Comprehensive Analysis of the Secreted Proteins of the Parasite *Haemonchus contortus* Reveals Extensive Sequence Variation and Differential Immune Recognition*

Received for publication, December 6, 2002, and in revised form, February 5, 2003
Published, JBC Papers in Press, February 7, 2003, DOI 10.1074/jbc.M212453200

Ana P. Yatsuda¶¶¶, Jeroen Krijgsveld§§**, Albert W. C. A. Cornelissen¶¶, Albert J. R. Heck***, and Erik de Vries‡‡‡

From the Division of Parasitology and Tropical Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht University, P.O. Box 80165, 3508 TD, Utrecht, The Netherlands and the Department of Biomolecular Mass Spectrometry, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA, Utrecht, The Netherlands

*Haemonchus contortus* is a nematode that infects small ruminants. It releases a variety of molecules, designated excretory/secretory products (ESP), into the host. Although the composition of ESP is largely unknown, it is a source of potential vaccine components because ESP are able to induce up to 90% protection in sheep. We used proteomic tools to analyze ESP proteins and determined the recognition of these individual proteins by hyperimmune sera. Following two-dimensional electrophoresis of ESP, matrix-assisted laser desorption ionization time-of-flight and liquid chromatography-tandem mass spectrometry were used for protein identification. Few sequences of *H. contortus* have been determined. Therefore, the data base of expressed sequence tags (dbEST) and a data base consisting of contigs from *Haemonchus* ESTs were also consulted for identification. Approximately 200 individual spots were observed in the two-dimensional gel. Comprehensive proteomics analysis, combined with bioinformatic search tools, identified 107 proteins in 102 spots. The data include known as well as novel proteins such as serine, metallo- and aspartyl proteases, in addition to *H. contortus* ESP components like Hc24, Hc40, Hc15, and apical gut GA1 proteins. Novel proteins were identified from matches with *H. contortus* ESTs displaying high similarity with proteins like cyclophilins, nucleoside diphosphate kinase, OV39 antigen, and undescribed homologues of *Caenorhabditis elegans*. Of special note is the finding of microsomal peptidase H11, a vaccine candidate previously regarded as a “hidden antigen” because it was not found in ESP. Extensive sequence variation is present in the abundant Hc15 proteins. The Hc15 isoforms are differentially recognized by hyperimmune sera, pointing to a possible specific role of Hc15 in the infectious process and/or in immune evasion. This concept and the identification of multiple novel immune-recognized components in ESP should assist future vaccine development strategies.

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¶ Both authors contributed equally to this work.

§ Supported by European Union Project QLK2-CT-1999-00565.

** Supported by the Center for Biomedical Genetics and the Dutch Organization for Scientific Research (NWO).

†† To whom correspondence should be addressed. Tel.: 31-30-253-2582; Fax: 31-30-254-0784; E-mail: e.vries@vet uu.nl.

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Gastrointestinal (GI) nematodes are currently controlled by the use of chemicals (anti-helminthics), but there is great interest in development of vaccines, mainly because of the emergence of drug-resistant parasites. Among the GI nematodes, *Haemonchus contortus* is economically important because of its blood feeding characteristics in the abomasum; it is able to cause severe losses in production of small ruminant herds. The parasitic stages of the developmental cycle occur in a single host, and during each phase the nematode releases a variety of molecules into the host or in *vitro* culture environment. These are usually referred to as excretory/secretory products (ESP).

ESP are of practical value as a source of potential vaccine components. Obtained after *in vitro* cultivation of adult worms in serum-free medium, ESP or its partially purified fractions are able to induce 65 to 90% protection against *H. contortus* in sheep (1, 2), but protective properties have not conclusively been attributed to individual proteins.

Regardless of its practical use in vaccination studies, there is hardly any conclusive evidence on the biological function of ESP. For some proteins, functional roles have been proposed on the basis of similarity to other proteins in sequence data bases (3, 4), or roles in immune evasion or in molting have been implied (5, 6). Furthermore, the surface of the cuticle may be constituted of proteins derived from the secretory system (7). The secretion of some proteins has been linked in time to the transition of free-living stages to parasitism (8).

The identification of the proteins present in ESP of *H. contortus* using a proteomics approach will provide a basis for studies on the following matters. (i) Identification of ES proteins as the products of specific genes, enabling bioinformatic analyses and much more specific functional studies. (ii) The complexity of ES, which has hardly been addressed by the almost exclusive use of one-dimensional gel electrophoresis. (iii) The variability between batches of ESP, a topic highly relevant to vaccination studies. (iv) The recognition of specific spots by immune sera, especially in cases where multiple spots are derived from the expression of multigene families or from variations in post-translational modifications. (v) The cellular origin of ESP. Among others, ESP can contain proteins secreted by the pharyngeal glands, the excretory system, epithelial cells...
of the intestine (e.g. digestive enzymes), or rectal and vaginal cells (7). Moreover, cytosolic components of decaying cells (either by apoptosis or other causes of damage) may be present in addition to epithelial membrane proteins that are cleaved off.

