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Proteomic analysis of secretory products from the model gastrointestinal nematode *Heligmosomoides polygyrus* reveals dominance of Venom Allergen-Like (VAL) proteins

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Abstract

The intestinal helminth parasite, *Heligmosomoides polygyrus* offers a tractable experimental model for human hookworm infections such as *Ancylostoma duodenale* and veterinary parasites such as *Haemonchus contortus*. Parasite excretory-secretory (ES) products represent the major focus for immunological and biochemical analyses, and contain immunomodulatory molecules responsible for nematode immune evasion. In a proteomic analysis of adult *H. polygyrus* secretions (termed HES) matched to an extensive transcriptomic dataset, we identified 374 HES proteins by LC-MS/MS, which were distinct from those in somatic extract HEx, comprising 446 identified proteins, confirming selective export of ES proteins. The predominant secreted protein families were proteases (astacins and other metalloproteases, aspartic, cysteine and serine-type proteases), lysozymes, apyrases and acetylcholinesterases. The most abundant products were members of the highly divergent venom allergen-like (VAL) family, related to *Ancylostoma* secreted protein (ASP); 25 homologues were identified, with VAL-1 and -2 also shown to be associated with the parasite surface. The dominance of VAL proteins is similar to profiles reported for *Ancylostoma* and *Haemonchus* ES products. Overall, this study shows that the secretions of *H. polygyrus* closely parallel those of clinically important GI nematodes, confirming the value of this parasite as a model of helminth infection.

1. Introduction

Infection with intestinal nematode parasites such as hookworm, whipworm and *Ascaris* remains an enormous global health problem, with over 25% of the world's population infected [1]. Moreover, similar pathogens account for major morbidity and economic loss

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among livestock in temperate climates [2]. The high prevalence and longevity of these parasites in immunocompetent hosts reflects a sophisticated array of mechanisms to modulate, disrupt and divert the host immune response [3; 4]. However, the identification of molecular mediators of parasite immunomodulation is still at an early stage [5-7]. For these reasons, the recent expansion in genomic [8-11], transcriptomic [12-21] and proteomic [22-30] analyses of parasitic nematodes provides an exciting platform for new discoveries.

A major theme in helminth research is the analysis of products released by live parasites which are likely to fulfil the many biological imperatives faced by a pathogen, including invasion of the host, creation of a suitable niche, and evasion of host immunity. These molecules, termed excretory-secretory (ES) products, have been the particular target of proteomic studies aimed at characterising the “secretome” of the major human [25-27] and veterinary [22; 23; 28; 30] parasites. In addition, many prominent individual ES proteins have been identified, most notably members of a large multi-gene Venom Allergen-Like (VAL) family [28; 31; 32], first characterized in ES of the canine nematode *Ancylostoma caninum* and named *Ancylostoma* Secreted Protein (ASP) [33]. Members of this gene family include effective vaccine molecules in experimental models [34], indicating also the potential for ES proteins as new immunoprophylactics against helminth infections in man and animals.

Because the major human intestinal helminth species do not normally infect laboratory animals, model systems with natural rodent nematode parasites are invaluable in gaining insights into the factors regulating infection and immunity. The murine intestinal nematode parasite, *Heligmosomoides polygyrus*, provides a widely studied system [35], and much is now known of the immunology of infection and the immune components which combine to protect the host [36-40]. Parasite-infected mice feature multiple levels of immunosuppression, including amelioration of allergy [41; 42], autoimmune diabetes [43; 44] and colitis [45-48]. At least part of the immunosuppression can be accounted for by expanded regulatory T cell activity [42; 49-51] and suppressive B cell populations [52] in infected mice, which are also able to transfer immunosuppression to uninfected recipients [42; 52].

Significantly, the immunomodulatory effects of live *H. polygyrus* infection can be reproduced with the soluble products (HES) collected from adult parasites cultivated *in vitro*. HES converts naive murine T cells into suppressive regulatory T cells [53], interferes with the ability of dendritic cells to stimulate effector T cells and suppresses antibody responses to unrelated antigens [54], and can prevent the development of airway allergy in mice (O’Gorman, McSorley et al., manuscript in preparation). Hence the nature of the HES products is of intense interest for potential novel immunomodulators that might be exploited in therapy of allergy and autoimmunity. More broadly, intestinal nematodes co-habit a complex ecosystem with commensal microbes, and bacterial-parasite interactions are also likely to be important in the establishment of a long term nematode infection [55].

Despite the sophistication of the cellular immune analyses of *H. polygyrus* infection, few molecular products from this parasite have yet been described [24; 56-60]. Indeed, genomic and transcriptomic datasets are only now being developed for this organism (Harcus et al.,

manuscripts in preparation). Taking a proteomic approach, we have identified the majority of proteins secreted by adult *H. polygyrus*, and show that there is a predominance of VAL/ASP-like products, which demonstrates that the overall composition and functional profile of HES closely parallels those of *Ancylostoma* and *Haemonchus* parasites. These results pave the way to use the mouse model for more precise determination of the role of many individual proteins in the biological processes of infection, intestinal establishment, and manipulation of the host immune response.

2. Materials and Methods

2.1 Parasites and HES

The original stock of *H. polygyrus bakeri* used in these studies was kindly supplied to us by Professor J M Behnke, University of Nottingham, UK. The life cycle of *H. polygyrus* was maintained in CBAxC57BL/6 F1 mice infected with 500 infective larvae by gavage, and adult worms were recovered 14 days later. Adult worms were washed extensively before incubation in serum-free RPMI1640 medium supplemented with 1% glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 100 μg/ml gentamicin (Gibco). Culture supernatants were recovered at 3-4 day intervals and replaced each time with fresh medium over a 3 week period. Worms remained viable throughout this time frame. Pooled supernatants were diafiltrated into PBS over a 3,000 MWCO Amicon membrane, and the resultant HES (*H. polygyrus* excretory / secretory products) material stored at −80°C [59]. The profile of proteins released each week did not differ significantly (Supplementary Figure 1). Soluble somatic extracts of adult worms (*H. polygyrus* extract; HEx) were prepared by homogenisation in a ground-glass hand-held homogeniser (VWR-Jencons, UK) in ice-cold PBS, followed by centrifugation at 13,000 *g* for 30 mins, from which the supernatant was collected and stored at −80°C until use.

2.2 2-D Gel Electrophoresis and spot identification

HES and HEx (25 μg per gel) were separated and silver stained as previously described [25], then scanned with a Linoscan 1450 (Heidelberg). Protein spots of interest were prepared for mass spectrometry analysis as before [25], and positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron mode, equipped with a Nd:YAG smart beam laser. MS spectra were acquired over a mass range of m/z 800-4000, and monoisotopic masses were obtained using a SNAP averaging algorithm. The ten strongest peaks of interest, with a S/N greater than 30, were selected for MS/MS fragmentation in LIFT mode. Bruker flexAnalysis software (version 3.3) was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra.

2.3 LC-MS/MS

Tryptic HES peptides were prepared essentially as before [25] and then loaded onto a nanoAcquity UPLC system equipped with a nanoAcquity Symmetry C_{18}, 5 μm trap (180 μm × 20 mm) and a nanoAcquity BEH130 1.7 μm C_{18} capillary column (75 μm × 250 mm; all Waters). The trap was washed for 5 min with 0.1% (v/v) formic acid at 10 μL/min. Subsequently, flow was switched to the capillary column, and peptides were separated by gradient elution (Solvent A = 0.1% (v/v) formic acid; Solvent B = acetonitrile with 0.1%

(v/v) formic acid; Initial gradient conditions 5% solvent B (2 min), then a linear gradient to 35% solvent B over 120 min, followed by a linear gradient to 50% solvent B over 5 min, and finally wash with 95% solvent B for 10 min. Flow rate was 300 nL/min and column temperature was 60°C). The nanoLC system was interfaced with a maXis UHR-TOF mass spectrometer (Bruker Daltonics) with a nano-electrospray source fitted with a steel emitter needle (180 μm O.D. × 30 μm I.D.; Proxeon). Instrument control, data acquisition and processing were performed using Compass 1.3 SR3 software (microTOF control, Hystar and DataAnalysis; Bruker Daltonics). Positive ESI-MS & MS/MS spectra were acquired using AutoMSMS mode. Instrument settings were: ion spray voltage: 1,500 V, dry gas: 6 L/min, dry gas temperature 160°C, ion acquisition range: *m/z* 50-2,200. AutoMSMS settings were: MS: 0.5s (acquisition of survey spectrum), MS/MS (CID with N_2 as collision gas): ion acquisition range: *m/z* 300-1,500, 5 precursor ions, absolute threshold 1,000 counts, acquisition time: 0.1s for precursor intensities >=100,000 counts increasing linearly to 1s for precursor intensitities of 1,000 counts, collision energy and isolation width settings were calculated automatically using the AutoMSMS fragmentation table, preferred charge states: 2 – 4, singly charged ions excluded, one fragmentation spectrum was acquired for each precursor and former target ions were excluded for 30s.

2.4 Database Searching and Bioinformatics

Tandem mass spectral data were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.1), through the Bruker ProteinScape interface (version 2.1). Search parameters required trypsin specificity, the carbamidomethylation of cysteine, allowed a maximum of one missed cleavage, and the possible oxidation of methionine. Spectra were searched against an in-house database composed of >460,000 cDNA sequences from both normalised and non-normalised libraries made from adult worm mRNA (Harcus et al, manuscript in preparation; http://genepool.bio.ed.ac.uk/blast/hpoly.html). The database was supplemented with existing NCBI depositions for *H. polygyrus*. Sequencing was performed on a Roche 454 instrument yielding reads of ~200 nt, and assembled into isotigs each representing a distinct transcript using Newbler 2.5. For gel spot identifications a peptide tolerance of 250 ppm and MS/MS tolerance of 0.5 Da were employed. For LC-MS/MS Mascot searches, MudPit scoring was used with a peptide tolerance of 10 ppm and MS/MS tolerance of 0.1 Da. The significance threshold was set at $p<0.05$, and hits were manually inspected for the presence of open reading frames. All LC-MS/MS data were filtered to only accept peptides with expect values <0.05, and single peptide hits were further filtered requiring expect values <0.01. All protein matches were required to contain atleast one unique peptide sequence not matched in any higher ranked proteins. The LC-MS/MS data were also searched against a Mascot generated decoy database, containing a random set of sequences with the same average amino acid composition and sequence length as the target database. Comparison of the number of sequences identified in the target and decoy databases estimated a false discovery rate of 2.01% HES and 2.94% for HEx for peptide matches above identity threshold. Spectra were also searched against Swiss-Prot to identify potential murine or bacterial proteins present in HES and HEx. Here the false discovery rate was higher (33.1% for HES and 6.92% for HEx), likely reflecting the paucity of *H. polygyrus* sequences in the database, and their low level of sequence identity with other species. The exponentially Modified Protein

Abundance Index (emPAI) for each identification was calculated according to the ratio of observed and observable peptides for each protein.

Identified protein sequences were subject to analysis by SignalP3.0 to ascertain presence of predicted signal peptide [61]. In some instances, protein sequences were judged to be truncated, by the absence of a start methionine and/or by homology to known protein sequences from other organisms. Proteins and conserved domains were identified by BLAST, and gene ontology (GO) categories determined with Interproscan version 31.0 (http://www.ebi.ac.uk/Tools/pfa/iprscan/). Protein sequences lacking conserved domains, but with significant similarity (BLAST score >40) to nematode proteins were labelled conserved nematode proteins (CSN = Conserved Secreted, No signal peptide; CSP = Conserved Secreted with signal-Peptide; CXN = Conserved eXtract, No signal peptide; CXP = Conserved eXtract with signal-Peptide). Novel sequences (BLAST score <40) were labelled NSN, NSP, NXN and NSP as described above. ClustalW sequence alignments were performed using MacVector version 11.1.1. To identify potential *N*- and *O*-glycosylation sites, NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/) were used respectively. Accession numbers given for nucleotide and protein sequences are those deposited with NCBI.

