Revisiting the *Ancylostoma Caninum* Secretome Provides New Information on Hookworm–Host Interactions

Taylor Morante, Catherine Shepherd, Constantin Constantinoiu, Alex Loukas, and Javier Sotillo*

Hookworm infection is a major tropical parasitic disease affecting almost 500 million people worldwide. These soil-transmitted helminths can survive for many years in the intestine of the host, where they feed on blood, causing iron deficiency anemia and other complications. These parasites release a variety of molecules known as excretory/secretory products (ESPs) that are involved in many different biological processes that govern parasite survival. Using a combination of separation techniques such as SDS-PAGE and OFFGEL electrophoresis, in combination with state-of-the-art mass spectrometry we have reanalyzed the dog hookworm, *Ancylostoma caninum*, ESPs (*Ac*ESP). We identified 315 proteins present in the *Ac*ESP, compared with just 105 identified in previous studies. The most highly represented family of proteins is the SCP/TAPs (110 of the 315 proteins), and the most abundant constituents of *Ac*ESP are homologues of the tissue inhibitors of metalloproteases (TIMP) family. Interestingly, we identified new homologs of well-known vaccine candidates and immunomodulatory proteins. This study provides novel information about the proteins secreted by *A. caninum*, and constitutes a comprehensive dataset to study the proteins involved in host–hookworm interactions.

Soil-transmitted helminthiases (including trichuriasis, ascariasis, and hookworm infections) are debilitating parasitic diseases that affect more than two billion people worldwide, with increased incidence occurring in impoverished and underdeveloped societies. Hookworms alone affect almost 500 million people in tropical regions of South America, Africa, and Asia,[2] and chronic infections result in iron-deficiency anemia and even physical and intellectual retardation in young children.[4] Adult hookworms live in the intestine of vertebrate hosts where they feed on blood, and constantly release products into their surrounding environment through excretion and secretion mechanisms (excretory/secretory products, ESPs). The ESPs contain proteins that facilitate a parasitic existence, notably penetration of and migration within a host, feeding on host tissues, and evasion of the host immune response.[4] In addition, recently, hookworm ESPs have been shown to contain immunoregulatory properties that can protect mice against inflammatory diseases such as inflammatory bowel diseases and asthma.[5–8]

Due to the difficulty in obtaining *Necator americanus* adult worms from the human host, the dog hookworm *Ancylostoma caninum* has been extensively used as a model to study hookworm infections. The first proteomic characterization of the ESPs produced by *A. caninum* (*Ac*ESP) was performed by Mulvenna et al. [9]; however, herein we revisit this data since the *A. caninum* genome was not available at the time and the sensitivity of mass spectrometers has improved dramatically since this last study was conducted.

A total of $\approx 300$ *A. caninum* adult worms were obtained from the small intestine of five fresh cadaver dogs that had been naturally infected. Worms were divided into two different batches and incubated in 5× substrate (Dulbecco’s PBS (DPBS) (+) CaCl$_2$ (+) MgCl$_2$, 5% antimycotic/antibiotic, 1% Glutamax) for 2 h at 37 °C and 5% CO$_2$ to reduce bacterial contamination. Hookworms were then transferred to 2× substrate and incubated for a further 24 h at 37 °C and 5% CO$_2$ at a rate of $\approx 50$ worms per 25 mL of media.

A total of 50 μg of *Ac*ESP from batch 1 was separated by SDS-PAGE and 18 bands were excised from the gel, reduced using dithiothreitol (DTT), alkylated with iodoacetamide (IAM), and digested with trypsin overnight as described previously.[9] One hundred (100) micrograms of *Ac*ESP from batch 2 was reduced and alkylated using DTT and IAM, respectively followed by trypsin digestion overnight. Peptides were separated using an OFFGEL fractionator as previously described.[9] All samples were desalted using C18 ZipTips after SDS-PAGE or OFFGEL separation.

T. Morante
College of Public Health, Medical & Veterinary Sciences
James Cook University
Cairns, Queensland, Australia

C. Shepherd, Prof. A. Loukas, Dr. J. Sotillo
Centre for Biodiscovery and Molecular Development of Therapeutics,
Australian Institute for Tropical Health and Medicine
James Cook University
Cairns, Queensland, Australia
E-mail: javier.sotillo@jcu.edu.au

Dr. C. Constantinoiu
College of Public Health, Medical & Veterinary Sciences
James Cook University
Townsville, Queensland, Australia

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/pmic.201700186

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Peptides were analyzed using a Shimadzu Prominance Nano HPLC coupled to an AB SCIEX Triple TOF+ 5600 mass spectrometer and processed using the software Analyst TF 1.6.1. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [10] partner repository with the dataset identifier PXD006511 and https://doi.org/10.6019/PXD006511.