Here we have described 107 identifications in the ESP of adults of *H. contortus* from gene sequence information deposited in GenBank™ nr as well as from *H. contortus* dbEST tag sequence data. The identification of the identified proteins have previously been associated with a protective immune response, although in several cases their presence in ESP has not been reported. For many proteins we have now shown that they appear in truncated forms or have extensive sequence modifications, which may complicate the design of vaccination strategies. Furthermore, many proteins have been detected in ESP for the first time and will be discussed with regard to their potential function and relation to an immune response.

**EXPERIMENTAL PROCEDURES**

*H. contortus ESP*—Standard procedures for harvesting ESP have been used as described for *H. contortus* and other nematodes (3, 9–13). All batches (A to D) were derived from experimental infections initiated from different larval stocks established over a period of 2 years from new generations of the same isolate. *H. contortus* adult worms were harvested from the abomasum of infected donor lambs, washed several times in PBS, and kept in RPMI 1640 medium containing antibiotics (100 IU of penicillin, 0.1 mg/ml streptomycin, and 5 μg/ml gentamicin) at 37 °C under 5% CO2. The parasites were first incubated for 4 h, after which the medium was harvested and new medium containing 2% glucose was added for overnight incubation. The supernatant was collected, centrifuged, filter-sterilized (0.2 μm), concentrated, and desalted (10 μM Tris, NaCl pH 7.4) in 3-kDa filters (Centriprep YM-3, Millipore).

**Sample Preparation and Isoelectric Focusing**—Prior to isoelectric focusing, the ESP were precipitated in a final concentration of 10% trichloroacetic acid (dissolved in acetone) containing 10 μM diethyrtreitol. The pellet was washed once in acetone with 10 μM diethyrtreitol and resuspended in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 2% carrier ampholyte mixture, pH 3–10NL) and supplemented with protease inhibitors (Complete Protease Inhibitor mixture, Roche Molecular Biochemicals). The sample was rehydrated and focused in an automated overnight run (IPGerPhor™ using 10–14 h of rehydration (30 V), followed by a step voltage focusing procedure (1 h 500 V, 1 h 1000 V followed by 8000 V until a total of 35–40 Kvh was reached). Prior to isoelectric focusing, the ESP were separated in 12.5% SDS-PAGE standards, Bio-Rad). Protein staining and image analysis of spot volume after normalization of the image, using the total spot volume normalization method multiplied by the total area of all the spots (Image Master software). For the immunoblotting analysis, the image of the 2-min exposure was used for the quantification procedures.

**Second Dimensional Electrophoresis**—The strips were incubated in 10 ml of equilibration buffer (50 m M Tris, 6 M urea, 2% SDS, 30% glycerol, pH 8.8) containing 30 μM diethyrtreitol for the first 15 min and replaced by equilibration buffer with 135 μM iodoacetamide for another 15 min. Electrophoresis in second dimension gel SDS-gel was carried out in a Hoefer SE600 system. Silver staining or Coomassie Brilliant Blue R-250 was used to visualize proteins after second dimensional electrophoresis. The images of the gels were acquired using LabScan v3.0 software on an ImageScanner (Amersham Biosciences).

**Immunoblotting**—ESP of *H. contortus* were separated in 12.5% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) using a semi dry system (Novablot, Hoefer) in transfer buffer (30 mM glycine, 48 mM Tris, 0.0375% SDS, 20% methanol) at 1.1 mA/cm2 for 1 h. The transfer efficiency was checked by staining of the membranes with DB71 (14). The membranes were blocked with 5% skimmed milk in PBS/0.05% Tween 20 (PBS-T, 1%, 37 °C) and all the washing steps were done with PBS-T (1 × 15 min, 1 × 10 min, 2 × 5 min). Either a pool of sera from parasite-free animals or sera from infected animals were used as source of IgG coupled to horseradish peroxidase (1/75000 in PBS-T/2% milk) for 1 h at room temperature. The chemiluminescent development was performed with ECL Plus according to the manufacturer’s instructions (Amersham Biosciences). The blots were exposed to X-ray film (Amersham Biosciences) and scanned using an ImageScanner, and matching was done by comparing the films of the blots with the DB71-stained membrane image and later with the master gel.

**Mass Spectrometry**—Proteins were in-gel digested with trypsin (Roche Molecular Biochemicals) in 50 mM ammonium bicarbonate (Sigma). Before MALDI-TOF analysis, peptides were concentrated using μC18-ZipTips (Millipore) and eluted directly on the MALDI-target in 1 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile. Peptides were analyzed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems) operated in reflectron mode at 20 kV accelerating voltage. Tandem MS measurements were performed on an electrospray ionization (ESI) quadrupole time-of-flight instrument (Q-Tof, Micromass Ltd., Manchester, UK) operating in positive ion mode and equipped with a Z-spray nano-ESI source. Nano-ESI needles were prepared from borosilicate glass capillaries (KwikFile™, World Precision Instruments Inc., Sarasota, FL) on a P-97 puller (Sutter Instrument Co., Novato, CA). The needles were coated with a gold layer using an Edwards Saverco sputter-coater 501 (at 40 nA, 1 kV, for 200 s). The capillary voltage was set at 1500 V; the cone voltage was 40 V. For the characterization of He15, the collision energy was optimized for individual peptides for optimal fragmentation. In all other cases, instead of nano-ESI needles a nano-LC system was coupled to the Q-TOF essentially as described in Ref. 15. Peptide mixtures were delivered to the system using a Famos autosampler (LC-Packings, Amsterdam, The Netherlands) at 3 μl/min and trapped on an Aqua™ C18 RP column (Phenomenex, Torrance, CA) during a total injection of 30 μl.

