

1 DATASET BRIEF

2 **Revisiting the *Ancylostoma caninum* secretome provides new information on**
3 **hookworm-host interactions**

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23 **Word count:** 3,235

24

25 **ABBREVIATIONS**

26 *AcES*: *Ancylostoma caninum* excretory/secretory products

27 DTT: Dithiothreitol

28 ESPs: excretory/secretory products

29 GST: Glutathione-s-transferase

30 IAM: iodoacetamide

31 TIMPs: Tissue inhibitor of metalloproteases

32 SCPs: SCP/Tpx-1/Ag5/PR-1/Sc7 domain containing proteins

33

34 **KEYWORDS:** *Ancylostoma caninum*, hookworm, proteomics, secretome,
35 excretory/secretory products, vaccines, immunomodulation.

36

37 **ABSTRACT**

38 Hookworm infection is a major tropical parasitic disease affecting almost 500 million
39 people worldwide. These soil-transmitted helminths can survive for many years in the
40 intestine of the host, where they feed on blood, causing iron deficiency anaemia and
41 other complications. To avoid the host's immune response the parasite releases
42 excretory/secretory products (ESPs), a complex mixture of glycans, lipids and
43 proteins that represent the major host-parasite interface. Using a combination of
44 separation techniques such as SDS-PAGE and OFFGEL electrophoresis, in
45 combination with state-of-the-art mass spectrometry we have reanalysed the dog
46 hookworm, *Ancylostoma caninum*, ESPs (AcES). We identified 315 proteins present
47 in the AcES, compared with just 105 identified in previous studies. The most highly
48 represented family of proteins is the SCP/TAPs (90 of the 315 proteins), and the most
49 abundant constituents of AcES are homologues of the tissue inhibitors of
50 metalloproteases (TIMP) family. We identified putative vaccine candidates and
51 proteins that could have immunomodulatory effects for treating inflammatory
52 diseases. This study provides novel information about the proteins involved in host-
53 hookworm interactions, and constitutes a comprehensive dataset for the development
54 of vaccines and the discovery of new immunoregulatory biologics.

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57 Soil-transmitted helminthiases (including trichuriasis, ascariasis and hookworm
58 infections) are debilitating parasitic diseases that affect more than two billion people
59 worldwide [1], with increased incidence occurring in impoverished and
60 underdeveloped societies. Hookworms alone affect almost 500 million people in
61 tropical regions of South America, Africa and Asia [2], and chronic infections result
62 in iron-deficiency anaemia and even physical and intellectual retardation in young
63 children [3]. Adult hookworms live in the intestine of vertebrate hosts where they
64 feed on blood, and constantly release products into their surrounding environment
65 through excretion and secretion mechanisms (excretory/secretory products, ESPs).
66 The ESPs contain proteins that facilitate a parasitic existence, notably penetration of
67 and migration within a host, feeding on host tissues, and evasion of the host immune
68 response [4]. In addition, recently, hookworm ESPs have been shown to contain
69 immunoregulatory properties that can protect mice against inflammatory diseases
70 such as inflammatory bowel diseases and asthma [5-9].

71 Due to the difficulty in obtaining samples from the human hookworm *Necator*
72 *americanus*, the dog hookworm *Ancylostoma caninum* has been extensively used as a
73 model to study hookworm infections. The first proteomic characterisation of the ESPs
74 produced by *A. caninum* (AcESP) was performed by Mulvenna et al. in 2009 [10];
75 however, herein we revisit this data since the *A. caninum* genome was not available at
76 the time and the sensitivity of mass spectrometers has improved dramatically since
77 this last study was conducted.

78 A total of ~300 *A. caninum* adult worms were obtained from the small intestine of 5
79 fresh cadaver dogs that had been naturally infected. Worms were divided into two
80 different batches and incubated in 5x substrate (Dulbecco's Phosphate Buffered
81 Saline (DPBS) (+) CaCl₂ (+) MgCl₂, 5% antimycotic/antibiotic, 1% Glutamax) for 2 h

82 at 37°C and 5% CO₂ to reduce bacterial contamination. Hookworms were then
83 transferred to 2x substrate and incubated for a further 24 h at 37°C and 5% CO₂ at a
84 rate of ~ 50 worms per 25 ml of media.

