PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

Estudo de proteínas de *Angiostrongylus cantonensis* para o entendimento da relação parasito-hospedeiro e análise de alvos para o diagnóstico das angiostrongilíases

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TESE DE DOUTORADO

Porto Alegre – RS - Brasil

2011

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Agradecimentos

Desde 2008, ao iniciar o doutorado pensei várias vezes no momento de escrever esta página. Pensei em todas as pessoas que gostaria de agradecer. Então percebi que muitas pessoas já faziam parte do meu crescimento científico há muito tempo. Na verdade a história começa em 1996 quando entrei no Laboratório de Parasitologia da PUCRS. Meu orientador Carlos Graeff Teixeira me encantou desde o primeiro minuto quando o abordei na descida de uma escada e perguntei: - “prof. eu queria falar com o senhor”… ele prontamente respondeu com um sorriso, - “fale agora!” Naquele momento entendi que nos daríamos bem! Ele me encaminhou à então técnica do laboratório, dona Eva Medeiros quem, me ensinou a tratar os animais, processar amostras, e me apresentou o mundo fascinante de enxergar os ovos de parasitos ao microscópio.

O principal sentimento de gratidão vem à tona quando penso em TUDO o que não sabia! E agora sei!

Desde lá foram muitas as conquistas e aprendizados, por vezes alguns percalços, mas que hoje vejo, também serviram para o meu crescimento! Foram muitas pessoas com quem convivi e aprendi, fosse a fazer um gel, analisar uma sequência, ou ler um artigo (nunca vou me esquecer Rô, quando me ajudaste a ler os artigos das caspases!!). Aprendi a discutir, criticar e interpretar resultados (agradeço em especial ao amigo prof. Dr. Carlos Alexandre S. Ferreira, pelas várias discussões sobre as GSTs e resposta imune). Duas outras pessoas maravilhosas, João H.C. Kanan e Gustavo Chemale, meus orientadores do mestrado, foram

fundamentais para o meu crescimento, que com muita dedicação e carinho me ensinaram que não há nada neste mundo que eu não possa aprender!

Esta tese foi construída ao longo de quase quatro anos e mais uma vez contou com a ajuda e dedicação de muitas pessoas:

De novo ao meu querido orientador Carlos, ou C.! (Depois de um tempo entendi. Ele não tem tempo de escrever todo nome para assinar os e-mails!). Uma pessoa que não tem medo de ter idéias diferentes, partilha seu conhecimento com a maior simplicidade e usa sua sabedoria para conduzir um Grupo de sucesso. E principalmente promove, com suas Atitudes, oportunidade para as pessoas crescerem como cientistas e seres humanos!

Aos meus também orientadores do doutorado sanduíche no CDC (*Centers for Disease Control and Prevention*), Patty Wilkins pela confiança e reconhecimento do meu trabalho. E em especial ao Alex, Alexandre J. da Silva, meu mestre *Jedi*, uma pessoa simples e acessível que com muita competência conduz um laboratório de renome. Proporcionou excelentes oportunidades para a minha vida profissional e me levou para o convívio de sua família linda!

Ao Grupo de Parasitologia da PUCRS por ter esta atmosfera de luta conjunta para a realização dos projetos de pesquisa. Agradeço pela acolhida, ajuda e companheirismo de todos! Em especial aos amigos Juliano Romanzini, Priscilla S. Pedersen, Rafael L. Maurer, Ana Cristina A. da Silva, Renata Ben, Letícia Silva, Camila Krug, Renata Russo, Bianca Cognato, Joana Borges, Carla Müller, Bárbara Rodrigues, Carolina Veríssimo, Silvana L. Alves, Cristiane C. Franceschina e Nicolle F. Jovchelevich.

Aos grupos de pesquisa do CDC dos Laboratórios de Diagnóstico de Doenças Parasitárias em: Biologia Molecular, Imunoquímica e Morfologia. Pela carinhosa acolhida e ajuda na realização dos experimentos. Em especial ao Keith Levert, Lisa Rascoe, Sukwan Handali, Pat Lee, John Noah, Henry Bishop, Marcos Almeida, Janine Paulaskas e ao anjo Isabel McAuliffe que me acolheu como uma filha.

Ao grupo de pesquisa do Laboratório de Fisiologia da Conservação da PUCRS em especial à profa. Dra. Guendalina T. Oliveira e à Bibiana K. Dutra pelo apoio e discussões.

Ao grupo de pesquisa dos Laboratórios de Biologia Molecular de Cestódeos e de Genômica Estrutural e Funcional do Centro de Biotecnologia da UFRGS, em especial ao prof. Dr. Henrique B. Ferreira pela colaboração na pesquisa.

Aos amigos de fé, Larissa S. Heinzelmann, Maria Gabriela V. Gottlieb, Rosane S. Silva e Paulo M. Pinto.

Com muito amor e carinho agradeço aos meus pais, que são meu exemplo, minha raiz, minha força.

Aos meus amados irmãos, por serem essas pessoas tão profundamente minhas, que completam a energia que preciso para viver.

Ao Vlad, meu amado marido! Meu maior aliado e amigo, com quem brinco e cresço todos os dias!

