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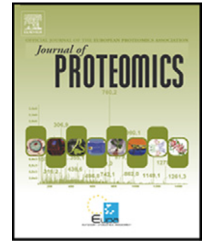

Comprehensive proteomic profiling of adult *Angiostrongylus costaricensis*, a human parasitic nematode

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ABSTRACT

Angiostrongylus costaricensis is a nematode helminth that causes an intestinal acute inflammatory process known as abdominal angiostrongyliasis, which is a poorly understood human disease occurring in Latin America. Our aim was to study the proteomic profiles of adult parasites focusing on immunogenic proteins. Total cellular extracts from both genders showed similar 2-DE profiles, with 60% of all protein spots focused between pH 5–7 and presenting molecular masses from 20.1 to 66 kDa. A total of 53 different dominant proteins were identified in our dataset and were mainly associated with the following over-represented Gene Ontology Biological Process terms: “macromolecule metabolic process”, “developmental process”, “response to stress”, and “biological regulation”. Female and male immunoblots showed similar patterns of reactive proteins. Immunoreactive spots identified by MALDI-PSD were found to represent heat shock proteins, a putative abnormal DAuer Formation family member, and galectins. To date, very few biochemical analyses have focused on the nematode *Angiostrongylus costaricensis*. As such, our results contribute to a better understanding of its biology and the mechanisms underlying the host–parasite relationship associated with this species. Moreover, our findings represent a first step in the search for candidate proteins for diagnostic assays and the treatment of this parasitic infection.

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1. Introduction

There are 15 *Angiostrongylus* species, of which only two represent a public health concern related to causing abdominal angiostrongyliasis and eosinophilic meningoencephalitis in humans: *A. costaricensis* and *A. cantonensis*, respectively [1,2]. *A. cantonensis*, a rat lungworm, frequently occurs in outbreaks with case numbers ranging from tens to hundreds [3]. Cases have been detected throughout Southeast Asia, the South Pacific, Madagascar, Africa, the Caribbean, and the continents of Australia and North America [4,5]. Over 2827 cases of *A. cantonensis* meningitis have been reported in approximately 30 countries [3,4]. This species was recently also detected in Brazil [6,7]. Additionally, *A. costaricensis* produces abdominal angiostrongyliasis; its biological cycle was described by Morera (1973) [8] (Fig. 1). This disease was first described in Costa Rica [9]; other cases have been reported from the United States to northern Argentina [10]. Although this infection is enzootic in Texas [11], the only case of autochthonous human infection reported in the United States [12] was later identified

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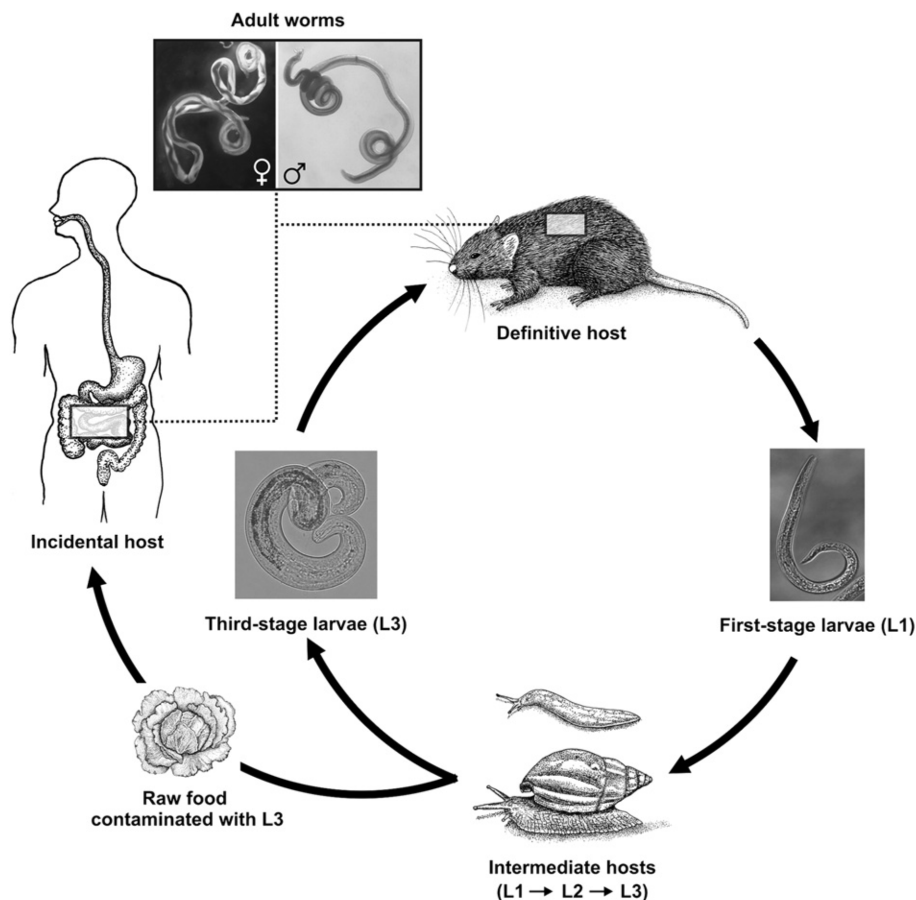

Fig. 1 – The biological life cycle of *Angiostrongylus costaricensis*. *A. costaricensis* is normally found in *Sigmodon hispidus*, *Rattus rattus* and other definitive hosts. The first stage larvae (L1) in the definitive hosts migrate into the intestinal lumen and are eliminated with the feces. Mollusks eat the contaminated feces or are externally infected via their epidermis, and second (L2) and third stage larvae (L3) develop in these hosts. The L3 are infectious to both definitive hosts and humans, who are incidental hosts. Infection with these parasites occurs through the ingestion of infected mollusks or unwashed vegetables contaminated with mucous of mollusks containing L3 *A. costaricensis* (see the Introduction for more details).

as a case of anisakiasis; however the authors replied maintaining the original diagnosis [13]. Even though abdominal angiostrongyliasis has been considered a public health problem in Costa Rica, its first reported outbreak occurred in Guatemala, where raw mint (eaten separately or as an ingredient in ceviche) was the likely vehicle of infection [14].