Database searches were performed against a database consisting of the *A. caninum* genome and proteins from the common Repository of Adventitious Proteins (cRAP, http://www.thegpm.org/crap/) with Mascot using Mascot Daemon (v.2.5.1, Matrix Science) and X! Tandem (v.2015.12.15.2) and Comet (v.2016.01 rev.2) using PeptideShaker (v.1.11.0). [11] Annotation of proteins was performed using Blast2GO, [12] Pfam, [13] PANTHER, [14] and InterProScan. [15]

A total of 237 and 289 proteins were identified with two or more peptides and FDR < 1% using PeptideShaker and Mascot (Supporting Information, Tables S1 and S2), respectively, from which 211 were common between both search programs, while 26 and 78 were uniquely found by PeptideShaker and Mascot, respectively, resulting in a final quantity of 315 AcESP proteins (Supporting Information, Table S3). A total of 67 out of the 105 proteins identified by Mulvenna et al. [9] were also found in the present study.

The top three most abundant proteins found by Mascot (Table 1) were ANCCAN_13497 (a previously described tissue inhibitor of metalloproteases; TIMP [16]), ANCCAN_25071 (a hypothetical protein), and ANCCAN_19759 (a sperm-coating protein; SCP/Tpx-1/Ag5/PR-1/Sc7 domain-containing proteins; SCP/TAPS). The top three proteins found by X! Tandem and Comet using PeptideShaker (Table 2) were ANCCAN_03259 and ANCCAN_01699, two SCP proteins, and ANCCAN_13497 a TIMP (also found by Mascot in the top three most abundant proteins). TIMP proteins are a multifunctional family of inhibitors of matrix metalloproteases associated with different functions in eukaryotic systems such as tissue remodeling, extracellular matrix turnover, cell proliferation, and angiogenesis among others. [17] However, in parasites, it has been previously suggested that TIMP-like proteins might not be functioning as matrix metalloproteases inhibitors. [18] The TIMP-like protein ANCCAN_13497 (previously identified as Ac-TMP-1 [16]) was already identified by Mulvenna et al. as the most abundant

### Table 1. Top 20 proteins found by Mascot in the excretory/secretory products of *Ancylostoma caninum* adult worms based on emPAI.

| Accession number | Description          | Number of validated unique peptides | emPAI    | Pfam                        |
|------------------|----------------------|-------------------------------------|----------|-----------------------------|
|                  |                      | SDS                                  | OGE      | SDS                         | OGE                   | NTR domain (PF01759) |
| ANCCAN_13497     | TIMP-like            | 13                                   | 9        | 60.86                       | 16.4                 |
| ANCCAN_25071     | Hypothetical protein | 10                                   | 5        | 26.08                       | 3.15                 |
| ANCCAN_19759     | SCP                  | 12                                   | 6        | 21.95                       | 2.54                 |
| ANCCAN_20483     | DOMON domain-like protein | 10                           | 2        | 18.18                       | 0.99                 | DOMON domain (PF03351) |
| ANCCAN_03257     | Platelet inhibitor   | 2                                    | –        | 16.71                       | –                    |
| ANCCAN_03259     | SCP                  | 3                                    | 3        | 8.03                        | 8.12                 |
| ANCCAN_26655     | TIMP                 | 7                                    | 4        | 10.05                       | 2.89                 |
| ANCCAN_23843     | Glu/Leu/Phe/Val dehydrogenase | 10                              | –        | 11.94                       | –                    | Glu/Leu/Phe/Val dehydrogenase domain (PF02812) |
| ANCCAN_26341     | Glutamate dehydrogenase | 11                          | 3        | 10.78                       | 0.86                 |
| ANCCAN_16282     | Hypothetical protein | 5                                    | 2        | 9.15                        | 1.54                 |
| ANCCAN_08479     | SCP                  | 8                                    | 3        | 9.19                        | 1.05                 |
| ANCCAN_01923     | Hypothetical protein | 11                                   | 3        | 9.61                        | 0.56                 | Protein of unknown function (DUF3270) (PF11674) |
| ANCCAN_17690     | SCP-like protein     | 17                                   | 7        | 8.21                        | 1.05                 | CAP domain (PF00188) |
| ANCCAN_21219     | SCP                  | 6                                    | 3        | 6.73                        | 2.14                 | CAP domain (PF00188) |
| ANCCAN_18161     | Galactoside-binding lectin family | 18                          | 8        | 7.12                        | 1.43                 | Galactoside-binding lectin domain (PF00337) |
| ANCCAN_06585     | SCP                  | 4                                    | –        | 6.84                        | –                    | CAP domain (PF00188) |
| ANCCAN_04963     | Apyrase              | 15                                   | 5        | 6.4                         | 0.7                  | Apyrase domain (PF06079) |
| ANCCAN_00673     | Hypothetical protein | –                                    | 3        | 5.81                        | –                    |
| ANCCAN_24968     | TIMP                 | 5                                    | 3        | 4.2                         | 1.04                 | TIMP domain (PF00965) |
| ANCCAN_03218     | Hypothetical protein | –                                    | 6        | 4.87                        | –                    |