2.5 Antibody generation and Western blotting

HES (1 μg) was separated by 2-D gel electrophoresis as described above, blotted as before [25], and then blocked in 5% skimmed milk powder (Marvel)-TBS with 0.05% Tween 20 (TBST) for 2 hours at room temperature. Polyclonal antibodies to *Hp*-VAL-1, -2 and -4 were generated to 6-His-tagged recombinant proteins expressed in pET21 (Novagen)-transformed *E. coli*, solubilised in 8M urea and purified by metal chelating chromatography under the same chaotropic conditions; rats were immunized with 100 μg of recombinant protein co-precipitated with alum, boosted on days 28 and 35 with 100 μg protein in alum, and serum collected on day 42. To assess binding to HES, membranes were probed with 1/1000 sera dilutions in block solution overnight at 4°C, washed extensively in TBST, and then with 1/2000 rabbit anti-rat Ig (1 hour room temperature; DakoCytomation). Following further washing in TBST, blots were developed using ChemiGlow West, according to the manufacturer's instructions (Alpha Innotech) and imaged using a FluorChem SP (Alpha Innotech).

2.6 Surface radio-iodination, immunoprecipitation and surface staining

Adult *H. polygyrus* were surface radio-labelled as described in earlier publications [62] but using Pierce Iodinination Reagent (Iodogen) as the catalyst for generating nascent iodine [63]. Eppendorf tubes (1.5ml) were coated with 200 μl of a 1 mg/ml solution of Iodination reagent (Pierce) in chloroform, dried, washed with PBS, before transfer of approximately 500 adult worms and 500 μCi ^{125}Iodine (Perkin Elmer) on ice. The sample was incubated with frequent agitation for 10 minutes, quenched by the addition of a saturated solution of L-tyrosine (Sigma), and radio-labelled parasite surface material produced as for HEx as described above, except that parasites were homogenized in PBS containing 1.5 % nOG detergent and 1% protease inhibitor cocktail (Sigma P8340). Surface labelled parasite proteins were then separated by 2-D gel electrophoresis as above, and then dried and

autoradiographed as before [62]. Immunoprecipitates were performed following pre-clearing of radiolabelled parasite extract with Protein G agarose beads (Millipore, 16-266) in the presence of MOPC 31C IgG1 isotype control (for mouse anti-VAL monoclonal antibodies) or naïve rat serum (for rat anti-VAL polyclonal serum) for 30 minutes at room temperature. Unbound parasite material was then incubated with 2 μg of mAb to VAL-1 (clone 3-36), VAL-2 (clone 4-S4), VAL-4 (clone 2-11) or MOPC 31 control IgG1 in non-denaturing IP buffer (20 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton-X100) for 2 hours, then with Protein G agarose beads overnight, at 4°C with rotation.

The production and specificity of anti-VAL mAb from the spleens of infected mice is to be described elsewhere [64]. Alternatively, 5 μl polyclonal anti-VAL-1, 2, 4 rat sera or naïve rat sera was used. Beads were washed 5 × 5 minutes in IP buffer, and bound proteins eluted by boiling in NuPAGE LDS sample buffer (Invitrogen) / 0.5 M 2-mercaptoethanol, before separation on 1-D SDS-PAGE and autoradio graph as before [64]. For sections, adult *H. polygyrus* worms were snap-frozen on dry ice in Cryo-M-Bed mountant (Bright Instruments), cryostat sections (5 μm; Leica) cut onto Polysine™ slides (VWR), dried and then fixed in 100% acetone for 10 min. Sections were washed twice with PBS for 10 min, and then incubated with the mouse mAb described above (50 μg/ml in 1% FCS / PBS) for 2 hours at room temperature, washed twice in PBS as before, and then incubated with secondary anti-mouse Ig TRITC (1/100 in PBS) for 1 hour at room temperature. Sections were washed extensively and then mounted in anti-fade Vectashield mountant (Vector Labs), before imaging with an Olympus fluorescent microscope.

3. Results

3.1 Mass Spectrometric identification of HES proteins from 2D PAGE gels

H. polygyrus adult excretory-secretory (HES) products were collected from parasites cultured in serum-free medium and concentrated over a 3,000 MW cut-off membrane. To determine the identity of HES proteins, we used both 2-dimensional gel electrophoresis (2DGE) and ‘shotgun’ proteomics approaches. HES contained over 100 discernable polypeptides when analyzed by silver staining of 2DGE (Figure 1 A), in a pattern clearly distinct from that of *H. polygyrus* somatic soluble protein extract (HEx, Figure 1 B). Analysis of 53 of the spots visible by 2DGE of HES (Figure 1 C) provided identities when matched against an in-house transcriptomic database of adult *H. polygyrus* mRNA (composed of 466,844 Roche 454 sequence reads, Harcus et al, manuscript in preparation). Additional spots were examined but did not provide sufficient material for MS identification (data not shown).

The most abundant products, as judged by intensity of silver staining, were found to be members of the VAL/ASP gene family (eg spots 4-9, 20-25, 35, 38-40), as well as apyrases, lysozymes, myoglobins and proteases. Table 1 summarises the full list of parasite proteins identified in this manner, which also includes a galectin, vitellogenin, chitinase, enolase and two novel gene sequences. We also observed that some proteins were present in multiple spots (e.g. variants of VAL-1 and VAL-2 were present in spots 20 - 24 and 5- 9, respectively, and apyrase-2 in spots 31 - 33). Consistent with this, a polyclonal rat serum generated against recombinant VAL-1.1 recognised a chain of at least 8 spots by Western blot (Figure

1 D). Although several secreted HES proteins show micro-variation at the amino acid level (see below), this does not fully account for the observed differences in pI, as the same variant may be present in several different spots (e.g. VAL-1.2 is present in spots 21 – 24), and conversely more than one sequence variant was often seen in the same spot (see Table 1 and Supplementary Figure 2), as the amino acid polymorphisms identified do not lead to large changes in predicted pI (data not shown).

3.2 Mass Spectrometric identification by LC-MS/MS

For a more exhaustive analysis of HES components, we employed LC-MS/MS on a total of 40 μg of HES, resulting in the identification of a total of 374 secreted HES proteins; 100 of the most abundant (as ranked by Mascot score) are presented in Table 2, with the full listing given in Supplementary Table 1. Among the HES proteins identified were a selection of proteases, particularly metalloproteases (zinc metalloproteases and a large number of astacins), cysteine proteases (cathepsin B, legumain and necpain), aspartyl proteases (necepsin), and various serine proteases (cathepsin A, dipeptidyl peptidase four, serine carboxypeptidases and trypsin family proteins). Several other classes of enzymes were abundantly secreted, including relatively high levels of acetylcholinesterases, apyrases, chitinases and lysozymes. Protease inhibitors (cystatins, Kunitz inhibitors and serpins), transthyretin-related (TTR) proteins, chondroitin proteoglycans, and lectins (both C-type lectins and galectins) were also detected, as were a large number of proteins of unknown function with homologues in other nematodes (conserved nematode proteins) and novel proteins as yet unidentified in other helminths. In addition, as discussed further below, no fewer than 25 distinct VAL proteins were identified. Most of the abundant proteins had been localized by 2DGE to specific spots, as indicated where appropriate in Table 2. Searching of LC-MS/MS data against Swiss-Prot revealed that HES contained two different Ig kappa light chains (accession numbers P01654 and P01837) and a single IgG1 heavy chain (P01869). No significant matches were found to any bacterial protein sequences. We confirmed the presence of trace amounts of host immunoglobulin by ELISA, which showed that 1 μg of HES contains 0.135 ± 0.010 ng murine IgG1 (data not shown). It is likely that this antibody is bound to the adult worm *in vivo*, and subsequently dissociates from the parasite during the *in vitro* culture period. No murine proteins were detected in HEx.

In parallel, the soluble somatic protein extract (HEx) of the worm was analysed, yielding 446 identities thereby greatly extending analysis beyond previously available information [24]. Table 3 presents 100 of the most abundant (ranked by Mascot score) and a full listing is given in Supplementary Table 2. Complete mass spectrometric data for HES and HEx are presented in Supplementary Tables 3 and 4 respectively. In contrast to HES, many of the HEx products were ribosomal proteins and protein synthesis factors, as well as cytoskeletal components (actin, tropomyosin and tubulin) and also cytosolic enzymes involved in glycolysis, lipid binding and redox reactions. GO annotation of HES and HEx (Figure 2 and Supplementary Table 5) indicated that translation (GO:0006412) was the most common “biological process” term for HEx, and structural component of ribosome (GO:0003735) was the most common “molecular function”, indicative of the large number of ribosomal proteins present. In contrast, proteolysis (GO:0006508) and metalloendopeptidase activity (GO:0004222) were the most common biological process and molecular function terms for

HES. It is important to note that a greater number of proteins in HES compared to HEx failed to match GO terms (molecular function 54.8% HES *Vs* 23.5% HEx; biological process 68.7% HES *Vs* 41.3% HEx), consistent with the specialised nature of the parasite secretions unique to its intestinal niche, and in contrast to the somatic extract which is generally composed of proteins common to most eukaryotic organisms.

3.3 Comparison of proteins in HES and HEx reveals preferentially secreted proteins

Only 104 of 374 (27.8%) HES proteins were detectable in HEx (Table 4). The most abundant of these "somatic" proteins present in HES were the myoglobins and vitellogenins, both of which are extremely highly expressed by adult worms, the former representing the dominant species on 2D profiles (Figure 1B). Hence, while the secreted components in HES in general represent a selective subset of the whole worm proteome, abundant somatic constituents such as myoglobin are also found in the *in vitro* parasite culture medium. In this regard it is noteworthy that both myoglobin and vitellogenin contain N-terminal signal sequences. It is also possible that as core egg proteins, vitellogenins diffuse from the eggs released by adult females during *in vitro* culture, or are present in intrauterine contents that accompany egg release. Comparison of exponentially modified Protein Abundance Index (emPAI) values for the 104 proteins common to HES and HEx indicated that there were large differences in the level of certain proteins between the two parasite preparations (e.g. VAL-1 and VAL-2 variants were highly abundant in HES and detected at only trace amounts in HEx; Table 4). This is not unexpected given that parasite secretions originate from the worm itself.

3.4 HES and HEx components differ significantly in proportion of predicted signal peptide sequences and in extent of novel gene products

Of the 374 HES proteins identified, 291 (77.8%) contained a predicted N-terminal signal peptide (Figure 3). In addition, 100 (26.7%) did not correspond to any annotated gene in the NCBI database, and of these 70 (18.7%) were novel proteins with no database match. The remaining 30 sequences matched predicted or hypothetical proteins of unknown function from *C. elegans* or other nematodes, and were classified as conserved nematode proteins. When these 100 secreted proteins of unknown function were examined for the presence of a potential signal peptide, approximately 85% of each (60/70 novel, 25/30 conserved) were signal peptide-positive, indicating that an important set of novel secreted proteins are present in HES. The novel proteins in particular were mostly <150 amino acids, with an average predicted molecular weight of 16.5 kDa (range 5.4 – 55.5 kDa). In contrast to HES, only 25.1% (112/446) of proteins identified in HEx encoded signal peptides, and HEx also contained noticeably fewer proteins of unknown function, with only 15 conserved nematode proteins (of which 4 were also detected in HES) and 9 novel proteins (4 detected in HES).

3.5 VAL proteins are associated with the parasite surface

In parallel with HES analysis, we also investigated the adult parasite surface proteins accessible to radio-iodination of live worms. When surface iodination was employed, solubilised proteins were analysed by 2D gel electrophoresis and autoradiography, and showed mobilities similar to VAL-1 and -2 (compare Fig. 4 A with Fig. 1 C) which could be specifically immunoprecipitated with monoclonal and polyclonal antibodies specific for

VAL-1 and VAL-2 (Fig. 4 B), indicating that both VAL-1 and VAL-2, highly enriched in HES, are present on the surface of the adult worm. In contrast, we did not detect surface expression of the similarly abundantly secreted VAL-4.