Several rodents are known to be definitive hosts of *A. costaricensis*, whereas its intermediate hosts are represented by mollusks of the *Veronicellidae* family [15]. More recently, it was shown that dogs can act as a reservoir host for *A. costaricensis* [16]. The parasite spreads to humans by means of the consumption of raw vegetables containing third-stage larvae (L3) developed in mollusks [17]. Currently, the explosive expansion of the giant snail *Achatina fulica* in many areas of Brazil has the potential to increase the transmission of *A. costaricensis* and *A. cantonensis* throughout the entire country [18–20]. A low number of infective L3 are apparently required to establish infection in vertebrate hosts, including humans [21]. According to serological studies, the number of clinical cases may be higher than the number of cases presenting symptomatic disease [22]. As shown by a preliminary evaluation, the antibody response to these infections gradually decreases over time, indicating that the worms do not survive for a long period in humans [23,24]. These observations suggest that abdominal angiostrongyliasis can spontaneously recede and should be better substantiated with a more sensitive and specific serum diagnostic test due to the existence of broad cross-reactivity among helminths of different species. Unfortunately, attempts to increase the specificity of immunodiagnostic tests usually lead to lower sensitivity, and vice-versa [23]. More extensive observations with clinical, parasitological and serological follow-ups are required for better evaluation of the prognostic value of serological and other molecular methods associated with abdominal angiostrongyliasis [24]. It is of note that some patients develop a severe abdominal disease that is only cured by surgery that removes the affected intestinal segment. The adult worms tend to aggregate in the more affected areas, and patients can be cured with this surgery. Longitudinal studies have not yet been performed due to the limitations of diagnostic tests and the inefficacy of antiparasitic drugs. In conclusion, abdominal angiostrongyliasis is clearly an underdiagnosed disease [25]. Better knowledge about the distribution of this human infection will depend on awareness of the

disease among medical personnel, on epidemiologic surveys of the infection in regional mollusk populations and on reliable serological tests based on well-defined antigens that are still not available [22–28]. Pathologists should be on constant alert during histopathological analysis of cecal appendix and intestinal segments with intense eosinophilia; a detailed analysis can sometimes reveal eggs, vasculitis and even adult worms in unexpected material [29].

The nematode species comprising the genus *Angiostrongylus* were initially grouped into two subgenera, *Angiostrongylus* and *Parastrongylus*; these two subgenera have been elevated to full genera status [30,31], but this taxonomic treatment has not been generally accepted [2]. Certain *Angiostrongylus* species, such as *A. cantonensis*, *A. costaricensis*, *A. dujardini* and *A. malaysiensis*, have been recategorized [31] as belonging to the genus *Parastrongylus* [32] based on differences in the morphology of the parasite male bursa and the final mammalian host [33]. All phylogenetic analyses that have been carried out to date do not support the assignment of the component species to two genera or subgenera, i.e., *Angiostrongylus* and *Parastrongylus* [2]. More recently, a molecular analysis using restriction fragment length polymorphisms (RFLPs) allowed the differentiation of *A. cantonensis*, *A. costaricensis* and *A. vasorum* [34]. The molecular differentiation and phylogenetic trees of *Angiostrongylus* species have been defined based on sequences from small-subunit ribosomal DNA [33], internal transcribed spacer 2 (ITS-2)[35], mitochondrial cytochrome-c oxidase subunit (COI) [2] and a 66-kDa protein gene of *A. cantonensis* [36]. Based on COI sequences and the 66-kDa protein gene of *A. cantonensis*, two major clades were defined: (1) *A. cantonensis* and *A. malaysiensis*, and (2) *A. costaricensis* and *A. vasorum* [2,36]. In the study based on COI analysis, a Costa Rican isolate of *A. costaricensis* was found to be quite different from a Brazilian isolate, with an uncorrected p-distance of 11.39%. The COI and ITS-2 results indicate the possibility that the Costa Rica and Brazil isolates could be cryptic species [2,35]. In both maximum-parsimony and maximum-likelihood analyses, *A. costaricensis* was found to be the most distant taxon and possibly to represent the earliest divergence group in evolutionary history [36].

Our group [37,38] showed the life cycle of *A. costaricensis* in its natural vertebrate host (*Sigmodon hispidus*) and in a mouse model to be much more complex than originally described by Morera [8]. These studies have revealed that the L3 stage alternatively goes through two migratory courses during its development into an adult worm: a lymphatic/venous–arterial pathway and a venous portal pathway. The former is considered to represent the primary pathway because it is used by most of the larvae. Like other metastrongylides, *A. costaricensis* passes over the pulmonary circulation to migrate from the lymphatic system to the arterial circulation, where they circulate for some days before reaching their definitive habitat in the mesenteric arteries of the terminal ileum and cecum. Oviposition by mature females begins 15 days after this and defines two important periods from the pathological point of view: pre- and post-oviposition. The former depends on worms in different stages of development, and the latter essentially depends on egg deposition in tissues.

Due to the disease characteristics associated with these nematodes, the incubation period in humans is highly variable; it can range from 14 days [39] to approximately 49–79 days [40] and even to more than one year [41].The main clinical signs and symptoms of the disease are also variable and include palpable abdominal masses (tumor-like masses), abdominal pain and rigidity, fever, anorexia, vomiting, diarrhea, intestinal obstipation, hepatomegaly, jaundice, abdominal distension, emaciation, unproductive cough, nausea, intestinal obstruction, perforation or bleeding and painful rectal examination. The radiologic findings related to the disease are intestinal dilatation or obstruction, hydro-air levels, tumor-like masses, intestinal wall thickening and rigidity and spasticity of the intestinal wall. Leukograms usually present leukocytosis with eosinophilia (>10–70%). The main areas of localization of the lesions are in the cecum, ascending colon, appendix, and small intestine [14,42–44]. Some cases can essentially evolve with hepatic lesions, such as nodules or focal necrosis [45–48], which are partially explained by the secondary portal pathway [37,38]. Using two different animal models (Swiss Webster mice and *Sigmodon hispidus* rats), we showed that during its life cycle, *A. costaricensis* presents an alternative migration to hepatic veins as a normal event in the venous portal pathway, in which the nematode matures and lays fertile eggs inside the liver. *A. costaricensis* adult worms can then reach the liver through branches of the hepatic artery and portal vein.

The proportion of cases that are oligosymptomatic or asymptomatic is unknown, and abdominal angiostrongyliasis appears not to always represent a persistent infection [22,44]. The mortality rate among symptomatic cases ranges from 1.8 to 7.4% [14,44]. It is important to note that even in the pre-oviposition phase, vascular lesions were observed to occur in *S. hispidus* expressed as an inflammatory reaction in the abdominal lymphatic circulation (lymphangitis and perilymphangitis constituted by macrophages, eosinophils, and neutrophils) and periarteritis with or without fibrosis, fibrinoid necrosis of the muscular layer, and micro-hemorrhages in the arterial wall [38]. These events could explain some rare human cases with a short incubation period. The diagnosis of abdominal angiostrongyliasis is confirmed by the identification of eggs, larva, or adult worms of *A. costaricensis* in surgical specimens [48]. Larvogenesis is not a frequent event in human cases and the eggs are sometimes limited to the morula stage.