Resumo

O gênero *Angiostrongylus* Kamensky, 1905 agrupa animais pertencentes ao filo Nematoda, cuja característica marcante é a forma corporal cilíndrica. Duas espécies possuem importância médica: *A. costaricensis* cujo habitat natural são as artérias mesentéricas de camundongos silvestres e na infecção humana pode levar ao desenvolvimento de angiostrongilíase abdominal; e *A. cantonensis* que habita as artérias pulmonares de roedores e na infecção humana pode causar meningoencefalite eosinofílica. O diagnóstico de ambas as doenças é dificultado pela ausência de formas parasitárias nas fezes, no caso de infecções por *A. costaricensis* e raramente encontradas no líquido cefaloraquidiano no caso de meningoencefalite eosinofílica. Muitos estudos vêm sendo desenvolvidos para o aprimoramento da detecção das angiostrongilíases visando testes que sejam capazes de discernir das diferentes infecções parasitárias de forma sensível e específica. O antígeno de 31kDa é considerado atualmente o principal antígeno para o diagnóstico da meningoencefalite eosinofílica, causada por *A. cantonensis,* entretanto é proveniente da purificação de extratos brutos do parasito o que acarreta num processo laborioso e dispendioso que em última análise gera quantidades insuficientes para que haja ampla distribuição e compartilhamento entre os centros de diagnóstico. Com o intuito de aprimorar o diagnóstico sorológico das angiostrongilíases e tornar os antígenos disponíveis globalmente o presente trabalho buscou identificar novos alvos antigênicos e caracterizar o antígeno de 31kDa para posterior propagação de formas recombinantes. Além disso, foram estudadas moléculas que podem ser fundamentais na manutenção do parasitismo, que futuramente poderão ser alvos para o tratamento das angiostrongilíases. Duas fontes de antígenos a partir de vermes adultos fêmeas foram empregadas: produtos de excreção e secreção (ES) e extrato bruto (TE). Nos ES foi detectada a atividade de enzimas antioxidantes como catalase e superóxido dismutase e identificadas, por western blot e espectrometria de massas (MS), 17 proteínas alvo para o diagnóstico e tratamento das angiostrongilíases dentre elas hemoglobinases, proteínas de choque térmico e inibidores de proteases. Nas amostras de TE além da identificação de enzimas antioxidantes, estavam presentes glutationas transferases (GST), outra classe de enzimas de defesa. Estas proteínas foram purificadas por cromatografia de afinidade e analisadas por MS o que revelou sequencias peptídicas homólogas a GST de classe sigma. Em TE também foi possível a caracterização do antígeno de 31kDa que quando submetido a eletroforese bidimensional mostrou-se ser composto por 4 *spots* com ponto isoelétrico (pI) em torno de 4,5 sendo reconhecidos pelo soro de pacientes infectados com *Angiostrongylus* spp. Os *spots* foram analisados por MS e três diferentes proteínas foram identificadas: 14-3-3; proteína com domínio NAC e a subunidade épsilon do coatamero. O antígeno de 31kDa foi caracterizado como uma glicoproteína através de estudos de oxidação de glicídeos, onde se observou que a antigenicidade dos 4 *spots* foi dependente de resíduos de açúcar. As sequências de DNA dos antígenos foram obtidas pelo sequenciamento aleatório do genoma pela plataforma 454 (Roche) e depositadas no Genbank. Os dados gerados no presente trabalho contribuem de forma significativa para o desenvolvimento de antígenos recombinantes que poderão ser amplamente distribuídos para validação e aplicação em diagnóstico.

Abstract

The genus *Angiostrongylus* Kamensky, 1905 belongs to the Phylum Nematode, with round shape as its main feature. Two species have medical importance, *A. costaricensis* living in mesenteric arteries of wild mice and causing abdominal angiostrongyliasis in human and *A. cantonensis* which lives in pulmonary arteries of rats and may cause eosinophilic meningoencephalitis in humans. The diagnosis of both diseases is difficult due to absence of parasite in feces in case of the infection by *A. costaricensis* and seldom detected larvae in the cerebrospinal fluid in case of eosinophilic meningoencephalitis. Several studies have been performed to improve the diagnosis of angiostrongyliasis which should be able to differentiate in a specific and sensitive way among other parasitic infections. The 31kDa antigen has been considered the main antigen for eosinophilic meningoencephalitis diagnosis due to *A. cantonensis* infection. However this antigen is obtained from crude extracts of the worm by a laborious process of purification with low yielding and insufficient amount for large distribution to other diagnostic centers. In order to improve the serologic diagnostic of angiostrongyliasis and make the antigens widely available the present work aimed to identify new antigenic targets and also characterize the 31kDa antigen for further recombinant production. Besides that, essential molecules for parasite survival were investigated which in the future may be targets for disease treatment. Two sources of antigen from female worms were used: excretion and secretion products (ES) and total extract (TE). In ES, sample antioxidant enzymes activity were detected such as catalase and superoxide dismutase. Also was identified by Western blot and Mass spectrometry (MS), 17 proteins target for disease diagnosis and treatment like hemoglobinases, heat shock proteins and proteases inhibitors. In TE sample antioxidant enzymes as well as glutathione transferases (GST) which is another kind of defense enzyme were also detected. GSTs were purified by affinity chromatography and analyzed by MS. Peptide sequences from this experiment matched with homologous sequences of sigma class GST. In TE samples was possible to characterize the 31kDa and after two-dimensional electrophoresis was shown to be composed of four spots around 4.5 of isoelectric point (pI) and being recognized by sera from patients infected with *Angiostrongylus* spp. The spots were analyzed by MS and three different proteins were identified: 14-3-3 protein, NAC domain containing protein, and epsilon subunit of the coatomer protein complex isoform 2. The 31kDa antigen was characterized as a glycoprotein through studies of oxidation of carbohydrate where it was observed that the antigenicity of four spots was dependent on sugar residues. The DNA sequences of the antigens were obtained by random sequencing of the genome for 454 platform (Roche) and deposited in Genbank. The data generated in this study contribute significantly to the development of recombinant antigens that may be widely distributed for independent diagnostic validation.

Apresentação

A presente tese é composta por quatro artigos científicos apresentados nos capítulos:

No capítulo 1 é apresentado o artigo publicado na revista *Experimental Parasitology* em fevereiro de 2011, Detection of anti-oxidant enzymatic activities and purification of glutathione transferases from *Angiostrongylus cantonensis*. Neste trabalho foi investigada a presença de moléculas em amostras de excreção e secreção e extrato total, com potencial papel nos mecanismos de evasão do sistema de defesa do hospedeiro.

No capítulo 2 é apresentado o artigo publicado na revista *Experimental Parasitology* em novembro de 2011, Characterization of *Angiostrongylus cantonensis* excretory-secretory proteins as potential diagnostic targets. Neste estudo foram identificados potenciais alvos para o diagnóstico das angiostrongiliases em amostras de ES.

No capítulo 3 é apresentado o artigo de revisão submetido em novembro de 2011 à revista *International Journal of Inflammation*, para a edição especial em *Inflammation in Eosinophilic Meningitis,* Interface molecules of *Angiostrongylus cantonensis* – their role in parasite survival and modulation of host defenses. Este estudo explorou o potencial papel das proteínas de ES, identificadas previamente no capítulo 2, no estabelecimento e desenvolvimento da meningite eosinofílica.

No capítulo 4 é apresentado o artigo submetido para publicação na revista *International Parasitology* em setembro de 2011, The 31 kDa antigen of

Angiostrongylus cantonensis comprises an antigenic glycoprotein complex. Neste trabalho o antígeno de 31kDa de *Angiostrongylus cantonensis* foi identificado e caracterizado.

Após os quatro artigos, se apresenta o tópico de conclusões gerais, onde são compilados os principais resultados obtidos nos artigos e também se discute perspectivas futuras.

Por fim a tese é complementada por três apêndices onde se apresenta: 1) o estudo do potencial uso das GSTs como antígeno diagnóstico; 2) clonagem e expressão de algumas proteínas de *A. cantonensis*; 3) artigo em preparação: *Angiostrongylus cantonensis* – The Dirty Genome Approach.

