



Characterisation of protective vaccine antigens from the thiol-containing components of excretory/secretory material of *Ostertagia ostertagi*

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ABSTRACT

Previous vaccination trials have demonstrated that thiol proteins affinity purified from *Ostertagia ostertagi* excretory-secretory products (*O. ostertagi* ES-thiol) are protective against homologous challenge. Here we have shown that protection induced by this vaccine was consistent across four independent vaccine-challenge experiments. Protection is associated with reduced cumulative faecal egg counts across the duration of the trials, relative to control animals. To better understand the diversity of antigens in *O. ostertagi* ES-thiol we used high-resolution shotgun proteomics to identify 490 unique proteins in the vaccine preparation. The most numerous ES-thiol proteins, with 91 proteins identified, belong to the sperm-coating protein/Tpx/antigen 5/pathogenesis-related protein 1 (SCP/TAPS) family. This family includes previously identified *O. ostertagi* vaccine antigens *O. ostertagi* ASP-1 and ASP-2. The ES-thiol fraction also has numerous proteinases, representing three distinct classes, including: metallo-; aspartyl- and cysteine proteinases. In terms of number of family members, the M12 astacin-like metalloproteinases, with 33 proteins, are the most abundant proteinase family in *O. ostertagi* ES-thiol. The *O. ostertagi* ES-thiol proteome provides a comprehensive database of proteins present in this vaccine preparation and will guide future vaccine antigen discovery projects.

1. Introduction

The gastrointestinal nematode *Ostertagia ostertagi* causes serious health and welfare issues as well as substantial economic losses in cattle. Control of this parasite is reliant on the use of anthelmintics, but the widespread and prolonged use of these drugs has resulted in the selection of resistant worms (Bartley et al., 2021; Cotter et al., 2015; Edmonds et al., 2010). Alternative control approaches, such as vaccination, are needed to provide robust and sustainable control of these parasites. Previous *O. ostertagi* vaccination trials have included immunisation of cattle with live attenuated larvae (Bürger and Pfeiffer, 1969), with somatic antigens (Herlich and Douvres, 1979) or unfractionated ES products (Hilderson et al., 1995). None of these approaches have resulted in significant levels of protection against *O. ostertagi* infection. However, vaccination of calves with thiol-binding proteins that have

been affinity purified from adult *O. ostertagi* ES (*O. ostertagi* ES-thiol) results in statistically significant levels of protection, with a 56 – 60% reduction in mean cumulative faecal egg counts (cFEC) in vaccinates, relative to control, unvaccinated cattle (Geldhof et al., 2004, 2002).

The *O. ostertagi* ES-thiol fraction contains activation-associated secreted proteins (ASPs) and, of these, *O. ostertagi* ASP-1 (Oo-ASP-1) and Oo-ASP-2 have been identified as protective antigens in their native form (Geldhof et al., 2003, 2002; Meyvis et al., 2007). Other ASPs from parasitic nematodes have also shown promise as vaccine antigens; for example, ASPs from the hookworms *Ancylostoma caninum* (Ghosh et al., 1996; Ghosh and Hotez, 1999; Hilderson et al., 1995) and *Ancylostoma ceylanicum* (Goud et al., 2004; Mendez et al., 2005) provide significant levels of protection against parasite challenge and the recombinant cocktail vaccine against *Teladorsagia circumcincta* also contains an ASP (Nisbet et al., 2013). The ASPs belong to an evolutionary conserved

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group of secreted proteins from the SCP/TAPS (sperm-coating protein/Tpx/antigen 5/pathogenesis-related protein 1) family, and are present in diverse eukaryotic organisms, including parasitic nematodes. The SCP/TAPS are secretory proteins, characterised by the presence of either one or two cysteine-rich, antigen 5, and pathogenesis-related 1 (CAP) domains (CAP, InterPro domain IPR014044). The first reported SCP/TAPS protein was Ac-ASP-1 from the secretome of the hookworm, *A. caninum* (Hawdon et al., 1996). Since then, SCP/TAPS proteins have been identified in the secretome of many parasitic nematodes, often as part of large expanded families, and are likely to play an important role in parasitism (International Helminth Genomes Consortium, 2019; Viney, 2017).

ASPs from the adult *O. ostertagi* ES-thiol fraction have shown great promise for vaccine development (Geldhof et al., 2008, 2003, 2002; González-Hernández et al., 2016; Meyvis et al., 2007). Purified native *O. ostertagi* ASP-1 protected cattle by reducing *O. ostertagi* cumulative FEC in vaccinates relative to control unvaccinated animals, with greater levels of protection compared to animals vaccinated with the ES-thiol fraction (Meyvis et al., 2007). However, the development of a recombinant version of Oo-ASP-1 that protects against *O. ostertagi* infection has been a challenge. Initial experiments demonstrated that recombinant Oo-ASP-1 produced in yeast failed to protect animals against homologous challenge, even though the protein antigen was correctly folded and had the same three-dimensional protein shape as the native molecule (Borloo et al., 2013; González-Hernández et al., 2016). This failure in protection is likely due to the recombinant expression system being unable to reconstruct native structures of the worm protein, including post-translational modifications, such as glycosylation. Recent success has been achieved by production of recombinant Oo-ASP-1 produced in plants, where the recombinant protein is decorated with worm-like glycans present on the native molecule. This refined, plant expressed Oo-ASP-1 recombinant antigen, reduced cumulative FECs in vaccinates by 39% relative to control vaccinated animals (Zwanenburg et al., 2023). Thus, for a successful recombinant antigen, both the selection of antigen, as well as ability to produce a recombinant version that reflects the native molecule is critical for development of a successful vaccine.

In addition to ASPs, it is clear that the *O. ostertagi* ES-thiol protein fraction contains other protective antigens (Meyvis et al., 2007) and these may offer a source of additional vaccine antigens. This is demonstrated by a study in which *O. ostertagi* ES-thiol was fractionated into three sub-fractions, with each protein fraction significantly reducing cFECs in vaccinates, relative to control animals (Meyvis et al., 2007). Although the ES-thiol fraction is a potential source of other protective vaccine antigens, the protein diversity within this fraction is largely unknown. Therefore, the objective of this present study was to apply high-resolution shotgun proteomics to discover the diversity of proteins within *O. ostertagi* ES-thiol.

2. Materials and methods

2.1. Ethics statement

All animal procedures were performed at Moredun Research Institute (MRI), UK under project licenses 70/7914 and P23F688B4 as required by the UK Animals (Scientific Procedures) Act 1986, with ethical approval from MRI Animal Welfare and Ethical Review Body. Animals were maintained at MRI under conditions designed to exclude accidental infection with helminth parasites and were confirmed helminth-naïve at the start of the experiments.

2.2. Production of parasite material

To generate *O. ostertagi* adult worms for excretory-secretory product (ESP) generation, helminth-free Holstein-Friesian male calves (4–10 months old) were challenged with 50,000 infective third-stage (L3)

O. ostertagi larvae (isolate MOo2). At 21 days post infection (dpi) animals were euthanised and *O. ostertagi* adults were recovered from the gastric stomach (abomasum) of individual animals. In brief, the abomasum was opened along the greater curvature and washed in physiological saline (0.9% NaCl (w/v)). The abomasal wash sample was poured over a 212 µm sieve and floated on physiological saline at 37 °C for four hours and *O. ostertagi* parasites that migrated through the sieve were collected. Following washing, the abomasum was pinned to a polystyrene board and floated on physiological saline at 37 °C for four hours and *O. ostertagi* parasites that migrated out of the tissue were collected.

2.3. Preparation of *O. ostertagi* excretory-secretory products

At 21 dpi, *O. ostertagi* adults were recovered from the abomasum of infected calves, as described above. For collection of ESPs, harvested parasites were washed three times in phosphate-buffered saline (PBS) before culturing at 37 °C with 5% CO₂ in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing: 20 mM HEPES pH 7.5; 10 mM L-glutamine; 1000 U/ml penicillin; 1000 µg/ml streptomycin; 200 µg/ml gentamycin and 5 µg/ml amphotericin B. Culture supernatants were harvested every 24 h and replaced with fresh media up to 96 h. At each time point the viability of the parasites was confirmed on the basis of structural integrity and motility. The culture supernatants containing *O. ostertagi* ESPs were filtered using Millipore Express 0.22 µm PES 500 ml filters and stored at –70 °C. Prior to chromatography, ESPs were concentrated, and buffer exchanged into PBS using Amicon Ultra-15 columns (Millipore) with a 10-kDa molecular mass cut-off. Protein concentrations were determined by BCA assay (Thermo Fisher Scientific) with BSA standards.

2.4. *O. ostertagi* excretory-secretory product fractionation

Thiol-binding proteins were purified from *O. ostertagi* ESPs according to the method of Geldhof et al., 2002. In brief, a 5 ml thiol-sepharose column (Cytiva) was equilibrated in 5 column volumes of running buffer (10 mM Tris, 0.5 M NaCl, pH7.4). Immediately prior to chromatography, approximately 5 mg of *O. ostertagi* day 21 ESPs were reduced by incubation with 2.5 mM DTT (final concentration) for 30 min at 37°C and excess DTT was removed by desalting on a HiPrep 26/10 column. The reduced desalted ESP sample was applied to the thiol-sepharose column at 0.1 ml/min, the column was then washed with 10 column volumes of running buffer. Bound proteins were eluted with running buffer plus 50 mM DTT. Equilibration, washing and elution steps carried out at 0.8 ml/min. Eluted fractions were desalted on a HiPrep 26/10 column using 10 mM Tris-HCl pH 7.4 as the running buffer. ES-thiol proteins were then concentrated using Amicon Ultra-4, 10 kDa centrifugal concentrators. Protein concentration was determined by BCA protein assay (Thermo Fisher Scientific) with BSA standards and proteins analysed by reducing SDS-PAGE gel.

2.5. SDS-PAGE

Proteins were analysed by SDS-PAGE using the NuPAGE® electrophoresis system (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, protein samples were prepared in NuPAGE LDS sample buffer with reducing agent and heated to 70°C for 10 min prior to loading on NuPAGE 4–12% Bis-Tris gels. Gels were run in MES SDS running buffer and stained with SimplyBlue™ SafeStain (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Vaccination trials

A series of four vaccine trials were performed between 2017 and 2021 (Trials 1–4) to test the efficacy of an ES-Thiol-based vaccine against *O. ostertagi* challenge. The design of each trial is summarised in

Supplementary Table 1. For Trials 1 and 2, calves were immunised with 30 µg ES-Thiol plus Quil A® (Brenntag Biosector) adjuvant or Quil A® only (adjuvant control) on days 0 (V1), 21 (V2) and 42 (V3) via the intramuscular route. For Trials 3 and 4, calves were immunised with 15 µg ES-thiol plus Vax Saponin® (Guinness) adjuvant or Vax Saponin® only (adjuvant control) on days 0 (V1), 21 (V2) and 42 (V3) via the subcutaneous route (Trials 3–4). Calves were between 4 and 7.5 months old at first vaccination and were randomly allocated to either vaccine or adjuvant control groups balancing for age, sex, breed, and source farm. At V3, calves were either trickle infected with 25,000 *O. ostertagi* L3 isolate MOo2 (1000 L3 per day *per os*, 5 days a week for 5 weeks) (Trials 1–2) or challenged once with an oral bolus of 50,000 *O. ostertagi* L3 isolate MOo2 (Trials 3–4). Blood samples were collected via jugular venepuncture at days 0 and 28 for Trial 1–2 and days 0 and 42 for Trials 3–4 to determine serum levels of ES-Thiol specific antibodies as detailed in Section 2.7. Faecal samples were collected twice weekly post-L3 challenge for *O. ostertagi* faecal egg count measurements. Calves were euthanized on day 91 relative to V1 for Trial 1, day 98 for Trial 2 and day 76 for Trials 3 and 4, for worm burden estimation as detailed in Section 2.7.

2.7. Parasitological measurements

Faecal egg count (FEC) analysis was performed twice weekly on up to 10 g of faeces collected from each animal from day 10 post-challenge as previously described (Christie and Jackson, 1982). FEC over time was analysed by fitting a Poisson generalised additive mixed model (GAMM) with FEC as response, group as fixed effect and animal nested within trial as random effect. Separate smoothing splines were considered for each group in order to describe their temporal pattern while allowing for heterogeneous variances and autocorrelation. Cumulative FEC (cFEC) was estimated over the course of each trial by calculating the area under the FEC curves for each animal using the composite trapezoid rule (Taylor et al., 1997). Differences in cFEC between vaccinated and control groups was analysed using a negative binomial generalised linear mixed model (NB GLMM), including cumulative FEC as response variable, treatment group as fixed effect, and trial as random effect. Enumerations of abomasal worm burdens were carried out at post-mortem following standard techniques (Halliday et al., 2007; Jackson et al., 1984). Differences in total worms (luminal and mucosal) between vaccines and controls was analysed using negative binomial generalised linear model (NB GLM) accounting for data over-dispersion by estimating a dispersion parameter.