The distribution of VAL-1 and -2 was then investigated using monoclonal antibodies specific for each protein [64] to stain frozen sections of adult worms (Fig. 4 C-F). Both antibodies gave a highly restricted punctate pattern of labelling the body wall, in a series of structures at contralateral sites that may represent longitiduinal neuronal fibres or secretory tissues. Interestingly, in *A. caninum*, different VAL (ASP) products showed distinct localization patterns, with anti-Ac-ASP-4 staining the cuticle of adult worms, while antibodies to ASP-3 and -6 binding to glandular structures, and anti-ASP-5 to the gut, yet all four proteins are also found in adult worm ES [65].

3.6 Sequence analysis of the H. polygyrus VAL gene family

The most striking characteristic of HES is the predominance of members of the VAL gene family, both in terms of the abundance of certain members, particularly VAL-1, 2, 3, 4 and 7, as well as the large number (25) of different VAL proteins detected. VAL proteins show a conserved overall structure built around the SCP modular domain of ~200 amino acids (sperm-coating protein; pfam accession PF00188), typically containing 5 disulphide bonds. Members of this gene family generally contain either a single SCP domain, or two tandem domains, which are not necessarily closely related to each other in sequence. Figure 5 shows a schematic of the 25 secreted VAL proteins of adult *H. polygyrus*, including 21 full length proteins, 8 of which (VAL-4, 7, 10, 15, 19, 21, 22 and 25) are single domain and 13 double domain (VAL-1, 2, 3, 5, 6, 8, 9, 12, 13, 14, 16, 17 and 20). Phylogenetically, the single- and double-domain proteins appear to have diversified independently, although the single-domain VAL-19 is most likely to have evolved from a double-domain ancestor (Figure 5). Double-domain VAL proteins include an inter-domain linker “hinge” region, which in the case of VAL-1, 2 and 5 comprise multiple Ser/Thr residues bearing a common antigenic *O*-glycan [64]. Immunodominant serum antibodies target this glycan early in infection, although anti-peptide antibodies such as those tested in Figure 4 are also represented [64]. Similar stretches of predicted *O*-glycosylation are present in other secreted VAL proteins, particularly VAL-11, 17 and 20 (Figure 5).

Analysis of individual *H. polygyrus* VAL amino acid sequences reveals extensive variation between genes: for example, within the C-terminal domains of the 13 double-domain proteins, only 15 / 189 amino acid residues are completely conserved (8%), and a further 10 altered in only 1 gene sequence, while in the N-terminal SCP-1 domain there are only 7 identical positions (of which 6 are cysteines). Similarly, for the single domain VAL proteins there are only 8 fully conserved amino acids, and 7 differing at only 1 position. Additionally, transcriptomic studies have identified significant micro-sequence variation within individual VAL proteins (Harcus et al manuscript in preparation), and this was evident with respect to proteomic data. For example, five alternative VAL-1 sequences verified by proteomic data are presented in Figure 6. Note the extensive sequence diversity, particularly in the first SCP domain, suggesting that domain 1 is either under diversifying selection, or that intragenic recombination is taking place. Similar sequence variation, with multiple variants matching

the peptide data, was observed for VAL-2 and VAL-7. As the full genome of this parasite has yet to be assembled, we cannot yet determine whether the microvariation is due to either allelic polymorphism or recent duplication of the relevant gene loci. Sequence variation has previously been reported for VALs of *A. caninum* [66], *Cooperia punctata* [67] and *H.contortus* [22].

3.7 Non-VAL gene families represented in HES

In addition to the 25 VAL proteins, a number of conserved gene family proteins are well represented in HES with particular prominence of the following functional groups.

3.7.1 Acetylcholinesterases—Three acetylcholinesterases are among the 100 most abundant HES proteins (Table 2), with Hpb-ACE-1 represented by 3 distinct spots (Table 1). Two additional proteins, ACE-2 and -3, differ by only 18 amino acids (3.1%) from each other but show ~32% divergence from ACE-1. The secretion of AChE by adult *H. polygyrus* has been previously reported [68] and is a general feature of most nematode ES products [69]. In *N. brasiliensis*, the expression of three AChE isoforms has been shown to be differentially regulated according to the immune status of the host [70-73], and the 3 *H. polygyrus* proteins show highest similarity to isoform A of *N. brasiliensis*.

3.7.2 Apyrases—Four distinct apyrases were identified, one of which (Hpb-APY-1) was found as three minor sequence variants, all but one in the most abundant 100 HES proteins. Apyrases are adenosine diphosphatases, similar to mammalian CD73-like proteins, which catalyze the hydrolysis of ATP/ADP to AMP, and can often act also on other NDPs. Two recent reports have identified arthropod (*Cimex*)-like apyrases from related trichostrongylid nematodes *T. circumcincta* [74] and *Ostertagia ostertagi* [75] sharing 92% amino acid identity; in the latter case the enzyme was localised to the oesophageal glands. While all 4 *H. polygyrus* apyrases are homologous to these enzymes, levels of amino acid identity are less than 60%, indicating considerable diversification since divergence of the murine and ruminant parasites.

3.7.3 Lipid-binding Proteins—Several structurally unrelated lipid-binding protein families are represented in HES. HES contains two distinct homologues of the Fatty acid/ Retinol-binding (FAR) protein that has been recorded in ES of many other parasitic nematodes [76; 77] and which in the case of *A. caninum* FAR-1 has been shown to functionally bind fatty acids and retinol [78]. A nematode polyprotein allergen (NPA) is also secreted by *H. polygyrus*, homologues of which bind the same ligands in *Ascaris* [79] and *Dictyocaulus viviparus* [80]. In addition, a total of 12 transthyretins are represented in HES, members of a widespread and diverse family of small proteins believed to recognize small hydrophobic ligands such as thyroid hormone, retinol or phosphatidylserine [81-83].

3.7.4 Lysozymes—Lysozymes or muramidases are present in multiple forms in HES, with 8 distinct gene products identified. All are related to *C. elegans* and the 7 full-length sequences contain a potential signal peptide. In other organisms, lysozymes degrade the glycosidic bond linking *N*-acetylglucosamine and *N*-acetylmuramic acid in the murein proteoglycan of bacterial cell walls. Since adult *H. polygurus* cohabit with microbial flora,

and gram-positive bacteria are more susceptible to lysozyme-mediated lysis, it is possible that these lysozymes modify the bacterial population sharing the intestinal niche of the worm.

3.7.5 Proteases—Five aspartyl proteases (PF00026) in HES correspond to a major enzymatic class from parasitic nematodes. Aspartyl proteases play a key role in the ability of *A. caninum* hookworms to degrade haemoglobin [84], and are the target of protective antibodies against the human parasite *Necator americanus* [85]. We identified no fewer than 20 astacins, Zn-metalloproteases distributed across the animal kingdom with particular frequency in nematodes [86; 87].

Cysteine proteases have a major role in nematode parasites, particularly within the hookworm family. However, while 7 ES cathepsin B products have been defined in *H. contortus* [88], part of a larger gene family which show a particularly high level of transcription in intestinal tissue [89], only 4 are found in HES, a cathepsin B-like cysteine protease, a homologue of *N. americanus* necpain and two legumains (asparaginyl endopeptidases). Serine proteases are also less prominent in HES than aspartyl or metallo-enzymes, but include Cathepsin A, aminopeptidase, serine carboxypeptidases, trypsin family proteases and dipeptidyl peptidase four (DPF) proteins.

3.7.6 Protease Inhibitors—Three broad classes of protease inhibitor are found in HES. Cystatins are a broadly conserved family of cysteine protease inhibitors found across plant and animal phyla, with an especial role in immune modulation by parasitic nematodes [90; 91]. Inhibition of the protease active site involves a QVVAG sequence which is perfectly conserved in the *H. polygyrus* homologue, as also in Nippocystatin from *N. brasiliensis* ES [92] and in *B. malayi* Bm-CPI-2 [93]. However, while *Bm*-CPI-2 has a second inhibitory motif (SND) that blocks the legumain (asparaginyl endopeptidase) in antigen-presenting cells, this is altered in *H. polygyrus (*SNA) and may therefore not function in an identical fashion. Seven Kunitz type serine protease inhibitors are represented in HES, and again this gene is found in multiple forms in many helminth products [94]. Notably, a related secreted product from adult *A. ceylanicum* (AceK1) acts against a broad range of serine proteases, including trypsin, chymotrypsin and pancreatic elastase [95; 96]. In *C. elegans*, a Kunitz-type inhibitor is important in collagen processing for cuticle formation, with mutations causing the blister-5 (*bli-5*) phenotype [97]. A structurally unrelated family of serine inhibitors are the serpins, large globular proteins that include most dominant ES antigen of *B. malayi* MF [98; 99]. Three HES serpins were identified but are only distantly related to Bm-SPN-2, all being most similar to a serpin from *Trichostrongylus vitrinus*.

3.8 Similarity to other Strongylid nematodes

The overall profile of gene sets represented in HES shows many similarities to those reported in Trichostrongylid nematode parasites of ruminant livestock such as *H. contortus* [22] and *T. circumcincta* [30] as well as the more distantly related hookworm *Ancylostoma caninum* [28], as shown in Table 5, and a number of specific homologies are noted above**.** In each species, VAL family members predominate, and similar findings have been reported for other members of the Trichostronglyid taxon such as *Ostertagia ostertagi* [100].

Discussion

The secretome of extracellular pathogens provides fascinating insights into the biological strategy of infectious organisms, in particular those such as long-lived helminth species that must attain an optimal physiological and immunological balance with their hosts [7]. The spectrum of parasite secreted proteins represents the elaborate adaptions demanded by the parasitic mode of life, over and above nematode-specific functions evident in free-living relatives such as *C. elegans*, many of which are likely to have evolved to interact with a precise host pathway, often with a specific ligand or receptor, and each of which may offer a novel and effective route to intervention and therapy.

The analytical power of modern proteomics enabled us to identify 374 ES products of adult *H. polygyrus*, holding open the prospect of a complete “worm pharmacopeia” of potential host-modulating proteins [101]. Comparisons with the whole worm somatic protein extracts indicate a high level of selectivity, with many proteins only detectable in the secretions of the parasite, and conversely most somatic products being absent from HES. The likelihood that HES collected in vitro faithfully reflects release in vivo, is supported by recent work detecting circulating HES antigen in the serum of infected mice (Hewitson, unpublished), and demonstrating that host antibody responses are predominantly directed against HES, rather than HEx, antigens [64]. Validation of the methodological focus on ES products is further offered by the contrast in signal peptide-positive sequences within HES compared to HEx. Moreover, the concept that ES proteins may have evolved more rapidly to interact with host systems is supported by the higher number of novel (and novel signal peptide-positive) gene sequences among HES proteins than in the general body components, as previously noted in *N. brasiliensis* [13].

We also established that two of the major HES proteins, VAL-1 and -2, are represented on the surface of adult parasites. This finding reiterates work with other nematode species that found concordance between surface and secreted proteins of adult *B. malayi* [63; 102] and larval *T. canis* [103; 104]. These studies raise interesting questions regarding the route of secretion by live parasitic nematodes: while they possess specialized secretory apparatus such as oesophageal glands [103; 105], there is also evidence of direct trans-cuticular secretion deriving from the syncytial hypodermal tissue underlying the extracellular cuticle [106]. Now that the adult ES proteins are well defined, and with a number of monoclonal antibodies to these proteins [64], these issues can be directly addressed at the microscopic level.