**Identification of 107 Proteins in ESP by Mass Spectrometry**—Under optimized conditions of sample preparation, aiming at the prevention of proteolytic breakdown and limitation of artificial modifications, 224 Coomassie Brilliant Blue-stained spots were detected in a 140-μg sample of *H. contortus* ESP (Fig. 1). This complexity substantially exceeds previous estimations derived from one-dimensional SDS-PAGE and was even more apparent after silver staining, resulting in the automated detection of about 950 spots. Fig. 2 shows a comparison of silver-stained gels of four batches of ESP obtained from different infections (see “Discussion”). Five Coomassie Blue-stained gels holding samples of the same batch of ESP were found by imaging software analysis to be nearly identical and were used for the reported experiments. 130 spots were judged to contain sufficient material for fin-
gerprinting by MALDI-TOF mass spectrometry. Fingerprints were used for searching the GenBank™ non-redundant protein data base with Mascot software and allowed the identification of 61 spots of *H. contortus* origin. Because relatively few *H. contortus* entries are present in GenBank™ nr, an attempt was made to employ EST data for peptide mass fingerprint searching. *H. contortus* ESTs were clustered and searched by EMOWSE (as described under “Experimental Procedures”) for matching the fingerprints. The top-score hits identified in 43 cases a contig matching one of the 61 spots identified by Mascot in GenBank™ nr. Additional EMOWSE hits, scoring within the range observed for the 43 confirmed Mascot hits, could represent significant identifications of proteins that are currently only present in the EST-derived data. To check for the reliability of these hits, selected spots were subjected to peptide fragmentation by LC-MS/MS to obtain sequences that could be used for searching both GenBank™ nr and EST data bases. In addition, manual comparison of peptide fingerprints of 12 spots, lacking LC-MS/MS data, revealed a very high similarity to the fingerprints of adjoining spots (marked as “identified by fingerprint similarity” in Supplementary Table S1 that includes all MW, PI, spot volume, and scoring data), which in most cases was confirmed by a matching EMOWSE top hit. These spots could represent post-translationally modified forms of the same protein.

In this way, a total of 107 identifications were made from 102 spots, of which 62 were confirmed as top hit by EMOWSE searches of the EST-derived dataset (average score of 0.293, ranging from 0.132 to 0.556). In addition, for 14 spots confirmation by EMOWSE was obtained by lower ranking hits. The fingerprints of 8 spots with high EMOWSE scores (*p* > 0.250, average 0.345) lacked confirmation by other methods. Confidence in these hits was enhanced by a manual reinspection demonstrating that the fingerprints matched within 50 ppm mass accuracy to the exact calculated MWs of the predicted peptides of their respective EMOWSE hits (EMOWSE tolerates...
a less accurate MW range of ± 0.5 dalton). They are taken up in the supplementary table as preliminary identifications.

In Fig. 3 a colored scheme is used for categorizing identified spots in functional groups. Immunoblotting results are also incorporated.

**Immunoblotting Identifies 193 Spots That Are Recognized by Hyperimmune Serum**—Immunoblotting of two-dimensional gels in combination with sensitive chemiluminescence detection permits quantification of relative immunogenicity (included in Supplementary Table S1) of individual spots by taking the ratio between the density of a Coomassie Blue-stained spot and its chemiluminescence signal. Reproducible blots of ESP batch A were incubated with a pool of hyperimmune sera obtained from five sheep experimentally infected with *H. contortus* several times. Spots on the blot and the gel were matched by imaging software; the final image is shown in Fig. 4. From 193 immune-recognized spots detected by image analysis, 52 have been identified above by mass spectrometry. Relative immunogenicity was observed to vary over a 2500-fold range (comparing spot 182 to spot 170) but is in fact larger because some high-density spots display no immunological detection at all, whereas several immunogenic spots cannot be detected by Coomassie Blue staining. This also reflects the specificity of immune recognition of the respective spots.

**Candidate Vaccine Components Hc15, Hc24, and GA1 Are Members of Protein Groups Displaying Differential Immune Recognition**—Table I provides a list of protein identifications; full data can be found in Supplementary Table S1. Identifications that have exclusively been made by matches to ESTs have been annotated on the basis of BLAST analysis.

Hc15, Hc24, and GA1 are the only proteins that have previously been shown to be ESP constituents (17, 18) by N-terminal peptide sequencing. These proteins will first be inspected in detail. Although for each a single gene has been cloned, we
identified multiple spots for each of the three proteins (all of unknown function), hinting at sequence modifications (post-translational or in primary sequence). This was investigated in more detail for the spots identified as Hc15.