Capítulo 1

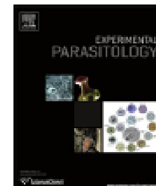
Detection of anti-oxidant enzymatic activities and purification of glutathione transferases from *Angiostrongylus cantonensis*

Experimental Parasitology 127 (2011) 365–369

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr



Detection of anti-oxidant enzymatic activities and purification of glutathione transferases from *Angiostrongylus cantonensis*

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A R T I C L E   I N F O

Article history:
Received 25 May 2010
Received in revised form 19 August 2010
Accepted 20 August 2010
Available online 31 August 2010

Keywords:
Anti-oxidant enzymes
Superoxide dismutase
Catalase
Glutathione peroxidase
Glutathione transferase
Angiostrongylus cantonensis

A B S T R A C T

There are several anti-oxidant enzyme families that play pivotal roles in facilitating the survival of parasites. Glutathione transferases (GSTs) are members of the anti-oxidant family that can detoxify a broad range of exogenous or endogenous compounds including reactive oxidative species. GSTs have been studied as vaccine candidates, immunodiagnostic markers and as treatment targets. Helminths of the genus *Angiostrongylus* live inside arteries of vertebrates and two main species are associated with accidental human infections: *Angiostrongylus costaricensis* adult worms live inside the mesenteric arteries and larvae of *Angiostrongylus cantonensis* become trapped in the central nervous system vasculature. Since the interactions between angiostrongylid nematodes and their vertebrate hosts are poorly understood, this study characterized the anti-oxidant enzymatic activities of *A. cantonensis* from female worms by collecting excreted and secreted (ES) and total extract (TE) molecules. Catalase (CAT) and superoxide dismutase (SOD) activities were found both in the ES and TE while glutathione peroxidase (GPX) and GST were found only in the TE. GSTs were purified by glutathione agarose affinity column (AcGST) and the pool of eluted GSTs was analyzed by mass spectrometry (LC-MS/MS) and *de novo* sequencing (Masslynx software). Sequences from two peptides (AcGSTpep1 and AcGSTpep2) present high identity to the N-terminal and C-terminal from sigma class GSTs of nematodes. It is known that these GST enzymes are associated with host immune regulation. Furthermore, understanding the role of parasite-derived anti-oxidant molecules is important in understanding host-parasite interactions.

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1. Introduction

Parasitism is dependent on the parasite's ability to subvert the host immune defenses, thereby facilitating long-term survival (Maizels et al., 1993; Henkle-Dürhsen and Kampkötter, 2001). In addition to subverting traditional immunologic responses, parasites also must avoid a number of potent cytotoxic and proinflammatory agents including reactive oxygen species (ROS) produced by activated macrophages (Behm and Ovington, 2000). ROS include O_2^-, H_2O_2, singlet oxygen ($^1O_2$), and the hydroxyl radical (OH.) that may cause damage to cell membranes, proteins and nucleic acids, eventually killing the parasite. As a consequence, parasites are endowed with the capacity of producing several enzymes with the potential of interfering with ROS activity *e.g.*, parasite-derived superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione transferases (GST), and catalase (CAT) (Dzik, 2006).

SODs (E.C. 1.15.1.1) are a group of metalloenzymes that catalyze the dismutation of the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2). CAT (E.C. 1.11.1.6) and GPX (E.C. 1.11.1.9) are implicated in the conversion of H_2O_2 into water. SODs are classified according to their active site binding metal *e.g.*, iron, manganese, nickel or copper/zinc. The Cu/Zn SOD can be extracellular or cytosolic, whereas the Mn SOD is mitochondrial. Fe SOD is typically found only in prokaryotes (Fridovich, 1995) and the Ni SOD has been described in *Streptomyces* species (Youn et al., 1996). The GST family (EC 2.5.1.18) includes phase II detoxification enzymes that catalyze the conjugation of the tripeptide glutathione (GSH) into a wide range of exogenous/endogenous toxic compounds, including those derived from oxidative burst (Hayes et al., 2005).

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0014-4894/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2010.08.018

These enzymes have already been used as vaccine candidates (Rao et al., 2003; Preyavichyapugdee et al., 2008), in immunodiagnostics (Cheng et al., 2007) or treatment targets *e.g.*, therapies aimed at interfering with their function (Torres-Rivera and Landa, 2008).

The genus *Angiostrongylus* has two species that can cause human diseases: *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis*. The former is neurotropic and causes eosinophilic meningoencephalitis. The latter parasitizes the mesenteric arteries and is the etiologic agent of abdominal angiostrongyliasis. The experiments presented in this report are focused on *A. cantonensis* that can lead to three main clinical presentations following infection: eosinophilic meningitis, eosinophilic encephalitis or the less common ocular angiostrongyliasis (Sawanyawisuth and Sawanyawisuth, 2008). Adult worms live in the pulmonary arteries of rats, where eggs hatch in pulmonary tissues and first-stage larvae (L1) are passed in the feces. Intermediate hosts, such as snails become infected by ingesting food contaminated with larvae or feces directly. The life cycle is completed when rats ingest third-stage larvae (L3) released from infected snails. Larvae migrate to the meningeal blood vessels, molt into fifth stage larvae and develop into adult worms in the lungs. In humans, the young adults die in the central nervous system, causing severe inflammation. Cases have been reported from Southeast Asia, Africa, Australia, and America (Wang et al., 2008) including recently detected transmission foci in Brazil (Caldeira et al., 2007) and Ecuador (Pincay et al., 2009).

Despite the public health importance of *A. cantonensis* infections, the biology of this parasite is poorly understood. The aims of this study were to identify anti-oxidant enzymes and to purify and characterize GTSs from *A. cantonensis*.

2. Materials and methods

2.1. Biological materials

Adult *A. cantonensis* worms were recovered from experimentally infected rats. *A. cantonensis* were obtained from the Department of Parasitology, Akita Medical School, Japan and have been maintained in our laboratory since 1997. Wistar rats served as definitive hosts and *Biomphalaria glabrata* as intermediate hosts. Rats were infected with 104 larvae by gavage inoculation. After 42 days the animals were euthanized for collection of the worms.

Animal handling was carried out according to Brazilian regulations, law 11794–08/10/2008, *Decreto* 6899–15/07/2009 and the recommendations issued by the *Conselho Nacional de Controle de Experimentação Animal* (CONEA), and the protocol was approved by the University Ethics Committee.

2.2. Excretion and secretion products (ES)

To identify the activity of anti-oxidant enzymes in ES products, adult worms were collected and washed three times with PBS (phosphate buffered saline) to eliminate host cell contamination. Three hundred female worms were maintained in 20 mL RPMI 1640 culture medium (Invitrogen, Carlsbad, CA) supplemented with 100 μg mL^{-1} penicillin and 100 U mL^{-1} streptomycin at 37 °C in 5% CO_2. Worms were placed in fresh medium every 24 h for 3 days. Exhausted medium was centrifuged at 15,000*g* for 10 min and supernatants were used as the source for ES products.

