N-glycome and N-glycoproteome of a hematophagous parasitic nematode *Haemonchus*

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**A B S T R A C T**

N-glycosylation is a physiologically vital post-translational modification of proteins in eukaryotic organisms. Initial work on *Haemonchus contortus* – a blood-sucking nematode of ruminants with a broad geographical distribution – has shown that this parasite harbors N-glycans with exclusive chitobiose modifications. Besides, several immunogenic proteins (e.g., amino- and metallo-peptidases) are known to be N-glycosylated in adult worms. However, an informative atlas of N-glycosylation in *H. contortus* is not yet available. Herein, we report 291 N-glycosylated proteins with a total of 425 modification sites in the parasite. Among them, many peptidase families (e.g., peptidase C1 and M1) including potential vaccine targets were enriched. Notably, the glycan-rich conjugates are distributed primarily in the intestine and gonads of adult worms, and consequently hidden from the host’s immune system. Collectively, these data provide a comprehensive atlas of N-glycosylation in a prevalent parasitic nematode while underlining its significance for infection, immunity and prevention.

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1. Introduction

N-linked glycosylation is one of the most prominent post-translational modifications of proteins in eukaryotes. It plays pivotal roles in numerous biological processes, including protein folding, receptor-ligand interactions, immune response, and disease pathogenesis [1–3]. Glycans have high structural diversity, and are covalently attached to multiple asparagine residues of a single polypeptide [4,5]. Various configurations of modified sites and oligosaccharide chains underlie different physiological roles of glycoproteins. Hence, the glycosylation profiling of proteins is imperative to elucidate their biological importance in a given organism.

Examination of N-glycosylation involves three primary workflows: glycan profiling, glycosylation site mapping, and glycoprotein analysis. In recent years, the advent of high-precision mass spectrometry (MS) has facilitated large-scale studies of glycome and glycoproteome. Usually, hydrophilic interaction chromatography (HILIC) combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-ToF MS) are deployed to study glycoproteomics and glycomics [6,7]. These technologies have contributed significantly to our understanding of the molecular structures and biological networks of protein glycosylation in rodent and human cells [8,9]. In many invertebrate
organisms, however, especially the nematode parasites, the N-glycosylation remains poorly studied. *Haemonchus contortus* is one such widespread parasite of ruminant animals, which causes significant socioeconomic impact and ecological perturbation worldwide [10].

*H. contortus* encodes a multitude of glycoproteins, many with high immuno-regulatory activity [11]. An earlier study of glycans catalytically released by PNGase A revealed a wide range of N-glycosylations that are not commonly present in vertebrate glycans, such as galactosylated fucose (Gal-Fuc) and core α1,3-fucosylation [12]. Such distinct glycans are also present in the excretory/secretory discharge of the adult worms [13] and markedly induced protection correlated with the high level of antibody titers against the glycan epitopes in a vaccination trial [14]. In accord, when the N-glycosylation sites of the H1-14 antigen – an isoform of the known protective antigen group (i.e., H11 complex) in *H. contortus* – were mutated, the recombinant protein displayed a significantly reduced affinity to antisera when compared to the wild-type antigen [15]. Hence, decoding the glycosylation profile in *H. contortus* is necessary to unravel protective immunity mechanisms and develop recombinant vaccines against the parasite. This work aimed to map the N-glycome and N-glycoproteome and provide a systematic scaffold for functional dissection of glycans and glycoproteins in *H. contortus* and related parasitic nematodes.

2. Material and methods

2.1. Biological reagents and chemicals

PNGase F and PNGase A were purchased from New England Biolabs (Ipswich, MA, USA). Fluorescein-conjugated lectins and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Vector Laboratories (Burlingame, CA, USA). The click maltose-HILIC tips were obtained from Dalian Institute of Chemical Physics (Dalian, China). Trypsin, sodium azide, phenylmethylsulphonyl fluoride (PMSF), porous graphitic carbon (PGC) cartridge, dimethyl sulfoxide (DMSO), 2,5-dihydroxybenzoic acid (DHB), trifluoroacetic acid (TFA) were procured from Sigma-Aldrich (St. Louis, MO, USA). Protease inhibitor cocktail, acetonitrile, and methanol were obtained from Merck KGaA (Darmstadt, Germany). Formic acid was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Parasite collection

All adult worms (mixed sexes) used herein were collected from goats at an abattoir in Hubei province (China) and were prepared as reported previously [16]. In brief, worms were identified, collected, and then extensively rinsed with sterile phosphate-buffered saline (PBS), followed by five additional washes in PBS containing 0.02% sodium azide. Three biological replicates were prepared and stored in liquid nitrogen until further use.