2.8. Measurement of vaccine-specific antibody response

Serum IgG1 and IgG2 levels against *O. ostertagi* ES-thiol were determined by ELISA on the day of the first vaccination (V1, day 0) and V2 +7days (day 28) (Trials 1 & 2) and on the day of the first vaccination (V1, day 0) and V3 (day 42) for Trials 3 & 4. Ninety-six-well plates were coated with 50 µl/well of ES-thiol diluted in 0.05 M carbonate buffer (pH 9.6) at a concentration of 1 µg/ml. Non-specific binding was blocked with 3% (w/v) fish gelatine in PBS (Sigma G7765). Individual cattle serum samples were applied in duplicate at 1:1000 (IgG1) or 1:500 (IgG2). Secondary antibodies used were BioRad MCA627GA mouse anti-bovine IgG1 (1:1000) and BioRad MCA626GA mouse anti-bovine IgG2 (1:2000) followed by the tertiary conjugate rabbit anti-mouse Ig conjugated to horseradish peroxidase, Dako P0260, (1:1000). Immunoreactivity was visualised by incubation with OPD substrate (SigmaFast OPD, P9187) at room temperature for 5–10 minutes, before stopping the reaction by addition of 25 µl 2.5 M H₂SO₄. All dilutions were made in PBS, 0.5% Tween80, 0.5 M NaCl and incubation steps carried out for 1 hr at 37 °C. Plates were washed six times between each step in PBS, 0.05% Tween 20 using a Bio-Tek ELx405 plate washer. A positive control sample was included in each plate to account for inter-plate variation. Optical density was measured at 492 nm using a Molecular Devices

SpectraMax ABS Plus plate reader. Statistical analysis was performed in GraphPad Prism by using two-way ANOVA with Tukey's multiple comparison test.

2.9. PacBio Iso-Seq library preparation and sequencing

At 21 dpi, *O. ostertagi* adults were recovered from the abomasum of infected calves, as described above. Male and female *O. ostertagi* parasites were viewed using a dissecting microscope and picked using a fine needle into separate tubes. Parasites were snap-frozen in liquid nitrogen and stored at –70 °C until RNA isolation. Total RNA was isolated from *O. ostertagi* male and female worms separately using RNeasy mini kits (Qiagen) with a DNaseI digest, according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop™ One spectrophotometer and quality assessed with an Agilent 2100 Bioanalyser using an RNA chip (Agilent Technologies). For library construction all RNA samples had a RIN value >7. Library preparation and sequencing were conducted at the University of Liverpool, Centre for Genomic Research. The sample was initially treated with Teloprime full length cDNA amplification kit v2 (Lexogen GmbH) following the manufacturer's protocol. This involved first strand cDNA synthesis by denaturation of sample (1000 ng) at 70°C with primer RTP and after cooling to 37°C mixing with reverse transcriptase and incubating at 46°C for 50 minutes. The sample was cleaned using the supplied columns. The sample was then ligated to a cap-dependent linker for 3 hours at 25°C and cleaned again with the same columns as before. The sample was incubated with 2nd strand synthesis mix for 90 sec at 98 °C, 60 sec at 62 °C, and 72 °C for 5 minutes with a hold at 10 °C. The sample was column cleaned again. Half of the sample was amplified thereby making ds-cDNA for 15 cycles (1x (95.8 °C 30 sec, 50 °C 45 sec, 72 °C 20 minutes), 14x (95.8 °C 30 sec, 62 °C 30 sec, 72 °C 20 minutes) and cleaned with a column as before. The amplified cDNA products were made into SMRTbell template libraries according to the Iso-Seq protocol by Pacific Biosciences. Sequencing was performed on the PacBio Sequel System, and 1 SMRT Cell was run for each sample with a movie run-time of 600 min for each SMRT Cell.

2.10. Sequence processing

For each sample, the CCS module of the IsoSeq v3 program (<https://github.com/PacificBiosciences/IsoSeq>) was used, with default parameter settings, to generate circular consensus sequence (CCS) reads from the sub-reads generated from each sequencing run. The CCS module provides full-length reads spanning entire transcript isoforms all the way from the polyA-tail to the 5' end. High quality isoforms were further clustered using CD-HIT version 4.6 (Fu et al., 2012) with the following parameters: -c = 0.97 -G = 0 -aL = 0.8 -AL = 100 -aS = 0.99 -AS = 30 -g = 1. Open reading frames (ORFs) were predicted using ANGEL ORF prediction software (<https://github.com/PacificBiosciences/ANGEL>) and annotated using Blast2GO through OmicsBox software (ver. 3.1, BioBam Bioinformatics Solutions).

2.11. Transcriptome completeness

Transcriptome completeness was determined using Benchmarking Universal Single-Copy Orthologs (BUSCO; v5.2.2; -mode proteins -lineage metazoa_odb10) gene completeness metrics (Simão et al., 2015).

2.12. Proteomic analysis of *O. ostertagi* ES-thiol

2.12.1. Liquid digest

ES-thiol samples were digested using an S-Trap micro column (ProtiFi) kit, following the manufacturer's instructions. Briefly, 1–2 µg of *O. ostertagi* ES-thiol was mixed with the supplied 2X SDS lysis buffer, proteins were reduced with 5 mM Tris (2-carboxyethyl) phosphine

(TCEP) and alkylated with 20 mM methyl methanethiosulfonate (MMTS). Proteins were then acidified with 2.5% phosphoric acid and bound to the S-Trap column in 6 volumes of S-Trap binding/washing buffer (100 mM TEAB in 90% Methanol). The column was centrifuged (4000 xg for 30 s) and then washed a further 4 times in wash buffer. Proteins were digested using sequencing grade trypsin (in 50 mM TEAB) in a 1:10 ratio to trapped protein for 16 hrs at 37°C. Tryptic peptides were eluted in three steps, firstly, 40 µl 50 mM triethylammonium bicarbonate (TEAB) buffer followed by 40 µl 0.2% formic acid (FA), and finally 40 µl 50% acetonitrile (ACN) in water and 0.2% FA. Elutions were pooled and dried in a vacuum drier. Dried peptides were reconstituted in 20 µl of 0.1% FA prior to analysis by Mass Spectrometry.

2.12.2. Gel piece digest

A single SDS-PAGE gel lane containing *O. ostertagi* ES-thiol proteins was excised and sliced horizontally from top to bottom to yield a series of 26 equal gel slices. The resulting gel slices were destained with 50 mM ammonium bicarbonate in 50% acetonitrile (ACN). Proteins were reduced with 10 mM DTT in 100 mM ammonium bicarbonate and alkylated with 55 mM Iodoacetamide in 100 mM ammonium bicarbonate was added and placed in the dark for 30 min. Proteins were digested with trypsin (Promega Porcine trypsin, 10 ng/µl trypsin in 25 mM ammonium bicarbonate) at 37 °C overnight.

2.12.3. LC-MS/MS analysis

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis was performed using an Ultimate 3000 nano-LC system coupled to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific). The LC system was equipped with an Acclaim Pepmap nano-trap column (C18 PepMap100, 300 µm × 5 mm, 5 µm, 100 Å, Thermo Fisher Scientific) and an Acclaim Pepmap RSLC analytical column (C18, 100 Å, 75 µm × 50 cm EasySpray, Thermo Fisher Scientific). The tryptic peptides were loaded onto the trap column at an isocratic flow of 5 µL/min of 0.05% v/v trifluoroacetic acid for 6 min, peptides were then separated on the Easy Spray analytical column. The eluents were 0.1% v/v formic acid (solvent A) in H₂O and 80% v/v CH₃CN in 0.1% v/v formic acid (solvent B). A 65 min gradient was used at 300 nL/min from (i) 0–5 min, 5% B; (ii) 5–40 min, 5–40% B; (iii) 40–45 min, 40–80% B; (iv) 45–50 min, 80% B; (v) 50–65 min, 80–5% B.

Data were acquired in positive mode using a data dependent approach, MS1 scans were acquired at 70,000 resolution over a mass range of *m/z* 380–1500 with an AGC of 3e6 and a maximum IT of 100 ms. In each cycle, the 10 most intense ions with charge states of ≥2 and intensity thresholds of ≥2.0e5 were selected for MS/MS and subjected to high-energy collision dissociation (HCD) at a normalized collision energy of 30%. The isolation window was set at 1.2 *m/z*, at a resolution of 17,500, an AGC target of 1e5 and a maximum IT time of 100 ms. The dynamic exclusion time was set to 30 s.

2.12.4. Proteomic data analysis

The MS raw data was processed using the Proteome Discoverer platform (version 2.4, Thermo Fisher Scientific) and Sequest HT algorithm. The MS data was searched against the translated *O. ostertagi* male and female Iso-Seq database. A maximum of two missed tryptic cleavages were allowed for and the minimum peptide length was six amino acids. The oxidation of Methionine and protein N-terminal acetylation were set as variable modifications, while carbamidomethylation of Cysteine was set as a fixed modification. MS and MS/MS ion tolerances were set at 10 ppm and 0.02 Da, respectively. A maximum false discovery rate (FDR) of 1% at both the peptide and the protein levels was set and only proteins identified with ≥2 peptides were accepted. The lists of accepted proteins from the 26 Gel-LC slices were compiled into one list using Proteome Discoverer. The Gel-LC output was then merged with the output from 3 liquid digest loadings to give a final list of identified and accepted proteins. The relative protein abundance was calculated based on the Exponentially Modified Protein Abundance

Index (emPAI). The emPAI score of a given protein is proportional to its abundance in the sample (Ishihama et al., 2005).

2.12.5. Protein database compilation

To generate a PacBio Iso-Seq derived database for MS searching, we grouped high-quality isoforms from *O. ostertagi* male (15,521 isoforms) and female (13,387 isoforms) and clustered isoforms using CD-HIT version 4.6 (Fu et al., 2012) with the following parameters: “-c = 1.0 -n = 5 -G = 1 -g = 1 -b = 20 -l = 10 -s = 0.0 -aL = 0.0 -aS = 0.0”. The resultant protein coding sequence database, containing 22,698 unique isoforms, formed the reference proteome.

2.12.6. Annotation of protein sequences

For annotation, the FASTA protein sequences were imported into OmicsBox (ver. 3.1, BioBam Bioinformatics Solutions) and annotated using blastp search against NCBI nr database (E-value ≤ 1.0 × 10⁻³). Subsequent GO mapping was performed using the Blast2GO mapping against the latest version of the GO database to obtain the functional labels (Götz et al., 2008). Prediction of transmembrane domains and signal peptide sequences was done using the TOPCONS prediction server (Tsirigos et al., 2015).

2.13. Phylogenetic analysis of CAP domain proteins

For phylogenetic analysis, ninety-one *O. ostertagi* CAP domain proteins identified in the *O. ostertagi* ES-thiol fraction were analysed in a framework of previously characterised CAP domain containing proteins from clade V parasitic nematodes. This included four ASPs from *O. ostertagi* (Oo-ASP-1 [CAD23183.1] from (Vercauteren et al., 2003); Oo-ASP-2 [CAD56659.1] from (Geldhof et al., 2003); Oo-ASP-3 [CAO00416.1] and Oo-ASP-4 [CAO00417.1] from (Visser et al., 2008)) and one ASP from *T. circumcincta* (Tci-ASP-1 [CBJ15404.1] from (Nisbet et al., 2010)). In addition, we included nine ASPs from *A. caninum*, which have become the canonical set of hookworm ASPs (Acan-NIF [AAA27789.1] from (Moyle et al., 1994); Acan-PI [AAK81732.1] from (Del Valle et al., 2003); Acan-ASP-1 [Q16937.1] from (Hawdon et al., 1996); Acan-ASP-2 [AAC35986.1] from (Hawdon et al., 1999); Acan-ASP-3 [AAO63575], Acan-ASP-4 [AAO63576], Acan-ASP-5 [AAO63577], Acan-ASP-6 [AAO63578] from (Zhan et al., 2003) and Acan-ASP-7 [AEJ86344] from (Datu et al., 2008)). Further CAP domain proteins were retrieved from the proteomes of *Haemonchus contortus* (Doyle et al., 2020) (bioproject: PRJEB506; 229 CAP domain proteins) and *T. circumcincta* (bioproject: PRJNA72569; 203 CAP domain proteins) based on the presence of Interpro CAP domain (IPR014044) using an E-value threshold of <10⁻³. The retrieved protein set contained a total of 537 CAP domain proteins, the full list is available in Supplementary File 1.