Mascot and EMOWSE searches of MALDI-TOF fingerprints in combination with manual comparison of the fingerprints from this gel region identified 21 potential Hc15 protein spots distributed over a wide range of pI and MW (pI \(5.53\) – \(7.04\); \(16.1\) – \(19.0\) kDa) as indicated in Fig. 3. Tandem mass spectrometry was pursued on these spots until high sequence coverage was obtained. All spots appear to be Hc15-related. In Fig. 5 the single published sequence of Hc15 (AAC47713) is taken as a basis, and all sequenced parts of the individual spots are shaded.

Amino acid substitutions, all of which can be explained by single nucleotide changes, are observed in 10 of 21 spots. One to 5 residues are substituted out of a set restricted to 6 positions (Fig. 5, shaded in black) and occur in 9 different combinations. Four of 6 (T46A, R64P, T73A, and V90I) are also observed to be encoded by one or more of the 19 ESTs matching Hc15 and are thus confirmed to be present at the protein level in ESP. Two additional substitutions (H47R and N72S), detected by de novo sequencing using the MS data, were not found in ESTs.

Spectra of six spots contained a 1362-Da mass peak, the full sequence (SGNQVMFENINK) of which does not correspond to a tryptic fragment and therefore possibly represents the N terminus (Fig. 5). This N terminus exactly matches the end of an 11-amino acid deletion (between Glu-20 and Gly-30) in three ESTs (BF059795, BF422966, BF423305). Both in these three ESTs and in all other sequences, signal peptide cleavage is predicted to occur after Gly-19 (SignalP). The predicted N-terminal tryptic peptide of AAC47713 (ESQLNTK) and of the other 16 ESTs was not observed in any of the spots, suggesting alternative cleavage at the N terminus at Gly-30. A total of 17 of the 19 available Hc15 ESTs contain the stop codon and, as concluded from an alignment of these EST sequences, the 35-amino acid C-terminal tryptic peptide seems to be the most conserved part of the protein. Thus, spots 188 and 190 on the one hand and spots 161, 162, 165, and 167 on the other hand are almost fully sequenced by MS/MS and predicted to have an identical number of amino acids but, nevertheless, display a \(\sim 2000\)-Da mass difference. Possibly post-translational modifications are involved in such differences.

Collectively, the residue substitutions and alternative N-terminal sequences result in considerable variation among Hc15 proteins. A direct relationship between sequence variation and immune recognition, as measured by the relative immunogenicity described in Fig. 6, is far from obvious. Only 2 of the 10 spots in which substitutions have been found are recognized by hyperimmune serum (Fig. 5, spots 177 and 189), and even spots in which no sequence variation is detected by MS/MS display a 5-fold difference in immunogenicity (e.g. spots 180 and 183) or are not detected at all (e.g. prominent spots 175 and 193).

Variation in relative immunogenicity is even more pronounced within the groups identified as Hc24-like (9 spots) and GA1-like (21 spots). For the Hc24 group a 32-fold difference was observed (comparing spot 128 to 131), whereas one of nine was not recognized at all (spot 119). Similar to the Hc15 group,
spots are distributed over a wide pI and MW range (4.80–6.71; 26.2–34.3 kDa). Spots of the GA1 group are located in a more restricted area (see below) accommodating many other, sometimes co-migrating, spots (more clearly demonstrated after silver staining). This increases fingerprint complexity and might explain the low, but significant, Mascot scores of a number of GA1 identifications (Table S1). In addition, low scores may point at considerable sequence modification between spots and the published GA1 sequence, a feature that can only be clarified by an extensive MS/MS approach as used above for Hc15. GA1 protein is a 92-kDa membrane-associated polyprotein from which p46GA1 and p52GA1 subunits are released into solution after cleavage by glycosylinositol-specific phospholipase C (18). The p52GA1 subunit has a glycosylphosphatidylinositol anchor, and only the p46GA1 subunit was reported to be present in ESP. Here we have identified 9 spots with similarity to p52GA1 (pI 5.76–6.79; 45.1–51.7 kDa) and 12 spots similar to p46GA1 (pI 5.84–5.96; 40.4–42.2 kDa). A total of 18 of 21 GA1 spots are recognized within a broad range (730-fold for p52GA1) of relative immunogenicity for both subunits. However, the demonstrated co-localization of some proteins in this area (e.g. spots 69, 76, and 96) emphasizes that even two-dimensional separation cannot always definitely resolve which component is actually immune-recognized.