2.3. Total extracts (TE)

Protein extracts from *A. cantonensis* were obtained by homogenizing adult worms in lyses buffer (50 mM Tris, pH 7.4, 0.1% Triton-X100, 5 mM DTT) in liquid nitrogen. After homogenization, samples were centrifuged at 15,000*g* for 1 h at 4 °C to separate soluble proteins defined as the TE. Protein concentrations were estimated using the Bradford method.

2.4. Anti-oxidant enzyme activities

SOD activity was assayed as described by Misra and Fridovich (Misra and Fridovich, 1972). Briefly, the activity of SOD present in the homogenate was assessed spectrophotometrically at 480 nm by measuring SOD-mediated inhibition of epinephrine auto-oxidation. The amount of enzyme that inhibited 50% of the maximum epinephrine auto-oxidation is defined as 1U of SOD activity. CAT activity was determined by measuring the exponential disappearance of H_2O_2 at 240 nm and was expressed as pmoles per milligram of protein (Boveris and Chance, 1973). GPX activity was assessed as described by Wendel (Wendel, 1981). NADPH (5 mM) oxidation was monitored at 340 nm and expressed in nmol NADP per min per protein. The GST activity was measured according to Boyland and Chasseaud (Boyland and Chasseaud, 1969) by measuring the conjugation of 1-chloro 2,4 dinitrobenzene (CDNB) with reduced glutathione (GSH) activity as a function of increasing absorbance values at 340 nm. Data are expressed as mean ± standard error of mean of triplicate samples.

2.5. GST Purification

A. cantonensis GSTs were purified from the protein extract using a glutathione-agarose (Sigma–Aldrich, St. Louis, MO) affinity column at 4 °C. The sample was passed through the column overnight and eluted with reduced glutathione (10 mM) according to manufacturer's instructions. The eluted protein was applied onto 12% SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (1-DE) and stained with Coomassie blue.

2.6. Two dimensional electrophoresis (2-DE)

Proteins eluted from the GST columns were precipitated with an equal volume of ice-cold 20% TCA in acetone (v/v) and washed twice in ice-cold acetone before solubilization with sample buffer consisting of 7 M urea, 2 M thiourea, 4% w/v CHAPS, 66 mM DTT and 0.5% carrier ampholytes (v/v) (Biolyte 3–10, Bio-Rad, Hercules, CA). Samples were in-gel rehydrated and focused on 7 cm pH 3–10NL IPG strips (GE Healthcare, Piscataway, NJ). The focusing was performed using a Multiphor II (GE Healthcare) under the following conditions: 60 V 30 min, 340 V 60 min, 1200 V 90 min and 3500 V 145 min.

After focusing, strips were equilibrated for 15 min in reducing equilibration buffer (30% v/v glycerol, 6 M urea, 1% DTT) followed by a 15 min incubation in alkylation equilibration buffer (30% v/v glycerol, 6 M urea, 4% iodoacetamide). IPG strips were run in the second dimension on 10 × 10 cm SDS–PAGE gels (12.5% acrylamide) at 60 V for the first 30 min and 100 V for 120 min. Gels were stained with Coomassie blue.

2.7. Mass spectroscopy

The manually excised spots from 1-DE and 2-DE gels were in-gel digested with trypsin (Promega, Madison, WI) according to the following protocol: three washing steps with 50% acetonitrile (ACN) and 50 mM ammonium bicarbonate for 15 min, followed by ACN. Gels were dried by vacuum centrifugation and the proteins were digested for 18–24 h at 37 °C. Peptides were extracted in 1 mL of 50% ACN and 5% TFA for 1 h. The peptides were then dried and re-suspended in 10 μL of 1% formic acid. The resulting peptide mixture was desalted using a ZipTip® column (Millipore, Billerica, MA).

For MALDI-TOF analysis, a 1 µL crude sample digest was mixed with 1 µL of α-cyano-4-hydroxycinnamic acid (10 mg mL^{-1} in 0.1% TFA in 1:1 ACN/methanol) and an aliquot of 0.5 µl was delivered to the target plate and dried at room temperature. MS was performed on MALDI Micro MX (Waters Corp., Milford, MA), operated in the reflectron mode for MALDI-TOF peptide mass fingerprint (PMF). Each spectrum was produced by accumulating data from 2000 consecutive laser shots. Peptides were identified by matching the measured monoisotopic masses to theoretical monoisotopic masses generated using the MASCOT search engine (http://www.matrixscience.com). Maximum mass errors of 100 ppm were allowed for validation of PMF protein identifications.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was made using an ESI-Q-TOF ultima API mass spectrometer (Micromass, Manchester, UK) coupled to a nanoACQUITY UltraPerformance LC® (UPLC®, Waters Corp.). A nanoflow ESI source was used with a lockspray source for lockmass measurements during all the chromatographic runs. The peptides were separated in a Nanoease C18 (75-m ID) capillary column by elution with a water/acetonitrile 0.1% formic acid gradient. Data were acquired in data-dependent mode (DDA) and multiple charged peptide ions (+2 and +3) were automatically mass selected and dissociated in MS/MS experiments. Typical LC and ESI conditions consisted of a flow of 600 nl/min, nanoflow capillary voltage of 3.3 kV, block temperature of 100 °C and cone voltage of 100 V. Each sample was analyzed three times to demonstrate the reproducibility and consistency of the technique.

The MS/MS spectra were processed using Proteinlynx 2.0 software (Waters Corp.) and the generated PKL file was used to perform database searches using Mascot software (Matrix Science, London, UK). Search parameters allowed a maximum of one missed cleavage, cysteine modifications, methionine oxidation, peptide tolerance of 100 ppm and MS/MS tolerance of 0.1 Da. The significance threshold was set at $p < 0.05$, and identification required that each protein contain at least one peptide with an expected value < 0.05. The similarity searches were done using the BLAST platform and alignments were performed using the CLUSTAL W (1.83) multiple sequence alignment algorithm.

Table 1
Antioxidants enzymatic assays.

Enzyme	TE	ES	Unity
Superoxide dismutase	23.68 ± 2.95	28.31 ± 3.37	U/mg of protein
Catalase	2.14 ± 0.05	4.34 ± 0.13	pmoles/mg of protein
Glutathione S-transferase	32.29 ± 10.42	ND	U/mg of protein
Glutathione peroxidase	631.319 ± 0.40	ND	nmol NADP/min/mg of protein

In order to access the antioxidant profile of *A. cantonensis* SOD, CAT, GPX and GST were assayed both in TE and ES samples. ND: not detected.