A comparative histopathological study of confirmed and suspected cases of *A. costaricensis* infection revealed two types of macroscopic features: a predominant thickening of the intestinal wall (pseudoneoplastic pattern) and congestive necrotic lesions (ischemic-congestive pattern). Microscopically, three fundamental histopathological findings were detected, defining a triplet that establishes the diagnosis of probable abdominal angiostrongyliasis: (1) a massive infiltration of eosinophils in all layers of the intestinal wall; (2) a granulomatous reaction; and (3) eosinophilia vasculitis affecting arteries, veins, lymphatic structures and capillaries. The eosinophilic arteritis is usually centripetal, originating in the adventitia [44]. A definitive diagnosis relies on the identification of adult worms in arterial vessels (more rarely in veins) following surgical intervention (Fig. 2A–F). No treatment has thus far proven to be effective against the disease; moreover, treatment with some antiparasitic drugs can even worsen the course of the disease through unknown mechanisms [10]. Recently, it was shown that intranasal vaccination against

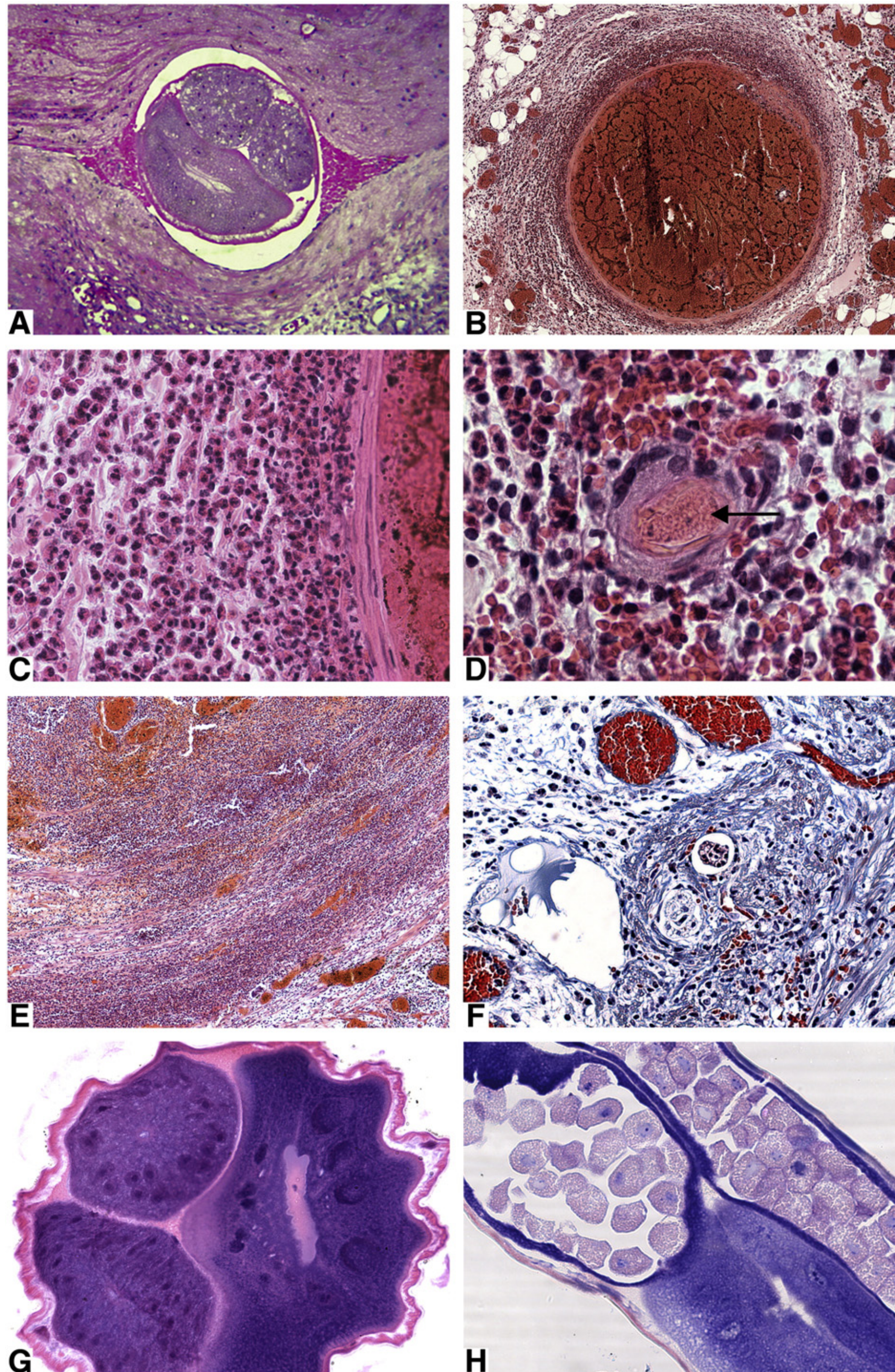

Fig. 2 – Histological lesions of abdominal angiostrongyliasis caused by *Angiostrongylus costaricensis*. (A) Female adult worm in a mesenteric artery of an infected *Sigmodon hispidus* individual showing the intestine (on the left) and the two ovaries (on the right).The parasite is located in the interface between the arterial endothelial layer and a thrombus [Hematoxylin–eosin (HE) 80×]. (B) Eosinophilic periarteritis with thrombus on human cecal appendix (HE 10×). (C) Detail of eosinophilic periarteritis full of eosinophils (HE 63×). (D) Immature egg (arrow head) in a human cecal wall, surrounded by a giant cell in the middle of the inflammatory infiltrate (HE 100×). (E) Injury of a human cecal muscle layer by intense inflammatory infiltrate (HE 10×). (F) Eggs in morula stage surrounded by inflammatory cells in a human cecal subserosa layer; the blood vessels are dilated and congested (Masson's trichrome stain 10×). (G) Transversal section of an isolated female adult worm surrounded by a clear muscle layer under the cuticle; the structure on the right is the intestine with its central lumen, and on the left, two anterior ovaries (immature segment) can be observed (Lennert's Giemsa 63×). (H) Longitudinal section of an isolated female adult worm presenting the two uteri full of eggs; the darker structure corresponds to the intestine (Lennert's Giemsa 40×).

A. costaricensis with synthetic antigens and recombinant peptides belonging to the catalytic region of the serine/threonine phosphatase 2A (PP2a) protein of the parasite results in a protective immune response in C57Bl/6 mice [49].

Even though nematodes are one of the most numerous and diverse phyla of animals on earth, including several human parasitic helminths, few studies have employed proteomic approaches to study their biology. One of the main limitations to performing such studies is the scarcity of genomic information available, which may hamper faster progress in this area [50]. In addition to the model organisms *Caenorhabditis elegans* [51] and *C. briggsae* [52], only five nuclear genomes from nematodes have been published: *Brugia malayi* [53], a major human filarial parasite; *Meloidogyne incognita* [54] and *M. hapla* [55], plant pathogens; *Pristionchus pacificus* [56], a beetle-associated species used as a model system in evolutionary biology; and *Trichinella spiralis* [57], a food-borne zoonotic parasite.

Most of the proteomic studies on nematodes have been performed on the free-living soil worm *C. elegans*, which is a convenient model system for *in vivo* studies of various physiological problems relevant to human diseases. Proteomics has contributed to the characterization of *C. elegans* nematodes by improving genome annotation and allowing analyses of phenotypic changes following RNAi treatment (targeted gene suppression), the performance of quantitative studies under various biological conditions and the profiling of protein expression during development and aging (for review, see Ref. [58]). In addition to revealing new diagnostic and therapeutic targets, high-throughput technologies could provide key insights related to comprehending mechanisms such as how the parasites invade host tissues and modulate their protective immune response [59,60].