2.3. Protein extraction and glycopeptide enrichment

Proteins were extracted from each of three replicates. In brief, each 0.5 g of adult worms was immersed in 500 μL of ice-cold lysis buffer (8 M urea, 1% protease inhibitor cocktail) and then homogenized in a glass homogenizer for 20 min. The homogenates, kept on ice, were sonicated in 30 s bursts for 5 min. Samples were centrifuged at 10,000 g for 25 min to remove the remaining debris. Finally, the supernatants were collected, and protein concentrations were detected using a BCA assay. Individual protein sample (400 μg) was reduced with 5 mM dithiothreitol at 56 °C for 35 min and then alkylated with 11 mM iodoacetamide at room temperature for 20 min in a dark room. The urea concentration of the sample was diluted to less than 1 M with 100 mM NH₄HCO₃ solution. Trypsin was added at a mass ratio of 1:50 (trypsin/protein) for the first digestion (overnight) and a ratio of 1:100 (trypsin/protein) for an additional 5 h. Finally, tryptic peptides were evaporated to dryness using a centrifugal evaporator (Eppendorf, Hamburg, Germany). Glycopeptides were collected using a HILIC enrichment method. Briefly, the dried peptides were reconstituted in 40 μL of loading buffer (80% ACN with 1% TFA) and then pipetted into a HILIC tip. After centrifugation (4000g, 20 min), glycopeptides were washed three times with 40 μL of loading buffer and centrifuged to remove the residual non-glycopeptides. Afterward, glycopeptides were eluted three times with 20 μL of 10% ACN. Finally, enriched glycopeptides were divided into two equal parts and respectively dried by the centrifugal evaporator.

2.4. Release, purification, and permethylation of N-glycans

N-glycan preparation workflow was based on a previous report with some modifications [17] (see Fig. 1). Briefly, an aliquot of glycopeptides was incubated with 2 μL of PNGase F in 50 μL of sodium phosphate buffer (pH 7.5) at 37 °C for 24 h. PNGase F-cleaved glycans were then desalted and purified by PGC cartridges, as described elsewhere [17,18]. Glycopeptides (remaining after PNGase F treatment) were dried and redissolved in 50 μL of sodium acetate solution (pH 6.0) for PNGase A (2 μL) digestion at 37 °C for 24 h. PNGase A-released glycans were isolated by PGC cartridges, as described above. Permethylation of glycans was performed following an established protocol [19]. In brief, native glycan pools released by PNGase F and PNGase A were dissolved in 50 μL DMSO, followed by the addition of DMSO–NaOH slurry (100 μL). Subsequently, 50 μL of methyl iodide was added, and the reaction mixture was shaken for 15 min at room temperature. The reaction was terminated by adding 500 μL of ultrapure water and 250 μL chloroform to separate liquid phases. The lower organic phase containing permethylated glycans was collected and washed 3x with ultrapure water. The two organic phases were transferred to separate vials and dried by the centrifugal evaporator.

2.5. MALDI-ToF and MALDI-ToF/ToF analysis

Spectra were obtained using the 5800 MALDI-MS instruments in the positive ionization mode (SCIEX, Concord, Canada). The dried glycan residues were reconstituted in 15 μL methanol solution. The matrix was prepared with 10 mg/mL DHB in 50% acetonitrile and 10 mM sodium acetate. 1 μL of permethylated glycan was mixed with 1 μL of freshly-prepared DHB before spotting on a MALDI-target plate and allowed to crystallize at room temperature. In total, 1000 laser shots were applied on each sample spot. MSI/MS analyses were performed with air as CID gas using 2 kV collision energy. The final data were processed by DataExplorer 4.0 (SCIEX, Concord, Canada), and the selected spectra were annotated using GlycoWorkbench (2.1 build 146).