3. Results

3.1. Anti-oxidant enzymatic assays

The anti-oxidant enzyme profile for SOD, CAT, GPX and GST from *A. cantonensis* was measured (Table 1). CAT displayed activity in both extracts but was higher in the ES than in the TE. Similarly, SOD displayed activity in both extracts with higher activity reported in the ES extract. The activity of GPX and GST was seen only in the TE extract samples.

3.2. Purification of GSTs

A. cantonensis soluble protein extracts (4 mg) were applied to a glutathione agarose affinity column for GST purification. After GST elution from the column and electrophoresis migration, the proteins were found between 20–25 kDa which is expected for this family of proteins (Fig. 1A).

The purified *A. cantonensis* are referred to as AcGSTs which comprises 2% of the total soluble protein extracts (0.102 mg). To analyze the pattern of AcGST, 0.02 mg were applied to 7 cm IPG strips for 2-DE analysis. Nine spots were detected after electrophoresis (Fig. 1B).

3.3. Mass spectroscopy and protein identification

AcGSTs were excised from the gel and subjected to MALDI-TOF analysis. Since this approach did not result in the successful identification of the target protein homologs, the pool of eluted AcGSTs was extracted from 1D gel and digested as previously mentioned. The AcGSTs were then analyzed by LC-MS/MS and Masslynx software was used for *de novo* sequencing. This analysis identified two peptides (designated AcGSTpep1 and AcGSTp2) whose sequences were subjected to BLAST analysis for the identification of related sequences.

The BLAST analysis identified similarity between the AcGSTpep1 peptide and a C-terminus sequence present in the glutathione transferase from *Oesophagostomum dentatum* and *Ancylostoma caninum* and a similarity between the AcGSTpep2 peptide and the N-terminus of the glutathione transferase from *Heligmosomoides polygyrus*, *Caenorhabditis elegans*, *Haemonchus contortus* and *O. dentatum* (Table 2).

The respective sequences were further subjected to CLUSTAL W (1.83) multiple sequence alignment algorithm analysis for the

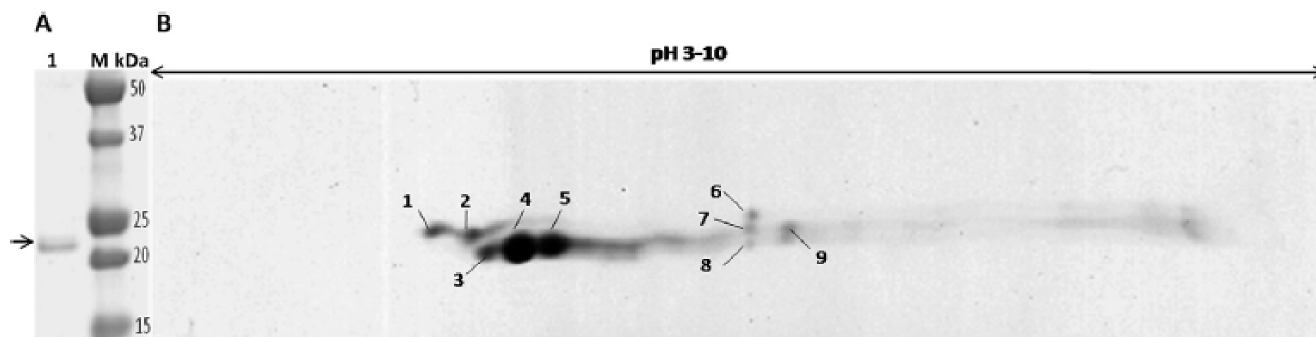

Fig. 1. Purification of GSTs from *A. cantonensis*. (A) SDS–PAGE was carried out to visualize the purity of eluted proteins. (Lane 1) 0.3 ug of AcGSTs and M are the protein standard markers showed in kDa. (B) 2D Electrophoresis from *A. cantonensis* GSTs. Isoelectric focusing was carried out in 7-cm immobilized pH gradient strips pH 3–10NL. The second dimension was carried out in SDS–polyacrylamide gels. The gels were stained with Coomassie blue reagent. The arrow indicates AcGST.

Table 2
Protein identification.

Peptide	Sequence	Organism matched	Protein matched	Identity	GeneBank access
Acgstpep1	ESFPFGQVPVLEVDGK	*O. dentatum*	GST-2	100%	gb|ACA30415.2
		C. elegans	GST-9	100%	gb|AAA83164.1
		H. contortus	GST	86%	gb|AAF81283.1
		H. polygyrus	GST-2	100%	gb|AAF36480.1
Acgstpep2	RVPGFLDGFPEVK	*O. dentatum*	GST-2	84%	gb|ACA30415.2
		A. caninum	GST	75%	gb|AAT37718.1

The obtained peptides from the pool of GSTs eluted proteins were searched for amino acid similarity on BLASTp public platform.

```
AcGSTpep1          -------------------------------------------ESFPFGQVPVLEVDGK- 16
C. elegans       1 MVSYKLIYFQSRGNGEIARQVFAFAGQEFIDERISKEQWAEIKNMTPFGQVPVLEVDGRQ 60
H. contortus     1 MVHYKLTYFNGRGAAEIIRQVFVLAGQDYEDVRLTHEEWPKHKASMPFGQLPVLEVDGKQ 60
H. polygyrus     1 MVHYKLTYFNGRGNGECARQIFAVAGQQYEDVRLTHEQFAPMKPNLPFGQVPVLEVDGKQ 60
A. caninum       3 --HYKLTYFNGRGAGECARQIFALADQKYEDVRLTQETFAPLKATFPFGQVPVLEVDGQQ 58
                                                                ****:*******::

AcGSTpep2          ----------------------------------RVPGFLDGFPEVK------------- 13
O. dentatum    137 ITKFLKKSKSGFLVGDKVTWVDLLISEHMADMSARIPEFLDGFPEVKAHMEKVRSIPALK 196
A. caninum     137 ITKFLKNNPSGFLVGDSVTWIDLLLAEHASDIQSKVPEYLEGFPEVKAHMEKVRSIPKLK 196
                                                     ::*::*:******
```

Fig. 2. Multiple alignments of GSTs from different species. The two peptides from *A. cantonensis* were aligned by CLUSTAL W (1.83) algorithm. The GeneBank access of all used sequences can be found on Table 2. Gray amino acids as well as the symbol (:) indicate not matched amino acid and the symbol (*) indicate perfect aligned amino acids related to each peptide. The species are: *Caenorhabditis elegans*; *Haemonchus contortus*; *Heligmosomoides polygyrus*; *Ancylostoma caninum* and *Oesophagostomum dentatum*.

identification of conserved sequences. This analysis revealed conserved amino acid sequences between AcGSTpep1 and two sequences from *A. cantonensis* to related proteins in other nematodes, suggesting that AcGSTpep1 and AcGSTpep2 belonged to the sigma class GST enzymes (Fig. 2).