For sequence alignment we used a single domain CAP protein from *O. ostertagi* (transcript/11662) that had been trimmed to its CAP domain (amino acids 129–274) to extract an alignment of CAP domains from the protein set. Briefly, CAP domains were extracted using jackhmmer (from HMMER package 3.3.2) which was run for 10 iterations, or to convergence before then, at maximum sensitivity, and with E-value thresholds of 10⁻⁶, with the following parameters “-N 10 -E 1e-6 -domE 1e-6 -incE 1e-6 -incdomE 1e-6 -max -A <output alignment file>”. The resulting protein domain sequences were extracted from the output alignment file and realigned with MUSCLE 5.1 (Edgar, 2021) and trimmed with ClipKit 1.3.0 (Steenwyk et al., 2020). Aligned and trimmed CAP domain sequences were used to create maximum-likelihood phylogenies using FastTree 2.1.11 (Price et al., 2010) using the LG amino acid substitution model and the reliability of the tree was tested using the Shimodaira-Hasegawa test with 1000 resamples. Trees were viewed and annotated using iTOL v.5 (Letunic and Bork, 2021).

2.14. Phylogenetic analysis of astacin-like metalloproteinases

For phylogenetic analysis, thirty-eight *O. ostertagi* astacin-like metalloproteinases identified in the *O. ostertagi* ES-thiol fraction were analysed in a framework of M12A domain proteins from clade V parasitic nematodes. This included three astacin-like metalloproteinases from *O. ostertagi* (CAD19995; CAD28559 and CAD11605) and one from *T. circumcincta* (Tci-MEP-1, CCR26658). Further astacin-like metalloproteinases were retrieved from the proteomes of *H. contortus* (Doyle et al., 2020) (bioproject: PRJEB506; 120 proteins); *T. circumcincta* (bioproject: PRJNA72569; 244 proteins) and *Heligmosomoides polygyrus* (bioproject: PRJEB15396; 78 proteins) based on the presence of Interpro peptidase M12A domain (IPR001506) using an E-value threshold of $<10^{-3}$. The retrieved protein set contained a total of 484 astacin-like metalloproteinases, the full list is available in Supplementary File 2. For sequence alignment we used an astacin-like metalloproteinase from *T. circumcincta* (Tci-MEP-1, CCR26658) that had been trimmed to its peptidase M12A domain (amino acids 142–339) to extract an alignment of these domains from the protein set. Protein domains were extracted, aligned and phylogenies reconstructed using the same pipeline for phylogenetic analysis of CAP domain proteins.

3. Results

3.1. Vaccine trial

To confirm the protective effects of *O. ostertagi* ES-thiol-based vaccines and consistency of the effect, four independent vaccination trials were performed. Calves were immunised via the intramuscular or subcutaneous route with either *O. ostertagi* ES-thiol adjuvanted with a saponin-based vaccine adjuvant or a saponin-based vaccine adjuvant alone, and subsequently challenged with either a trickle infection (Trials 1 and 2) or bolus infection (Trials 3 and 4) of *O. ostertagi* L3. Daily and cumulative FEC data from immunised and adjuvant-only control groups are shown in Fig. 1. Across all four trials, immunisation with ES-thiol resulted in a significant reduction in daily and cumulative FEC relative to the adjuvant only control group ($p < 0.0001$ for both daily and cumulative FEC). The reductions in mean cumulative FEC for Trial 1, 2, 3 and 4 were 64%, 71%, 60% and 47%, respectively (Fig. 1b). No reduction in worm burden was observed in ES-thiol vaccinated calves in Trials 1, 2 and 3, however, a significant 26% reduction in mean worm burden in vaccinates relative to control animals was observed in Trial 4 (Supplementary Figure 1). Furthermore, ES-thiol vaccination resulted in a significant increase in serum levels of ES-thiol specific IgG1 and IgG2 in all four trials, relative to control adjuvant only vaccinates (Supplementary Figure 2).

3.2. Generation of an *O. ostertagi* protein database

To generate a sequence database against which to search proteomic spectra data, we used Pac-Bio isoform sequencing (Iso-Seq) of *O. ostertagi* mRNA isolated from adult male and female parasites. This resulted in the generation of 15,521 and 13,387 unique open reading frames (ORFs) for male and female parasites, respectively (Fig. 2). For each dataset, the average read length was 1300 bp (males) and 1459 bp (females). Completeness of each transcriptome database was assessed using Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis using the metazoa lineage as the reference dataset. We identified 352 of 954 (36.9%) and 420 of 954 (44.0%) metazoan BUSCOs for the male and female transcript datasets, respectively, which is likely due to the relatively low depth of sequencing in Iso-seq.

Finally, to produce a non-redundant dataset that represented both male and female *O. ostertagi* encoded proteins, protein sequences derived from both datasets were merged and redundant sequences removed by clustering using CD-HIT, based on an identity threshold of 100% across the length of each protein. This resulted in an *O. ostertagi*

adult male and female database containing 22,698 unique proteins. Raw PacBio Iso-Seq data generated in this study have been deposited in the Sequence Read Archive (SRA) (<https://www.ncbi.nlm.nih.gov/sra>) with BioProject ID: PRJNA898386.

3.3. Preparation of *O. ostertagi* ES-thiol proteins

For purification of *O. ostertagi* ES-thiol proteins, adult worms (21 dpi) were cultured *in vitro* and supernatants containing excreted/secreted proteins were purified using thiol-chromatography. For production of sufficient material for small scale vaccination studies, 11 mg of *O. ostertagi* ESP was used as starting material, from which 0.9 mg were recovered after thiol-affinity chromatography, representing an 8.2% protein recovery. SDS-PAGE analysis of *O. ostertagi* ES and ES-thiol is shown in Supplementary Figure 3. In both protein fractions the range of stained proteins was similar (approx. 3–98 kDa.), however the pattern of stained proteins differed between the two preparations. In particular, proteins at approx. 30 kDa, 45 kDa and 48 kDa in the ES-thiol fraction were enriched relative to unfractionated ES (Supplementary Figure 3). For vaccination trials presented in this study (Trial 1 – 4), four separate batches of *O. ostertagi* ES-thiol were used. Each batch was analysed by SDS-PAGE and no visible differences in Coomassie stained protein profiles were observed. The ES-thiol preparation used in vaccine Trial 3 was subsequently subjected to proteomic analysis detailed in Section 3.4 below.

3.4. Proteomic analysis of *O. ostertagi* ES-thiol

To identify *O. ostertagi* ES-thiol proteins we used two complementary approaches; a proteomic analysis of tryptic peptides from a Coomassie stained SDS-PAGE gel and a proteomic analysis of tryptic peptides from an *in vitro* liquid trypsin digest. Peptides from both approaches were analysed by LC-MS/MS and resulted in the identification of 490 unique proteins in *O. ostertagi* ES-thiol (Supplementary Table 2).

Of the 490 *O. ostertagi* ES-thiol proteins identified, the majority (476, 97.1%) had blast hits against proteins from other parasitic nematodes. The most frequent top hits were against *T. circumcincta* proteins (226 hits) and *H. contortus* proteins (110 hits). A total of 14 sequences (2.9%) encoded novel proteins with no database match (Supplementary Table 2). When examined for the presence of a secretion peptide, 246 proteins (50.2%) contained a predicted signal peptide (Supplementary Table 2 and Fig. 3A), while 12 proteins (2.4%) contained one or more transmembrane domains (Supplementary Table 2).

All identified ES-thiol proteins were classified by gene ontology (GO) analysis and assigned to categories, which included biological processes (BP), molecular function (MF) and cellular compartment (CC) (Fig. 3, panel B and C). Interestingly, 182/490 (37.1%) proteins did not return GO hits in either BP, MF or CC categories. This lack of GO annotation highlights a significant knowledge gap surrounding secreted proteins produced by parasitic helminths. Of the proteins with an associated GO term, within the BP category, the top GO term was “proteolysis”, which contained 83 unique proteins (Fig. 3, panel C). Proteinases were also represented in the MF classification, with “metalloendopeptidase activity” and “aspartic endopeptidase activity” containing 43 and 17 unique proteins, respectively (Fig. 3, panel C). Within CC category, the top GO terms were “extracellular region” and “integral membrane protein”, with 25 and 11 proteins, respectively (Fig. 3, panel C).

To get a deeper insight into protein function, the 490 *O. ostertagi* ES-thiol proteins were analysed using Interpro domain classification (Fig. 3, panel D). The most numerous protein family in the *O. ostertagi* ES-thiol preparation was the SCP/TAPS family and accounted for 91/490 proteins, containing either a single or double Interpro CAP domain (IPR014044) (Fig. 3, panel D). The top-20 most numerous protein families are shown in Fig. 3, panel D. Within the top-20 most frequently observed domains, proteases were highly represented, including domains from: metalloproteinases (peptidase M13, N-terminal domain

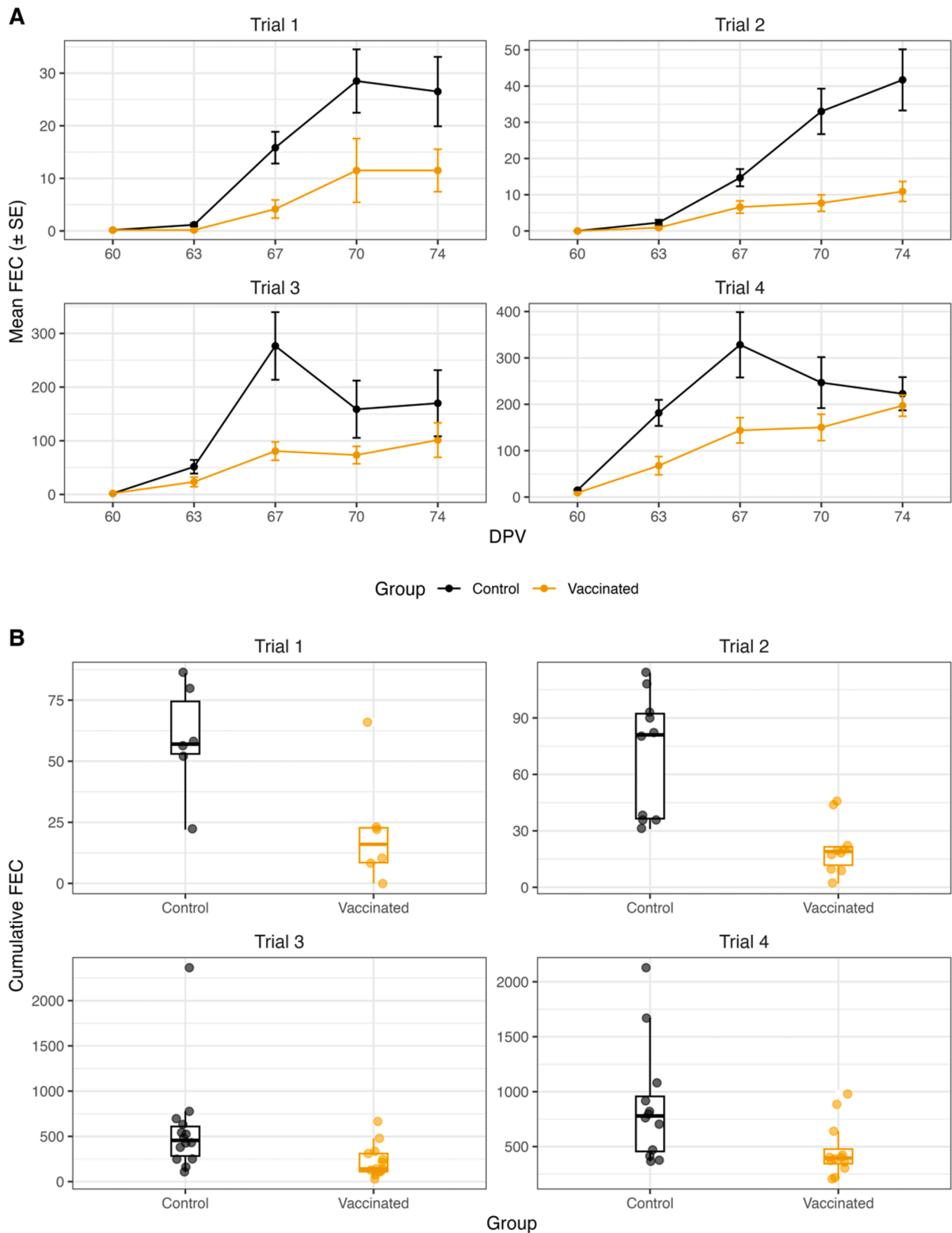


Fig. 1. *O. ostertagi* ES-thiol vaccine trial data. (A) Daily mean faecal egg counts over time shown as days post vaccination (DPV) \pm standard error of the mean for Trials 1–4. Trial 1 (n = 6, each group), Trial 2 (n = 10, each group), Trial 3 (n = 14 for control, n = 13 for vaccinate) and Trial 4 (n = 12, each group). Animals were either trickle infected (Trials 1 & 2; oral challenge with 1000 *O. ostertagi* L3 per day, 5 days/week from days 42–76.); or bolus infected (Trials 3 & 4; oral challenge with 50,000 *O. ostertagi* L3 on day 42). (B) Boxplots of cumulative faecal egg count (cFEC) data for Trials 1–4. Individual data points represent cFEC calculated for each individual calf and boxplots indicate the median cFEC and interquartile range. Control = calves vaccinated with a saponin-based vaccine adjuvant alone. Vaccinated = calves vaccinated with ES-thiol plus a saponin-based vaccine adjuvant.