2.6. Lectin-fluorescence microscopy

The adult worms were flat-fixed in 4% paraformaldehyde and sliced for paraffin embedding. Samples for light microscopy (thickness, 4 μm) were incubated with fluorescein-labeled lectins overnight at 4 °C. The source and binding specificity of the lectins used are listed in Table 1. After three PBS washes, the lectin-treated sections were stained with DAPI (room temperature, 5 min) to visualize the cell nuclei and then washed 3x, each for 5 min. Samples were mounted with an anti-fading solution for fluorescence microscopy (Olympus, Tokyo, Japan). Image files were
scanned and analyzed using ImageJ software to semi-quantify the mean fluorescence intensity.

2.7. Deglycosylation of glycopeptides and LC-MS/MS analysis

For N-glycoproteome analysis (see Fig. 1, three replicates), deglycosylation of glycopeptides was performed by sequential digestion with PNGase F and PNGase A, as described previously [20]. Briefly, an aliquot of enriched glycopeptides was dissolved in 50 μL of 50 mM NH₄HCO₃ buffer (prepared using H₂¹⁸O, pH 7.5), followed by deglycosylation with 2 μL PNGase F (37 °C, 24 h). As noted, the procedure was carried out in ¹⁸O-water. The isotope-labeled glycosylation site-specific tagging can be distinguished from non-enzymatic deamidation [21]. The deglycosylated fraction was subjected to HILIC enrichment, as outlined above. Peptides deglycosylated by PNGase F were collected from the HILIC tip effluent. The HILIC tip-bound glycopeptides were eluted and dried by the centrifugal evaporator and finally reconstituted in 50 μL of 50 mM sodium acetate solution (in H₂¹⁸O, pH 6.0). 2 μL of PNGase A enzyme was supplemented, followed by incubation at 37 °C for 24 h. The PNGase F- and PNGase A-deglycosylated peptides were desalted using C18 ZipTips (manufacturer’s protocol), dissolved in 0.1% formic acid (solvent A), and loaded onto a reversed-phase analytical column equipped with an EASY-nLC 1000 UPLC system. A gradient at a constant flow rate of 400 nL/min was programmed to increase the solvent B (0.1% formic acid in 98% acetonitrile) from 6 to 23% in the first 28 min, followed by another 8 min from 23 to 35%, and then 3 min to reach 80%, and after that staying constant at

Table 1

The source and binding specificity of lectins used in this study.

| Abbreviation | Source | Oligosaccharide specificity | Reference |
|--------------|--------|----------------------------|-----------|
| Con A        | Canavalia ensiformis | α-linked Man, branched and terminal | [50] |
| WGA          | Triticum vulgare | Terminal GlcNAc | [51] |
| SBA          | Glycine max | Terminal α, β-GalNAc | [52] |
| UEA-I        | Ulex europaeus | α-Fuc | [53] |

Man: Mannose; GlcNAc: N-acetylgalactosamine; GalNAc: N-acetylgalactosamine; Fuc: Fucose.

Fig. 1. Workflow of N-glycome and N-glycoproteome analysis in Haemonchus contortus. Trypsin-digested and HILIC-enriched glycopeptides were divided into two equal aliquots for preparing N-glycome (blue box) and N-glycoproteome (red box) samples. For N-glycome analysis, glycopeptides remaining after PNGase F treatment were further digested by PNGase A to release N-glycans with core α1,3-modified fucose. Free glycans were permethylated before MALDI-ToF MS and MS/MS analyses. For N-glycoproteome studies, glycopeptides were deglycosylated by PNGase F in heavy water. The remaining glycopes were further enriched by HILIC and subject to PNGase A digestion. The ¹⁸O-tagged deglycosylated peptides were analyzed by LC-MS/MS. N-glycans were deduced from the recorded spectra and computational fragmentation using the GlycoWorkbench suite, whereas N-glycopeptides were identified through bioinformatic tools (see methods). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
80% for the last 3 min. After UPLC, peptides were ionized by a nanospray ionization source (2.0 kV) and analyzed by MS/MS in Orbitrap Fusion™ Tridend™ (Thermo Fisher Scientific, Bremen, Germany).