The most striking feature of the HES analysis is the dominance of multiple VAL proteins. The VAL family is an extraordinary one in being so widely distributed in nature, with alternative names from diverse systems including Cysteine-RIch Secretory Protein (CRISP), Sperm Coat Protein (SCP) in mammals and plant Pathogenesis-Related protein (PR), without any clear indication as to their functional role(s) [107; 108]. Within the nematodes, the archetypal VAL protein is the *Ancylostoma* secreted protein (ASP), a homologue of which (ASP-2) has been taken forward for human hookworm vaccine trials [109]. Interestingly, in *Ancylostoma* species from dogs and humans, multiple and divergent ASPs are known. For example, some 25 distinct VAL family transcripts were identified by Mitreva

and colleagues from *A. caninum* [16]. For potential vaccines, it is important to target products of the initial infective stages as well as of established adults; thus future work with *H. polygyrus* will investigate VAL gene expression in immature as well as adult stages of the parasite. Additionally, we are currently assessing the protective potential of both the total adult secretions and individual VAL proteins.

VAL genes are also expressed in non-parasitic organisms, but with an intriguing association with the interface between different species, as for example in snake and insect venoms and haematophagous insect saliva, as well as in the response of plants to microbial infection. Moreover, numerous homologues exist in the free-living nematode *C. elegans* [108], arguing that these genes are must have many functions outside the frame of host-parasite interactions. Indeed, *lon-1* is such a gene that controls body length downstream of TGF-β signalling [110]. It is plausible, therefore, that the SCP domain is simply an adaptable protein framework that facilitates the evolution of diverse specialized functions, and that such diversity is accentuated by inter-species interactions; certainly the extensive radiation within the *H. polygyrus* lineage is consistent with this notion. Intriguingly, VAL proteins found in HES differ from those of *C. elegans* in terms of both the enrichment of double domain proteins and in the presence of a serine/threonine rich linker region between SCP domains, the site of highly antigenic *O*-glycans in the HES proteins [64].

The comparison with related nematode species (Table 5) is instructive, not only in confirming that dominant VAL secretion is shared among different members of the taxon, but also identifying many common, and some particular, gene sets associated with intestinal parasitism. Most conspicuous across all these species is the level of protease production, predominantly astacins and other metalloproteases, but also including the aspartyl, cysteine and serine protease classes. Together with the release of a series of protease inhibitors (cystatins, serpins, Kunitz inhibitors etc) this suggests that nematodes can reset the hydrolytic and proteolytic environment of the gastrointestinal tract, possibly to avoid enzymatic attack but also quite probably to degrade host mediators and obstacles such as mucins, immunoglobulins and innate defence molecules.

A further set of parallels are seen with the production of apyrases, with four different proteins identified. Whilst apyrases have been associated with the inhibition of blood clotting by blood feeding insects [111], they do not appear to be secreted by blood feeding hookworms [28]. An alternative role may be immunomodulatory through conversion of pro-inflammatory ATP/ADP to anti-inflammatory AMP [112]. ATP, released by intestinal bacteria, can activate dendritic cells (DC) to secrete the pro-inflammatory cytokines IL-6 and IL-23, resulting in the induction of inflammatory Th17 cells [113]. Similarly, ATP-dependent DC activation is essential for Th2-dependent lung pathology in asthma models, and this can be inhibited through the administration of an apyrase [114]. In this context, adenosine generation may both induce Foxp3+ regulatory T cells and be used by this cell type as a mechanism of suppression [115]. Finally, in *C. elegans*, an endo-apyrase, APY-1, fulfills a more homeostatic role in the stress response of the organism [116], a role that cannot be excluded for *H. polygyrus* homologues.

Whilst we have catalogued the identity of several hundred of the most abundant proteins in the search for helminth immunomodulators, we did not detect expression of the TGFβ family member encoded by *H. polygyrus*, and expressed by all mammalian stages [60]. Such activity appears linked to the ability of HES to induce de novo Foxp3-positive regulatory T cells [53]. This may be because TGFβ is active at sub-nM levels, which may be beyond the level of detection of our MS analysis. Alternatively, it is not known whether the TGFβ activity in HES is due to a true-TGFβ homolog. We are currently fractionating HES to determine this. Another potential immunomodulator previously considered is calreticulin, which has been reported to drive the Th2 response that is strongly provoked in *H. polygyrus* infection [58]; as calreticulin is present at only low levels in HES, but is readily detectable in worm extract, it remains to be determined if this product emanates from active secretion or leakage from compromised parasites.

In conclusion, we have embarked on a fine-detail molecular dissection of an important nematode parasite, which serves as an excellent model for both human and veterinary helminth diseases. Whilst RNAi has not yet been successfully demonstrated with *H. polygyrus* [117], more recent studies have established that secretory proteins may be optimally positioned for silencing in this way [118], which should allow functional testing of the various potentially immunomodulatory proteins described here. It is important to note that so far we have analysed only adult HES, but that infection is initiated by L3 which embed in the intestinal submucosa and clearly elaborate a set of equally fascinating mediators; hence in due course we hope to analyse the ES of immature stages and complete the proteomic characterization of this organism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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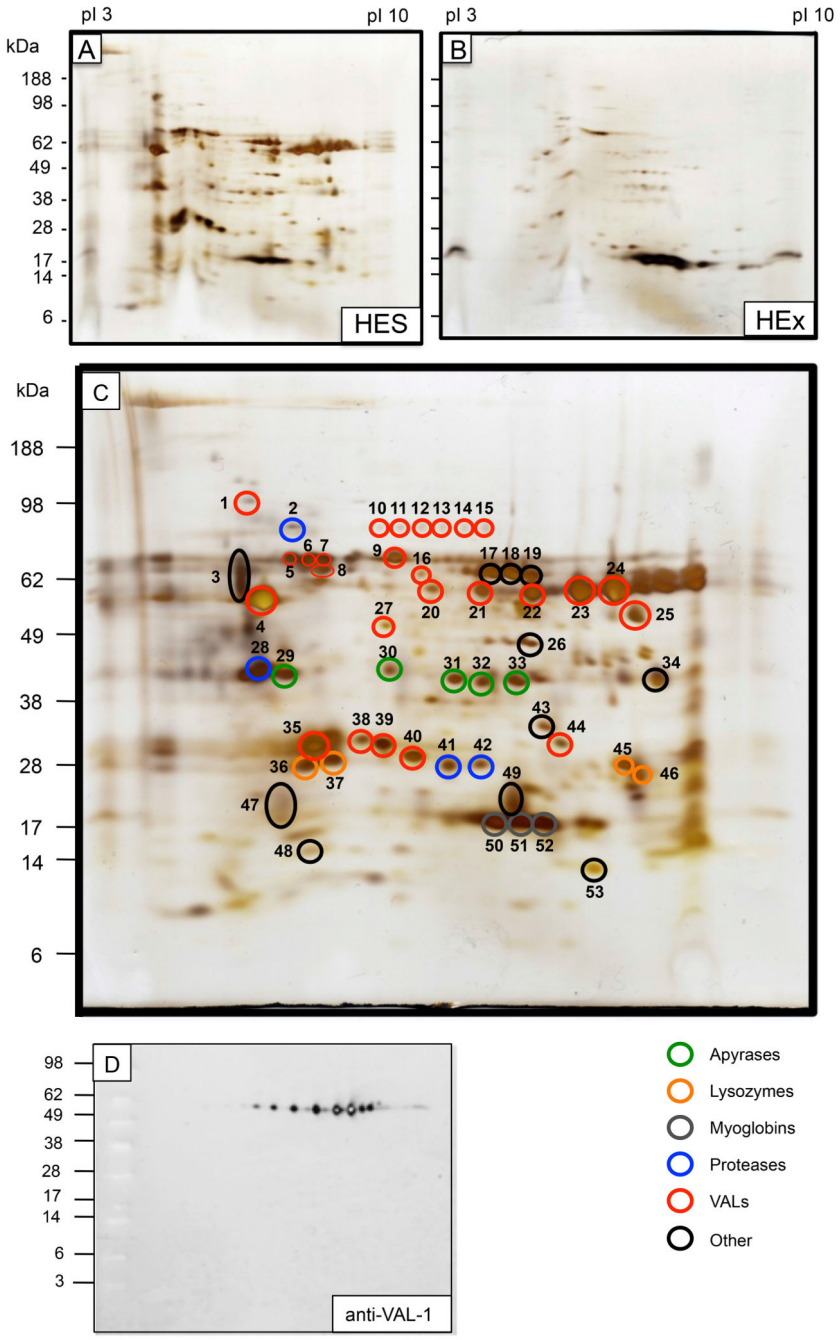

Figure 1. 2-Dimensional analysis of *H. polygyrus* secreted proteins (HES) and soluble somatic extract (HEx)

A. HES Silver stain

B. HEx Silver Stain. Note that the major spots of 15-18 kDa have previously been identified as myoglobins [24].

C. HES annotated with spots analysed by MS/MS as presented in Table 1

D. Anti-VAL-1 rat polyclonal antibody on Western blot

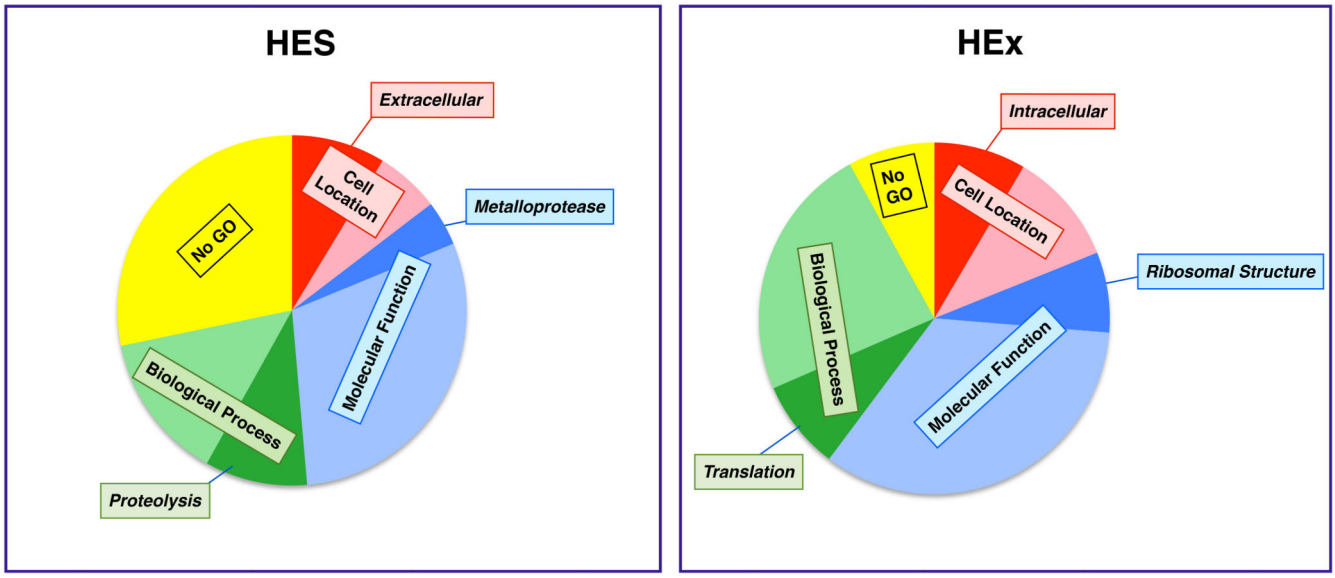

Figure 2. GO distribution of the proteins in HES and HEx

All identified proteins in HES and HEx were analysed by Gene Ontology and categorised firstly into 4 broad categories: cell localation (Red), molecular function (Blue), biological process (Green), and no recognized GO similarity (Yellow). Within each category, the most frequent term is shown (darker colours). Some proteins are included in more than one category. A more detailed listing of GO identifications is given in Supplementary Table 3