**DISCUSSION**

**General**—Control programs for GI nematodes in ruminants are currently based on the use of anthelmintics. These interventions are seriously hampered by the occurrence and rapid spread of drug-resistant parasites (e.g. Refs. 19–21). Part of the research community has, for more than a decade, focused its efforts on the development of vaccines with the ultimate aim of controlling helminth infections and GI disease. This research was confronted with two basic problems. First, the immunological toolbox to study and describe host-parasite interactions in ruminants was limited, certainly when compared with similar infections in humans or rodent models. Second, identification and purification of promising unique antigens have been difficult because of the complex life cycle of GI nematodes, the absence of in vitro culture methods, and the inherent large genetic heterogeneity of the population used. In this study we describe a large scale, high accuracy mass spectrometric proteome analysis of the ESP of *H. contortus*, a very important pathogenic GI nematode of small ruminants. *H. contortus* was selected because resistance to all classes of anthelmintics has been reported for this species on all continents. In addition, several approaches have been undertaken with the aim of developing a vaccine against this parasite, some of which are based on ESP. ESP of nematodes are produced by a standardized method of incubating parasitic stages in protein-free medium (3, 9–13), resulting in a protein pool of largely unresolved composition, origin, and function. Considering composition, we revealed the pattern of 224 ES proteins of *H. contortus* within a 3–10 pH range. Making use of GenBank™nr and dbEST data, we have

**Figure 4.** Immunoblotting of *H. contortus* ESP. ESP was probed with a pool of sera from five animals protected from *H. contortus* after several infections. The image is composed of two exposures (A is 30 s, and B is 5 min) to prevent overexposure of the upper part. Some of the spot ID numbers are indicated in the figure (some non-recognized spots are in circles). Markers are indicated on the right (in kDa).
assigned 107 identities from 102 spots. A major concern about ESP has been its nematode-specific origin, especially with regard to bacterial and host contamination. However, among the 130 most abundant protein spots, not a single bacterial protein was detected. The identified host proteins (serpins and complement factor C3) could be in ESP because of their high binding affinity for nematode proteins (proteases and antigens, respectively), thus revealing potential host-pathogen interactions. The presence of a small number of intracellular enzymes of nematode origin (five glycolytic enzymes and one glutamate dehydrogenase) indicates protein leakage from cells. Serious cell damage or decay is not likely, because no traces were found of abundant intracellular components like ribosomal and cytoskeletal proteins, which probably do not exit their cell through small leaks because of their association in high molecular weight complexes. Factors discussed above can be considered as a standard condition because a comparison of ESP batches obtained from four different isolations displayed a high degree of similarity in spots (Fig. 2).

This leaves us with a large set of specifically secreted products for which a function, and possibly a purpose, should be resolved. The purpose refers to potential applicability in vaccine development, whereas function and applicability could find a common theme in the diversity that we observed within a number of protein groups in association with highly variable parasitic diversity or variation counteracting host-immune responses. Both options have hardly been addressed at all in metazoan parasites. The outcome of our analyses has major implications for the present set of vaccine candidates. These will be discussed in line with future vaccine development. We will also discuss a set of newly identified proteins in ESP.

| Protein description                  | Number of IDs | Accession number |
|--------------------------------------|---------------|------------------|
| H. contortus ESP proteins            |               |                  |
| Hc15                                 | 21            | AAC47713, BF423205, BF059785, BF059790, BF066301, BF060216, BF060110 |
| Hc24                                 | 5             | AAC47714, BF059884, BF422751 |
| Hc24                                 | 1             | BF060998 |
| Hc24                                 | 1             | BF059857 |
| Hc24                                 | 2             | BF060283 |
| Hc40                                 | 4             | AAC03562, BF060098 |
| GA1- apical gut membrane protein     | 21            | CAB60199, AAB01192, BE496641, BF422939 |
| Proteases                            |               |                  |
| Microsomal aminopeptidase*           | 4             | CAB57358, CAC39009 |
| Metalloproteinase mep1*              | 6             | AAC31568, BE496726 |
| Metalloproteinase mep2*              | 2             | AAC28740 |
| Metalloproteinase mep1B              | 2             | AAC03561, AI723449 |
| Serine protease*                     | 3             | BF422843 |
| Serine protease*                     | 2             | AI723355, BF422889, BF059836, BF423241, BF060014, BF060421 |
| Aspartyl protease*                   | 3             | BF422825 |
| Antioxidant enzymes                  |               |                  |
| Superoxide dismutase*                | 1             | Q76666, BG734232 |
| Glutathione S-transferase*           | 1             | BM138779 |
| Cytosolic enzymes                    |               |                  |
| Glutamate dehydrogenase              | 4             | AAC19770, BM281185, BM280602, BM283265, BG733747, BG733758, Amblyomma variegatum BM290097, C. elegans NP 504656 |
| Enolase                               | 1             | BM281185, BM280602, BM283265, BG733747, BG733758, Amblyomma variegatum BM290097, C. elegans NP 504656 |
| Lactate/malate dehydrogenase*        | 1             | Aascaris suum, BE496726 |
| Glutathione S-transferase*           | 1             | BM280602, BM283265, BG733747, BG733758, Amblyomma variegatum BM290097, C. elegans NP 504656 |
| Aldolase*                            | 1             | BM280602, BM283265, BG733747, BG733758, Amblyomma variegatum BM290097, C. elegans NP 504656 |
| Triosephosphate isomerase*           | 1             | BM280602, BM283265, BG733747, BG733758, Amblyomma variegatum BM290097, C. elegans NP 504656 |
| Aldehyde dehydrogenase*              | 1             | BM139041, BM139053, BM138866 |
| Enzymes                              |               |                  |
| Peptidyl-prolyl-cis-transamidase*    | 3             | BM139041, BM139053, BM138866 |
| Peptidyl-prolyl-cis-transamidase*    | 3             | BM139041, BM139053, BM138866 |
| Nucleoside diphosphate kinase*       | 1             | BM139041, BM139053, BM138866 |
| Potential host proteins              |               |                  |
| Complement C3 precursor              | 1             | Mus musculus, P01027, Sus scrofa AAA40565 |
| Serpin                               | 1             | Bos taurus, AAF23888 |
| Serpin                               | 3             | Ovis aries, CAA3561, Bos taurus, AAA50448 |
| Others                               |               |                  |
| Transferrin-like domain*             | 1             | BF423163, BF060057 |
| Transferrin-like domain, IC8.8 protein| 1          | BM138822, BM138926, BM138889, BM139052, BM139052, AW670763, BF059890, BM139192, BF060064, BM138977 |
| Protein C. elegans protein*          | 1             | BF423000, BE222825, and 73 other H. contortus ESTs matching |
| F54D3.3 C. elegans protein*          | 1             | BM138822, BM138926, BM138889, BM139052, BM139052, AW670763, BF059890, BM139192, BF060064, BM138977 |
| Y105C5B.5 C. elegans protein*        | 1             | BM138822, BM138926, BM138889, BM139052, BM139052, AW670763, BF059890, BM139192, BF060064, BM138977 |
| OV39 antigen*                        | 1             | BM138822, BM138926, BM138889, BM139052, BM139052, AW670763, BF059890, BM139192, BF060064, BM138977 |
| Globin-like                          | 1             | BM138822, BM138926, BM138889, BM139052, BM139052, AW670763, BF059890, BM139192, BF060064, BM138977 |
| Total of IDs (total identified spots)| 107 (102)     |                  |