2.8. Database analysis

The MS/MS data were evaluated by the MaxQuant software package (v1.5.2.8). Tandem mass spectra were searched against H. contortus entries in the UniProt database (24277 proteins) concatenated with reverse decoy database. Trypsin was specified as the cleavage enzyme allowing up to 2 missed cleavages. The mass tolerance for precursor ions was set at 20 ppm in the first search and 5 ppm in the main search. The mass tolerance for fragment ions was set at 0.02 Dalton. Carbamidomethyl on the cysteine residue was specified as a fixed modification, and oxidation on methionine was defined as a variable modification. The false discovery rate was adjusted to ≤1%, and the minimum score for modified peptides was set at ≥40. Glycosylated sites identified herein are based on the consensus of ≥2 biological replicates.

2.9. Bioinformatic methods

The Gene Ontology Annotation (GOA) was based on the UniProt-GOA database (http://www.ebi.ac.uk/GOA/). Proteins were classified according to molecular function, cellular component, and biological process. Amino acid sequence motifs comprising at least 20 residues within +/-10 residues of the N-glycosylation sites were analyzed by the MoMo suite (http://meme-suite.org/tools/momo/). Secondary structure analysis was done using the NetSurfP program with settings as described previously [22]. The PSORTb v3.0 software package was used for predicting the subcellular location of proteins (http://www.psort.org/) [23]. The functional domains were annotated based on the InterPro database (http://www.ebi.ac.uk/interpro/) [24].

2.10. Statistics and data availability

The graphs are presented as the mean with SE from at least three independent assays unless specified otherwise. Statistical analyses were performed using GraphPad Prism software (v8.0). Significance was tested by unpaired two-tailed Student’s t-test (p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). The data generated or analyzed during this work are provided in the main article and figures. The source data for the N-glycoproteomics and N-glycomics can be found in supplementary data and accessed through the PRIDE repository (PXD023379). All resources are available from the authors upon reasonable request.

3. Results

3.1. Analysis of N-glycans in Haemonchus contortus

To determine the N-glycan profile of adult worms, we digested enriched glycopeptides by PNGase F, followed by PNGase A treatment to cleave the PNGase F-resistant N-glycans with core α1,3-modified fucose residues [25]. Native N-glycans were permethylated and subsequently analyzed by MALDI-ToF MS and MS/MS. Glycan species are displayed primarily as [M + Na]+ adducts on the MS spectra. The spectrum of PNGase F-released glycans (Fig. 2A) revealed the pauci-mannosidic structures with or without fucose (Hex3,HexNAc2,Fuc0,2) and high-mannose structures (Hex5,HexNAc2). A significant fraction of complex-type glycans (Hex3,HexNAc3,Fuc0,2) was also identified (Fig. 2A, Table S1).

The MS data of the PNGase A-released glycans revealed an enriched pool of structures with multiple fucose residues, appearing at m/z 1316.0, 1346.0, 1520.1, 1694.3, and 1724.3 (Hex2,HexNAc2,Fuc1,3, Fig. 2B and Table S1). Besides, many pauci-mannosidic and high mannose structures (Hex4,HexNAc2) were detected in the PNGase A-digested pool, indicating incomplete removal of such moieties from glycopeptides by the PNGase F.

The glycan ions of selected MALDI-ToF MS spectra were further subject to MS/MS fragmentation. We observed the presence of a Gal-Fuc-like disaccharide unit on some N-glycan structures as described elsewhere [12], especially the ones possessing pauci-mannosidic and truncated backbones. The representative MS/MS spectrum of a PNGase F-released glycan at m/z 1549.9 (Hex4,HexNAc2,Fuc1, Fig. 3A) displayed three diagnostic fragment ions at m/z 433 (C-type ion), 678 (Y-type ion), and 894 (B-type ion), which indicated the presence of a Galβ1,4Fuc epitope linked to GlcNAc residue at the reducing end. As judged by the co-fragmentation ions (m/z 474, 871, 1098), the spectrum suggested the occurrence of another dominant isomer, which carries fucose on the 6-position of the proximal GlcNAc and four hexoses at the non-reducing termini. The m/z 433 fragments representing the Gal-Fuc-like unit appeared on the spectra of at least 7 different N-glycan moieties in both PNGase F and A released glycan pools (Table S1).