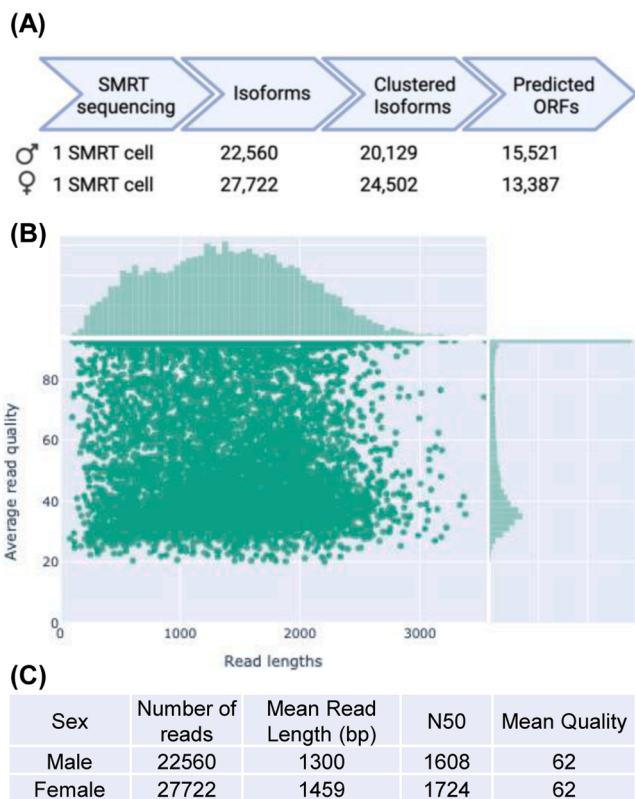


Fig. 2. Summary of PacBio Iso-Seq sequencing for adult *O. ostertagi* males and females. (A) Bioinformatic pipeline and number of full-length isoforms generated for *O. ostertagi* male and female parasites. (B) Dot plot showing distribution of read length (bp, x-axis) and sequence quality (Phred score, y-axis) for all male and female isoforms (50,282 isoforms in total). Histograms show distribution of read length (bp) and quality (Phred score) for all reads. Phred score of 40 equates to a 0.01% error rate. (C) Sequencing summary statistics for *O. ostertagi* male and female Iso-Seq libraries. Mean quality = Phred score.

[IPR008753]; peptidase M12A [IPR001506]; aspartyl proteases (Peptidase family A1 domain [IPR033121]; pepsin-like domain [IPR034164]; astacin-like metallopeptidase domain [IPR034035]) and cysteine proteinases: (peptidase C1A, papain C-terminal [IPR000668]) (Fig. 3, panel D).

To determine relative protein abundance in the *O. ostertagi* ES-thiol preparation, identified proteins with at least two peptides, were ranked by their Exponentially Modified Protein Abundance Index (emPAI) score (Ishihama et al., 2005). The emPAI score of a given protein is based on observed to observable peptides detected by mass spectrometry and the score is proportional to its abundance in the sample (Ishihama et al., 2005). The full-ranking of proteins identified in a liquid-digest is shown in Supplementary Table 3. In summary, the top-20 proteins includes previously described *O. ostertagi* ASPs, including Oo-ASP-1 (transcript/16135) and Oo-ASP-2 (transcript/14958, transcript/15874) (Geldhof et al., 2003; Vercauteren et al., 2003). Also within the top-20 other known parasite ES proteins, including: ES antigen protein 17 (ES17, transcript/20119) and ES20 (transcript/17737); an aspartyl proteinase (transcript/16200) and a peroxiredoxin (transcript/22849).

3.5. Phylogenetic analysis of *O. ostertagi* CAP domain proteins

The proteomic analysis of *O. ostertagi* ES-thiol demonstrated that there is a large number of CAP domain proteins within this protein fraction. In addition, previous studies have shown that Oo-ASP-1 and Oo-ASP-2 (both CAP domain proteins) are the most abundant proteins within *O. ostertagi* ES-thiol (Geldhof et al., 2003; Meyvis et al., 2007).

Thus, given the importance of CAP domain proteins in the *O. ostertagi* ES-thiol fraction we performed a comprehensive phylogenetic analysis of this protein family.

Within the *O. ostertagi* ES-thiol fraction we identified 91 CAP domain containing proteins which match the InterPro CAP domain (IPR014044) (Fig. 4 and Supplementary Table 4). The majority of *O. ostertagi* CAP domain proteins (75/91, 82.4%) have a predicted N-terminal secretion signal, with 43/91 containing a single CAP domain and 48/91 with a double CAP domain (Supplementary Table 4). Phylogenetic analysis of CAP domain proteins identified in *O. ostertagi* ES-thiol, along with CAP domain proteins derived from whole genomes of *A. caninum*, *H. contortus* and *T. circumcincta* is shown in Fig. 4. Within this phylogeny, previously characterised *O. ostertagi* ASP vaccine candidates [ASP-1 (Vercauteren et al., 2003) and ASP-2 (Geldhof et al., 2003)] and other *O. ostertagi* ASPs [Oo-ASP-3 and Oo-ASP-4 (Visser et al., 2008)] are highlighted. In addition, we have highlighted nine ASPs from *A. caninum*, which have become the canonical set of hookworm ASPs (these include: Acan-NIF (Moyle et al., 1994); Acan-PI (Del Valle et al., 2003); Acan-ASP-1 (Hawdon et al., 1996); Acan-ASP-2 (Hawdon et al., 1999); Acan-ASP-3, Acan-ASP-4, Acan-ASP-5, Acan-ASP-6 from (Zhan et al., 2003) and Acan-ASP-7 (Datu et al., 2008) (Fig. 4).

The phylogenetic reconstruction shows several well supported clades that contain large numbers of homologous CAP domain proteins from the same nematode genus, which indicates recent duplication and diversification (Fig. 4). Within the phylogeny, Oo-ASP-1 and Oo-ASP2 (both single domain CAP proteins) resolve together in a well-supported clade (Fig. 4). The phylogeny demonstrates that Oo-ASP-1 (CAD23183) is closely related to *T. circumcincta* ASP-1 (CBJ15404) plus 3 other identified proteins in *O. ostertagi* ES-thiol, represented by transcripts including: transcript/16135; transcript/14620 and transcript/15497 (Fig. 4). In contrast, in a separate clade, OoASP-2 (CAD56659) is closely related to *T. circumcincta* TELCIR_11365 and TELCIR_18835, plus six related proteins in *O. ostertagi* ES-thiol, represented by transcripts: transcript/15383; transcript/15960; transcript/18805; transcript/14958; transcript/15874 and transcript/15062 (Fig. 4).

3.6. Phylogenetic analysis of *O. ostertagi* astacin-like metallopeptidase

In our proteomic analysis, the second most numerous family of proteins in *O. ostertagi* ES-thiol were the astacin-like metalloproteinases, with hits to peptidase M12A domain (IPR001506, 38 proteins) and astacin-like metallopeptidase domain (IPR034035, 33 proteins) (Fig. 5). Prior to this analysis, three *O. ostertagi* astacin-like metalloproteinases have been identified in *O. ostertagi* ES (including CAD19995; CAD28559 and CAD11605) by immunoscreening with mucosal antibodies from cattle rendered immune to *O. ostertagi* by continuous exposure to the parasite (De Maere et al., 2002). In addition, we have previously shown that an astacin-like proteinase from *T. circumcincta* (Tci-MEP-1, CCR26658) is present in *T. circumcincta* ES material of infective stage parasites, and recognised by both IgA and IgG responses of immune sheep (Nisbet et al., 2019; Smith et al., 2009). In addition Tci-MEP-1 is a component of a recombinant cocktail vaccine that protects sheep against *T. circumcincta* infection (Nisbet et al., 2013). Thus, given that astacin-like metallopeptidases are abundant in the protective native *O. ostertagi* ES-thiol vaccine, and are a protective antigen in *T. circumcincta* vaccine, we investigated this family further in *O. ostertagi*.

Within the *O. ostertagi* ES-thiol fraction there were 38 putative astacin-like metalloproteinases which match the InterPro Peptidase M12A domain (IPR001506) (Fig. 5 and Supplementary Table 2 and 5). Relatively few of the *O. ostertagi* astacin-like metalloproteinases (9/38, 23.7%) had a predicted N-terminal secretion signal, and a single protein had a predicted transmembrane domain (Supplementary Table 2 and 5). The identified astacin-like metalloproteinases had a multi-domain structure that is common within this family of proteinases, with 31

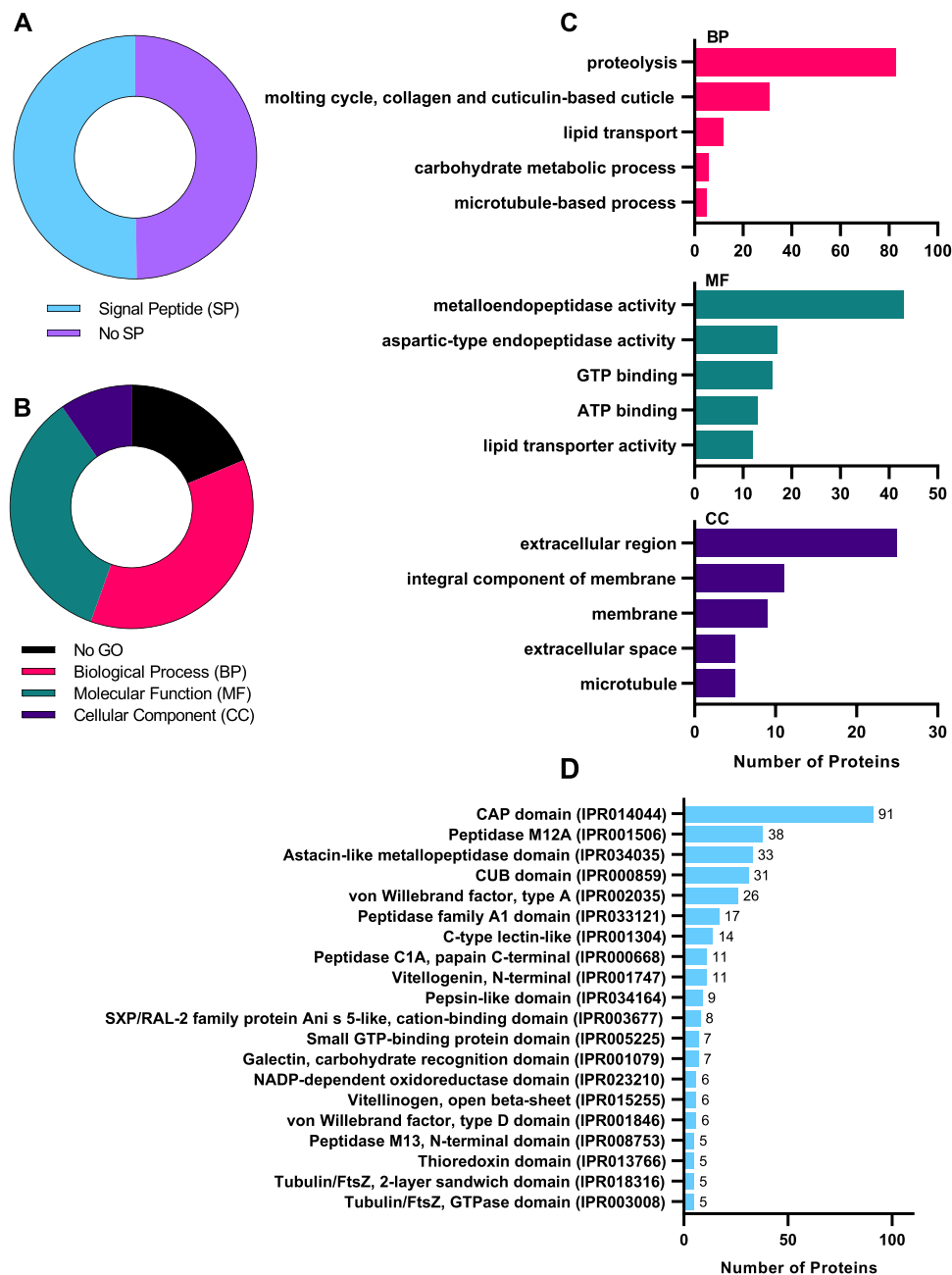


Fig. 3. GO classification and Interpro domain classification of identified *O. ostertagi* ES proteins. (A) Proportion of ES proteins containing a secretion signal (n=490). (B) GO distribution of identified ES proteins. Proteins are classified into three broad GO categories: biological process (red); molecular function (green) and cellular component (purple). (C) Direct GO count showing the top five GO terms for each category. Some proteins are included in more than one category. (D) Interpro domain classification of identified *O. ostertagi* ES proteins showing top twenty categories. Some proteins are included in more than one category.