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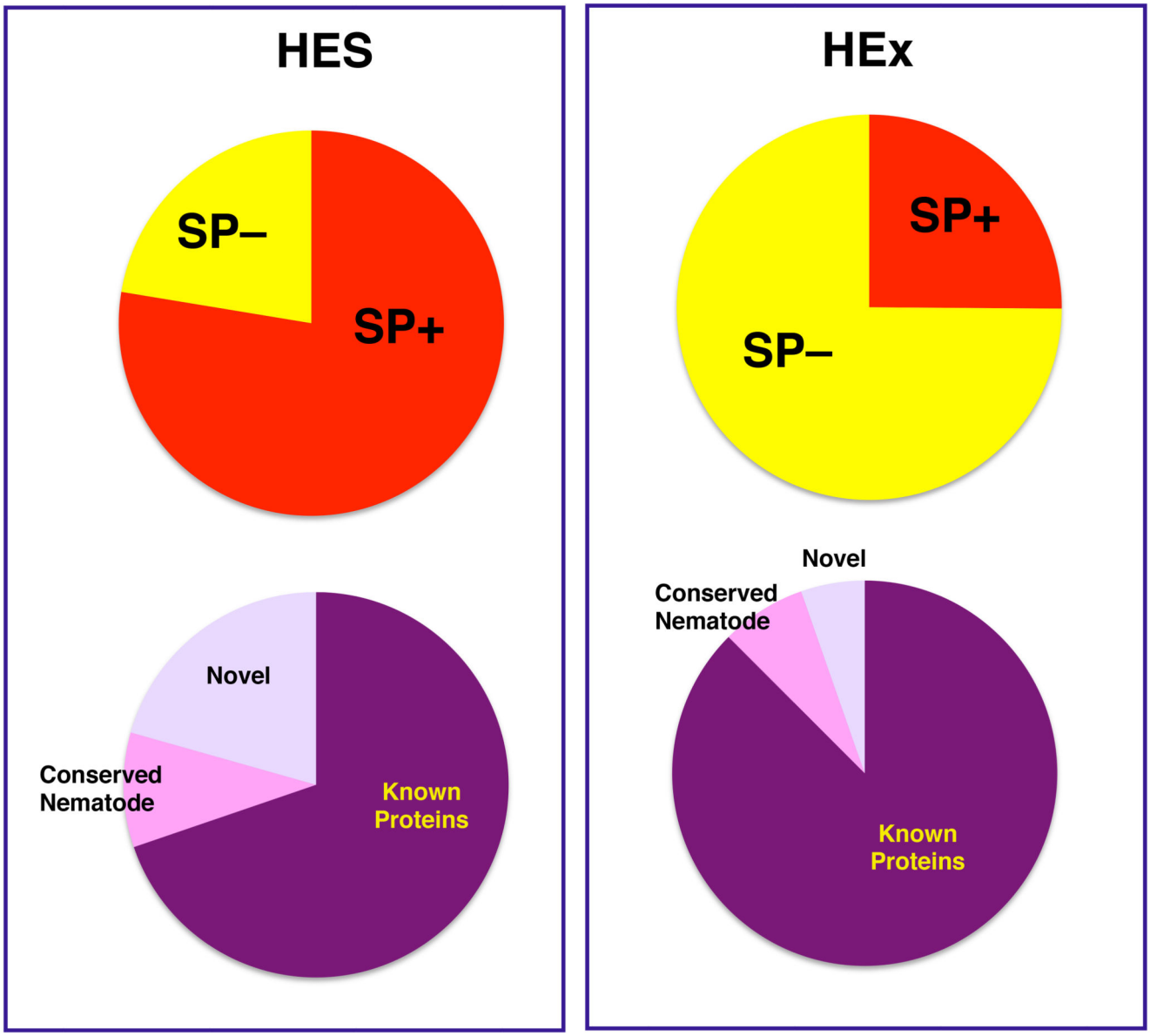

Figure 3. Novel genes and signal peptides of the proteins in HES and HEx

A. Distribution of signal peptide-containing protein sequences

B. Proportions of novel and nematode-conserved genes containing signal peptides

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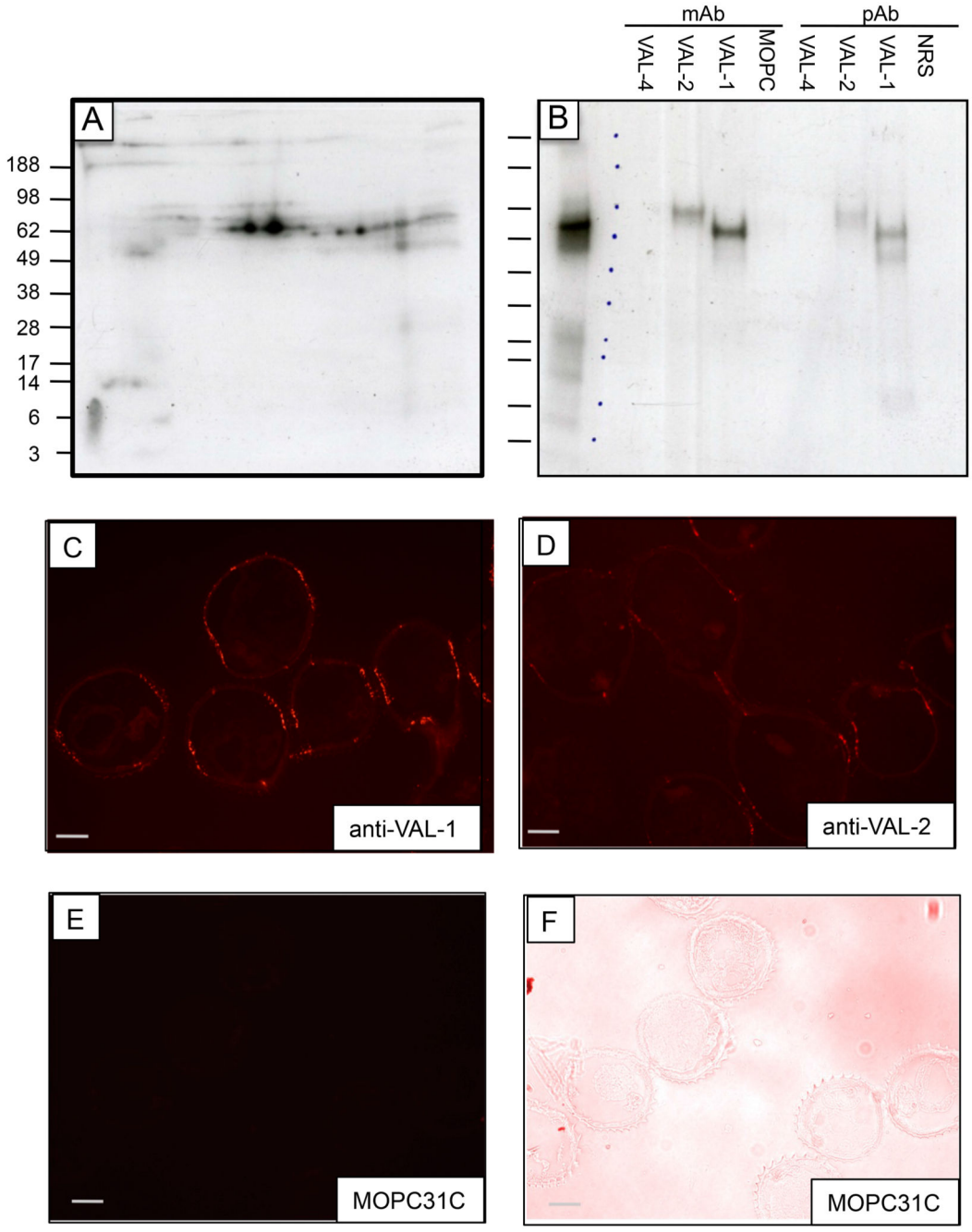

Figure 4. Surface labelling of adult *H. polygyrus* reveals VAL-1 and VAL-2 are surface associated

A. Surface iodination, 2D gel

B. Immunoprecipitation of surface labelled VAL-1 and -2 with specific antibodies

C. Anti-VAL-1 monoclonal antibody on adult worm section

D. Anti-VAL-2 monoclonal antibody on adult worm section

E, F. MOPC control antibody on adult worm section with corresponding bright field image

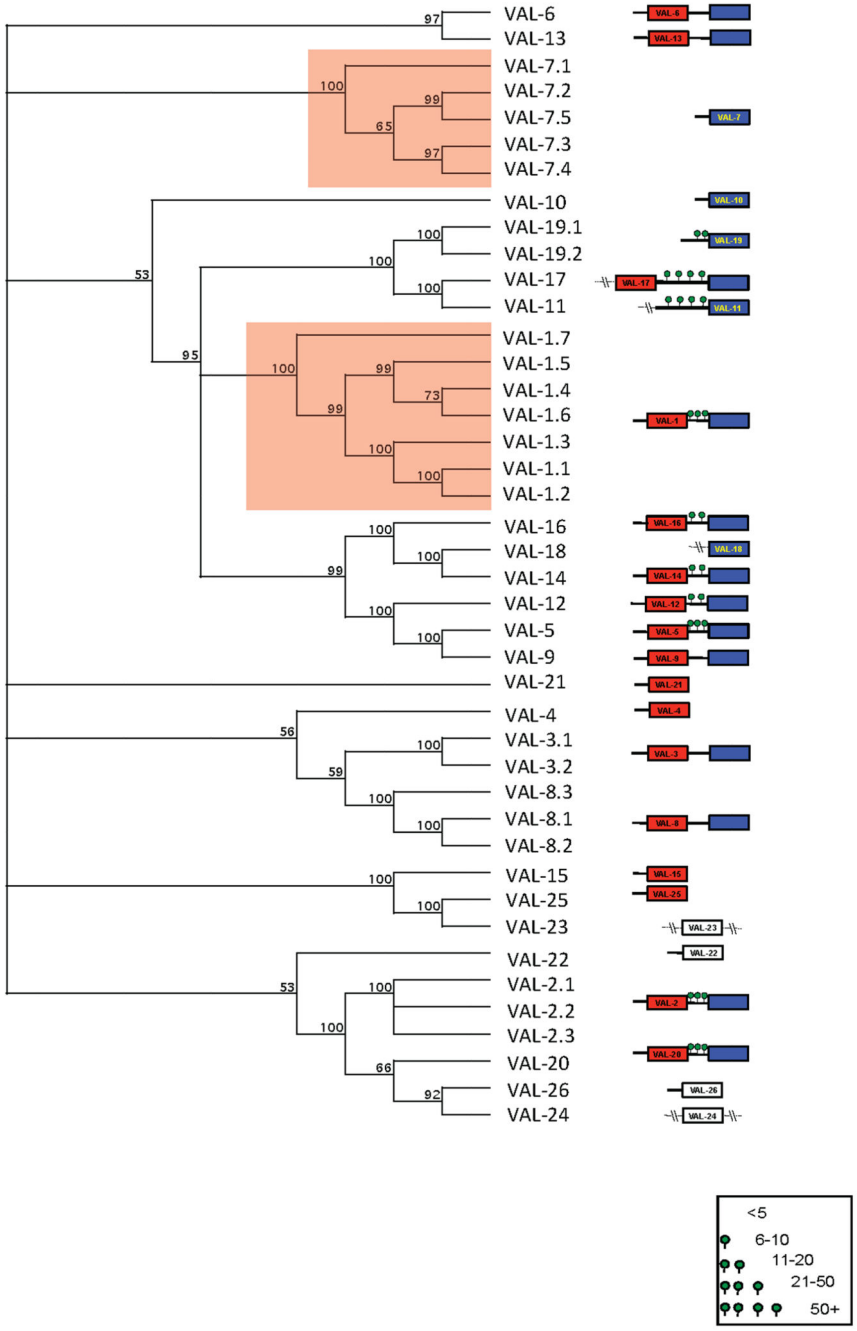

Figure 5. Schematic of relatedness of *H. polygyrus* VAL-1-25

The domain structure of Hpb-VAL-1 to -25 are depicted including signal sequences, linker regions (predicted O-glycosylation is indicated with green circles) and SCP homology domains (N-terminal red, C-terminal blue). Single SCP domain proteins are coloured according to whether they are related to N (red) or C-terminal (blue) SCP domains. Divergent sequences equally distinct from both are white. Sequence truncation is indicated by (−\−).