In studies on parasitic nematodes, a widely adopted approach is to focus on the investigation of their secretomes. Apart from mediating interactions with the host (including modification of defense signaling pathways), excretory/secretory proteins may be an important source of potential immunogens to be used for diagnostics and vaccine development [61]. Several studies have thus far employed electrophoresis and/or liquid chromatography followed by MS/MS to identify secreted proteins from the helminths *Haemonchus contortus* [62], *Trichinella spiralis* and *T. pseudospiralis* [63,64], *Teladorsagia circumcincta* [65] and *Brugia malayi* [66–68]. Other proteomic studies on nematodes have focused on analyzing gender- and/or species-specific antigens [69–71], as well as the plasticity of protein expression patterns under different environmental conditions [72,73].

The aim of the present study was to comparatively identify the most abundant proteins in crude extracts from female and male *Angiostrongylus costaricensis*, particularly their immunogenic proteins.

2. Materials and methods

2.1. Parasites

The life cycle of the parasites was maintained at the laboratory using *Sigmodon hispidus* rodents and the snail *Biomphalaria glabrata* as definitive and intermediate hosts, respectively. Three-month-old rats were orally infected with 30 L3 larvae/animal. Adult worms were recovered by dissection of the mesenteric arteries of cotton rats after 40 days of infection [38]. They were extensively rinsed in PBS, segregated according to gender, weighted, and then stored at −80 °C until further use. Discrimination between genders was based on classical morphological criteria: females are usually longer and thinner than males and present an intestine full of blood, and males exhibit typical copulatory bursa with several rays and two copulatory spicules [8,74,75]. All procedures with animals were approved by the Animal Ethics Committee at Fiocruz (CEUA license # P0246/05) and were carried out in accordance with the *International Guiding Principles for Biomedical Research Involving Animals*, as issued by the *Council for the International Organizations of Medical Sciences*.

2.2. Optimization of protein extraction procedures

Protein extraction was performed after maceration of the worms (10 mg) in microcentrifuge tubes containing an abrasive resin (Sample Grinding Kit, GE Healthcare) and 150 μL of one of the following extraction solutions: (A) 1% SDS, 60 mM DTT and 40 mM Tris base; (B) 8 M urea, 4% CHAPS, 60 mM DTT, 40 mM Tris base and 1% v/v IPG buffer (same pH range of the IPG strip); (C) 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris base, 60 mM DTT and 1% v/v IPG buffer (same pH range of the IPG strip). Extraction with solution C was also performed in the presence of the Complete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland), following the manufacturer's instructions. After incubation for 1 h at room temperature with gentle shaking, cellular debris and resin were spun out (16,000 ×*g*, 15 min), and proteins were precipitated from the supernatant overnight with cold ethanol/acetone [1(protein extract):4 (ethanol):4 (acetone) v/v] at −20 °C. The precipitated proteins were sedimented at 16,000 ×*g* for 30 min, washed 3 times with ethanol/acetone/water (4:4:2 v/v) and solubilized overnight at 4 °C in extraction solution C without Tris base. Aliquots collected at each extraction step were assayed for total protein content using the 2-D Quant Kit (GE Healthcare).

2.3. SDS-PAGE

Protein extracts were initially analyzed by homogeneous SDS-PAGE (12%) in the Mini-Protean II system (Bio-Rad Laboratories) under reducing conditions using 4% stacking gels [76]. Additionally, low molecular weight markers from GE Healthcare were used and gels were stained with 0.2% CBB R-250.

2.4. 2-DE

Total cellular extracts of female and male adult worms were fractionated first on Immobiline DryStrips (IPG 11 cm pH 3–11 NL or 11/18 cm pH 4–7)(GE Healthcare) and then by homogeneous 15% SDS-PAGE as previously described [77]. Following in-gel sample rehydration at 30 V for 12 h, the following IEF electric conditions for 18 cm IPG strips were used: 200 V/1 h, 500 V/1 h, 1000 V/1 h, 1000–8000 V/30 min, and 8000 V/7 h (60,000 VhT). For 11 cm IPG strips, the maximum voltage was limited to 6000 V/6 h (44,000 VhT). Gels were stained with colloidal CBB G-250 or Sypro Ruby (Invitrogen) for total protein

visualization or incubated with the glycan-specific stain ProQ-Emerald (Invitrogen) for the detection of glycoproteins, as specified by the manufacturer's instructions. CBB-stained gels were scanned using an Image Scanner (GE Healthcare), and image analysis was performed using Image Master 2D Platinum 7.0 software (GE Healthcare). Spot detection was automatically performed with minimal manual editing. For each gender, three independent sample preparations were analyzed by 2-DE. Protein spot abundances were expressed as a mean ± standard deviation. Comparisons of spot abundances between female and male groups were performed using Student's t-test ($p \leq 0.01$). Fluorescent images were acquired on a Typhoon Trio scanner (GE Healthcare) with a resolution of 100 μm and photomultiplier (PTM) values adjusted to optimize sensitivity and avoid oversaturation. The excitation/emission wavelengths for Sypro Ruby and ProQ-Emerald were 488/610 and 532/520, respectively.

2.5. *Characterization of immunogenic proteins*

Immediately after electrophoresis, the proteins on 2-DE gels were transferred to PVDF membranes (Immun-Blot™ 0.2 μm, BioRad) at 270 mA for 3 h using the TE77 PWR semi-dry blotter (Amersham Biosciences). Two 2-DE gels were transferred at the same time by stacking them vertically in a multi-layered stack. After blocking unoccupied membrane sites overnight with TBS containing 0.05% Tween 20 and 5% skim milk, the PVDF membrane was incubated for 2 h with pooled serum taken from Swiss Webster mice 28 days after experimental infection with *A. costaricensis* (1/1000 v/v dilution in freshly prepared blocking solution). After washing 3× for 10 min with TBS containing 0.05% Tween 20, the membranes were further incubated for 2 h with the secondary antibody HRP-conjugated sheep anti-mouse IgG (whole antibody, GE Healthcare)(1/25,000 dilution in TBS + 0.05% Tween 20). The membranes were washed again with TBS + Tween 20 and then incubated between two cellophane sheets with the SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific) prepared according to the manufacturer's instructions. Each membrane/cellophane "sandwich" was exposed to Hyperfilm ECL Film (GE Healthcare) for 3 min. The spots on 2-DE blots were matched to their homologues in 2-DE gels using Image Master 2D Platinum 7.0 software (GE Healthcare).