85 A total of 50 µg of AcESP from batch 1 was separated by SDS-PAGE and 18 bands
86 were excised from the gel, reduced using dithiothreitol (DTT), alkylated with
87 iodoacetamide (IAM) and digested with trypsin overnight as described previously
88 [10]. One hundred (100) micrograms of AcESP from batch 2 was reduced and
89 alkylated using DTT and IAM, respectively followed by trypsin digestion overnight.
90 Peptides were separated using an OFFGEL fractionator as previously described [10].
91 All samples were desalted using C18 ZipTips after SDS-PAGE or Offgel separation.
92 Peptides were analysed using a Shimadzu Prominane Nano HPLC coupled to an AB
93 SCIEX Triple TOF+ 5600 mass spectrometer and processed using the software
94 Analyst TF 1.6.1. The mass spectrometry proteomics data have been deposited to the
95 ProteomeXchange Consortium via the PRIDE [11] partner repository with the dataset
96 identifier PXD006511 and doi:10.6019/PXD006511.

97 Database searches were performed against a database consisting of the *A. caninum*
98 genome and proteins from the common Repository of Adventitious Proteins (cRAP,
99 <http://www.thegpm.org/crap/>) with Mascot using Mascot Daemon (v.2.5.1, Matrix
100 Science) and X! Tandem (v.2015.12.15.2) and Comet (v.2016.01 rev.2) using
101 PeptideShaker (v.1.11.0) [12].

102 A total of 237 and 289 proteins were identified with 2 or more peptides and FDR <1%
103 using PeptideShaker and Mascot (Supplementary Table 1, 2), respectively, from
104 which 211 were common between both search programs, while 26 and 78 were
105 uniquely found by PeptideShaker and Mascot, respectively, resulting in a final
106 quantity of 315 AcESP proteins (Supplementary Table 3). A total of 67 out of the 105

107 proteins identified by Mulvenna et al. [10] were also found in the present study
108 (Figure 1A).

109 The top three most abundant proteins found by Mascot (Table 1) were
110 ANCCAN_13497 (a previously described tissue inhibitor of metalloproteases; TIMP
111 [13]), ANCCAN_25071 (a hypothetical protein), and ANCCAN_19759 (a sperm-
112 coating protein; SCP, also called SCP/Tpx-1/Ag5/PR-1/Sc7 domain containing
113 proteins; SCP/TAPS). The top three proteins found by X! Tandem and Comet using
114 PeptideShaker (Table 2) were ANCCAN_03259 a platelet inhibitor,
115 ANCCAN_01699 an SCP protein, and ANCCAN_13497 a TIMP (also found by
116 Mascot in the top three most abundant proteins). TIMP proteins are a multifunctional
117 family of inhibitors of matrix metalloproteases (MMP) associated with different
118 functions in eukaryotic systems such as tissue remodelling, extracellular matrix
119 turnover, cell proliferation and angiogenesis among others [14, 15]. However, in
120 parasites, it has been previously suggested that TIMP-like proteins might not be
121 functioning as MMP inhibitors [16]. The TIMP-like protein ANCCAN_13497
122 (previously identified as *Ac*-TMP-1 [13]) was already identified by Mulvenna et al. as
123 the most abundant (and only TIMP-like) protein in the *Ac*ES [10]. Interestingly, other
124 *A. caninum* TIMP-like protein, the renamed Anti-Inflammatory Protein-2 (AIP-2), has
125 been shown to be a potent immunomodulatory protein that suppresses airway
126 inflammation in a mouse model of asthma [7]. AIP-2 has extensive homology with
127 other TIMPs found in *Ac*ES, including the highly abundant ANCCAN_26655 and
128 ANCCAN_24968, suggesting that these two proteins could have similar
129 immunomodulatory properties. Four different TIMPs are present in *Ac*ES, and are
130 highly abundant based on the emPAI and spectral count.

131 The SCP proteins are highly represented in the infective larval stage of hookworms
132 [17], where a role in larval penetration and infection has been hypothesized (reviewed
133 by [18]). They have also been speculated to be involved in immunomodulation [19].
134 For instance, an *A. caninum* SCP-like protein (known as Neutrophil Inhibitory factor;
135 NIF) is able to inhibit neutrophil function and oxidative stress [20]. Although we
136 didn't find this protein in the adult secretions, we have found two SCPs having
137 extensive homology with the NIF sequence deposited in NCBI (accession number
138 AAA27789.1): ANCCAN_04194 (88% identity and E-value = 6.9×10^{-89}) and
139 ANCCAN_22933 (94.3% identity and E-value = 1.5×10^{-65}). This could refer to a
140 missannotation of the genome, since a blast search against the *A. caninum* genome
141 using the deposited NIF sequence doesn't return any sequence with 100% homology.
142 SCP-related proteins (including SCPs and SCP-like proteins) contributed to 90 out of
143 315 (31%) of the overall protein families and were by far the most highly represented
144 family of proteins in the AcES (Figure 1B). These results suggest that the SCP family
145 might play a key role in orchestrating a parasitic existence and modulating the host's
146 immune response, and further studies should focus on this family of proteins.