* IDs, identifications.
Variation in and Validation of Vaccine Candidates Hc15, Hc24, and GA1—For three proteins (Hc15, Hc24, and GA1), a definite proof for their ES origin had been previously obtained. The successful use of these proteins in vaccination trials when in native partially purified fractions (17, 22), followed by poor results with their corresponding recombinant version (2), suggests that a single gene often yields many products (e.g. Ref. 23). Sequence variations among the 21 Hc15 spots, as established here by MS/MS, do not fully explain the wide pI and MW ranges observed. Post-translational modifications in the C-terminal tryptic peptide (as nearly all other peptides were resolved) or largely anomalous migration behavior can thus also contribute to this diversity.

N-linked oligosaccharides of *H. contortus* have different structures in comparison to the host (24) and have been shown to contribute to immune recognition (25). In combination with the presence of gene families, as has been demonstrated for an Hc15 homologue of the nematode *Cooperia punctata* (26), this has clear implications for vaccination trials.

Whereas the Hc15 family has no homologues in any organism outside the Trichostrongyloidea superfamily of gastrointestinal nematodes, Hc24 and Hc40 are related antigenic proteins (27, 28) and part of a venom allergen antigen homologue/associated secreted protein (VAAH/ASP) family present in vertebrates, yeast, plants, nematodes, and hymenopteran venoms (8, 29–30). Only single Hc24 and Hc40 genes have been characterized in *H. contortus*, but it might be a complex gene family with the clustering of ESTs in 22 different contigs and 5–13% sequence variation between contigs (nema.cap.ed.ac.uk/nematodeESTs/Hemonchus/Hemonchus.html).

In *C. punctata* multiple Hc24 and Hc40 homologues have recently been identified (31). This is the first direct identification of Hc40 in ESP, and the occurrence of multiple spots is clearly a shared property of immunogenic proteins. GA1 proteins show similarity to bacterial TolB proteins, which are supposed to be involved, among other functions, in outer membrane integrity and membrane transport of colicins (32). Although only the 46PGA1 fragment has previously been detected in ESP (18), we also consistently detected the 52PGA1 fragment in different batches.

A large variation in immunogenicity is detected among members of all three families. Some spots of the Hc24 group (spots 128 and 137) and the GA1 group (spots 89 and 90) belong to the most immunogenic proteins of ESP, but other members of the groups are not recognized at all. Only six of the Hc15 spots are recognized. Hc15 has been cloned because of a prominent immune reaction of the 15-kDa band in a one-dimensional SDS-PAGE gel (17). After the identification of several other (novel) immune reacting proteins in the same MW region, it has become clear that previous interpretations correlating Hc15 to protection may have been premature. The most immunogenic protein by far is spot 170, which matches a homologue of *C. elegans* Y105C5B.5. It belongs to the transthyretin-like nematode-specific protein family (52 members in *C. elegans*) with weak similarity to transthyretin, a thyroid hormone transport protein in vertebrates. Interestingly, two other spots (179 and

**FIG. 5. Peptide sequences of Hc15 spots.** Alignment of the Hc15 protein (AAC47713) and other homologous ESTs with the identified peptide sequences (shaded in gray) of the 21 spots based on the template AAC47713. The predicted signal peptide cleavage site is indicated by an arrow.