containing a CUB domain (IPR000859) and a single proteinase with a ShKT domain (IPR003582) (Möhrlén et al., 2003). Phylogenetic analysis of astacin proteinases identified in *O. ostertagi* ES-thiol, along with astacins derived from whole genomes of *H. contortus*, *H. polygyrus* and *T. circumcincta* is shown in Fig. 5. The phylogenetic analysis shows several well supported clades that contain large numbers of homologous astacin-like metalloproteinases from the same nematode genus, which indicates recent duplication and diversification (Fig. 5). Within the phylogeny, the *O. ostertagi* astacins group most closely with those from *T. circumcincta*. Of the previously described *O. ostertagi* astacins, metalloproteinase I (CAD19995) is most closely related to *T. circumcincta* MEP-1 (CAD19995) along with six astacins within the same clade (including those encoded by transcript/9188; transcript/11276; transcript/7232; transcript/11922; transcript/9025 and transcript/11256)

(Fig. 5).

3.7. Other vaccine candidates in *O. ostertagi* ES-thiol

We have previously generated a recombinant subunit vaccine against *T. circumcincta* that protects sheep from *T. circumcincta* infection (Nisbet et al., 2013). As *T. circumcincta* and *O. ostertagi* are both clade V nematodes, and phylogenetically closely related, we searched *O. ostertagi* ES-thiol proteins for orthologues of *T. circumcincta* vaccine antigens (Table 1). The *T. circumcincta* recombinant vaccine contains eight proteins, including: Tci-SAA-1; Tci-MIF-1; Tci-ASP-1; Tci-TGH-2; Tci-CF-1; Tci-ES20; Tci-MEP-1 and Tci-APY-1 (for accession numbers and description of proteins see Table 1). Of these proteins, homologues of Tci-ASP-1, Tci-CF-1, Tci-ES20, Tci-MEP-1 and Tci-APY-1, which meet

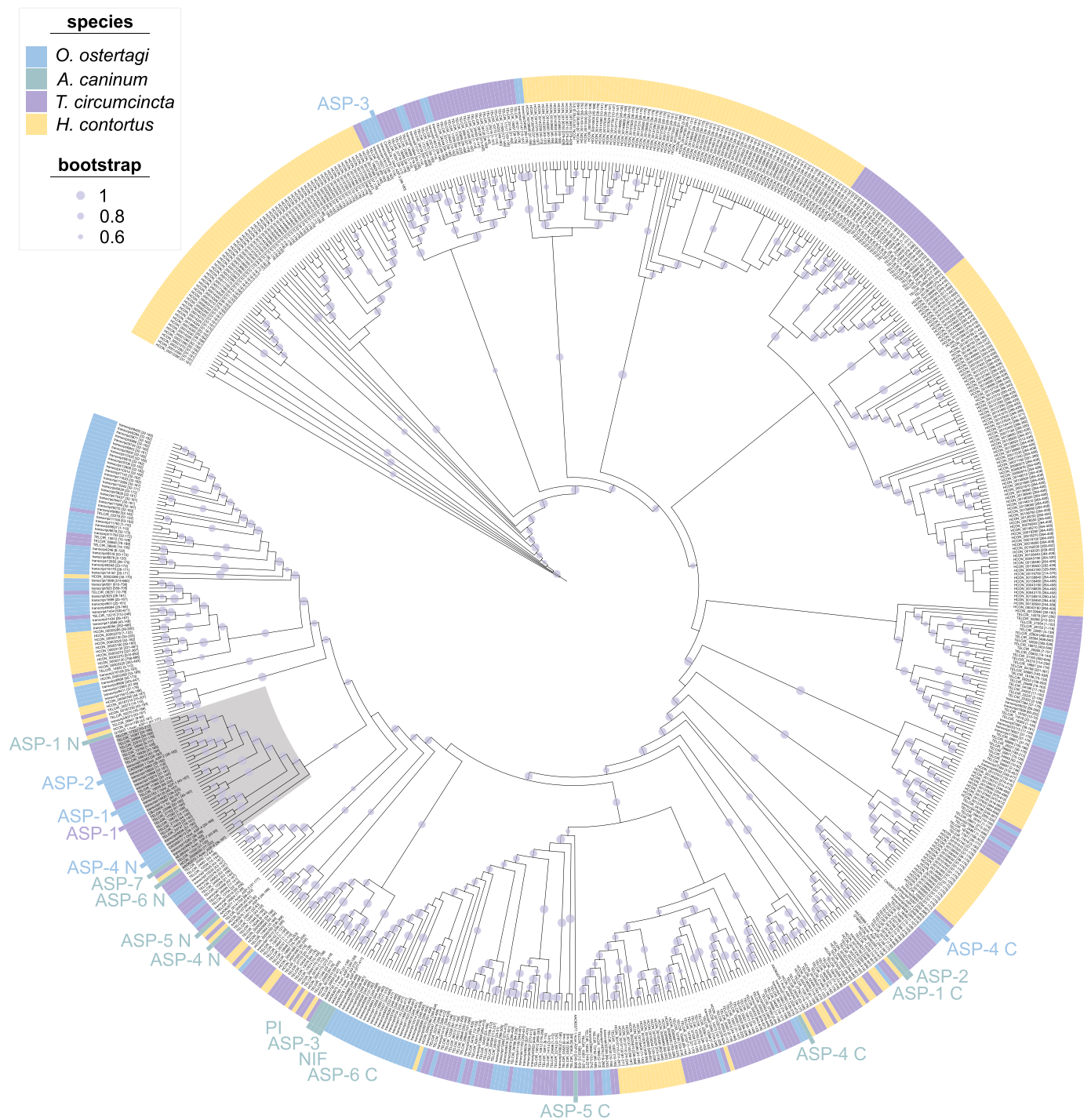


Fig. 4. Maximum likelihood phylogeny of CAP domain proteins identified in Oost ES-thiol and other parasitic nematodes. Due to the structure of CAP domain proteins, which contain either a single or double domain, only the CAP protein domain was used for phylogenetic reconstruction. CAP domain proteins from *O. ostertagi* (blue), *T. circumcincta* (purple), *A. caninum* (green) and *H. contortus* (yellow) were included in the analysis. Accession numbers for each identified CAP protein are shown in the tree and the coordinates of the CAP domain that was used for phylogenetic reconstruction is shown in square brackets. Known ASPs are highlighted and the N- and C- terminal CAP domains from two-domain ASPs are noted as “N” or “C”. A subtree (38 nodes) that contains *O. ostertagi* ASP-1 and *T. circumcincta* ASP-1 is highlighted grey. Confidence values >0.6 are shown on branches of the tree.

the criteria of reciprocal best blast-hits, were detected in *O. ostertagi* ES-thiol proteins (Table 1).

4. Discussion

Our current study confirms that antigens derived from *O. ostertagi* ES-thiol significantly reduce *O. ostertagi* egg output in vaccinated cattle relative to adjuvant only vaccinated control animals. This suggests that

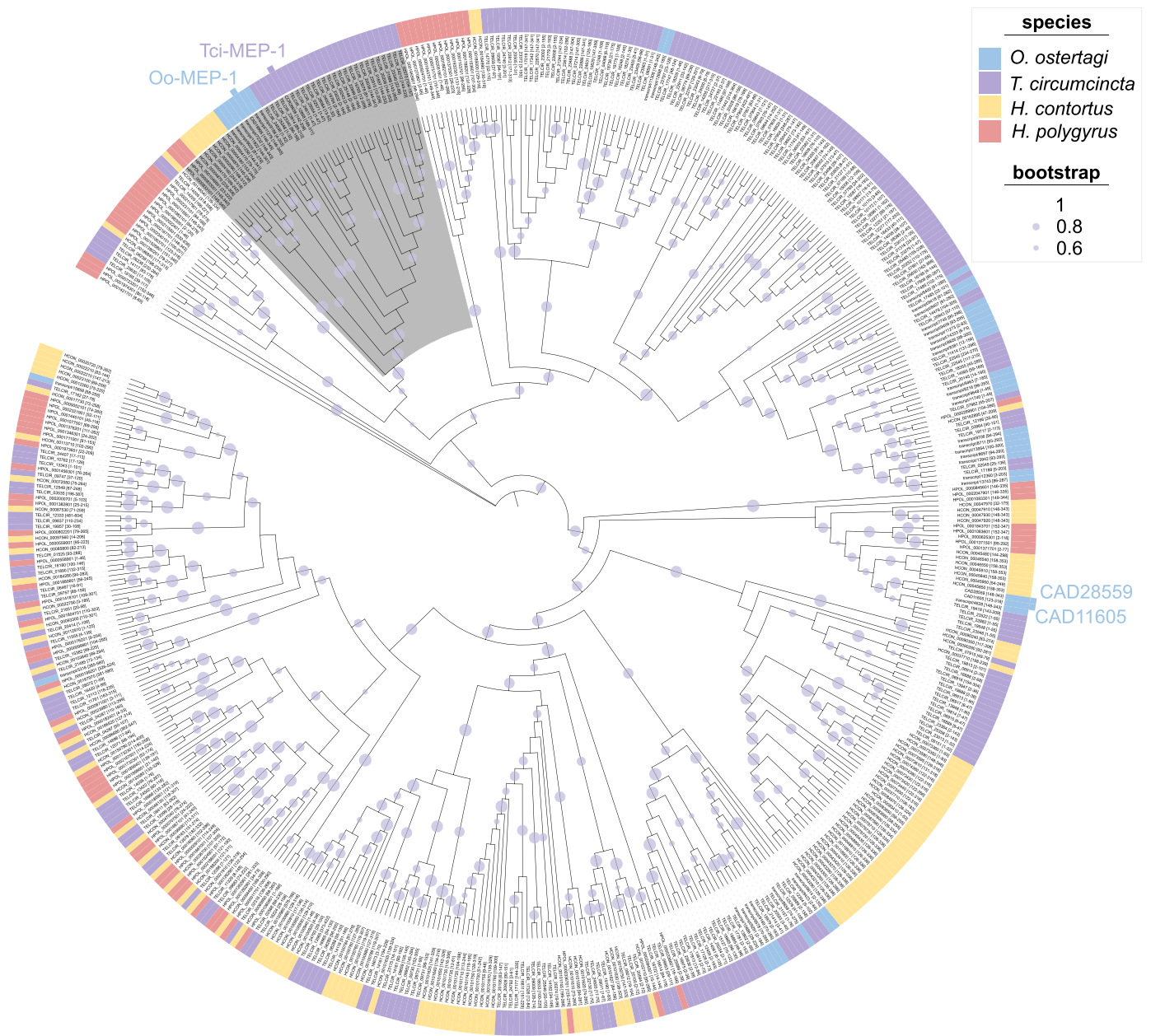


Fig. 5. Maximum likelihood phylogeny of astacin-like metalloproteinases in Oost ES-thiol and other parasitic nematodes. Astacin-like peptidase M12A domain proteins from *O. ostertagi* (blue), *T. circumcincta* (purple), *H. contortus* (yellow) and *H. polygyrus* (pink) were included in the analysis. Accession numbers for each astacin-like metalloproteinase are shown in the tree and the coordinates of the peptidase M12A domain that was used for phylogenetic reconstruction is shown in square brackets. Previously characterised astacin-like proteinases from *O. ostertagi* and *T. circumcincta* are highlighted. A subtree (43 nodes) that contains *O. ostertagi* MEP-1 and *T. circumcincta* MEP-1 is highlighted grey. Confidence values >0.6 are shown on branches of the tree.

parasite proteins released during *in vitro* culture are also released during infection, as immunisation with proteins derived from *in vitro* parasite culture confers protection in terms of reduced egg production. In agreement with previous reports, the main protective effect is a significant reduction in cumulative faecal egg counts, while adult worm burdens between vaccinates and controls were not significantly different in three (Trial 1; Trial 2 and Trial 3) of the four trials. Across the four trials presented here, cumulative parasite egg output was reduced by 47 – 71% in vaccinated cattle relative to the control adjuvant only vaccinated groups. Similar reductions in parasite egg output have been previously reported in three ES-thiol vaccine challenge experiments, with cumulative FEC reduced by 56 – 62% in vaccinated relative to control adjuvant only groups (Geldhof et al., 2004, 2003, 2002; Meyvis et al., 2007). In summary, our four trials, plus the three previously published

ES-thiol vaccine challenge experiments, demonstrate that the effect of ES-thiol vaccination on cattle is reproducible, with vaccinates in all trials having significantly reduced *O. ostertagi* FECs relative to control animals.