4. Discussion

To date, there are no data describing the identification of *Angiostrongylus* species-derived anti-oxidant enzymes. Data presented in this report for the first time describe the identification of CAT, GPX, GST and SOD activities from *A. cantonensis*, the causative agent of eosinophilic meningoencephalitis (Wang et al., 2008).

Different parasite species utilize different enzyme-based defense strategies to combat the deleterious effects of ROS produced by the host, *e.g.*, catalase from *H. contortus* plays an important detoxification role since inhibiting its function increased the susceptibility of this nematode to hydrogen peroxide (Kotze, 2003). However, not all parasites posses these defensive enzymes and have not been identified, for example, in *Schistosoma mansoni* (Mkoji et al., 1988).

Our findings showed that catalase activity was present in both TE and ES extracts at very low levels similar to what has been described in other helminthes (Dzik, 2006), however, GPX activity was only detected in the TE preparation. Previously, GPX was thought to be absent in parasites (Callahan et al., 1988) but its activity has been shown to be very important for defense against ROS in *S. mansoni* (Mourão et al., 2009). The *A. cantonensis* anti-oxidant defense system comprised of CAT and SOD likely provide frontline defense, and cytosolic GPX, SOD, CAT and GST likely function in the maintenance of intracellular integrity. The control of H_2O_2 is essential since its breakdown results in the formation of OH. for which no specific scavenger molecule exists (Dzik, 2006), highlighting the increased importance of GPX activity. However, other anti-oxidant enzymes such as peroxiredoxin (PRX) should be further investigated in the context of H_2O_2 neutralization.

Several studies suggested that SOD levels were important in protecting parasites against oxidative injury and ROS generated by the host (Henkle-Dürhsen and Kampkötter, 2001; Maizels et al., 1993). SOD activity from *A. cantonensis* was found in both TE and ES extracts. It seems likely that high SOD levels would help facilitate immune evasion since the ES components are active at the host-parasite interface (Hewitson et al., 2008).

Analysis of purified GST enzymes has been identified in several parasites including *Taenia solium* (Vibanco-Pérez et al., 1999), *H. polygyrus* (Brophy et al., 1994) and *Fasciola hepatica* (Chemale et al., 2006), although, no study examined the presence of GSTs in *Angiostrongylus* species. Identification and characterization of these enzymes is very pertinent to understanding parasite immune evasion mechanisms since these proteins play key roles in defense against the oxidative stress response.

Anti-helminthic drugs may be used to treat cerebral angiostrongyliasis only in combination with corticosteroids in order to avoid negative side effects resulting from the simultaneous destruction of large numbers of parasites (Sawanyawisuth and Sawanyawisuth, 2008). For this reason, novel treatment strategies must be developed and GSTs represent a potential target for therapeutic interventions. Xu and coworkers demonstrated that interfering with the *S. mansoni* GST using a monoclonal antibody reduced female worm fecundity and egg viability (Xu et al., 1991). The possibility of inhibiting oviposition is of special interest as a new treatment alternative for abdominal angiostrongiliasis because eggs play a central role in pathogenesis (Bender et al., 2003). Trials with substances that inhibited oviposition in schistosomiasis did not show the same effects in the treatment of experimental *A. costaricensis* infections (Mentz et al., 2007). One explanation is that *A. costaricensis* GSTs could be interfering and detoxifying the substances tested thus far for the treatment of abdominal angiostrongyliasis. Therefore, selective inhibition of *Angiostrongylus* GSTs may provide a novel therapeutic target with the potential of inhibiting oviposition and may ultimately constitute a useful treatment option.

A. costaricensis infections are confirmed following the identification of either worms or their eggs in mesenteric tissues, neither possible in cases of *A. cantonensis* infections. Larvae are not detected in fecal examination of patients with abdominal angiostrongyliasis since the eggs become trapped in human tissues

as a consequence of host-mediated inflammatory responses (Graeff-Teixeira et al., 1997). For these reasons, GSTs have already been selected as immunodiagnostic targets (Cheng et al., 2007) and a GST from *A. costaricensis* has been identified as a potential antigen that can be used for the diagnosis of *A. costaricensis* infections by Western-blot analysis (Abraham et al., 2004).

This study described the purification, proteomic analysis and identification of *A. cantonensis* GSTs. Nine spots were visualized on 2-DE analysis. MALDI-TOF analyzes did not identify target proteins by PMF (Peptide mass fingerprint), a problem common with organisms with unsequenced genomes (Barrett et al., 2005) or missing EST data, as is the case with *Angiostrongylus* species. The use of ESI-Q-TOF analysis and *de novo* sequencing with Masslyx software identified target peptides that were subjected to BLAST analysis for the identification of similar sequences. Two peptides were obtained and were matched to the glutathione transferase from *O. dentatum*, *A. caninum*, *C. elegans* and *H. contortus*. The similarity to these proteins suggested that AcGSTpep1 and AcGSTpep2 probably corresponded to a sigma class GST that is well conserved in nematodes. These sigma class enzymes are also referred to as prostaglandin-D-synthase GSTs, a family of proteins implicated in mediating host immune suppression (Kubata et al., 2007).

Understanding the anti-oxidant mechanisms mediated by *A. cantonensis* GSTs can provide targets for the development of treatment modalities aimed at interfering with parasite reproduction and survival. An improved understanding of the biology and pathogenesis of these parasitic nematodes may result from a better understanding of the mechanism of action of their antioxidative enzymes, particularly GSTs which are also attractive new targets that can be used in the treatment and diagnosis of *Angiostrongylus* species infections.

Role of the funding source

Funding sources had no role in study design, collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

Acknowledgments

Financial support was provided by CNPq, CAPES and FAPERGS. C. Graeff-Teixeira is a recipient of a CNPq PQ 1D fellowship and grants 300456/2007-7 and 477260/2007-1 (Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico do Brasil).

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Capítulo 2

Characterization of *Angiostrongylus cantonensis* excretory- secretory proteins as potential diagnostic targets.