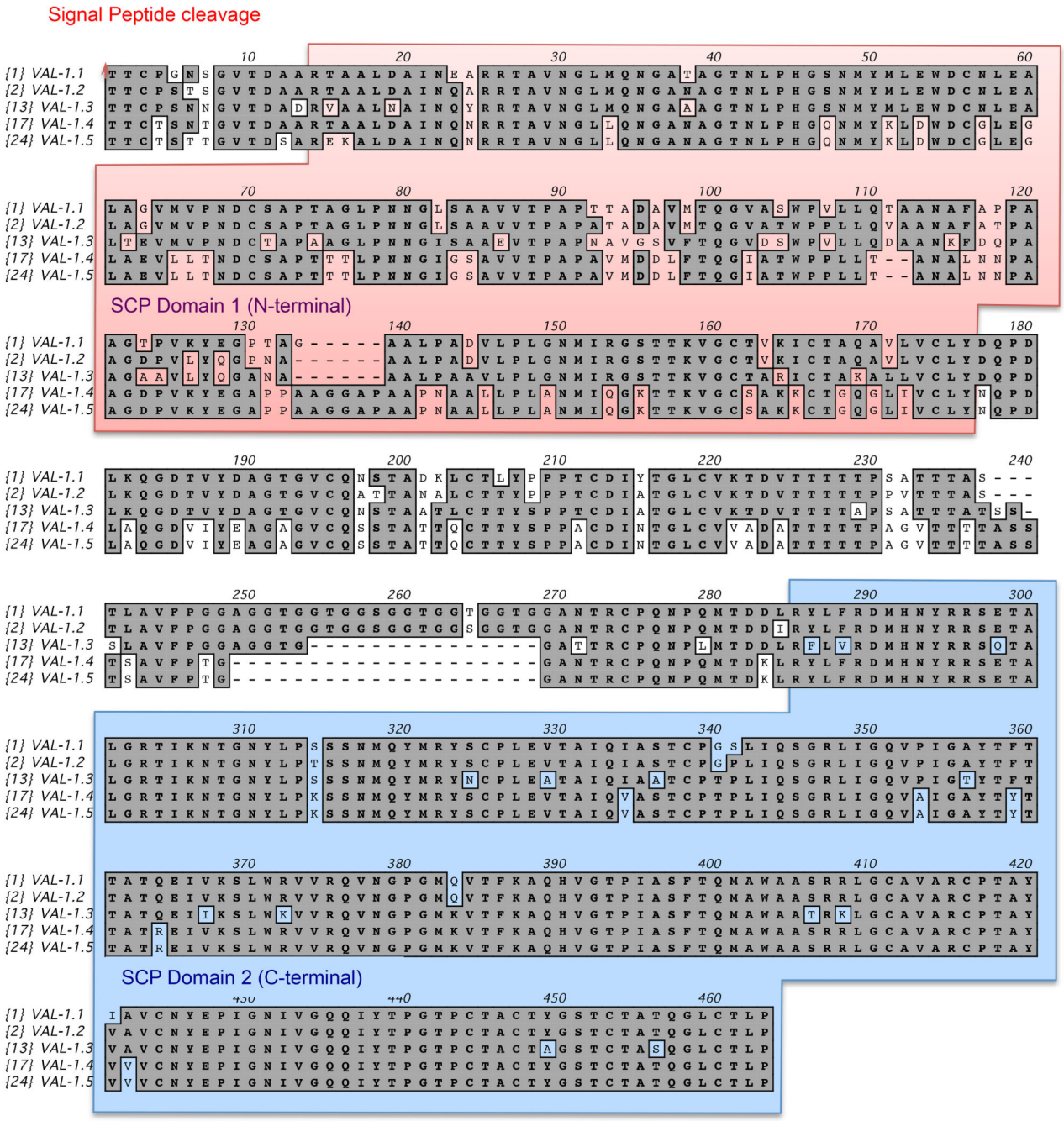

Figure 6. Sequence variation within *H polygyrus* VAL-1 proteins

Mature proteins with signal cleavage site and N and C-terminal SCP domains indicated.

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Table 1

Proteins identified in HES from spots

Tabulation of spot numbers and identifications

Spot	Identity	Domain	Hpb adult database	Score	Peptides	Accession No
1	VAL-3.1	Double SCP	isotig05790	64	2	JF914909
2	MEP-1 Zinc metalloprotease	Peptidase M13	isotig02967	144	5	(*truncated*)
3	CHI-1 Chitinase	Chitinase GH19	isotig04840	268	3	• • • • • • • •
4	VAL-3.1	Double SCP	isotig05790	462	6	JF914909
5	VAL-2.1/2.2/2.3 *(a)*	Double SCP	isotig03106	43	1	JF914906
6	VAL-2.1/2.2/2.3 *(a)*	Double SCP	isotig03106	266	7	JF914906
7	VAL-2.1/2.2/2.3 *(a)*	Double SCP	isotig03106	206	6	JF914906
8	VAL-2.1/2.2/2.3 *(a)*	Double SCP	isotig03106	32	1	JF914906
9	VAL-2.1/2.2/2.3 *(a)*	Double SCP	isotig03106	429	7	JF914906
10	VAL-5	Double SCP	isotig04839	186	8	JF914911
11	VAL-5	Double SCP	isotig04839	146	10	JF914911
12	VAL-5	Double SCP	isotig04839	132	8	JF914911
13	VAL-5	Double SCP	isotig04839	145	5	JF914911
14	VAL-5	Double SCP	isotig04839	169	7	JF914911
15	VAL-5	Double SCP	isotig04839	121	4	JF914911
16	VAL-9	Double SCP	isotig05765	495	10	JF914917
17	ACE-1 Acetylcholinesterase	Esterase lipase	isotig05694	21	1	JF439067
18	ACE-1 Acetylcholinesterase	Esterase lipase	isotig05694	32	2	JF439067
19	ACE-1 Acetylcholinesterase	Esterase lipase	isotig05694	53	2	JF439067
20	VAL-1.1	Double SCP	isotig06320	16	1	JF914902
21A	VAL-1.2	Double SCP	isotig03069	43	1	JF914903
21B	VAL-1.4	Double SCP	isotig01653	38	1	JF914905
22A	VAL-1.2	Double SCP	isotig03069	129	4	JF914903
22B	VAL-1.1	Double SCP	isotig06320	103	4	JF914902
22C	VAL-1.4	Double SCP	isotig01653	70	2	JF914905
23A	VAL-1.2	Double SCP	isotig03069	419	5	JF914903
23B	VAL-1.1	Double SCP	isotig06320	402	5	JF914902
24A	VAL-1.1	Double SCP	isotig06320	613	6	JF914902
24B	VAL-1.2	Double SCP	isotig03069	385	5	JF914903
25	VAL-6	Double SCP	isotig03505	401	4	JF914912
26	Enolase *(b)*	Enolase	isotig06965	103	3	(*truncated*)
27	VAL-8.1	Double SCP	isotig02308	257	6	JF914916
28	MEP-3 Zinc metalloprotease	Peptidase M13	isotig05155	177	3	(*truncated*)
29	APY-1.1 Apyrase	Apyrase	isotig02250	68	3	JF721961
30	APY-3 Apyrase	Apyrase	isotig03589	188	5	JF721966

Spot	Identity	Domain	Hpb adult database	Score	Peptides	Accession No
31	APY-2 Apyrase	Apyrase	isotig07051	213	6	JF721965
32	APY-2 Apyrase	Apyrase	isotig07051	323	3	JF721965
33	APY-2 Apyrase	Apyrase	isotig07051	185	5	JF721965
34	PHP-1, PHA-domain protein	PHA02954	isotig03547	180	4	• • • • • • • •
35	VAL-4	Single SCP	isotig10387	127	3	JF914910
36A	LYS-1 Lysozyme	Muramidase GH25	isotig08802	411	6	• • • • • • • •
36B	LYS-3 Lysozyme	Muramidase GH25	isotig08606	126	3	• • • • • • • •
37A	LYS-1 Lysozyme	Muramidase GH25	isotig08802	483	6	• • • • • • • •
37B	LYS-3 Lysozyme	Muramidase GH25	isotig08606	170	3	• • • • • • • •
38A	VAL-7.3	Single SCP	isotig02284	94	3	JF914915
38B	VAL-7.2	Single SCP	isotig01525	61	2	JF914914
39	VAL-7.2	Single SCP	isotig01525	48	1	JF914914
40	VAL-7.1	Single SCP	isotig01524	296	6	JF914913
41	NAS-1.1/1.2 Nematode Astacin *(c)*	ZnMc astacin-like	isotig04178	280	4	(*truncated*)
42	NAS-3.3 Nematode Astacin *(d)*	ZnMc astacin-like	isotig01791	141	2	(*truncated*)
43	Galectin	Double GLECT	isotig09082	219	4	(*truncated*)
44	VAL-10	Single SCP	isotig04979	27	1	JF914918
45	LYS-2 Lysozyme	Muramidase GH25	isotig05074	220	4	• • • • • • • •
46	LYS-2 Lysozyme	Muramidase GH25	isotig05074	179	3	• • • • • • • •
47	Vitellogenin	VWD	contig00471	64	2	(*truncated*)
48	NSP-4 Novel Secreted Protein	None	isotig11873	60	1	• • • • • • • •
49	NSP-16 Novel Secreted Protein	None	isotig05257	39	2	• • • • • • • •
50	Myoglobin	Globin	isotig04274	64	1	• • • • • • • •
51	Myoglobin	Globin	isotig04274	125	3	• • • • • • • •
52	Myoglobin	Globin	isotig04274	94	3	• • • • • • • •
53	TTR-1 Transthyretin-related	DUF290	isotig04612	452	6	• • • • • • • •

Accession numbers from NCBI GenBank are given where full-length sequences are available.

(a) Peptides are common to all three variants of VAL-2; isotig and Accession number given are for VAL-2.1. Isotig and accession numbers for VAL-2.2 and -2.3 are isotig07425/JF914907 and isotig03105/JF914908.

(b) 1 additional peptide matches isotig18996 coding for N-terminal segment of Enolase separated from isotig 06965 by gap of ~27 nt.

(c) Peptides are common to NAS-1.1 and -1.2; latter is isotig04179; peptides common to both

(d) Two additional peptide matches to each of variants NAS 3.1 (isotig01799) and NAS-3.4 (isotig 01793)

Table 2
Top 100 Proteins identified in HES by LC-MS/MS

Top 100 HES by mascot score

Rank	Identity	Conserved domains	Code	Score	SP
1	VAL-1.1	SCP (Double)	isotig06320	6546	SP
2	VAL-1.2	SCP (Double)	isotig03069	5788	SP*
3	VAL-2.2	SCP (Double)	isotig07425	5149	SP*
4	VAL-2.3	SCP (Double)	isotig03105	5039	SP
5	VAL-3.1	SCP (Double)	isotig05790	4754	SP
6	VAL-2.1	SCP (Double)	isotig03106	4003	SP
7	Vitellogenin	Vitellogenin, DUF1943	contig00471	3997	SP
8	LYS-1 Lysozyme	Muramidase GH25	isotig08802	3615	SP
9	VAL-7.2	Single SCP	isotig01525	3369	SP
10	VAL-7.5	Single SCP	isotig01526	3367	SP
11	VAL-7.3	Single SCP	isotig02284	3051	SP
12	LYS-2 Lysozyme	Muramidase GH25	isotig05074	2947	SP
13	VAL-1.3	Double SCP	isotig06456	2668	SP
14	VAL-7.4	Single SCP	isotig02282	2604	SP
15	VAL-7.1	Single SCP	isotig01524	2593	SP
16	TTR-1 Transthyretin-related	DUF290	isotig04612	2130	SP
17	VAL-1.4	Double SCP	isotig01653.	1841	SP
18	APY-1.1 Apyrase	Apyrase	isotig02250	1833	SP
19	APY-1.2 Apyrase	Apyrase	isotig07986	1818	SP
20	VAL-13	Double SCP	isotig06642	1809	SP
21	VAL-4	Single SCP	isotig10387	1770	SP
22	MEP-1 Zinc metalloprotease	Peptidase M13	isotig02967	1624	SP*
23	VAL-1.5	Double SCP	isotig01652	1596	SP*
24	NSP-1 Novel Secreted Protein with SP	None	isotig11973	1584	SP
25	APY-1.3 Apyrase	Apyrase	isotig05261	1583	SP
26	Myoglobin	Globin	isotig04274	1579	SP
27	Peritrophin-A-like protein	Chitin-binding type 2	Isotig05677	1561	SP*
28	Vitellogenin	DUF1943	contig00207	1552	SP*
29	ACE-1 Acetylcholinesterase	Esterase lipase	isotig05694	1527	SP
30	NPA-1 Nematode polyprotein allergen	None	isotig02438	1498	SP
31	APY-2 Apyrase	Apyrase	isotig07051	1474	SP
32	Myoglobin	Globin	isotig11742	1446	SP
33	VAL-9	Double SCP	isotig05765	1431	SP
34	Myoglobin	Globin	isotig04273	1390	SP
35	VAL-11	Single SCP	isotig05330	1303	SP*
36	MSP-1 Major Sperm Protein	Motile Sperm	isotig01565	1278	NO