Proteins were identified by SDS-PAGE, Offgel, or both. CAP: Cysteine-rich secretory protein family; DOMON: dopamine beta-monooxygenase N-terminal; NTR: UNC-6/NTR/C345C module; SCP: sperm-coating protein; TIMP: tissue inhibitor of metalloproteases.
### Table 2. Top 20 proteins found by X! Tandem and Comet in the excretory/secretory products of *Ancylostoma caninum* adult worms based on spectrum counting.

| Accession number | Description                  | Number of validated unique peptides | Spectrum count      | Pfam                        |
|-------------------|------------------------------|-------------------------------------|---------------------|-----------------------------|
|                   |                              | SDS       | OGE     | SDS   | OGE     |                                  |
| ANCCAN_03259      | SCP                          | 4         | 7       | 12605.4  | 3157.95  | CAP domain (PF00188)            |
| ANCCAN_01699      | SCP                          | 3         | –       | 9940.4  | –       | CAP domain (PF00188)            |
| ANCCAN_13497      | TIMP-like                    | 17        | 15      | 1674.2  | 4779.2  | NTR domain (PF01759)            |
| ANCCAN_03254      | SCP-like protein             | 7         | 5       | 2278    | 1622.4  | CAP domain (PF00188)            |
| ANCCAN_25071      | Hypothetical protein         | 8         | 6       | 1395.2  | 1587.6  | CAP domain (PF00188)            |
| ANCCAN_26655      | TIMP-like                    | 3         | 2       | 1567.9  | 1196.3  | NTR domain (PF01759)            |
| ANCCAN_03218      | Hypothetical protein         | –         | 5       | –      | 2280.6  | –                                |
| ANCCAN_03214      | Hypothetical protein         | 1         | 7       | 18.78   | 2244.7  | –                                |
| ANCCAN_11008      | Secreted protein 4 precursor | 2         | 3       | 517.8   | 2046.8  | –                                |
| ANCCAN_20841      | Unknown                      | 4         | 3       | 495.5   | 1982.6  | –                                |
| ANCCAN_25718      | Excretory secretory protein 1| 2         | 3       | 1060.7  | 1394.7  | –                                |
| ANCCAN_23152      | Hypothetical protein         | –         | 3       | –      | 2060.2  | AnfO_nitrog domain (PF009582)   |
| ANCCAN_13591      | Hypothetical protein         | –         | 4       | –      | 1869.4  | –                                |
| ANCCAN_16282      | Hypothetical protein         | 5         | –       | 1737.3  | –       | –                                |
| ANCCAN_10664      | TIMP-like                    | –         | 2       | –      | 1453.6  | TIMP domain (PF00965)           |
| ANCCAN_05485      | SCP-like protein             | 3         | 2       | 564.8   | 1093.2  | CAP domain (PF00188)            |
| ANCCAN_19423      | Hypothetical protein         | 2         | 4       | 60.76   | 1238.3  | –                                |
| ANCCAN_05778      | Copper zinc superoxide       | 6         | 5       | 525.6   | 947.6   | Copper/zinc superoxide dismutase domain (PF00080) |
| ANCCAN_18168      | Kunitz Bovine pancreatic trypsin inhibitor domain | – | 3 | – | 1082.6 | Kunitz/Bovine pancreatic trypsin domain (PF00014) |
| ANCCAN_19037      | Hypothetical protein         | –         | 4       | –      | 881.3   | –                                |

Proteins were identified by SDS-PAGE, OFFGEL, or both. AnfO_nitrog: Iron only nitrogene protein AnfO; CAP: Cysteine-rich secretory protein family; NTR: UNC-6/NTR/C345C module; SCP: sperm-coating protein; TIMP: tissue inhibitor of metalloproteases.