The MS/MS spectra of PNGase F-released N-glycans suggested that a significant portion carries either an LDN-like or an LDNF-like motif, as reported previously [17]. As exemplified by m/z of 1662.0 (Hex3,HexNAc4) (Fig. 3B) and 2010.2 (Hex,HexNAc4,Fuc2) (Fig. 3C), the spectrum of the former glycan displayed diagnostic fragments at m/z 527 (B-type ion) and 1157 (Y-type ion), indicating a GalNAc-GlcNAc (LDN) unit. The latter glycan (m/z 2010.2) showed key fragments at m/z 701, 905, 1127, and 1332, suggesting the fuscoylated LDN (LDNF) motif on its antennae. In contrast to the PNGase F, a difucoylated isomeric moiety was released by PNGase A at m/z 1520.1 (Hex,HexNAc,Fuc2) (Fig. 3D). Another fragment ion at m/z 648 and 894 signified that the proximal GlcNAc residue is substituted at the 3- and 6-OH positions. Also, as advocated by m/z 433 (C-type ion) and 1109 (Z-type ion) on the same spectrum, another dominant isomer with a Gal-Fuc-like motif may exist. This isomer also carries fucose residue on the innermost reducing-end GlcNAc, as shown by the m/z 474 and 1068 fragments. Compared to previous findings [12,17,26], our N-glycome data mapped a much higher diversity of N-glycans in H. contortus, many of which contained potentially typical structures such as Gal-Fuc motifs, terminal LDN and LDNF, and core α1,3-fucosylation.

3.2. Anatomical distribution of glycosylation in Haemonchus contortus

The above data suggested the presence of pauci/high-mannose and complex N-glycans, which harbor mannose and GlcNAc/GalNAc termini, and core fucosylated structures with or without galactose capping. We, therefore, selected plant lectins with affinities to recognize these glycan moieties to discern their distribution patterns in H. contortus (Table 1). While differences were observed in the binding patterns (Table 2), all lectins revealed strong fluorescent intensity in the adult worms (Fig. 4). Both mannose- and GlcNAc-binding lectins (Con A and WGA) were detected in the intestine and gonad of the adult female and male worms. Con A-treated samples displayed a clear punctate signal distributed in the cytoplasm and microvilli of the intestine (Fig. 4B-C). In contrast, WGA, which favors terminal GlcNAc, highlighted glycoconjugates located in the intestinal...
microvilli of both sexes, ovaries of females, and male adults’ cement gland. The latter appeared to superimpose with the distribution pattern of Con A-bound glycoconjugates. SBA, having broad specificity to the terminal GalNAc, recognized the ovaries in female worms (Fig. 4A), which suggests the occurrence of N-glycoproteins with β-linked GalNAc termini, presumably due to the presence of the LDN and/or LDNF epitope. Notably, fucose residues were detected (by UEA-I) mainly in the intestinal cytoplasm of the female adult worms (Fig. 4A-C). Collectively, our lectin binding assays indicated an abundant expression of mannose-, terminal GlcNAc/GalNAc- and fucose-rich glycoconjugates and thereby endorsed the N-glycome analysis, as described elsewhere in this work.

3.3. Identification of N-glycoproteome and N-glycosylation sites

Next, we performed HILIC enrichment in conjunction with LC-MS/MS to identify PNGase F- and PNGase A-deglycosylated...
peptides. We identified 380 sites located on 257 proteins using PNGase F-deglycosylated peptide mixtures (Table S2), and 109 sites present on 96 proteins using PNGase A-released peptide samples (Table S3). Most N-glycosylated sites (316 sites) could be entirely released by the PNGase F enzyme. Only 45 N-glycosylated sites were unique to the PNGase A-deglycosylated sample (Fig. 5A, Table S4), indicating the occurrence of a 1,3-fucose in asparagine-linked GlcNAc residues. After attaching the shared subset (64 sites), a total of 425 N-glycosylated sites on 291 unique proteins composing the N-glycoproteome data were identified with high confidence (Fig. 5A, Table S4).