147 Among the most represented protein domains in AcES was the metallopeptidase
148 family M13 (11 proteins), the transthyretin-like family (10 proteins), astacin
149 metalloproteases (7 proteins) and glutathione-s-transferases (GSTs; 6 proteins). The
150 role of peptidases in the secretome of helminths have been linked to important roles in
151 parasitism [21]; however, the roles of transthyretin-like proteins are still unknown
152 (despite their abundance in nematodes in particular). Astacins are a family of
153 metallopeptidases highly abundant in the secretome of helminths [22]. Indeed, the
154 human hookworm *N. americanus* has 82 astacin-encoding genes [23], and astacins are
155 abundantly represented in ESPs of the rat hookworm *Nippostrongylus brasiliensis*

156 [24] and other nematodes [25]. The role of astacins is not fully understood, although,
157 in nematodes, they have been shown to participate in host invasion and parasite
158 development [26, 27]. It is also noteworthy to highlight the presence of GSTs in the
159 *AcES* proteome. *Na*-GST-1, a GST secreted by adult stage *N. americanus* that is
160 thought to play a role in feeding by detoxifying the free heme produced after
161 hemoglobin ingestion, is being currently tested as a vaccine against *N. americanus*
162 [28]. Other molecules that have been tested as vaccines against hookworms include
163 aspartic proteases [29] and cysteine proteases [30], which are protein families also
164 represented in our study (Figure 1B). In the present study we found 5 different
165 cysteine proteases (ANCCAN_06649, ANCCAN_06644, ANCCAN_30567,
166 ANCCAN_06619 and ANCCAN_06647) and four aspartic proteases
167 (ANCCAN_18339, ANCCAN_13546, ANCCAN_29459 and ANCCAN_25067)
168 Thus, it is tempting to speculate that the GSTs, cysteine proteases and aspartic
169 proteases identified in the present study could be potential vaccines against the dog
170 hookworm.

171 In the present study we have reanalysed the protein constituents of *AcES* in order to
172 gain a more comprehensive snapshot of the hookworm secretome and how this
173 impacts on host-pathogen interactions. We have identified almost three times as many
174 proteins as previously reported in the ESP of this important parasite. In addition, new
175 proteins of interest with potential as both novel immunoregulatory biologics and
176 vaccine candidates have been identified, and clearly warrant future exploration.

177

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271 diminishes the fecundity and growth of worms. *J Infect Dis* 2004, *189*, 1952-1961.

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274 **FIGURE LEGENDS**

275 **Fig. 1.** (A) Comparison between the number of *Ancylostoma caninum*
276 excretory/secretory proteins (*AcES*) identified by Mulvenna et al. [10] and the present
277 study. (B) Top ten most represented protein families in the *AcES* after a Pfam
278 analysis. CAP: Cysteine-rich secretory protein family.

279

280 **Table 1.** Top 20 proteins found by Mascot in the excretory/secretory products of
 281 *Ancylostoma caninum* adult worms based on emPAI. Proteins were identified by
 282 SDS-PAGE, Offgel, or both. CAP: Cysteine-rich secretory protein family; DOMON:
 283 dopamine beta-monooxygenase N-terminal; NTR: UNC-6/NTR/C345C module; SCP:
 284 sperm-coating protein; TIMP: tissue inhibitor of metalloproteases.

Accession Number	Description	No. Validated Unique Peptides		emPAI		Pfam
		SDS	OGE	SDS	OGE	
ANCCAN_13497	TIMP-like	13	9	60.86	16.4	NTR domain (PF01759)
ANCCAN_25071	Hypothetical protein	10	5	26.08	3.15	CAP domain (PF00188)
ANCCAN_19759	SCP	12	6	21.95	2.54	CAP domain (PF00188)
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	CAP domain (PF00188)
ANCCAN_26655	TIMP-like	7	4	10.05	2.89	NTR domain (PF01759)
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	Glu/Leu/Phe/Val dehydrogenase domain (PF02812)
ANCCAN_16282	Hypothetical protein	5	2	9.15	1.54	-
ANCCAN_08479	SCP	8	3	9.19	1.05	CAP domain (PF00188)
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-like	5	3	4.2	1.04	TIMP domain (PF00965)
ANCCAN_03218	Hypothetical protein	-	6	-	4.87	-