Experimental Parasitology xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

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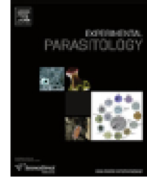

Characterization of *Angiostrongylus cantonensis* excretory–secretory proteins as potential diagnostic targets

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A R T I C L E I N F O

Article history:
Received 9 June 2011
Received in revised form 25 August 2011
Accepted 3 October 2011
Available online xxxx

Keywords:
Angiostrongylus cantonensis
Angiostrongylus costaricensis
ES antigens
Eosinophilic meningoencephalitis
Heterologous antigens

A B S T R A C T

Angiostrongyliasis results from infections with intra-arterial nematodes that accidentally infect humans. Specifically, infections with *Angiostrongylus cantonensis* cause eosinophilic meningitis and *Angiostrongylus costaricensis* infections result in eosinophilic enteritis. Immunological tests are the primary means of diagnosing infections with either pathogen since these parasites are usually not recoverable in fecal or cerebrospinal fluid. However, well-defined, purified antigens are not currently available in sufficient quantities from either pathogen for use in routine immunodiagnostic assays. Since *A. costaricensis* and *A. cantonensis* share common antigens, sera from infected persons will recognize antigens from either species. In addition to their potential use in angiostrongyliasis diagnosis, characterization of these proteins that establish the host–parasite interphase would improve our understanding of the biology of these parasites. The main objective of the present work was to characterize *A. cantonensis* excretory–secretory (ES) products by analyzing ES preparations by two-dimensional gel electrophoresis coupled with immunoblotting using pools of positive sera (PS) and sera from healthy individuals (SC). Protein spots recognized by PS were excised and analyzed by electrospray ionization (ESI) mass spectrometry. MASCOT analysis of mass spectrometry data identified 17 proteins: aldolase; CBR-PYP-1 protein; beta-amylase; heat shock protein 70; proteosome subunit beta type-1; actin A3; peroxiredoxin; serine carboxypeptidase; protein disulfide isomerase 1; fructose-bisphosphate aldolase 2; aspartyl protease inhibitor; lectin-5; hypothetical protein F01F1.12; cathepsin B-like cysteine proteinase 1; hemoglobinase-type cysteine proteinase; putative ferritin protein 2; and a hypothetical protein. Molecular cloning of these respective targets will next be carried out to develop a panel of *Angiostrongylus* antigens that can be used for diagnostic purposes and to further study host–*Angiostrongylus* interactions.

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1. Introduction

Intra-arterial worms from two *Angiostrongylus* species cause disease in humans: *A. cantonensis* is the primary causative agent of eosinophilic meningoencephalitis and *A. costaricensis* causes eosinophilic ileocolitis (Graeff-Teixeira et al., 1991; Wang et al., 2008a).

Cerebral angiostrongyliasis is endemic in Southeast Asia and the Pacific Islands but an increasing number of cases have been reported in Africa, Australia, and Central, North and South America (Graeff-Teixeira et al., 2009; Wang et al., 2008a), including a recently detected transmission foci in Brazil (Caldeira et al., 2007; Maldonado et al., 2010) and Ecuador (Pincay et al., 2009). Ongoing human expansion into geographic areas with active *A. cantonensis* transmission has raised public health concerns from this emerging pathogen (Diaz, 2008). Abdominal angiostrongyliasis cases have been reported throughout the Americas, from the southern United States to northern Argentina (Pena et al., 1995) with some sporadic cases reported in Europe and the United States (Vázquez et al., 1993; Jeandel et al., 1988).

Confirmed diagnosis of either cerebral or abdominal angiostrongyliasis is seldom made because larvae are usually retained in infected tissues as a result of host inflammatory responses (Céspedes et al., 1967; Graeff-Teixeira et al., 1991; Punyagupta et al., 1975). Therefore, molecular diagnostic methods are needed for accurate diagnosis and to conduct epidemiological studies. In the context of *A. cantonensis* infections, several studies have

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doi:10.1016/j.exppara.2011.10.003

Please cite this article in press as: Morassutti, A.L., et al. Characterization of *Angiostrongylus cantonensis* excretory–secretory proteins as potential diagnostic targets. Exp. Parasitol. (2011), doi:10.1016/j.exppara.2011.10.003

focused on the identification of *Angiostrongylus* antigens that can be used for diagnostic purposes (Eamsobhana and Yong, 2009) while the immunodiagnosis of abdominal angiostrongyliasis has been routinely carried out using crude antigens (Geiger et al., 2001).

Since harvesting significant numbers of *A. costaricensis* worms for the purpose of antigen production is hindered by the need to use non-conventional laboratory rodents, i.e., the wild mouse *Oligoryzomys nigripes*, *A. cantonensis* proteins have been utilized as heterologous antigens for the immunodiagnosis of *A. costaricensis* infections (Ben et al., 2010). The use of cross-reactive antigens for the diagnosis of both infections with *Angiostrongylus* species is possible since these two infections present distinct symptomology, e.g., gastroenteritis versus meningoencephalitis.

ES parasite proteins are important as both diagnostic target antigens and as a means of better understanding the host–parasite interaction at the molecular level. In this study, an ES fraction of *A. cantonensis* was analyzed using proteomics and blots probed with sera obtained from *A. costaricensis*-infected patients in order to identify novel immunodiagnostic angiostrongyliasis diagnostic targets and to improve our understanding of the *Angiostrongylus*–host relationship.

2. Material and methods

2.1. Biological materials

Adult *A. cantonensis* worms were harvested from experimentally infected *Rattus norvegicus*. *A. cantonensis* were originally obtained from the Department of Parasitology, Akita Medical School, Japan and have been maintained in our laboratory since 1997. Wistar rats served as definitive host and *Biomphalaria glabrata* as intermediate host. Rats were infected with 104 larvae by gavage inoculation. After 42 days, animals were sacrificed and worms collected.

Animal handling was carried out according to regulations set forth by Brazilian law 11794-08/10/2008, Decreto 6899-15/07/2009 and the recommendations issued by the Conselho Nacional de Controle de Experimentação Animal (CONEA) and the protocol approved by the University Ethics Committee.

2.2. Excretory–secretory products (ES)

ES products were obtained by *in vitro* cultivation of adult worms. Three hundred female worms were carefully collected from pulmonary arteries using histological forceps under a stereomicroscope. Worms were washed three times with PBS (phosphate buffered saline, pH 7.4) to eliminate host cell contaminants and maintained in 20 mL RPMI 1640 culture medium (Invitrogen, Carlsbad, CA) supplemented with 100 μg mL^{-1} penicillin and 100 U mL^{-1} streptomycin at 37 °C in 5% CO_2. Worms were placed in fresh medium every 24 h for 3 days. Exhausted medium was centrifuged at 15,000*g* for 10 min and supernatants concentrated 25 times using Amicon Millipore filters (5 kDa MWCO). Collected material was used as the ES product source and protein concentrations were determined by the Bradford assay using bovine serum albumin as a standard.