The number of N-glycosylated sites assigned on each protein ranged from 1 to 7 (average degree of glycosylation, 1.5). More than half of glycoproteins (210/291, 72%) carried only a single N-glycosylation site, 51 (18%) of them harbored double N-glycosylation sites, and the rest 30 (10%) contained three or more sites (Fig. 5B). The neighborhood residues of glycosylated asparagines may help determine the specificity of *H. contortus* oligosaccharyltransferase (OST), which transfers the dolichol-linked oligosaccharide precursor to the nascent polypeptides. The characteristic sequences of modified sites and their enrichment statistics were obtained by MoMo [27]. As shown in Fig. 5C, three conserved amino acids flanking the glycosylated asparagine residues (from −10 to + 10) were defined. These motifs included (N-x-T-*-, Y), (N-x-T), and (N-x-S), where x represents any amino acid except for proline, and the asterisk denotes a random amino acid. Based on the hierarchical cluster analysis (Fig. 5D), threonine and serine displayed the highest probability at the position + 2, while the frequency of a proline residue in the proximity was markedly underrepresented. Therefore, the N-glycosylation sites in *H. contortus* matched with the canonical motif (N-x-T/S, x ≠ proline) while resonating with an earlier study on the free-living nematode *Caenorhabditis elegans* [28].

We tested the secondary structure and surface accessibility of glycosylated and non-glycosylated asparagines using the NetSurfP algorithm [22]. The analysis found that N-glycosylation was less likely to occur on the alpha-helix, as judged by a high probability of non-glycosylated asparagine (p = 1.39e−5). In contrast, N-glycosylated residues were more likely to be positioned on the beta-strand (p = 0.0477) and disordered coil (p = 7.06e−6) (Fig. 5E).

### Table 2

| Lectin | Intestinal cytoplasm | Intestinal microvilli | Ovaries | Intestinal cytoplasm | Intestinal microvilli | Cement gland | Outer cuticle |
|--------|----------------------|----------------------|---------|----------------------|----------------------|--------------|--------------|
| Con A  | +++                  | +++                  | +++     | +++                  | +++                  | +++          | +            |
| WGA    | +++                  | +++                  | +++     | +++                  | +++                  | +++          | +            |
| SBA    | ++                   | ++                   | ++      | ++                   | ++                   | +            | *            |
| UEA-I  | ++                   | ++                   | ++      | ++                   | ++                   | +            | *            |

*Intensity means fluorescence intensity from lectin-stained images, as quantified using the ImageJ program.*
left). As expected, the surface accessibility analysis suggested that the probability of modified asparagines on the protein surface was notably higher than non-N-glycosylated residues ($p = 1.39 \times 10^{-7}$) (Fig. 5E, right). These results, taken together, suggest a preference of the parasite OST for the beta-strand and coiled regions exposed to the surface of glycoproteins.

3.4. Functional analysis and predicted localization of glycosylated proteins

We finally examined the identified glycoproteins for predicted molecular function and subcellular localization to discern their physiological relevance (Fig. 6, Table S5-6). Notably, 23% and 13% of glycoproteins were associated with the binding function and hydrolase activity, respectively (Fig. 6A). Similarly, a sizeable fraction was predicted to localize in the extracellular space (51%), cytoplasm (15%), and plasma membrane (14%) (Fig. 6B). Other major glycoproteins supposedly reside in the endoplasmic reticulum (9%) and mitochondria (4%). The gene ontology (GO) enrichment (Table S7) further demonstrated an association of identified glycoproteins with the terms ‘extracellular matrix’ and ‘basement membrane’ (Fig. 6C), further adding to the reliability of our current understanding of this important parasitic nematode. The N-glycome of *H. contortus*, achieved by a fast atom bombardment mass spectrometer, was first reported well over two decades ago [17]. Compared to *state-of-the-art* technologies, the method had limited sensitivity and mass accuracy in detecting and classifying N-glycans. We deployed the MALDI-ToF-MS, which has become a method of choice for N-glycomics due to its high resolution, tolerance to impurities, and its ability to detect large complex glycans with high molecular weight [33]. Consequently, in conjunction with HILIC-LC-MS/MS, we identified a total of 425 N-glycosylated sites in 291 unique proteins in *H. contortus*. Our N-glycan data indeed obtained additional ion signals not reported in earlier FAB-MS analysis [17] that included Gal-Fuc-like modifications and complex-type N-glycans appearing at $m/z$ 1386.9, 1591.0, 1662.0, 1795.1, 2040.2, and 2255.4. Moreover, when compared to recent MALDI-MS data on PNGase A-released glycans [12], we observed glycans with a terminal GalNAc/GlcNAc at $m/z$ 1662.0, 1795.1, 1836.1, 1907.1, 2040.2, 2214.3, and 2255.4 (Table S1), many of which may carry LDN or LDNF epitopes on antennae. Such differences are likely due to different amidase utilized for releasing glycans and detection instruments.