The commercial success of vaccines depends not only on robust and reproducible levels of protection, but the ability to produce the vaccine that is economically viable to use. There are currently two commercial vaccines, based on native parasite antigens, which meet these criteria. The first, commercially known as Bovilis® Huskvac (produced by MSD animal health), is a live attenuated vaccine that protects against the cattle lungworm, *Dictyocaulus viviparus* (Jarrett et al., 1960). The second, commercially known as Barbervax®, protects sheep against infection by the barber pole worm, *H. contortus*, and is based upon native parasite gut antigens isolated by lectin affinity chromatography (Nisbet

Table 1Identification of *T. circumcincta* vaccine orthologs in *O. ostertagi* ES-thiol, based on reciprocal blast best hits.

<i>T. circumcincta</i> vaccine antigen	Accession	Interpro Family	Interpro Domain	<i>O. ostertagi</i> orthologue
Tci-SAA-1	CAQ43040	Uncharacterised conserved protein UCP207779 (IPR016859)	none predicted	none
Tci-MIF-1	CBI68362	Macrophage migration inhibitory factor (IPR001398)	none predicted	none
Tci-ASP-1	CBJ15404	Cysteine-rich secretory protein-related (IPR001283)	CAP domain (IPR014044)	transcript/15497
Tci-TGH-2	ACR27078	Transforming growth factor-beta-related (IPR015615)	Transforming growth factor-beta, C-terminal (IPR001839)	none
Tci-CF-1	ABA01328	none predicted	Cathepsin propeptide inhibitor domain (IPR013201); Peptidase C1A, papain C-terminal (IPR00668); Papain-like cysteine endopeptidase (IPR039417)	transcript/17432
Tci-ES20	CCR26659	none predicted	none predicted	transcript/25725
Tci-MEP-1	CCR26658	Metallopeptidase, nematode (IPR017050)	CUB domain (IPR00859); Astacin-like metallopeptidase domain (IPR034035); Peptidase M12A (IPR001506); Peptidase, metallopeptidase (IPR006026)	transcript/11256
Tci-APY-1	CBW38507	Apyrase (IPR009283)	none predicted	transcript/17184

et al., 2016; Smith et al., 1994). Although the *O. ostertagi* ES-thiol vaccine is able to significantly reduce *O. ostertagi* fecundity, its costly and time-consuming production negates its use as a commercial product. The high cost associated with this vaccine result from complexities of its production, which includes: isolation of adult parasites from infected cattle; *in vitro* culture of the parasite to generate ESPs and recovery of low amounts of protective ES-thiol antigens from ESPs. For these reasons, commercial exploitation of this vaccine is not feasible.

The aim of this study was to characterise the proteome of *O. ostertagi* ES-thiol using high-resolution shotgun proteomics to inform future vaccine antigen discovery projects, with the capacity to complement the current best, ASP-based, vaccine candidates (Geldhof et al., 2003, 2002; Meyvis et al., 2007). Previous proteomic analysis of the ES-thiol, and sub-fractions of the ES-thiol, have demonstrated that the single domain ASPs Oo-ASP-1 and Oo-ASP-2 are the dominant proteins in these preparations (Geldhof et al., 2003; Meyvis et al., 2007). However, in addition to Oo-ASP-1 and Oo-ASP-2, it is likely there are other protective antigens within this protein preparation. This was demonstrated by an *O. ostertagi* vaccine trial using three sub-fractions of ES-thiol, which included: an ASP-enriched fraction; a cysteine proteinase (CP) enriched fraction and a “remaining proteins” fraction (Meyvis et al., 2007). Following *O. ostertagi* challenge, all three fractions were protective and resulted in significantly reduced cumulative FEC relative to control animals (Meyvis et al., 2007). Importantly, it was shown that antibodies from animals vaccinated with the CP enriched fraction and the “remaining proteins” fraction did not bind antigens from the ASP enriched fraction. Thus, demonstrating that in addition to the ASPs, there are other protective antigens within ES-thiol (Meyvis et al., 2007).

To conduct a comprehensive proteomic assessment of *O. ostertagi* ES-thiol, we first needed a protein sequence database, against which proteomic data could be searched. As there is currently no available *O. ostertagi* genome, we used isoform sequencing (Iso-Seq) to generate a library of full-length transcripts from male and female *O. ostertagi* parasites. Using this approach, we generated 15,521 male and 13,387 female high quality unique full-length transcripts, significantly increasing the number of *O. ostertagi* sequences available in the NCBI database. With the database in place, we used high-resolution shotgun proteomics to identify 490 proteins within *O. ostertagi* ES-thiol. Our analysis significantly extends previous proteomic characterisation of *O. ostertagi* ES-thiol (Geldhof et al., 2003), and demonstrates that the fraction is relatively complex. The number of identified ES-thiol proteins is likely an underestimate of the actual total, as the database against which the protein spectra were searched against is partial (with BUSCO score of 36.9% and 44% for males and females, respectively). Work is underway to complete the *O. ostertagi* genome through the Darwin Tree of Life Project (Darwin Tree of Life Project Consortium, 2022), and when the genome becomes available, this resource will facilitate further antigen

discovery in the *O. ostertagi* ES-thiol protein preparation. In addition, a high-quality genome will allow us to determine which proteins are derived from different gene models, or which result from alternative splicing or heterozygosity within the population. In terms of vaccine development, this will allow a better understanding of population level variation of important *O. ostertagi* vaccine antigens.

Importantly, from the proteins that we identified, previously described protective antigens Oo-ASP-1 and Oo-ASP-2 (Geldhof et al., 2003; Meyvis et al., 2007) as well as cysteine proteinases (Geldhof et al., 2002) were present. Of the identified proteins approx. 50% contained a signal peptide, indicating these are likely secreted via the classical secretory pathway. The remaining proteins lack a signal peptide, but were secreted or excreted nonetheless. It is possible that these proteins are packaged for secretion via alternative pathways. Such pathways include direct transfer across the plasma membrane and packaging of protein cargo within plasma membrane derived extracellular vesicles (EVs) (Cohen et al., 2020). It has been shown that many secreted parasite proteins, especially those contained within EVs, lack a classical N-terminal secretion signal (Silverman et al., 2008). The cellular mechanism of protein secretion in parasitic nematodes, and biological significance of partitioning proteins across different secretory pathways awaits further investigation.

In terms of protein abundance within *O. ostertagi* ES-thiol, it has been shown previously that Oo-ASP-1 and Oo-ASP-2 are the dominant CAP proteins (Geldhof et al., 2003). Our analysis shows that in addition to Oo-ASP-1 and Oo-ASP-2 there is a large diversity of CAP domain proteins within this protein preparation (Supplementary Table 2 and 4 and Fig. 4). In agreement with previous reports, our protein abundance ranking, based on protein emPAI score, confirms that ASP-1 (ranked 19th), ASP-2 (ranked 7th) and ASP-3 (ranked 23rd) are the most abundant ASPs within the *O. ostertagi* ES-thiol fraction. The contribution of each of these ASPs, plus that additional lower ranked ASPs, towards vaccine efficacy remains to be determined. It is known that CAP-domain proteins are ubiquitously present in ESPs of parasitic nematodes, and often upregulated in parasitic phases of their lifecycle (Heizer et al., 2013; Hewitson et al., 2011; International Helminth Genomes Consortium, 2019; Viney, 2017). Expression profiles and the secreted nature of *O. ostertagi* CAP domain proteins point to a role in host-parasite interactions, yet specific roles for most CAP domain proteins expressed by helminths remain unknown, with few exceptions. The hookworm CAP domain protein Na-ASP-2 from *Necator americanus*, has been shown to have immunomodulatory function, and is able to suppress B cell receptor signalling and reduce neutrophil recruitment and therefore plays an important role in host-parasite interactions (Bower et al., 2008; Tribolet et al., 2015). Given the abundance of CAP domain proteins in *O. ostertagi* ES-thiol, and that they are expressed and secreted in parasitic life stages of the parasite, it is likely that they also play an important role in

host-parasite interactions.

Proteases and proteinase inhibitors are also numerous in the secretomes of parasitic nematodes (Hewitson et al., 2011; International Helminth Genomes Consortium, 2019; Martín-Galiano and Sotillo, 2022; Viney, 2017) and this is also true of *O. ostertagi* ES-thiol. Our proteomic analysis reveals an abundance of proteinases in this fraction, representing three distinct classes, including: metallo-; aspartyl- and cysteine proteinases (Fig. 3, panel D). In parasitic nematodes it is thought that secreted proteinases and proteinase inhibitors perform diverse tasks linked to parasitism, including immunomodulation, host tissue penetration and modification of the environment and digestion of blood (McKerrow et al., 2006). Within *O. ostertagi* ES-thiol, M12 astacin-like metalloproteinases are the most numerous family of proteinases, with 38 proteins containing a peptidase M12A domain (IPR001506) and 33 proteins with an astacin-like metalloproteinase domain (IPR034035) (Supplementary Table 2 and 5 and Fig. 5). M12 astacins are one of the most abundant proteins in the secretome of parasitic nematodes (Martín-Galiano and Sotillo, 2022). This family is extensively expanded in nematode clades IVa and V, where they have been suggested to play a role in tissue migration and immune modulation (International Helminth Genomes Consortium, 2019). Given the abundance of astacins in *O. ostertagi* ES thiol, and the importance of these proteinases in other parasitic nematodes, this family is likely to play a key role in host-parasite interactions. Again, as with the other protein families described here, the physiological role of secreted proteinases, as well as the role they play in generation of protective immunity in the *O. ostertagi* ES-thiol vaccine, remains to be determined.

The mechanism of immune protection in the ES-thiol QuilA vaccine group is currently unknown. However, previous studies have shown that protection is likely antibody mediated, as high levels of ES-thiol antigen-specific mucosal IgG1, IgG2 and IgA antibodies are associated with protection (Geldhof et al., 2004, 2002; González-Hernández et al., 2016; Meyvis et al., 2007). As we have demonstrated, *O. ostertagi* ES-thiol is a complex mix of proteins, with 490 proteins identified, and further work is needed to identify which antigens are associated with protection. Currently the most promising vaccine antigen from this complex mix is Oo-ASP-1. ASPs are known vaccine candidates in other parasitic nematodes and have been shown to protect against parasite infection (Ghosh and Hotez, 1999; Sen et al., 2000). In *O. ostertagi* ES thiol, previous studies have shown that Oo-ASP-1 is the dominant antigen, and recognised by sera from immune animals (Geldhof et al., 2003, 2002). Furthermore, native Oo-ASP-1 purified from ES-thiol is protective against homologous infection, reducing parasite cumulative FEC by 74% and 59% relative to control adjuvant only vaccines in two separate trials (González-Hernández et al., 2016; Meyvis et al., 2007). Protection is also observed when animals are immunised with recombinant Oo-ASP-1 that has been engineered to display native surface glycans, with parasite fecundity reduced by approx. 39% relative to control vaccinated animals (Zwanenburg et al., 2023). Although the immune mechanism is unknown, in both ES-thiol and native Oo-ASP-1 vaccines, it is tempting to speculate that mucosal antibodies against Oo-ASP-1 interfere with reproduction and egg production. This is a plausible hypothesis, as it is known that *O. ostertagi*, ASP-1 is expressed in adult parasites and Oo-ASP-1 localises to the reproductive tracts in both sexes (Visser et al., 2008). Thus, it is tempting to speculate that mucosal antibodies localise to these tissues *in vivo* and interfere with reproduction, resulting in decreased parasite fecundity.