(and only TIMP-like) protein in the AcESP.[9] Interestingly, other *A. caninum* TIMP-like protein, the renamed Anti-Inflammatory Protein-2 (AIP-2), has been shown to be a potent immunomodulatory protein that suppresses airway inflammation in a mouse model of asthma.[7] AIP-2 has extensive homology with other TIMPs found in AcESP, including the highly abundant ANCCAN_26655 and ANCCAN_24968, suggesting that these two proteins could have similar immunomodulatory properties. Four different TIMPs are present in AcESP, and are highly abundant based on the emPAI and spectral count.

The SCP proteins are highly represented in the infective larval stage of hookworms, where a role in larval penetration and infection has been hypothesized (reviewed by [19]). They have also been speculated to be involved in immunomodulation.[20] For instance, an A. caninum SCP-like protein (known as Neutrophil Inhibitory factor; NIF) is able to inhibit neutrophil function and oxidative stress.[21] Although we did not find this protein in the adult secretions, we have found two SCPs having extensive homology with the NIF sequence deposited in NCBI (accession number AAA27789.1): ANCCAN_04194 (88% identity and E-value = 6.9 x 10^-89) and ANCCAN_22933 (94.3% identity and E-value = 1.5 x 10^-65). This could refer to a misannotation of the genome, since a blast search against the *A. caninum* genome using the deposited NIF sequence does not return any sequence with 100% homology. SCP-related proteins (including SCPs, SCP-homolog proteins, and SCP-like proteins) contributed to 110 out of 315 (31%) of the overall protein families and were by far the most highly represented family of proteins in the AcESP (Figure 1). These results suggest that the SCP family might play a key role in orchestrating a parasitic existence and modulating immune response of the host, and further studies should focus on this family of proteins.

Among the most represented protein domains in AcESP was the metallopeptidase family M13 (11 proteins), the transtatrin-like family (ten proteins), astacin metalloproteases (seven proteins), and glutathione-s-transferases (GSTs; six proteins). The role of peptidases in the secretome of helminths has been linked to important roles in parasitism[22]; however, the roles of transtatrin-like proteins are still unknown (despite their abundance in nematodes in particular). Astacins are a family of metallopeptidases highly abundant in the secretome of helminths.[23] Indeed, the human hookworm *N. americanus* has 82 astacin-encoding genes,[24] and astacins are abundantly represented in ESPs of the rat hookworm.
Nippostrongylus brasiliensis\(^{[25]}\) and other nematodes\(^{[26]}\). The role of astacins is not fully understood, although, in nematodes, they have been shown to participate in host invasion and parasite development.\(^{[23]}\) It is also noteworthy to highlight the presence of GSTs in the AcESP proteome. Na-GST-1, a GST secreted by adult stage \emph{N. americanus} that is thought to play a role in feeding by detoxifying the free heme produced after hemoglobin ingestion, is being currently tested as a vaccine against \emph{N. americanus}.\(^{[28]}\) Other molecules that have been tested as vaccines against hookworms include aspartic proteases and cysteine proteases (reviewed by \(^{[29]}\)), which are protein constituents of the hookworm.\(^{[11]}\)

In the present study, we have reanalyzed the protein constituents of AcESP in order to gain a more comprehensive snapshot of the hookworm secretome and how this impacts on host–pathogen interactions. We have identified almost three times as many proteins as previously reported in the ESP of this important parasite. In addition, new proteins of interest with potential (based on their homology to other molecules) as both novel immunoregulatory biologics and vaccine candidates have been identified, and clearly warrant future exploration.

**Abbreviations**

AcESP, \emph{Ancylostoma caninum} excretory/secretory products; DTT, dithiothreitol; ESPs, excretory/secretory products; GST, Glutathione-S-transferase; IAM, iodoacetamide; TIMPs, Tissue inhibitor of metalloproteases; SCPs, SCP/Tpx-1/Ag5/PR-1/Sc7 domain containing proteins

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

**Conflict of Interest**

The authors declare no competing financial interests.

**Keywords**

\emph{Ancylostoma caninum}, excretory, hookworm, proteomics, secretome, secretory products

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