2.6. *Protein analysis by mass spectrometry*

In-gel protein digestion, N-terminal chemical derivatization of tryptic peptides with 4-sulphophenyl isothiocyanate (SPITC), and sample desalting with C18 ZipTip micropipette tips (Millipore) were performed as previously described [78]. All MS spectra were acquired in positive ion reflector mode on an AB Sciex MALDI-TOF/TOF 5800 Mass Spectrometer using Explorer software, version 4.0.0. An aliquot (0.3 μL) of the desalted tryptic digest was deposited onto the target plate immediately before the addition of an equal volume of a saturated matrix solution [10 mg/mL α-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) in 50% acetonitrile/0.1% trifluoroacetic acid]. After sample drying at room temperature, both MS and MALDI-PSD data were acquired with a 1 kHz laser. Typically, 2040 and 2000 shots were accumulated for spectra in MS mode and PSD mode, respectively. Up to 20 of the most intense ion signals with a signal-to-noise ratio above 30 were selected as precursors for MALDI-PSD acquisition, excluding common trypsin autolysis peaks and matrix ion signals. External calibration in MS mode was performed using a mixture of five peptides: des-Arg1-Bradykinin (*m/z*) 904.4680; angiotensin I (*m/z*) 1296.6850; Glu1-fibrinopeptide B (*m/z*) 1570.6770; ACTH (1–17) (*m/z*) 2093.0870 and ACTH (18–39) (*m/z*) 2465.1990. MALDI-PSD spectra were externally calibrated using known fragment ion masses observed in the spectrum of angiotensin I.

2.7. *Database searching and gene ontology analysis*

Following data acquisition, peak lists from uninterpreted spectra were created using the Peaks-to-Mascot script of 5800 Explorer software (Applied Biosystems) and uploaded to the online Mascot search engine (Matrix Science). The search considered carbamidomethylation as a static modification and methionine oxidation, propionamide cysteine and N-terminal derivatization with SPITC as variable modifications. Up to two missed cleavages were accepted. The spectra were searched against NCBInr. Peaks Studio 5.2 [79] was used as an extra measure to confirm the interpretation of tandem spectra identified as described above; the same modification settings and protein database were used. We used PatternLab's Gene Ontology Explorer (GOEx) module [80,81] to further interpret our list of identified proteins. First, we used Goanna [82] to

Table 1 – Quantitative analysis of extraction yields and protein recovery after different sample preparation methods. Protein concentration was measured using the 2D-Quant-kit assay.

	Extraction solutions	Extraction yield (μg ptn/mg worm)			% Recovery after EtOH precipitation		
		Mean	SD	n	Mean	SD	n
Female	A	105.74	2.77	4	ND		
	B	86.11	9.06	4	60.78	6.86	4
	C	84.10	11.71	11	91.52	8.83	10
	C + inhibitor cocktail	81.34	8.58	2	ND		
Male	C	87.12	11.24	7	95.67	4.02	6

ND, not determined; n, number of independent replicates.

assign GO terms to each identified protein by Blasting [83] them against the online SwissProt, TrEMBLE, and UniProt databases. This generated a text file containing each protein's accessions number and the corresponding GO terms. This file, together with the Gene Ontology database [84] (OBO v 1.2 downloaded from geneontology.org in March 1st 2011), served as an input to GOEx so that statistically over-represented GO terms ($p \leq 0.01$) could be determined according to the hypergeometric distribution.

3. Results and discussion

3.1. Optimization of protein extraction

Female specimens of *A. costaricensis* are longer than male worms [8,74] and were recovered in higher numbers in infected *Sigmodon hispidus*. Therefore, the optimization of protein extraction conditions was performed only in females. Table 1 shows the quantitative results from grinding these parasites under different extraction conditions. Assuming SDS-based solution A as the gold standard (100% extraction efficiency), we showed that both solutions B (containing urea as caothropic agent) and C (containing urea/thiourea) were efficient in extracting approximately 80% of whole worm proteins. Qualitatively, SDS-PAGE profiles from all extraction conditions showed comparable patterns of protein bands with different staining intensities over the entire range of molecular masses from 14.4 kDa to more than 97 kDa (Fig. 3). After the addition of a protease inhibitor cocktail to solution C, no changes were observed in the extraction yield or the SDS-PAGE profile, indicating that proteolysis is not a major concern under the denaturing conditions used here. Because protease inhibitors can additionally modify proteins and cause charge artifacts, they were not used in this study. Proteins were precipitated with a mixture of cold ethanol/acetone so that contaminants that might impair subsequent 2-DE analysis, such as salts and nucleic acids, could be removed [85]. No qualitative differences were observed in the unidimensional protein profiles of samples extracted with solution C before and after precipitation (Fig. 3). Regarding the recovery of proteins from ethanol/acetone

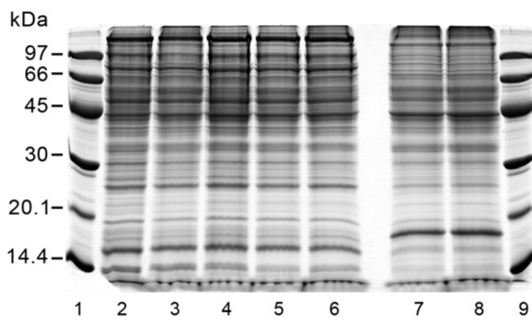

Fig. 3 – SDS-PAGE of protein extracts from adult *Angiostrongylus costaricensis* nematodes. Whole worms were ground in different solutions to optimize extraction conditions. The composition of solutions A–C is described in the Materials and methods section. Lanes 1 and 9, molecular mass markers; lane 2, female proteins extracted with solution A; lane 3, female proteins extracted with solution B; lane 4, female proteins extracted with solution C; lane 5, female proteins extracted with solution C +Complete™ protease inhibitor cocktail; lane 6, female proteins extracted with solution C and precipitated with ethanol/acetone; lane 7, male proteins extracted with solution C; lane 8, male proteins extracted with solution C and precipitated with ethanol/acetone. Gels (12%) were run under reducing conditions and stained with CBB R-250. The same amounts of protein (20 μg/10 μL) were applied in lanes 2–8.