Rank	Identity	Conserved domains	Code	Score	SP
37	MEP-2 Zinc metalloprotease	Pep0, Peptidase M13 N	isotig05366	1232	SP
38	Vitellogenin	Vitellogenin N, LPN N	contig00203	1205	SP*
39	Vitellogenin	VWD	contig00477	1204	SP*
40	VAL-8.1	Double SCP	isotig02308	1200	SP
41	TTR-2 Transthyretin-related	DUF290	isotig13558	1156	SP
42	NAS-1.1 Nematode Astacin protease	ZnMc astacin-like	isotig04178	1148	SP*
43	ACE-2 Acetylcholinesterase	Esterase lipase	isotig00868	1122	SP
44	PHP-1 PHA domain protein	PHA02954	isotig03547	1120	SP
45	NAS-1.2 Nematode Astacin protease	ZnMc astacin-like	isotig04179	1095	SP*
46	Myoglobin	Globin	isotig05169	1094	SP
47	VAL-8.2	Double SCP	isotig02308	1040	SP*
48	MEP-3 Zinc metalloprotease	Peptidases M13, M13 N	isotig05155	1007	SP*
49	ACE-3 Acetylcholinesterase	Esterase lipase	isotig00869	1006	SP
50	Deoxyribonuclease II	Dnase_II	isotig08122	1006	SP*
51	VAL-6	Double SCP	isotig03505	996	SP
52	CHI-1 Chitinase	Chitinase GH19	isotig04840	988	SP
53	Astacin protease (fragment)	None	isotig01219	956	SP*
54	Trypsin family protein	Trypsin SPc	isotig03474	919	NO
55	Vitellogenin	VWD	contig00212	918	SP*
56	Aspartyl protease (necepsin)	Pepsin/retropepsin-like	isotig06497	884	SP
57	Trypsin family protein	Trypsin SPc	isotig03473	883	NO
58	VAL-1.6	Double SCP	isotig01655	867	SP
59	NAS-2.1 Nematode Astacin protease	ZnMc astacin-like	isotig01336	858	SP*
60	MFH-1 Ascaris MFP2b homologue	MFP2b	isotig07283	845	NO
61	NAS-2.2 Nematode Astacin protease	ZnMc astacin-like	isotig01334	836	SP*
62	NAS-3.1 Nematode Astacin protease	ZnMc astacin-like	isotig01799	832	SP*
63	NAS-4 Nematode Astacin protease	ZnMc astacin-like	isotig00214	830	SP*
64	Enolase	Enolase	isotig06965	826	NO
65	VAL-17	Double SCP	isotig05765	825	SP*
66	TTR-3 Transthyretin-related	DUF290	isotig14171	821	SP
67	MEP-4 Zinc metalloprotease	Peptidases M13, M13 N	isotig05402	806	SP
68	VAL-12	Double SCP	isotig06637	803	SP
69	NSP-2 Novel Secreted Protein with SP	None	isotig12022	802	SP
70	NSP-3 Novel Secreted Protein with SP	None	isotig12137	802	SP
71	NAS-5.1 Nematode Astacin protease	ZnMc astacin-like, CUB	isotig01596	779	SP*
72	NSN-1 Novel Secreted Non-signal-peptide protein	None	isotig18405	777	NFL
73	VAL-5	None	isotig04839	746	SP
74	Chondroitin-like protein	None	isotig01493	710	SP
75	Vitellogenin	Vitellogenin N	contig00199	696	SP

Rank	Identity	Conserved domains	Code	Score	SP
76	NAS-5.2 Nematode Astacin protease	ZnMc astacin-like, CUB	isotig01598	691	SP*
77	VAL-14	Double SCP	isotig04896	689	SP
78	LYS-3 Lysozyme	Muramidase GH25	isotig08606	683	SP*
79	NAS-6 Nematode Astacin protease	ZnMc astacin-like, CUB	isotig07866	681	SP*
80	Myoglobin	Globin	isotig11666	683	SP
81	NSP-4 Novel Secreted Protein	None	isotig05257	681	SP
82	MFH-2 Ascaris MFP2b homologue	MFP2b	isotig07373	647	NO
83	APY-3 Apyrase-3	Apyrase	isotig03589	644	SP
84	Complement regulatory protein	CCP	isotig13827	632	SP
85	Aldolase	FBP Aldolase 1a	isotig04915	627	NO
86	Complement regulatory protein	CCP	isotig12552	611	SP*
87	NSP-5 Novel Secreted Protein	None	isotig12800	599	SP
88	NSP-6 Novel Secreted Protein	None	isotig12832	596	SP
89	NSP-7 Novel Secreted Protein	None	isotig12576	592	SP
90	NSN-2 Novel Secreted Non-signal-peptide protein	None	isotig14195	589	NFL
91	FAR-1 Fatty acid/retinol binding protein	Gp-FAR-1	isotig11475	577	SP*
92	HEX-1 Hexokinase	Hexokinases 1, 2	isotig05973	563	NO
93	NSP-8 Novel Secreted Protein	None	isotig16252	557	SP
94	NSP-9 Novel Secreted Protein	None	isotig09200	529	SP
95	SCP-1 Serine carboxypeptidase	Esterase lipase	isotig05975	529	SP
96	CSP-1 Conserved nematode Secreted protein	None	isotig13235	528	SP*
97	SPN-1 Serpin	Serpin	isotig08233	512	NFL
98	NAS-3.2 Nematode Astacin protease	ZnMc astacin-like	isotig01792	509	SP*
99	CSP-2 Conserved nematode Secreted Protein	None	isotig09016	502	SP
100	NAS-3.3 Nematode Astacin protease	ZnMc astacin-like	isotig01791	498	SP*

SCP denotes the PFAM Sperm Coat Protein domain found in either single or double formation in VAL proteins. SS denotes presence of predicted signal sequence; SS* indicates that the sequence is truncated but homologous to known proteins containing signal sequences; NFL denotes transcripts which are not full length and for which no database homologues were found with signal sequences (in the case of novel proteins, no homologues were found at all). Note that VAL-8.1 corresponds to a sequence similar but not identical to isotig02308, while VAL-8.2 matches exactly.

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Table 3
Top 100 Proteins identified in HEx by LC-MS/MS

Top 100 HEx by mascot score

Rank	Identity	Code	Score	SS
1	Vitellogenin	contig00471	3369	
2	Myoglobin	sotig11742	3366	
3	Myoglobin	sotig13336	3098	
4	Actin	sotig01642	2585	
5	Actin	sotig01640	2251	
6	Myoglobin	sotig04274	2208	
7	Myoglobin	sotig11356	2113	
8	Myoglobin	sotig05169	1977	
9	Myoglobin	sotig04273	1833	
10	HSP90	sotig04714	1831	
11	Major sperm protein	sotig01565	1820	
12	Phosphoenolpyruvate carboxykinase	sotig05532	1713	
13	Aldolase	sotig04915	1611	
14	Myoglobin	sotig11666	1607	
15	Nematode polyprotein allergen NPA-1	sotig02438	1562	
16	Enolase	sotig06965	1476	
17	Protein disulfide isomerase	sotig05787	1465	
18	Vitellogenin	contig00207	1301	
19	Vitellogenin	contig00212	1295	
20	Eukaryotic translation elongation factor 1A	isotig06220	1223	
21	14-3-3 family member	isotig07975	1218	
22	Actin	isotig08179	1212	
23	Eukaryotic translation elongation factor 1A	isotig02523	1187	
24	Beta-tubulin	isotig06421	1179	
25	HSP70	isotig07134	1167	
26	Vitellogenin	contig00203	1040	
27	Vitellogenin	EST Hp_ADY001C04	1025	
28	Eukaryotic translation elongation factor 2	isotig05363	1015	
29	Ribosomal protein 60S P0	isotig01358	965	
30	HSP60	isotig06093	958	
31	Protein disulfide isomerase	isotig01916	902	
32	Vitellogenin	contig00199	884	
33	AAA family ATPase	isotig05378	857	
34	HSP90	isotig05404	851	
35	Serpin	isotig02225	788	
36	Ribosomal protein 40S S8	isotig10482	785	

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Rank	Identity	Code	Score	SS
37	Thioredoxin peroxidase	isotig05188	771	
38	Alpha-tubulin	isotig06550	736	
39	Arginine kinase	isotig06694	736	
40	Fumarase	isotig02533	717	
41	Myoglobin	isotig12632	698	
42	Actin	isotig08340	695	
43	Cystathionine beta-synthase	isotig05473	681	
44	Glyceraldehyde 3-phosphate dehydrogenase	isotig03503	646	
45	Calreticulin	Hpb-CRT	636	
46	Glutamate dehydrogenase	isotig06500	634	
48	HSP70	isotig05458	585	
49	Tropomyosin	Hpb-TRP	585	
50	Nucleosome assembly protein	isotig04959	573	
51	Chondroitin family member	isotig01493	571	
52	Malate dehydrogenase	isotig05076	561	
53	Phosphoglycerate_kinase	isotig06484	533	
54	Myoglobin	isotig13442	531	
55	Ribosomal protein 60S L6	isotig11208	524	
56	Vitellogenin	EST Hp_ADY_001 G11	523	
57	Myoglobin (fragment)	isotig19571	521	
58	Fatty acid and retinol binding protein	isotig11475	515	
59	Alpha-tubulin	isotig06165	512	
60	C-type lectin	isotig02337	499	
61	Phosphatidylethanolamine-binding protein	isotig05126	469	
62	Ribosomal protein 60S L18	isotig10630	465	
63	Ribosomal protein 40S S4	isotig05116	463	
64	CSN-2 Conserved secreted protein, No signal sequence	isotig04423	449	
65	Chondroitin family member	isotig06559	433	
66	Eukaryotic translation initiation factor 4A	isotig06491	432	
67	Retinol binding protein	Hpb-RBP	419	
68	Enolase (fragment)	isotig18996	404	
69	Nucleoside diphosphate kinase	isotig05236	401	
70	Ribosomal protein 60S L7a	isotig09109	397	
71	CSN-5 Conserved secreted protein, No signal sequence	isotig03664	393	
72	Glutathione S-transferase	isotig10292	389	
73	Macrophage migration inhibitory factor	isotig14093	383	
74	Vitellogenin	contig00211	372	
75	Cyclophilin	isotig11124	364	
76	Dehydrogenase	isotig03780	363	