**FIG. 6. Comparison of immune recognition of the Hc15 spots.** Volumes of the Hc15 spots present in the Coomassie Blue-stained gel and on the immunoblot (normalized as described under “Experimental Procedures”). The spot with the highest normalized volume in the gel or on the blot were taken as 100%; the relative volume of all other spots is expressed in percentages on the y-axis. Spots are ordered by increasing pI on the x-axis. Open bars indicate the relative volumes in the Coomassie Blue-stained gel and solid bars the volumes on the immunoblot.
184) match another EST cluster that is also homologous to Y105CSB.5 but, distinct from spot 170, does not contain a signal peptide and is not immune reactive. Another identified transport protein is represented by spot 158, matching a cuticular globin-like protein. Nematode globins have been isolated from *Nippostrongyulus brasiliensis* (33) and *Trichostrongylus colubriformis* (34) and have been detected in *H. contortus* (35) and *Osieragia ostertagia* (36). The secreted form is present in the cuticle of the parasite and might be involved in oxygen transport for muscular activity of the worm, because nematode globins have higher affinity for oxygen than the host globins (100 and 1000 times higher in *N. brasiliensis* and *Ascaris suum*, respectively) (37, 38). Two spots represent homologues of OV39, a protein from the filarial nematode *Onchocerca volvulus* (39) that is possibly involved in the autoimmune tectorial components because of its cross-reactivity with a retinal auto-antigen (hr44). OV39 is supposed to be involved in the autoimmune pathogenesis of this parasitic disease that causes river blindness in humans (40, 41). One spot matches *C. elegans* F54D5.3 protein, which in *H. contortus* is represented by a single contig containing 194 ESTs, indicating a high expression level. A possible function has not been described.

**ESP Contains Zinc Metalloproteases, Serine and Aspartic Proteases**—Protease activity has been detected and partially characterized by substrate specificity in ESP of *H. contortus* (42–44). Here we identified multiple spots matching four different types of proteases belonging to three different protease classes (metallo-, serine and aspartic proteases). Surprisingly, no cysteine proteases were detected. Cathepsin-B-like proteases constitute almost 5% of the 4843 ESTs presently analyzed and 17% of the ESTs from gut tissue (45). All clusters contain a putative signal peptide, indicating their targeting for the secretory pathway (including lysosomal proteins). Purification of ESP, using recombinant *H. contortus* cystatin affinity chromatography, identified only a few spots displaying high protease activity (not shown), indicating that cysteine proteases may indeed be among the low-density spots in ESP, not included for analysis by MS in the present study.

Four spots were identified as an amino-peptidase of the metalloprotease class, previously identified as the antigenic protein H11 in microsomal membrane fractions of *H. contortus*. H11 is taken as the most effective *H. contortus* immunogen, inducing up to 93% protection (46-48). The presence of H11 in ESP was unexpected, because it has been cloned from the insoluble gut membrane fraction (microvilli) of *H. contortus* and is classified as hidden antigen, i.e., not presented to the host immune system. Protection would be based on the binding of antibodies to the gut of the worm, which would die from starvation. We have consistently observed H11 in four different batches of ES.

In addition, three 85- to 95-kDa predicted endo-proteolytic zinc metalloproteases of *H. contortus* (meP1, meP1B, meP2) were identified by matching peptide mass fingerprints from ten spots of 40–46 kDa. Strikingly, all the MS fingerprints matched with either the N-terminal or the C-terminal half of the protein. LC-MS/MS data confirmed that the N-terminal half was represented by the three more acidic (as predicted) and slightly smaller spots, whereas the other seven spots matched the C-terminal domain. These observations suggest that the protease is cleaved in two parts. None of the few studies on metalloproteases from parasitic nematodes has described proteolytic cleavage, although smaller sized bands have been implied as degradation products (49, 50). Human metalloproteases are associated with a membrane complex from which the active protease can be shed as an inactive form by autocatalytic processing. A non-autocatalytic process, taking place intracellularly, gives rise to the release of active fragments (51). In *H. contortus*, the cellular location of processing is unknown, and it remains to be determined whether the cleavage products are proteolytically active.

A similar phenomenon was observed for seven spots matching either the N- or the C-terminal part of a protein composed of a duplicated domain strongly resembling serine proteases (as predicted from a cluster of 30 ESTs). In addition, the protein is predicted to be glycosylphosphatidylinositol-anchored and the observed molecular mass of ~62 kDa (C-terminal half, spots 40, 41, 42) and ~52 kDa (N-terminal half, spots 60, 63, 65, 69) fit to a predicted 127.6 kDa for the full-length protein (including signal peptide and anchor peptide). Based on sequence comparisons, both halves may very well encode an active serine protease, thereby classifying the full-length protein as a polyprotein. *C. elegans* has three genes encoding a serine protease double-domain version, apparently constituting a new type inside the family of serine carboxypeptidases (GenBank™ Accession nr NP_501598, NP_501599, NP_507841).