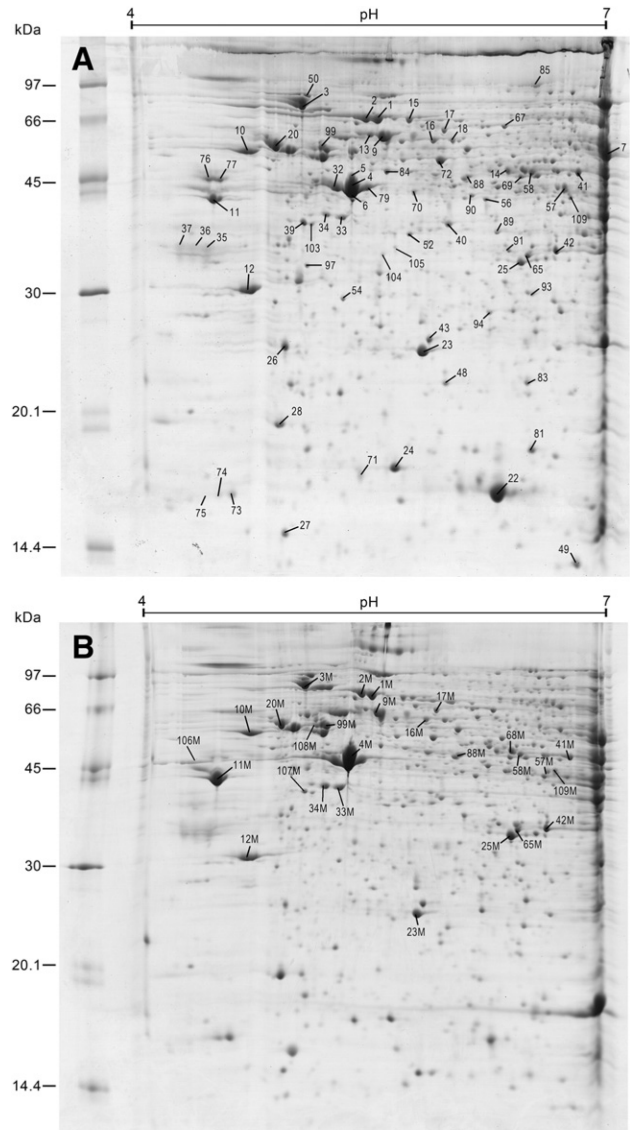

Fig. 4 – 2-DE gels of total protein extracts from adult *Angiostrongylus costaricensis* nematodes. Proteins (0.5 mg) from female (A) and male (B) worms extracted with solution C were separated by 2-DE on 18 cm IPG strips pH 4–7, followed by 15% SDS-PAGE. Gels were run under reducing conditions and stained with colloidal CBB G-250. The migration of molecular mass markers is shown on the acidic side of the gel. Numbers refer to the spot identity used in the tables. The letter M placed after the spot number indicates male proteins. Numbers without a letter refer to female proteins. Representative images of three independent replicates are shown.

pellets, the use of thiourea in combination with high concentrations of urea [86] dramatically increased the solubilization power of solution C as compared to solution B (Table 1). Under optimized conditions, male samples ground in solution C showed approximately the same extraction yield and percentage of protein recovery as females (Table 1). SDS-PAGE profiles were similar for both genders, although differences in the intensity distribution of the protein bands were evident (Fig. 3).

3.2. *Two-dimensional analysis of protein extracts*

To improve electrophoretic separation, extracts of female or male adult worms were independently fractionated by 2-DE. Using 11 cm IPG strips, pH 3–11 NL, approximately 800 protein spots were visualized by colloidal CBB staining (Fig. 1, Supplementary data). Although the body morphology and size are significantly different in female and male worms [87], their 2-DE profiles were similar, with less than 30% of unmatched spots being observed (which were concentrated in the more basic regions of the gels). Most protein spots (≈ 60%) from both genders were focused between pH 5 and 7, with molecular masses ranging from 20.1 to 66 kDa. In such broad-range IPG strips, more than one protein may be focused within the same gel spot [88]. Therefore, we decided to further improve the proteome analysis by using 18 cm medium-range IPG strips, pH 4–7 (Fig. 4). Approximately 1000 protein spots were detected between pH 4–7 on each individual gel. No significant gender-specific differences in expression levels were observed for 75% of these spots. Of the remaining 25% of protein spots, 7.5% and 10.4% were uniquely detected in female or male worms, respectively. These could represent true gender-specific proteins or quantitative differences between males and females (with the least abundant spots falling under the sensitivity threshold of the detection method used). Whether such differences represent biologically important variations remains to be determined.

3.3. *Protein identification by MALDI-PSD MS*

In an attempt to better characterize the protein profiles of *A. costaricensis* parasites, the most abundant spots shown in Fig. 4 were excised and analyzed by MALDI-PSD (Table 2 and Supplementary Table 1). It is important to note that only 12 gene sequences can be retrieved from the NCBInr database for *A. costaricensis* nematodes, all of which code for mitochondrial proteins. Hence, most of the uninterpreted experimental tandem spectra were matched to predicted fragment patterns from homologous species. To simplify the interpretation of the MALDI-PSD spectra, tryptic peptides were chemically derivatized with SPITC before MS analysis. This simple N-terminal sulfonation reaction leads to the formation of a much cleaner spectrum (almost exclusively) comprised of y-series ions, as b-series products are neutralized by a strongly negative modifying group [78,89]. Because the derivatization reaction is not 100% efficient, both derivatized and non-derivatized peptides were observed in most cases. Of the 106 dominant protein spots excised from 2-DE gels from females, 72 (68%) were identified by mass spectrometry. For male samples, 27 out of the 46 processed spots (59%) were positively identified. Although 16 spots gave rise to good quality MALDI-PSD spectra, they could not be identified; these peptide ions probably correspond to genes that have yet to be described.

3.4. *Interpretation of the identification results*

The GOEx tool [81] was used to search for associations between our MS data and Gene Ontology (GO) terms [84]. Several GO terms were statistically over-represented in our dataset, from which we highlight the following biological process terms: a) related to "macromolecule metabolic process" (GO:0009059-macromolecule biosynthetic process, GO:0019538-protein metabolic process, GO:0006508-proteolysis); b) related to "developmental process" (GO:0002164-larval development, GO:0048513-organ development, GO:0055115-entry into diapose); c) related to "response to stress" (GO:0006979-response to oxidative stress, GO:0006986-response to unfolded protein) and d) related to "biological regulation" (GO:0040008-regulation of growth, GO:0048518-positive regulation of biological process). The corresponding proteins for each enriched GO term are listed in Supplementary Table 2.

Not surprisingly, several of the most abundant proteins identified in *A. costaricensis* extracts were cytoskeleton-associated proteins, such as actin, myosin light chain, alpha tubulin, tropomyosin and collagen. These proteins play important roles in maintaining the body shape and muscle integrity of the nematodes [71,90,91]. The somatic musculature in nematodes is technically a part of the body wall, and it functions together with the pseudocoel and the cuticle as a hydrostatic skeleton [92] (Fig. 2G). Identified proteins involved in energy metabolism included, but were not limited to, cytochrome c oxydase, ATP synthase, enolase, glutamine synthetase, glutamate ammonia ligase, methionine adenosyltransferase and ABC transporter. Enolase is a multifaceted glycolytic protein that was traditionally thought to be restricted to the cytosol. Interestingly, some years ago, it was described on the surface of some helminths, where it binds to plasminogen and may be involved in the degradation of the host's extracellular matrix [93–96]. Proteins that directly interfere with host effector mechanism were also detected in the present proteomic study. Some of these are anti-oxidant proteins, such as peroxiredoxin, thioredoxin, translationally controlled tumor protein and aldehyde dehydrogenase. They effectively detoxify host-generated reactive oxygen species that could otherwise damage parasite cellular components, such as proteins, lipids and nucleic acids. Therefore, antioxidant proteins constitute a key factor favoring parasite survival inside the intravascular (mainly arterial) system, and thus contribute to the host–parasite relationship. These proteins are being investigated as putative protective anti-parasite vaccines [97]. Other noteworthy identified proteins include As37 and cyclophilins, which are members of the immunoglobulin family. The latter is a folding helper enzyme belonging to the peptidyl-prolyl cis–trans isomerase class [98]. Both proteins have previously been described in other parasitic nematodes, such as *Haemonchus contornus* [62] and *Brugia malayi* [67], although their role in the parasites' immune evasion remains unknown. Finally, we identified a 14-3-3 protein, a 30 kDa polypeptide belonging to a highly conserved family of molecules that regulate intracellular signal transduction and the cell cycle [99]. This protein has also been

observed in other helminths, such as *Echinococcus multilocularis*, *E. granulosus*[100] and *Schistosoma mansoni*. In the last species, the 14-3-3 protein is believed to be involved in parasite growth and survival [101] and is being evaluated as a vaccine candidate against schistosomiasis [102].