A commercially viable approach to produce anti-parasite vaccines is through recombinant expression of critical parasite antigens that are important for host-parasite interactions. An example of this includes the vaccine against the tapeworm *Taenia solium*, which is based on a single recombinant parasite antigen, designated TSOL18 (Flisser et al., 2004). This vaccine, commercially sold as Cysvax™, is highly efficacious, and provides 100% protection against the disease (Flisser et al., 2004; Jayashi et al., 2012). Recombinant vaccines also have potential to protect against parasitic nematodes. We have previously shown that a

recombinant subunit vaccine based on worm ES proteins protects sheep against *T. circumcincta* infection. The vaccine contains recombinant versions of eight proteins and protects sheep against *T. circumcincta* infection, reducing both egg count (58–70%) and adult worm burden (56% - 75%) in vaccinated lambs (Nisbet et al., 2013). Of the eight antigens included in this recombinant vaccine, orthologues of five are found in *O. ostertagi*-ES-thiol (Table 1). It remains to be determined if these *O. ostertagi* orthologues are recognised by the host immune response and if they contribute towards ES-thiol vaccine efficacy.

In summary, our work demonstrates that the *O. ostertagi* ES-thiol vaccine consistently reduces *O. ostertagi* fecundity in vaccinated animals across four independent vaccine trials. Using high-resolution shotgun proteomics, we demonstrate that *O. ostertagi* ES-thiol is relatively complex, with 490 identified proteins, belonging to diverse protein families. Analysis of the *O. ostertagi* ES-thiol proteome reveals that CAP domain proteins and astacin-like metalloproteinases, which are highly abundant in the secretome of parasitic nematodes, are also highly abundant in the *O. ostertagi* ES-thiol fraction. Within the ES-thiol fraction, CAP-domain proteins dominate, and include previously identified protective antigens Oo-ASP-1 and Oo-ASP-2. Our proteomic analysis of ES-thiol will facilitate future *O. ostertagi* vaccine antigen discovery projects to complement existing *O. ostertagi* vaccine antigens.

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CRediT authorship contribution statement

Philip Steele: Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **David Frew:** Investigation. **Kevin McLean:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Dorota Androskuc:** Methodology, Investigation, Formal analysis, Data curation. **Peter Geldhof:** Writing – review & editing, Methodology. **Jimmy Borloo:** Methodology. **Javier Palarea Albaladejo:** Writing – review & editing, Formal analysis, Data curation. **Alasdair J. Nisbet:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Tom N. McNeilly:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Daniel R.G. Price:** Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

PS and TMN conceived the study. All authors designed the research. DRGP, PS, TNM, KML, DA performed research. PG and JB trained PS in *O. ostertagi* ES-thiol purification. DRGP, PS, KML, DA, JPA, AJN, TMN analysed data. DRGP wrote the paper with contributions from all

authors. All authors read and approved the final manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2024.110154.

References

- Bartley, D.J., Jewell, N.J., Andrews, L.M., Mitchell, S., Morrison, A.A., 2021. Molecular and phenotypic characterisation of fenbendazole resistance in a field-derived isolate of *Ostertagia ostertagi*. *Vet. Parasitol.* 289, 109319 <https://doi.org/10.1016/j.vetpar.2020.109319>.
- Borloo, J., Geldhof, P., Peelaers, I., Van Meulder, F., Ameloot, P., Callewaert, N., Vercruyse, J., Claerebout, E., Strelkov, S.V., Weeks, S.D., 2013. Structure of *Ostertagia ostertagi* ASP-1: insights into disulfide-mediated cyclization and dimerization. *Acta Crystallogr. D. Biol. Crystallogr.* 69, 493–503. <https://doi.org/10.1107/S0907444912050019>.
- Bower, M.A., Constant, S.L., Mendez, S., 2008. *Necator americanus*: the Na-ASP-2 protein secreted by the infective larvae induces neutrophil recruitment in vivo and in vitro. *Exp. Parasitol.* 118, 569–575. <https://doi.org/10.1016/j.exppara.2007.11.014>.
- Bürger, H.J., Pfeiffer, A., 1969. [Experimental vaccination of calves with irradiated larvae of *Ostertagia ostertagi* and *Cooperia oncophora*]. *Zent. Vet. B* 16, 357–367.
- Christie, M., Jackson, F., 1982. Specific identification of strongyle eggs in small samples of sheep faeces. *Res. Vet. Sci.* 32, 113–117.
- Cohen, M.J., Chirico, W.J., Lipke, P.N., 2020. Through the back door: unconventional protein secretion. *Cell Surf.* 6, 100045 <https://doi.org/10.1016/j.tscw.2020.100045>.
- Cotter, J.L., Van Burgel, A., Besier, R.B., 2015. Anthelmintic resistance in nematodes of beef cattle in south-west Western Australia. *Vet. Parasitol.* 207, 276–284. <https://doi.org/10.1016/j.vetpar.2014.11.019>.
- Darwin Tree of Life Project Consortium, 2022. Sequence locally, think globally: the darwin tree of life project. *Proc. Natl. Acad. Sci. USA* 119, e2115642118. <https://doi.org/10.1073/pnas.2115642118>.
- Datu, B.J.D., Gasser, R.B., Nagaraj, S.H., Ong, E.K., O'Donoghue, P., McInnes, R., Ranganathan, S., Loukas, A., 2008. Transcriptional changes in the hookworm, *Ancylostoma caninum*, during the transition from a free-living to a parasitic larva. *PLoS Negl. Trop. Dis.* 2, e130 <https://doi.org/10.1371/journal.pntd.0000130>.
- De Maere, V., Vercauteren, I., Saverwyns, H., Claerebout, E., Berx, G., Vercruyse, J., 2002. Identification of potential protective antigens of *Ostertagia ostertagi* with local antibody probes. *Parasitology* 125, 383–391. <https://doi.org/10.1017/S003118200202196>.
- Del Valle, A., Jones, B.F., Harrison, L.M., Chadderton, R.C., Cappello, M., 2003. Isolation and molecular cloning of a secreted hookworm platelet inhibitor from adult *Ancylostoma caninum*. *Mol. Biochem. Parasitol.* 129, 167–177. [https://doi.org/10.1016/S0166-6851\(03\)00121-X](https://doi.org/10.1016/S0166-6851(03)00121-X).
- Doyle, S.R., Tracey, A., Laing, R., Holroyd, N., Bartley, D., Bazant, W., Beasley, H., Beech, R., Britton, C., Brooks, K., Chaudhry, U., Maitland, K., Martinelli, A., Noonan, J.D., Paulini, M., Quail, M.A., Redman, E., Rodgers, F.H., Sallé, G., Shabbir, M.Z., Sankaranarayanan, G., Wit, J., Howe, K.L., Sargison, N., Devaney, E., Berriman, M., Gilleard, J.S., Cotton, J.A., 2020. Genomic and transcriptomic variation defines the chromosome-scale assembly of *Haemonchus contortus*, a model gastrointestinal worm. *Commun. Biol.* 3, 656. <https://doi.org/10.1038/s42003-020-01377-3>.
- Edgar, R.C., 2021. MUSCLE v5 enables improved estimates of phylogenetic tree confidence by ensemble bootstrapping. *bioRxiv* 2021.06.20.449169. <https://doi.org/10.1101/2021.06.20.449169>.
- Edmonds, M.D., Johnson, E.G., Edmonds, J.D., 2010. Anthelmintic resistance of *Ostertagia ostertagi* and *Cooperia oncophora* to macrocyclic lactones in cattle from the western United States. *Vet. Parasitol.* 170, 224–229. <https://doi.org/10.1016/j.vetpar.2010.02.036>.
- Flisser, A., Gauci, C.G., Zoli, A., Martínez-Ocaña, J., Garza-Rodríguez, A., Domínguez-Alpizar, J.L., Maravilla, P., Rodríguez-Cañul, R., Avila, G., Aguilar-Vega, L., Kyngdon, C., Geerts, S., Lightowlers, M.W., 2004. Induction of protection against porcine cysticercosis by vaccination with recombinant oncosphere antigens. *Infect. Immun.* 72, 5292–5297. <https://doi.org/10.1128/IAI.72.9.5292-5297.2004>.
- Fu, L., Niu, B., Zhu, Z., Wu, S., Li, W., 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28, 3150–3152. <https://doi.org/10.1093/bioinformatics/bts565>.
- Geldhof, P., Claerebout, E., Knox, D., Vercauteren, I., Loosova, A., Vercruyse, J., 2002. Vaccination of calves against *Ostertagia ostertagi* with cysteine proteinase enriched protein fractions. *Parasite Immunol.* 24, 263–270. <https://doi.org/10.1046/j.1365-3024.2002.00461.x>.
- Geldhof, P., Meyvis, Y., Vercruyse, J., Claerebout, E., 2008. Vaccine testing of a recombinant activation-associated secreted protein (ASP1) from *Ostertagia ostertagi*. *Parasite Immunol.* 30, 57–60. <https://doi.org/10.1111/j.1365-3024.2007.01001.x>.
- Geldhof, P., Vercauteren, I., Gevaert, K., Staes, A., Knox, D.P., Vandekerckhove, J., Vercruyse, J., Claerebout, E., 2003. Activation-associated secreted proteins are the most abundant antigens in a host protective fraction from *Ostertagia ostertagi*. *Mol. Biochem. Parasitol.* 128, 111–114. [https://doi.org/10.1016/S0166-6851\(03\)00044-6](https://doi.org/10.1016/S0166-6851(03)00044-6).
- Geldhof, P., Vercauteren, I., Vercruyse, J., Knox, D.P., Van Den Broeck, W., Claerebout, E., 2004. Validation of the protective *Ostertagia ostertagi* ES-thiol antigens with different adjuvantia. *Parasite Immunol.* 26, 37–43. <https://doi.org/10.1111/j.0141-9838.2004.00681.x>.
- Ghosh, K., Hawdon, J., Hotez, P., 1996. Vaccination with alum-precipitated recombinant *Ancylostoma*-secreted protein 1 protects mice against challenge infections with infective hookworm (*Ancylostoma caninum*) larvae. *J. Infect. Dis.* 174, 1380–1383. <https://doi.org/10.1093/infdis/174.6.1380>.
- Ghosh, K., Hotez, P.J., 1999. Antibody-dependent reductions in mouse hookworm burden after vaccination with *Ancylostoma caninum* secreted protein 1. *J. Infect. Dis.* 180, 1674–1681. <https://doi.org/10.1086/315059>.
- González-Hernández, A., Van Coppennolle, S., Borloo, J., Van Meulder, F., Paerewijck, O., Peelaers, I., Leclercq, G., Claerebout, E., Geldhof, P., 2016. Host protective ASP-based vaccine against the parasitic nematode *Ostertagia ostertagi* triggers NK cell activation and mixed IgG1-IgG2 response. *Sci. Rep.* 6, 29496 <https://doi.org/10.1038/srep29496>.
- Götz, S., García-Gómez, J.M., Terol, J., Williams, T.D., Nagaraj, S.H., Nueda, M.J., Robles, M., Talón, M., Dopazo, J., Conesa, A., 2008. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.* 36, 3420–3435. <https://doi.org/10.1093/nar/gkn176>.
- Goud, G.N., Zhan, B., Ghosh, K., Loukas, A., Hawdon, J., Dobardzic, A., Deumic, V., Liu, S., Dobardzic, R., Zook, B.C., Jin, Q., Liu, Y., Hoffman, L., Chung-Debose, S., Patel, R., Mendez, S., Hotez, P.J., 2004. Cloning, yeast expression, isolation, and vaccine testing of recombinant *Ancylostoma*-secreted protein (ASP)-1 and ASP-2 from *Ancylostoma ceylanicum*. *J. Infect. Dis.* 189, 919–929. <https://doi.org/10.1086/381901>.
- Halliday, A.M., Routledge, C.M., Smith, S.K., Matthews, J.B., Smith, W.D., 2007. Parasite loss and inhibited development of *Teladorsagia circumcincta* in relation to the kinetics of the local IgA response in sheep. *Parasite Immunol.* 29, 425–434. <https://doi.org/10.1111/j.1365-3024.2007.00959.x>.
- Hawdon, J.M., Jones, B.F., Hoffman, D.R., Hotez, P.J., 1996. Cloning and characterization of *Ancylostoma*-secreted protein. A novel protein associated with the transition to parasitism by infective hookworm larvae. *J. Biol. Chem.* 271, 6672–6678. <https://doi.org/10.1074/jbc.271.12.6672>.
- Hawdon, J.M., Narasimhan, S., Hotez, P.J., 1999. *Ancylostoma* secreted protein 2: cloning and characterization of a second member of a family of nematode secreted proteins from *Ancylostoma caninum*. *Mol. Biochem. Parasitol.* 99, 149–165. [https://doi.org/10.1016/S0166-6851\(99\)00011-0](https://doi.org/10.1016/S0166-6851(99)00011-0).
- Heizer, E., Zarlenga, D.S., Rosa, B., Gao, X., Gasser, R.B., De Graef, J., Geldhof, P., Mitreva, M., 2013. Transcriptome analyses reveal protein and domain families that delineate stage-related development in the economically important parasitic nematodes, *Ostertagia ostertagi* and *Cooperia oncophora*. *BMC Genom.* 14, 118. <https://doi.org/10.1186/1471-2164-14-118>.
- Herlich, H., Douvres, F.W., 1979. Gastrointestinal nematode immunization trials in cattle. *Am. J. Vet. Res.* 40, 1781–1782.
- Hewitson, J.P., Harcus, Y., Murray, J., van Agtmaal, M., Filbey, K.J., Grainger, J.R., Bridgett, S., Blaxter, M.L., Ashton, P.D., Ashford, D.A., Curwen, R.S., Wilson, R.A., Dowle, A.A., Maizels, R.M., 2011. Proteomic analysis of secretory products from the model gastrointestinal nematode *Heligmosomoides polygyrus* reveals dominance of venom allergen-like (VAL) proteins. *J. Proteom.* 74, 1573–1594. <https://doi.org/10.1016/j.jprotp.2011.06.002>.
- Hilderson, H., Berghen, P., De Graaf, D.C., Claerebout, E., Vercruyse, J., 1995. Immunisation of calves with *Ostertagia ostertagi* fourth stage larval antigens failed to protect calves from infection. *Int. J. Parasitol.* 25, 757–760. [https://doi.org/10.1016/0020-7519\(94\)00196-u](https://doi.org/10.1016/0020-7519(94)00196-u).
- International Helminth Genomes Consortium, 2019. Comparative genomics of the major parasitic worms. *Nat. Genet.* 51, 163–174. <https://doi.org/10.1038/s41588-018-0262-1>.
- Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., Mann, M., 2005. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol. Cell Proteom.* 4, 1265–1272. <https://doi.org/10.1074/mcp.M500061-MCP200>.
- Jackson, E., Jackson, F., Smith, W.D., 1984. Comparison of saline incubation and pepsin digestion as methods for recovering *Ostertagia circumcincta* larvae from the abomasum of sheep. *Res. Vet. Sci.* 36, 380–381.
- Jarrett, W.F., Jennings, F.W., McINTYRE, W.I., Mulligan, W., Urquhart, G.M., 1960. Immunological studies on *Dictyocaulus viviparus* infection; immunity produced by the administration of irradiated larvae. *Immunology* 3, 145–151.
- Jayashi, C.M., Kyngdon, C.T., Gauci, C.G., Gonzalez, A.E., Lightowlers, M.W., 2012. Successful immunization of naturally reared pigs against porcine cysticercosis with a recombinant oncosphere antigen vaccine. *Vet. Parasitol.* 188, 261–267. <https://doi.org/10.1016/j.vetpar.2012.03.055>.
- Letunic, I., Bork, P., 2021. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* 49, W293–W296. <https://doi.org/10.1093/nar/gkab301>.
- Martin-Galiano, A.J., Sotillo, J., 2022. Insights into the functional expansion of the astacin peptidase family in parasitic helminths. *Int. J. Parasitol.* 52, 243–251. <https://doi.org/10.1016/j.ijpara.2021.09.001>.
- McKerrow, J.H., Caffrey, C., Kelly, B., Loke, P., Sajid, M., 2006. Proteases in parasitic diseases. *Annu. Rev. Pathol.* 1, 497–536. <https://doi.org/10.1146/annurev.pathol.1.110304.100151>.
- Mendez, S., Zhan, B., Goud, G., Ghosh, K., Dobardzic, A., Wu, W., Liu, S., Deumic, V., Dobardzic, R., Liu, Y., Bethony, J., Hotez, P.J., 2005. Effect of combining the larval antigens *Ancylostoma* secreted protein 2 (ASP-2) and metalloprotease 1 (MTP-1) in protecting hamsters against hookworm infection and disease caused by *Ancylostoma ceylanicum*. *Vaccine* 23, 3123–3130. <https://doi.org/10.1016/j.vaccine.2004.12.022>.

