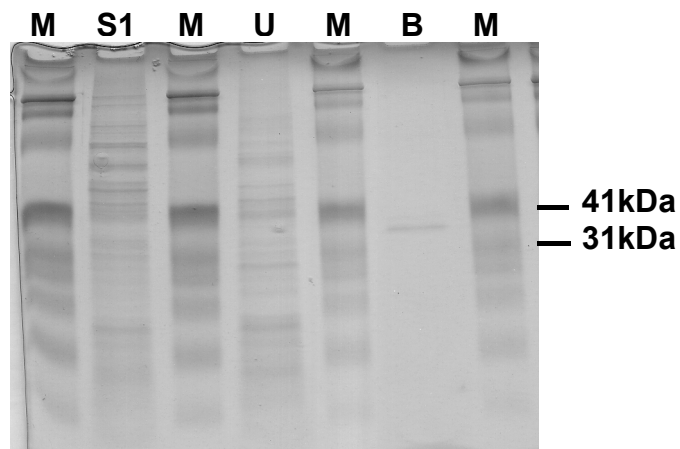


Table – Turner *et al*

Table 1. Summary of the LC-ESI-MS/MS analysis of the single SDS-PAGE gel band from the lactosyl sepharose-bound *H. contortus* S1 fraction.

Accession Number	Mowse Score	Peptide Matches	% Coverage	MS/MS	Ion Scores
gi 3341814	336	9	11	K.LTEPFEPGQTLTVK.G	3
				K.LTEPFEPGQTLTVK.G	46
				K.FQIFVDQK.E	43
				K.YYPVPYESGLAGEGLAPGK.S	79
				K.SIFLYGMPEK.K	48
				K.NGDIALHFNPR.F	35
				K.AVGFDLEIK.N	28
				K.NEEYAFQIMVNGER.F	64
gi 27475449	132	4	12	K.LGQPLMAGQTIDIHGAINEDANR	23
				R.MPFDQIEYLQIK.G	68
				K.VPWESAFPDGHLK.A	2
				R.WNLDLLGR.D	41
CB038029	44	2	8	K.IEPGQTLIVK.G	9
gi 2687412	41		2	R.NGDIALHFNPR.F	35
gi 7542331	41		1		
gi 7542333	41		2		
gi 30039163	41		3		
gi 30039165	41		3		

Footnote: Mass tolerances were set at 1.5Da for peptides and 0.5Da for MS/MS. Peptides were searched against the in house nematode EST database. Acceptance criteria for confirmation of protein identity were at least two peptides per protein and a minimum of 4 contiguous “b” and/or “y” ions for at least one peptide.