The systematic profiling of *A. costaricensis* proteins described above contributes to our understanding of the parasite's physiology. For example, this comprehensive molecular characterization may eventually help to explain why traditional anthelmintic drugs seem to induce erratic migration of these parasites, instead of killing them, which may exacerbate the consequences of the infection [10]. Proteomics could additionally unveil important molecules involved in host–parasite crosstalk, leading to the development of more effective therapeutic interventions for controlling the disease. For example, immunoreactive proteins from *A. costaricensis* nematodes are largely unknown, contributing to the difficulty involved in specifically diagnosing abdominal angiostrongyliasis in humans. A number of severe cases are confirmed through histopathological examination of specimens obtained after surgical treatment. Such drastic intervention may be necessary for the correction of intestinal perforations or obstructions that are eventually observed in angiostrongyliasis infections [25,29]. The first immunochemical investigations have used antigen preparations made from crude adult worm [22–24,103,104] or egg [28,105] extracts from *A. costaricensis*. However, it is well known that crude antigenic preparations are not suitable for immunodiagnosis due to their broad cross-reactivity with other helminth species. Ideally, purified antigens specific to the parasite should be used in immunodiagnostic tests [23].

3.5. *Analysis of immunogenic proteins*

In the present study, we exploited proteomic tools to specifically identify immunogenic proteins in *A. costaricensis*. These proteins were recognized after blotting 2-DE gels loaded with male or female total protein extracts onto PVDF membranes probed with antisera from Swiss Webster mice experimentally infected with *A. costaricensis* (Fig. 5). Overall, the immunoblots for both sexes showed similar profiles of reactive proteins, although some inter-gender variations were detected. One of the most striking differences observed was a stronger response for a group of 30–40 kDa female antigens focused between pH 4.5–5.5. Accordingly, when comparing adult worm antigens obtained under mild (non-denaturing) conditions in ELISA tests, Graeff-Teixeira et al. [103] reported that whole female extracts were twice as sensitive as male extracts in recognizing a proven acute human *A. costaricensis* infection. It was suggested that the strong antigenicity of eggs produced by female worms may contribute to explaining such differences [28]. In fact, each female presents a large number of eggs inside two uteri, which were obligatorily included in the proteomic analysis of the female pool (Fig. 2H).

Identifying immunoreactive spots on Western blots corresponding to CBB-stained proteins was not simple, mainly due to the poor correlation between immunogenicity and protein abundance, as described previously for other helminth parasites [62,67]. For example, actin spots were not recognized by antisera from infected mice although they represent the most abundant protein in the worm extracts. This was not unexpected because actin is a major constituent of eukaryotic cells and is widely observed throughout the animal kingdom, usually together with myosin [106,107]. It is unknown whether actin plays a critical role in *A. costaricensis* intestinal epithelial endocytosis [108]. On the other hand, strongly immunogenic proteins focused in the central region of the male blot corresponded to regions of the gel where several faintly CBB-stained spots (or no spots at all) could be detected (Fig. 2, Supplementary data). To further improve these results, we are presently carrying out assays for the direct detection of antigens in the polyacrylamide gels and/or immunoprecipitation (pull-down) followed by nLC–MS/MS analysis.

The only immunoreactive protein spots detected by mice antisera that could be unequivocally identified by MALDI-PSD were heat shock proteins (HSPs)[spots # 1(M), 2(M) and 3], a putative abnormal DAuer Formation family member [spot # 3 M] and galectins [spots # 25(M), 42(M) and 65(M)] (Fig. 5). HSPs and galectins, as well as several other non-immunogenic proteins of *A. costaricensis*, were found in multiple protein spots, indicating the presence of protein isoforms. Indeed, when staining the gels with ProQ-Emerald, a glycan-specific reagent, several protein spots were shown to be glycosylated (Fig. 3, Supplementary data), a common feature among helminth parasite antigens [109]. HSPs act as molecular chaperones, regulating protein folding in the cell. These proteins are related to the adaptive response of the parasite to the host immune system. Furthermore, in various infectious disease models, vaccination strategies using HSPs have induced significant protection [110]. Although HSPs also present a particularly high degree of structural conservation during evolution that must reflect the perpetuation of functions necessary for cell survival [111], their immunogenicity is highly dependent on the presence of functional phagocytic cells in the host [112]. Calreticulin and disulfide isomerase are other proteins related to protein folding that were identified in *A. costaricensis* extracts. In contrast to the HSPs, they were not immunogenic. Calreticulin is a well conserved 46 kDa protein that plays important roles in the regulation of key cellular functions [113]. This protein has been identified as a potent virulence factor in *Trypanosoma cruzi* [114], as necessary for stress responses and fertility in *C. elegans* [115] and as involved in immune responses in *Hekigmosomoides polygyrus* [116] and in *Necator americanus* [117]. Protein disulfide isomerase is a multi-functional enzyme that, in addition to its enzymatic activity involved in protein folding, seems to be essential for viability and extracellular matrix formation in *C. elegans* nematodes [118].

In male blots, immunogenic spot #3M was identified as a DAuer formation protein. In female blots, the corresponding less reactive spot matched an HSP. A BLASTp search in the NCBInr database indicated high sequence similarity (91%) between a Dauer formation protein and Heat Shock Protein 90. Dauer formation (*daf*) genes have been described as controlling both larval development and adult longevity in *C. elegans* [119,120]. These genes can prolong larval development under adverse environmental conditions, such as a lack of food and/or high temperature [121]. They also extend the adult lifespan during restricted nutrition periods and changes in temperature [122].

Table 2 – Summary list of the most abundant protein spots of *A. costaricensis* adult extracts identified by MALDI-PSD MS. The letter M placed after the spot number indicates male proteins. Numbers without a letter refer to female proteins. Protein analysis was performed by running the Mascot search engine against the NCBInr database. For a more detailed description of all identified proteins, see Supplementary Table 1.