PNGase A enzyme can release PNGase F-resistant glycans modified with core $\alpha$1,3-fucose, but it is limited in cleaving complex oligosaccharides [4,25]. We here combined PNGase F and PNGase A enzymes to fully release N-glycans from glycopeptides, providing a complete N-glycome profile for *H. contortus*. Several findings (Table S1), such as the presence of highly fucosylated glycans, pauci-mannosidic, and high mannose structures resonate with earlier studies [12,17]. Notably, *H. contortus* harbors a range of N-glycan modifications absent in mammalian glycans, especially the Gal-Fuc, LDNF motifs and $\alpha$1,3-fucosylation. Such 'non-mammalian' glycans are critical for the parasite invasion and counter-regulation of the host immune response [34]. Immunological studies have shown that the core $\alpha$1,3-fucose and LDNF epitopes are recognized by IgG antibodies in sheep vaccinated with *H. contortus* excretory/secretory glycoproteins [13,14]. Besides, $\alpha$1,3-fucose is a target of IgE during *H. contortus* infection [35].

**Fig. 4.** Anatomical distribution of glycoconjugates in *Haemonchus contortus*. Paraffin-embedded sections of the adult female (A) and male (B) worms were probed with fluorescein-labeled lectins, as indicated (green). Nuclei were stained by DAPI (blue). Major tissues are outlined. Images shown here represent three independent assays. (C) The intensity of fluorescence signals from lectin-stained images in panel A-B. Graphs show the mean fluorescence intensity, as quantified using the ImageJ program (NIH, USA). Abbreviations: ic, intestinal cytoplasm; mv, microvilli; int, intestine; ov, ovaries; gn, male gonad; cg, cement gland; cu, cuticle (outer). Scale bars: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

This study describing the N-glycome and N-glycoproteome of *H. contortus* complements the existing genomic, transcriptomic, and proteomic studies [29–32] and thereby fills a significant gap in our current understanding of this important parasitic nematode.
These glycan elements have also been reported in other parasitic helminths, e.g., *Trichuris suis* and *Schistosoma mansoni* [36,37], and are being explored as novel targets for vaccines and diagnostics.

A majority of glycans are secreted and/or located on the parasite surface, forming a protective glycocalyx against the host defense systems [34]. Previous lectin-based localization data in the parasitic trematode *Fasciola hepatica* have shown that most glycoproteins are distributed on the spines, suckers and tegumental coat, which are continually replaced to escape the immune attack by the host [38]. We discovered that glycoconjugates in *H. contortus* are primarily hidden in the intestine and gonad, which may also impede their recognition by the host’s immune system. Notably, initial works on *H. contortus* have suggested many immunogenic glycoproteins in the intestine (termed “hidden antigens”) [39,40], e.g., H11 and H-gal-GP, which may carry high mannose, LDNF and multiple fucose residues on N-glycans [17,41]. Staining with Con A, WGA and UEA-I lectins confirmed that glycoconjugates in *H. contortus* are primarily hidden in the intestine and gonad, which may also impede their recognition by the host’s immune system. Notably, initial works on *H. contortus* have suggested many immunogenic glycoproteins in the intestine (termed “hidden antigens”) [39,40], e.g., H11 and H-gal-GP, which may carry high mannose, LDNF and multiple fucose residues on N-glycans [17,41]. Staining with Con A, WGA and UEA-I lectins confirmed that glycoconjugates in