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286

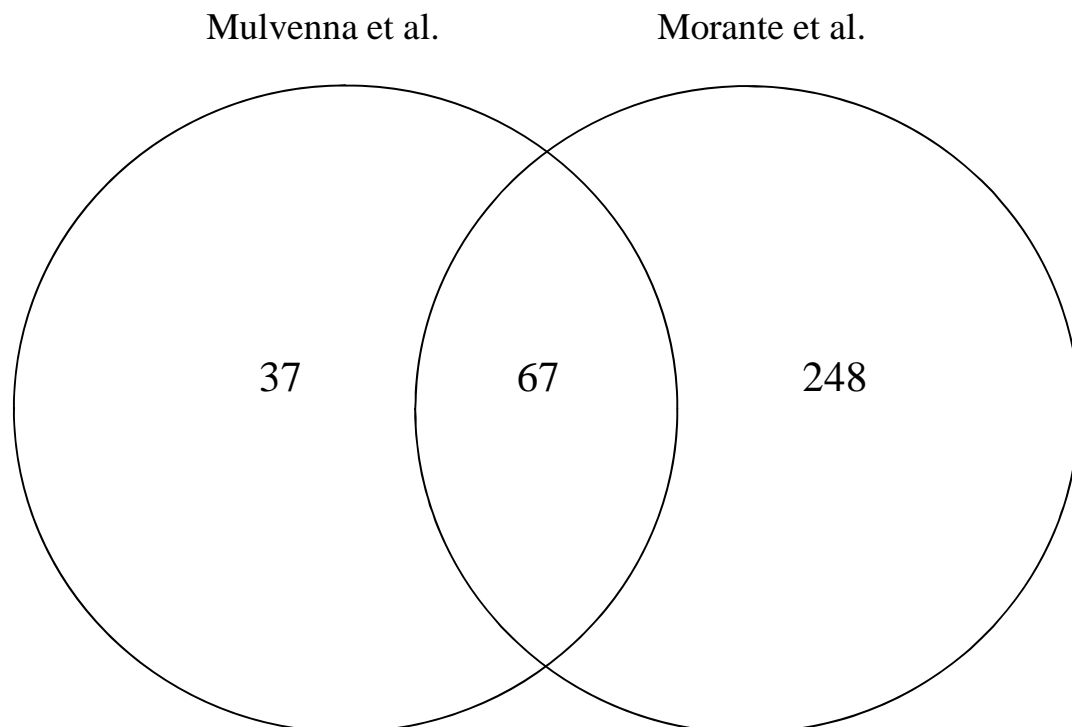
287 **Table 2.** Top 20 proteins found by X! Tandem and Comet in the excretory/secretory
 288 products of *Ancylostoma caninum* adult worms based on spectrum counting. Proteins
 289 were identified by SDS-PAGE, Offgel, or both. AnfO_nitrog: Iron only nitrogenase
 290 protein AnfO; CAP: Cysteine-rich secretory protein family; NTR: UNC-
 291 6/NTR/C345C module; SCP: sperm-coating protein; TIMP: tissue inhibitor of
 292 metalloproteases.

Accession Number	Description	No. Validated Unique Peptides		Spectrum Count		Pfam
		SDS	OGE	SDS	OGE	
ANCCAN_03257	Platelet Inhibitor	4	7	12605.4	3157.9	-
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_03218	Hypothetical protein	-	5	-	2280.6	-
ANCCAN_03214	Hypothetical protein	7	-	-	2244.7	-
ANCCAN_23152	Hypothetical protein	3	-	-	2060.2	AnfO_nitrog domain (PF09582)
ANCCAN_11008	Secreted protein 4 precursor	2	3	517.8	2046.8	-
ANCCAN_20841	Unknown	4	3	495.5	1982.6	-
ANCCAN_13591	Hypothetical protein	-	4	-	1869.4	-
ANCCAN_16282	Hypothetical protein	5	-	1737.3	-	-
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_10664	TIMP-like	-	2	-	1455.6	TIMP domain (PF00965)
ANCCAN_19423	Hypothetical protein	-	4	-	1238.5	-
ANCCAN_05485	SCP-like protein	3	2	564.8	1093.2	CAP domain (PF00188)

ANCCAN_18168	Kunitz Bovine pancreatic trypsin inhibitor domain	-	3	-	1082.6	Kunitz/Bovine pancreatic trypsin domain (PF00014)
ANCCAN_25718	Excretory secretory protein 1	2	3	1060.7	1394.7	-
ANCCAN_05778	Copper zinc superoxide dismutase	6	5	525.6	947.6	Copper/zinc superoxide dismutase domain (PF00080)
ANCCAN_19037	Hypothetical protein	-	4	-	881.3	-

293

A



B