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Rank	Identity	Code	Score	SS
77	Cytochrome C	isotig11144	361	
78	Ribosomal protein 60S L14	isotig14543	359	
79	RACK family member	isotig07754	358	
80	Aspartyl protease inhibitor	isotig05063	355	
81	Glutathione S-transferase	isotig07073	351	
82	VAL-3.1	isotig05790	349	
83	Ribosomal protein 60S L10	isotig12696	346	
84	Transketolase	isotig02953	346	
85	HSP70	isotig13132	345	
86	Ribosomal protein 40S S3	isotig09688	345	
87	Aldehyde dehydrogenase family member	isotig04738	339	
88	Oxidoreductase	isotig08193	323	
89	Glutathione S-transferase	isotig11588	322	
90	Adenosylhomocysteinase	isotig08907	308	
91	TTR-13 Transthyretin-related	isotig13284	304	
92	Glutathione S-transferase	isotig04128	302	
93	Ureidopropionase	isotig08079	300	
94	HSP70	isotig08182	296	
95	CSN-6 Conserved secreted protein, No signal sequence	isotig04424	288	
96	Fructose-1,6-bisphosphatase	isotig07764	284	
97	NAC domain-containing protein	isotig10507	283	
98	Alpha-tubulin	isotig06090	282	
99	Isocitrate lyase-malate synthase	isotig05351	282	
100	Ribosomal protein 60S L12	isotig11621	278	

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Table 4

Proteins Common to both HES and HEx

Rank	NAME	CODE	HES score	HES emPAI	HEx score	HEx emPAI	HES/HEx emPAI
1	VAL-2.1	isotig03106	5149	8.35	103	0.12	69.6
2	TTR-1 Transthyretin-related	isotig04612	2130	29.24	144	0.86	34.0
3	VAL-1.3	isotig06456	2668	1.97	47	0.06	32.8
4	VAL-7.5	isotig01526	3367	11.23	100	0.35	32.1
5	VAL-1.1	isotig06320	6546	4.05	77	0.18	22.5
6	NAS-3.1 Astacin protease	isotig01799	832	2.02	47	0.12	16.8
7	VAL-3.1	isotig05790	4754	3.66	349	0.25	14.6
8	VAL-7.1	isotig01524	2593	8.84	129	0.64	13.8
9	NSN-1 Novel secreted protein, No SP	isotig18405	777	4.03	40	0.31	13.0
10	Astacin protease family member	isotig01791	419	1.41	67	0.12	11.8
11	CSP-4 Conserved secreted protein with SP	isotig13290	482	1.82	37	0.16	11.4
12	VAL-4	isotig10387	1770	4.08	53	0.42	9.7
13	Aspartyl protease (necepsin)	isotig06497	884	1.17	136	0.18	6.5
14	Kunitz inhibitor	isotig01217	273	0.76	88	0.12	6.3
15	VAL-12	isotig06637	803	1.02	130	0.19	5.4
16	Astacin protease family member (fragment)	isotig01219	956	3.05	89	0.59	5.2
17	Superoxide dismutase	isotig09104	235	0.47	48	0.10	4.7
18	C-type lectin mannose receptor-like	isotig00496	366	0.97	61	0.23	4.2
19	MFH-1 Ascaris MFP2b homologue	isotig07283	845	1.17	137	0.29	4.0
20	Cystatin	isotig02700	261	0.41	71	0.12	3.4
21	CSP-2 Conserved secreted protein with SP	isotig09016	502	0.57	434	0.20	2.9
22	Motile sperm domain containing protein	isotig16097	175	0.50	144	0.22	2.3
23	Galectin	isotig09082	120	0.20	44	0.09	2.2
24	TIL domain-containing protein	isotig00779	98	0.27	42	0.13	2.1
25	Arginine kinase	isotig05009	212	0.65	193	0.33	2.0
26	Motile sperm domain containing protein	isotig16375	220	0.89	160	0.53	1.7
27	ERM family member	isotig02815	79	0.08	100	0.05	1.6

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Rank	NAME	CODE	HES score	HES emPAI	HEx score	HEx emPAI	HES/HEx emPAI
28	Cyclophilin	isotig09088	111	0.31	71	0.20	1.6
29	Actin depolymerising factor	isotig07522	145	0.23	144	0.15	1.5
30	Macrophage migration inhibitory factor	isotig14093	256	0.96	383	0.65	1.5
31	TTR-5 Transthyretin-like	isotig13207	397	0.89	88	0.61	1.5
32	MFH-3 Ascaris MFP2b homologue	isotig07980	463	0.84	148	0.58	1.4
33	TTR-10 Transthyretin-like	isotig11792	108	0.67	84	0.47	1.4
34	NSP Novel isotig12701	isotig12701	194	0.99	166	0.73	1.4
35	Chondroitin family member	isotig01493	710	0.48	571	0.38	1.3
36	Ferritin	isotig15727	117	0.74	140	0.73	1.0
37	NSP-39 Novel secreted protein with SP	isotig16333	108	0.78	187	0.77	1.0
38	Vitellogenin	contig00207	1552	5.73	1301	5.70	1.0
39	Myoglobin (fragment)	isotig19571	415	3.10	521	3.09	1.0
40	Calmodulin	isotig07710	53	0.07	57	0.07	1.0
41	Cathepsin-B like cysteine protease	isotig07017	147	0.06	136	0.06	1.0
42	Galectin	isotig09713	81	0.10	69	0.10	1.0
43	Kunitz inhibitor	isotig02721	82	0.13	82	0.13	1.0
44	Kunitz inhibitor	isotig02378	35	0.20	36	0.20	1.0
45	ML-domain containing protein	isotig10098	110	0.23	88	0.23	1.0
46	Motile sperm domain containing protein	isotig16212	138	0.22	92	0.22	1.0
47	NSP-59 Novel secreted protein with SP	isotig13319	38	0.16	35	0.16	1.0
48	Triose phosphate isomerase	isotig05016	276	0.44	198	0.44	1.0
49	VAL-15	isotig02149	133	0.20	200	0.20	1.0
50	Chondroitin family member	isotig08745	82	0.21	92	0.22	1.0
51	NPA-1 Nematode polyprotein allergen	isotig02438	1498	0.67	1562	0.75	0.9
52	Vitellogenin	contig00471	3997	3.05	3369	3.74	0.8
53	Nucleoside diphosphate kinase	isotig05236	462	1.18	401	1.55	0.8
54	TTR-6 Transthyretin related	isotig05212	304	0.62	210	0.83	0.7
55	Rab GDP dissociation inhibitor	isotig06129	54	0.16	168	0.22	0.7
56	Chondroitin family member	isotig06559	284	0.48	433	0.66	0.7

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Rank	NAME	CODE	HES score	HES emPAI	HEx score	HEx emPAI	HES/HEx emPAI
57	Vitellogenin	contig00199	696	2.38	884	3.29	0.7
58	Myoglobin	isotig11742	1446	3.31	3366	4.60	0.7
59	Enolase	isotig06965	826	2.13	1476	3.02	0.7
60	Transaldolase	isotig07176	59	0.14	62	0.21	0.7
61	Vitellogenin	contig00203	1205	4.87	1040	7.34	0.7
62	Aldolase	isotig04899	67	0.13	114	0.20	0.7
63	Vitellogenin (fragment)	sing00711	131	1.60	179	2.57	0.6
64	Myoglobin	isotig05169	1094	1.64	1977	2.64	0.6
65	Myoglobin	isotig04274	1579	3.13	2208	5.07	0.6
66	TTR-11 Transthyretin related	isotig04258	83	0.29	95	0.47	0.6
67	Ribosomal protein 60S L40 / Ubiquitinn	isotig13199	108	0.34	116	0.56	0.6
68	Serpin	isotig02225	281	0.33	788	0.59	0.6
69	Vitellogenin	contig00212	918	2.24	1295	4.24	0.5
70	Vitellogenin	contig00211	263	1.29	374	2.46	0.5
71	Chondroitin family member	isotig04525	51	0.13	75	0.27	0.5
72	Fatty acid/retinol binding protein	isotig11475	577	1.47	515	3.12	0.5
73	Fatty acid/retinol binding protein (fragment)	sing12372	71	0.27	78	0.61	0.4
74	Phosphatidylethanolamine-binding protein	isotig05126	339	0.64	469	1.67	0.4
75	Independent phosphoglycerate mutase	isotig01699	48	0.05	136	0.14	0.4
76	Vitellogenin	Hp_ADY_001G11	144	2.05	523	5.75	0.4
77	Cyclophilin	isotig11124	256	0.87	364	2.48	0.4
78	Thioredoxin peroxidase	isotig05188	498	1.79	771	5.19	0.3
79	Enolase (fragment)	isotig18996	326	1.08	404	3.30	0.3
80	Glutathione S-transferase	isotig11588	219	0.47	322	1.45	0.3
81	HSP70 (fragment)	isotig13132	148	0.35	345	1.11	0.3
82	Myoglobin	isotig11356	398	0.63	2113	2.00	0.3
83	Calreticulin	Hpb-CRT	74	0.21	636	0.67	0.3
84	Lipocalin domain-containing protein	isotig05316	104	0.34	229	1.09	0.3
85	Myoglobin	isotig11666	683	4.48	1607	14.53	0.3

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Rank	NAME	CODE	HES score	HES emPAI	HEx score	HEx emPAI	HES/HEx emPAI
86	CSN-5 Conserved secreted protein, No SP	isotig03664	43	0.08	393	0.27	0.3
87	Aldolase	isotig04915	627	0.77	1611	3.00	0.3
88	Major sperm protein	isotig01565	1278	2.10	1820	8.59	0.2
89	HSP60	isotig06093	195	0.43	958	1.76	0.2
90	Piwi domain-containing protein	isotig04875	77	0.06	167	0.25	0.2
91	14-3-3 family member	isotig07975	324	0.94	1218	3.98	0.2
92	CSN-2 Conserved secreted protein, No SP	isotig04423	127	1.08	449	4.75	0.2
93	Actin	isotig08340	102	0.27	695	1.38	0.2
94	Cytochrome C	isotig11144	50	0.13	361	0.86	0.2
95	Actin	isotig01640	340	0.47	2251	3.21	0.1
96	Eukaryotic translation elongation factor 2	isotig05363	86	0.09	1015	0.87	0.1
97	Protein disulfide isomerase	isotig01916	81	0.16	902	1.62	0.1
98	Eukaryotic translation elongation factor 1A	isotig02523	44	0.12	1187	1.64	0.1
99	Protein disulfide isomerase	isotig05787	133	0.24	1465	3.42	0.1
100	HSP70	isotig07134	100	0.22	1167	3.18	0.1
101	Glutathione S-transferase	isotig10292	47	0.24	389	3.88	0.1
102	Malate dehydrogenase	isotig05076	44	0.07	561	1.31	0.1
103	Ribosomal protein 60S P0	isotig01358	38	0.04	965	0.77	0.1
104	Retinol binding protein	Hpb-RBP	29	0.16	419	5.15	0.0

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Table 5

Table of common ES proteins between H. polygyrus, Ancylostoma caninum, Haemonchus contortus and *Teladorsagia circumcincta*.

	A. caninum	***H. contortus***	***H. polygyrus***	***T. circumcincta* (L4 not adult)**
	[28]	**[22]**	**Hewitson, this paper**	**[30]**
n	**105**	**107**	**375**	**32**
VAL/ASP	**24**	**13**	**25**	**8**
Metalloproteases/Astacins/ Aminopeptidases	**4**	**14**	**25**	**2**
Transthyretins	**5**	**2**	**12**	**0**
Lysozymes	**2**	**0**	**8**	**0**
Serine proteases	**0**	**8**	**8**	**0**
Galectins	**2**	**0**	**5**	**0**
Aspartyl proteases	**1**	**1**	**5**	**0**
CTLs	**3**	**0**	**3**	**0**
15-kDa ES	**8**	**21**	**2**	**0**
Cysteine proteases/necpains	**3**	**0**	**2**	**13**
Cyclophilins	**0**	**2**	**2**	**0**
Protein disulphide isomerases	**1**	**0**	**2**	**2**
Nucleoside diphosphate kinases	**0**	**1**	**1**	**0**
GA1	**0**	**21**	**0**	**0**