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**Fig. 5.** Identification and topological features of N-glycosylated sites in *Haemonchus contortus*. (A) Venn diagram of the number of N-glycosylated sites released by PNGase F and PNGase A. (B) Distribution of N-glycosylated sites in detectable glycoproteins. (C) Sequence motifs located nearby the target asparagine in enriched glycosylation sites. (D) Heatmap showing the relative frequency of amino acids in the proximity of asparagine (enrichment, red; depletion, green). (E) Distribution of non-glycosylated and glycosylated asparagine residues in alpha-helix, beta-strand, and disordered coil (left), as well as the predicted surface topology of glycosylation sites (right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
the intestine are mostly glycosylated by mannose, GlcNAc and fucose-rich motifs. In brief, lectin-binding assays reveal the parasite intestine as a rich source of glycosylated antigens.

A vast majority of identified N-glycosylated sites matched the canonical motif (N-x-T/S, x ≠ proline), located primarily in the disordered coil regions on the protein surface, which echoes with the N-glycoproteome profiles of other model organisms, C. elegans, Drosophila melanogaster and Danio rerio [42]. We also predicted the secondary structure and surface accessibility of glycosylated and non-glycosylated motifs, revealing significant enrichment of the modified N-x-T in the alpha-helix regions (Fig S1). Several glycoproteins identified herein, e.g., proteolytic peptidases, including peptidase C1 (cysteine peptidases, n = 32) and M1 (aminopeptidases, n = 8), were also found in a previous study of excretory/secretory proteins in H. contortus [43]. These molecules occur primarily in the intestine, facilitate hemoglobin degradation and nutrient acquisition, and are therefore expected to be essential for the growth, development and survival of hematophagous nematodes, H. contortus, Ancylostoma caninum and Necator americanus [44–46]. Due to critical physiological roles in parasitic worms, these peptidases are actively explored as prospective vaccine targets. Especially, the cysteine peptidases and aminopeptidases have been investigated widely in several immune protection trials against H. contortus [47].

The mutation of three predicted N-glycosylation sites on aminopeptidase was shown to significantly dampen the antibody response (about 95% reduction) compared with the native antigen [15], which suggested the antigenic importance of glycosylation sites. In this regard, we found that cathepsin B-like cysteine proteinase (P25793), cysteine proteinase (A0A6F7NM22) and aminopeptidase...
(A0A140EQKO) harbor two or more glycosylated sites including a potential α1,3-fucosylated site. The α1,3-fucosylated glycoproteins of \textit{C. elegans} are known to possibly induce Th2 type immune response in mice depending on the presence of intact glycans \cite{48}. There are indeed N-glycosylation sites modified by glycans bearing \textit{nematode-specific} motifs. The functional connectivity between unusual glycan structures and the specific glycosylation sites remain to be characterized by system-wide analysis of intact glycoproteins \cite{7,49}.

5. Conclusions

This study investigated the N-glycosylation profile of a parasitic nematode. Our glycoprotein enrichment-based mass spectrometry mapped the N-glycome and N-glycoproteome of \textit{H. contortus}, disclosing several exclusive features. In extended work, we revealed the parasite intestine and gonad as the major sites of glycoconjugates’ expression. Many glycoproteins with α1,3-fucosylation have been reported as potential vaccine candidates. Our work, therefore, lays the foundation to explore the biosynthesis and function of N-glycoproteins and thereby advance the therapeutic intervention against this widespread nematode parasite.

CRediT authorship contribution statement

Chunqun Wang: Methodology, Validation, Investigation, Data curation, Visualization, Writing - original draft. Wenjie Gao: Software, Formal analysis. Shi Yan: Formal analysis, Visualization. Xing-Quan Zhu: Resources, Xun Suo: Resources. Xin Liu: Software, Formal analysis. Nishith Gupta: Visualization. Min Hu: Conceptualization, Methodology, Validation, Visualization, Supervision.

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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Appendix A. Supplementary data

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