Aspartic proteases in schistosomes (trematodes) and in *H. contortus* are involved in the processing of hemoglobin (52, 53). Localized in the gut lumen, cross-reaction with polyclonal antisera suggested the presence of aspartic proteases in ESP at a lower concentration compared with gut tissue (53). Our experiment suggests the same with only one identification (spot 68).

**ESP Contain Polypeptides Involved in Antioxidant Stress and Extracellular Signaling**—Parasitic nematodes are sensitive to oxidative stress generated by their own cellular metabolism or by the host and therefore need an efficient oxidant defense system to ensure survival inside the host (54, 55). We have identified antioxidant enzymes such as superoxide dismutase (SOD) and glutathione S-transferase in our study. Their secreted forms were found in other nematodes (reviewed in Ref. 54), in trematodes (56), and were indirectly shown to be present in ESP (with polyclonal antibodies) for *H. contortus* (57).

Two types of proteins potentially involved in extracellular signaling were identified. Cyclophilins (CYP) are peptidyl-prolyl cis-trans-isomerases acting both as catalysts and chaperones in protein folding (58–60). For example, CYP-9 promotes the proper folding of collagen in parasitic nematodes, with direct implications for cuticle synthesis (61). CYPs have recently been classified as a pan-allergen family after demonstration of high sensitization to *Malassezia furfur* and *Aspergillus fumigatus* in human sera (62). The allergenic property of CYP has also been detected in the cestode *Echinococcus granulosus* (63). Secreted CYPs have extracellular signaling functions, such as induction of chemotaxis and adhesion of memory CD4 cells (64). Of 17 putative *C. elegans* CYPs, only CYP-3 (matching 2 spots) is demonstrated to be present exclusively in the single anterior mononuclear excretory cell (65). CYP-5, with a match to spot 150, is the only *C. elegans* CYP with a predicted signal peptide. Nucleoside diphosphate kinase (NDK) (spot 157) is responsible for maintaining the levels of intracellular nucleotide pools but is also involved in cell growth, differentiation, and tumor metastasis (66). A secreted form has been found only in some bacteria (67–69) and in the nematode *Trichinella spiralis* (70). A function is suggested by observed A TP-dependent cytotoxicity for macrophages and mast cells and prevention of cells from apoptosis (67, 71).

**Indepedent Batches of ESP Have Similar Profiles**—Differential immune recognition and the occurrence of multiple related spots, representing the expression of multiple genes and/or variation between individual worms within a population, present a challenge for the comparison of ESP patterns...
and immune responses under variable conditions. The effects of differences in worm isolates, host breed, and immune status of the host should all be investigated. Four batches of ESP, obtained over two years from experimental infections of the same breed of sheep with the laboratory strain used in this study were analyzed for variation. Larval stocks used were different because they were continuously replaced by later generations produced for strain maintenance. Thus the distribution of genetic variants within the populations used may vary. Nevertheless, Fig. 2 shows very limited variation of two-dimensional patterns between four batches. Silver-stained gels of the same ESP batch (Fig. 2A) and three other batches analyzed by MS demonstrate that the identified proteins, including the Hc15, Hc24, and GA1 families, occur in similar ratios in all gels. It is evident that even the majority of weakly stained spots, not detectable by Coomassie Blue staining, are present at comparable density in all four batches. Limited batch-to-batch variation makes the use of two-dimensional ESP protein and immune recognition patterns ideal tools for evaluating the parameters mentioned above. Studies of ESP profiles of parasites propagated after selection from (partially) immune hosts should allow evaluation of the role of antigenic diversity or variation in immune evasion. A specific problem of helminth challenge is the need for many repetitive experiments to identify multiple isoforms of the same proteins, such as Hc15. This study clearly shows that two-dimensional electrophoresis is still the most powerful technique to resolve closely related proteins, such as the many Hc15 and Hc24 isoforms observed in this work.

The majority of abundant proteins have now been identified; the use of two-dimensional electrophoresis will allow more detailed studies on the triggers and kinetics of their secretion. In addition, it has become clear that many of the major immune-recognized proteins result from minor, as yet unidentified, spots. Intensified protein studies like those reported here will be required to reveal their identity. Rational approaches for design of possible vaccine components require a better understanding of parasite biology. In describing here the first proteome map from a parasitic nematode, we have provided an initial step into a clearer understanding of the H. contortus secretion profile.

Acknowledgments—We thank the collaborators at the laboratories of Dr. D. Knox, Scotland, and Prof. J. Vercruysse, Belgium. We thank Nicole Bakker for technical assistance.

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Comprehensive Analysis of the Secreted Proteins of the Parasite *Haemonchus contortus* Reveals Extensive Sequence Variation and Differential Immune Recognition

Ana P. Yatsuda, Jeroen Krijgsveld, Albert W. C. A. Cornelissen, Albert J. R. Heck and Erik de Vries

*J. Biol. Chem.* 2003, 278:16941-16951.
doi: 10.1074/jbc.M212453200 originally published online February 7, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212453200

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