Spot no.	Protein name
1, 1M, 2, 2M, 3	Heat shock protein
3M	Putative abnormal DAuer formation family member
4, 4M, 5, 6	Actin
7	Elongation factor 1 alpha
9, 9M	Heat shock protein
10, 10M	Calreticulin
11, 11M	Tropomyosin
12, 12M	Fourteen-three-three family member
13	Heat shock protein
14	Methionine adenosyltransferase
15	COLlagen family member
16, 16M	CCT-2
17, 17M, 18	Chaperonin containing TCP-1 family member
20, 20M	Protein disulfide isomerase
22, 23, 23M	Peroxiredoxin
24	Hypothetical protein Rsph17025_3168
25, 25M	Galectin
26	Translationally controlled tumor protein
27	D-aminoacylase domain protein
28	*C. briggsae* CBR-MLC-2.2 protein
32	Actin
33, 33M, 34, 34M	As37
35, 36, 37	Putative Lin-5 (five) interacting protein
39	Ribosomal protein, small subunit family member
40	Stress-induced-phosphoprotein 1
41, 41M	Enolase
42, 42M	Galectin
43	PREDICTED: similar to mitochondrial truncated thioredoxin-dependent peroxide reductase precursor
48	Predicted protein
49	ABC transporter related
50	Hypothetical protein T05E11.3
52	NAD-dependent epimerase/dehydratase
54	20S proteasome alpha5 subunit
56	Hypothetical protein F17C11.9
57, 57M	Glutamate–ammonia ligase
58, 58M	Enolase
65, 65M	Galectin
67	Chaperonin containing TCP-1 family member
68M	Ubiquinol-Cytochrome c oxidoReductase complex family member
69	CRE-AHCY-1 protein
70	Uracil-DNA glycosylase
71	Cytochrome C oxidase family member
72	Hypothetical protein BURPS1710b_A0185
73, 74	Alkali myosin light chain
75	SUMO (ubiquitin-related) homolog family member (smo-1)
76, 77	Putative nucleosome binding protein
79	Putative beta-actin
81	Hypothetical protein ckrop_1216
83	CalPoNin family member
84	PREDICTED: similar to aldehyde dehydrogenase 1A2 isoform 2
85	Primosomal protein N ’
88M	Hypothetical protein
89	Hypothetical protein Y24D9A.8
90	Activator of 90 kDa heat shock protein ATPase homolog 1
91	Galectin-1
93	*C. briggsae* CBR-PAS-6 protein
94	Heat shock protein
97	Predicted protein
99, 99M	Alpha tubulin
103	Protein farnesyltransferase/geranylgeranyltransferase putative
104	Hypothetical protein
105	Hypothetical protein Y46G5A.19

Table 2 (continued)

Spot no.	Protein name
106M	Putative nucleosome binding protein
107M	Putative histone-binding protein Caf1
108M	Alpha tubulin
109, 109M	Glutamate–ammonia ligase

Galectins were also recognized by mice antisera as immunogenic proteins in *A. costaricensis*. They were identified in several spots and are members of the galactoside-binding lectin family, being characterized by a typical motif of conserved amino acids in their carbohydrate recognition domain(s) [123]. The biological function of nematode galectins is not well understood, although they may be important for survival and interaction with the host [124]. Additionally, they seem to be involved in mediating immune recognition and modulation of the host response via an unknown mechanism, which may involve downregulation of the host's innate immunity [125]. Based on their primary structure and subunit architecture, galectins have been classified as proto (subunit molecular mass 14.5–16 kDa), chimera (29–35 kDa) and tandem repeat (32–36 kDa) types [126] or galectins 1–12 [127]. These proteins have been described in several organisms and in *C. elegans* galectin-1 appears to be associated with the cuticle and pharynx of the adult worm [128].

4. Conclusions

To our knowledge, this work represents the first systematic effort to characterize the proteome of male and female *A. costaricensis* worms. Several important features of these proteomes were uncovered, such as the identity of the dominant proteins in adult nematode extracts and the overall characteristics of antigens detected by antisera from infected rats. These results will certainly contribute to improving our understanding of the host–parasite relationship, as well as assisting searches for candidate proteins for diagnostic assays and the treatment of abdominal angiostrongyliasis.

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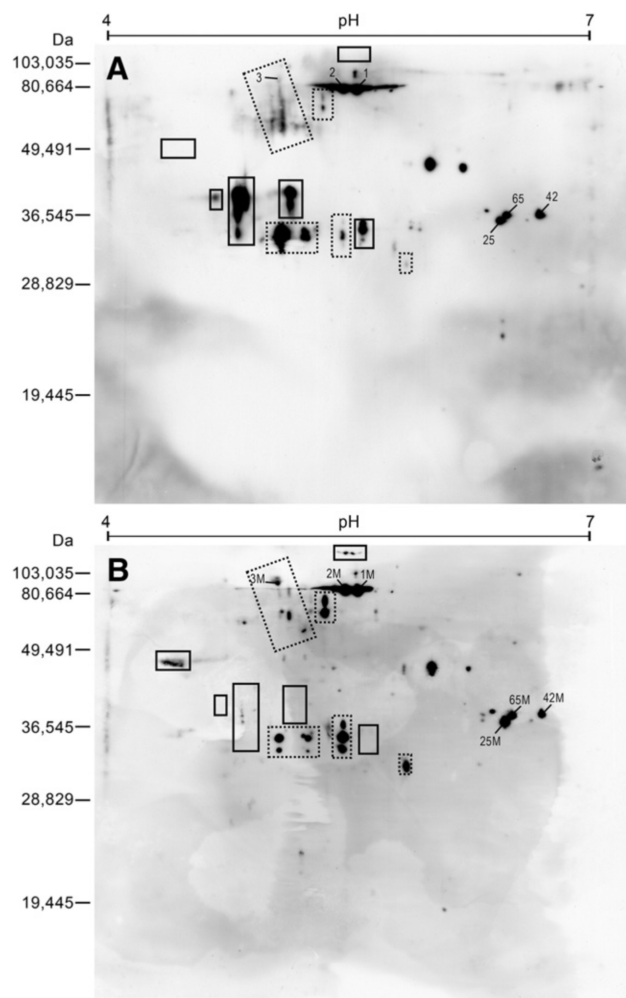

Fig. 5 – 2-DE/immunoblot of protein extracts from adult *Angiostrongylus costaricensis* nematodes. Proteins (0.1 mg) from female (A) and male (B) worms extracted with solution C were fractionated on 18 cm IPG strips pH 4–7, followed by 15% SDS-PAGE (reducing conditions), and further electroblotted onto a PVDF membrane that was probed with antisera from mice infected with *A. costaricensis*. After incubation with an anti-mouse secondary antibody conjugated to HRP, the membrane was developed by the addition of an ECL substrate. The migration of pre-stained molecular mass markers is indicated on the acidic side of the gel. Dotted-line boxes enclose protein spots with different signal intensities on female and male blots. Regions containing reactive spots exclusively found in one gender are indicated by solid-line boxes. Numbers refer to the spot identity used in the tables. The letter M placed after the spot number indicates male proteins. Numbers without a letter refer to female proteins.

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