- Meyvis, Y., Geldhof, P., Gevaert, K., Timmerman, E., Vercruyse, J., Claerebout, E., 2007. Vaccination against *Ostertagia ostertagi* with subfractions of the protective ES-thiol fraction. *Vet. Parasitol.* 149, 239–245. <https://doi.org/10.1016/j.vetpar.2007.08.014>.
- Möhrlen, F., Hutter, H., Zwilling, R., 2003. The astacin protein family in *Caenorhabditis elegans*. *Eur. J. Biochem* 270, 4909–4920. <https://doi.org/10.1046/j.1432-1033.2003.03891.x>.
- Moyle, M., Foster, D.L., McGrath, D.E., Brown, S.M., Laroche, Y., De Meutter, J., Stanssens, P., Bogowitz, C.A., Fried, V.A., Ely, J.A., 1994. A hookworm glycoprotein that inhibits neutrophil function is a ligand of the integrin CD11b/CD18. *J. Biol. Chem.* 269, 10008–10015.
- Nisbet, A.J., McNeilly, T.N., Price, D.R.G., Oliver, E.M., Bartley, Y., Mitchell, M., Palarea-Albaladejo, J., Matthews, J.B., 2019. The rational simplification of a recombinant cocktail vaccine to control the parasitic nematode *Teladorsagia circumcincta*. *Int. J. Parasitol.* 49, 257–265. <https://doi.org/10.1016/j.ijpara.2018.10.006>.
- Nisbet, A.J., McNeilly, T.N., Wildblood, L.A., Morrison, A.A., Bartley, D.J., Bartley, Y., Longhi, C., McKendrick, L.J., Palarea-Albaladejo, J., Matthews, J.B., 2013. Successful immunization against a parasitic nematode by vaccination with recombinant proteins. *Vaccine* 31, 4017–4023. <https://doi.org/10.1016/j.vaccine.2013.05.026>.
- Nisbet, A.J., Meeusen, E.N., González, J.F., Piedrafitra, D.M., 2016. Immunity to *haemonchus contortus* and vaccine development. *Adv. Parasitol.* 93, 353–396. <https://doi.org/10.1016/bs.apar.2016.02.011>.
- Nisbet, A.J., Smith, S.K., Armstrong, S., Meikle, L.I., Wildblood, L.A., Beynon, R.J., Matthews, J.B., 2010. *Teladorsagia circumcincta*: activation-associated secreted proteins in excretory/secretory products of fourth stage larvae are targets of early IgA responses in infected sheep. *Exp. Parasitol.* 125, 329–337. <https://doi.org/10.1016/j.exppara.2010.02.014>.
- Price, M.N., Dehal, P.S., Arkin, A.P., 2010. FastTree 2 – Approximately maximum-likelihood trees for large alignments. *PLoS One* 5, e9490. <https://doi.org/10.1371/journal.pone.0009490>.
- Sen, L., Ghosh, K., Bin, Z., Qiang, S., Thompson, M.G., Hawdon, J.M., Koski, R.A., Shuhua, X., Hotez, P.J., 2000. Hookworm burden reductions in BALB/c mice vaccinated with recombinant *Ancylostoma* secreted proteins (ASPs) from *Ancylostoma duodenale*, *Ancylostoma caninum* and *Necator americanus*. *Vaccine* 18, 1096–1102. [https://doi.org/10.1016/s0264-410x\(99\)00371-0](https://doi.org/10.1016/s0264-410x(99)00371-0).
- Silverman, J.M., Chan, S.K., Robinson, D.P., Dwyer, D.M., Nandan, D., Foster, L.J., Reiner, N.E., 2008. Proteomic analysis of the secretome of *Leishmania donovani*. *Genome Biol.* 9, R35. <https://doi.org/10.1186/gb-2008-9-2-r35>.
- Simão, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., Zdobnov, E.M., 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31, 3210–3212. <https://doi.org/10.1093/bioinformatics/btv351>.
- Smith, S.K., Nisbet, A.J., Meikle, L.I., Inglis, N.F., Sales, J., Beynon, R.J., Matthews, J.B., 2009. Proteomic analysis of excretory/secretory products released by *Teladorsagia circumcincta* larvae early post-infection. *Parasite Immunol.* 31, 10–19. <https://doi.org/10.1111/j.1365-3024.2008.01067.x>.
- Smith, W.D., Smith, S.K., Murray, J.M., 1994. Protection studies with integral membrane fractions of *Haemonchus contortus*. *Parasite Immunol.* 16, 231–241. <https://doi.org/10.1111/j.1365-3024.1994.tb00345.x>.
- Steenwyk, J.L., Buida, T.J., Li, Y., Shen, X.-X., Rokas, A., 2020. ClipKIT: a multiple sequence alignment trimming software for accurate phylogenomic inference. *PLoS Biol.* 18, e3001007. <https://doi.org/10.1371/journal.pbio.3001007>.
- Taylor, S.M., Kenny, J., Edgar, H.W., Ellison, S., Ferguson, L., 1997. Efficacy of moxidectin, ivermectin and albendazole oral drenches for suppression of periparturient rise in ewe worm egg output and reduction of anthelmintic treatment for lambs. *Vet. Rec.* 141, 357–360. <https://doi.org/10.1136/vr.141.14.357>.
- Tribolet, L., Cantacessi, C., Pickering, D.A., Navarro, S., Doolan, D.L., Trieu, A., Fei, H., Chao, Y., Hofmann, A., Gasser, R.B., Giacomini, P.R., Loukas, A., 2015. Probing of a human proteome microarray with a recombinant pathogen protein reveals a novel mechanism by which hookworms suppress B-cell receptor signaling. *J. Infect. Dis.* 211, 416–425. <https://doi.org/10.1093/infdis/jiu451>.
- Tsirigos, K.D., Peters, C., Shu, N., Käll, L., Elofsson, A., 2015. The TOPCONS web server for consensus prediction of membrane protein topology and signal peptides. *Nucleic Acids Res.* 43, W401–W407. <https://doi.org/10.1093/nar/gkv485>.
- Vercruyeren, I., Geldhof, P., Peelaers, I., Claerebout, E., Berx, G., Vercruyse, J., 2003. Identification of excretory-secretory products of larval and adult *Ostertagia ostertagi* by immunoscreening of cDNA libraries. *Mol. Biochem Parasitol.* 126, 201–208. [https://doi.org/10.1016/s0166-6851\(02\)00274-8](https://doi.org/10.1016/s0166-6851(02)00274-8).
- Viney, M., 2017. The genomic basis of nematode parasitism. *Brief. Funct. Genom.* 17, 8–14. <https://doi.org/10.1093/bfpg/elx010>.
- Visser, A., Van Zeveren, A.M., Meyvis, Y., Peelaers, I., Van den Broeck, W., Gevaert, K., Vercruyse, J., Claerebout, E., Geldhof, P., 2008. Gender-enriched transcription of activation associated secreted proteins in *Ostertagia ostertagi*. *Int. J. Parasitol.* 38, 455–465. <https://doi.org/10.1016/j.ijpara.2007.08.008>.
- Zhan, B., Liu, Y., Badamchian, M., Williamson, A., Feng, J., Loukas, A., Hawdon, J.M., Hotez, P.J., 2003. Molecular characterisation of the *Ancylostoma*-secreted protein family from the adult stage of *Ancylostoma caninum*. *Int. J. Parasitol.* 33, 897–907. [https://doi.org/10.1016/s0020-7519\(03\)00111-5](https://doi.org/10.1016/s0020-7519(03)00111-5).
- Zwanenburg, L., Borloo, J., Decorte, B., Bunte, M.J.M., Mokhtari, S., Serna, S., Reichardt, N.-C., Seys, L.J.M., van Diepen, A., Schots, A., Wilbers, R.H.P., Hokke, C. H., Claerebout, E., Geldhof, P., 2023. Plant-based production of a protective vaccine antigen against the bovine parasitic nematode *Ostertagia ostertagi*. *Sci. Rep.* 13, 20488. <https://doi.org/10.1038/s41598-023-47480-3>.