2.3. Two-dimensional gel electrophoresis (2DE)

ES proteins (90 μg) were desalted using the 2D Clean-Up Kit (GE Healthcare, Piscataway, NJ) followed by resolubilization in DeStreak Rehydration Solution (GE Healthcare) with 66 mM DTT and 0.5% carrier ampholytes (v/v). Samples were in-gel rehydrated on 11 cm pH 3–11 NL IPG strips (GE Healthcare) and isoelectric focusing was performed using an IPGphor Isoelectric Focusing System (GE Healthcare) with voltages increasing stepwise as follows: 500 V for 500 V h, linear gradient from 500 to 6000 V for 6500 V h, and a hold at 6000 V for 14,000 V h.

After focusing, strips were equilibrated for 15 min in fresh equilibration buffer (20% v/v glycerol, 6 M urea, 1% DTT, 2% SDS). IPG strips were run in the second dimension on a 4–12% acrylamide SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) Bis-Tris gels (Bio-Rad, Hercules, CA).

2.4. Antigen identification

Three gels were run simultaneously under identical conditions. One gel was stained with a mass spectrometry compatible silver nitrate staining (Mortz et al., 2001) and two gels were transferred onto nitrocellulose membranes. After proteins were transferred, blots were stained with a reversible stain (Novex® Reversible Membrane Protein Stain Kit, Invitrogen) that was applied directly onto the membranes, allowing for visualization of the proteins which were photo documented prior to stain removal. After immunodetection, membranes were again photo documented and images superimposed over the total protein image. This procedure was performed to match precisely the immunodetected proteins with proteins visualized in silver stained gels. Images from stained gels and from immuno assays were analyzed using Adobe Photoshop and membranes compared. As part of the analysis, the authenticity of respective protein spots was validated by visual examination.

2.5. Immunodetection

Blotted membranes were washed three times with fresh PBS-Tween (0.03% Tween) and blocked with 5% skim milk for 1 h at room temperature. Membranes were incubated for 2 h with a pool of sera (1:200 dilution) prepared from 20 patients with a confirmed histological diagnosis of abdominal angiostrongyliasis or pooled serum (1:200 dilution) from healthy individuals. Membranes were then probed with a peroxidase-conjugated anti-human IgG (Sigma, St. Louis, MO) (1:8000 dilution) for 1 h at room temperature. Antibody reactions were visualized using 0.05% 3-3′ Diaminobenzidine (DAB) (Sigma) plus 0.015% H_2O_2 in PBS, pH 7.4.

2.6. Mass spectrometry

Protein spots that specifically reacted with pooled serum from angiostrongyliasis patients (but not against serum collected from uninfected controls) were manually excised from 2DE gels and subjected to in-gel tryptic digestion (Promega, Madison, WI) and mass spectrometric analysis. Electrospray ionization (ESI) mass spectrometric analysis was performed using a Bruker model maXis ESI-Q-TOF instrument interfaced with an on-line nanospray source (Bruker Daltonics, Billerica, MA) to perform LC–MS/MS using a U-3000 HPLC configured for nanoliter per minute flows. The Dionex U-3000 nanobore HPLC was configured with dual ternary pumps with one output flow pump split using a calibrated 1:1000 splitter with active flow control. The system used a pulled-loop autosampler configured with a 20 μL sample loop. A desalting trap column (0.3 × 5 mm, 5 μm C18 PepMap 120 A, Dionex) was used and the analytical column used was a C18 PepMap (0.075 × 150 mm, 3 μm, 120A, Dionex). The solvents used were 0.1% formic acid in water and 80% acetonitrile/0.1% formic acid. The gradient was 2–55% in 90 min. The eluent from the analytical column was introduced into the maXis using the Bruker on-line nanospray source. The source was operated at a spray voltage of 900 V with a drying gas of nitrogen flowing at 6 L min^{-1}. The capillary temperature was set to 150 °C. The mass spectrometer was

set to acquire line spectra of 50–1900 m/z. MS/MS data were acquired in an automated fashion using the three most intense ions from the MS scan with precursor active exclusion for 90 s after three spectra were acquired for each parent ion. MS data were acquired at a scan speed of 3 Hz and MS/MS data were acquired at a scan speed of 1–1.5 Hz depending on the intensity of the parent ion. MS internal calibration was achieved by the use of a lock mass calibrant (HP-1222, Agilent Technologies).

Collected data were processed using Data Analysis (Bruker Daltonics) to produce deconvoluted and internally calibrated data that was saved as an xml peaklist that was uploaded to the MASCOT on line program (http://www.matrixscience.com).

3. Results

3.1. In vitro cultures

A. cantonensis ES proteins were obtained from culture supernatants pooled after three collections. Three hundred female worms were used to generate 480 μg ES proteins that were precipitated and subjected to 2DE analysis.

3.2. Two-dimensional gel electrophoresis (2DE)

ES proteins were applied to IPG strips (pH range 3–11 NL) and after isoelectric focusing, the second dimension was carried out using 4–12% acrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes and assayed against positive or normal human sera. Several protein spots were recognized only by positive sera but not by human sera from uninfected subjects. These spots were considered potential diagnostic targets and further analyzed by mass spectrometry (MS/MS). Most targets were obtained from acidic region of the IPG strip and the molecular weights of target proteins were between 20–50 kDa (Fig. 1).

3.3. Protein identification

Identified target proteins were excised from gels, digested with trypsin and the resulting peptides analyzed by mass spectrometry. Seventeen different proteins were identified using the Mascot program (Table 1). Since there was limited information regarding the *A. cantonensis* gene sequences, identification of the respective proteins identified was based on matches to homologous proteins from related organisms, e.g., *Caenorhabditis briggsae*, *Ascaris suum*, *Haemonchus contortus*, *Parelaphostrongylus tenuis* and *Caenorhabditis elegans* and non-related organisms such as *Perinereis aibuhitensis*, *Bombyx mori* and one plant sequence from *Oryza sativa*. The proteins identified were: aldolase; CBR-PYP-1 protein; beta-amylase; heat shock protein 70; proteosome subunit beta type-1; actin A3; peroxiredoxin; serine carboxypeptidase; protein disulfide isomerase 1; fructose-bisphosphate aldolase 2; aspartyl protease inhibitor; lectin-5; and hypothetical protein F01F1.12. Four proteins were identified as *A. cantonensis* proteins: cathepsin B-like cysteine proteinase 1; hemoglobinase-type cysteine proteinase; putative ferritin protein 2; and a hypothetical protein.

4. Discussion

Abdominal angiostrongyliasis is confirmed by detecting intra-arterial worms or the presence of eggs following histopathological examination of intestinal biopsies (Graeff-Teixeira et al., 1991). Corresponding confirmatory findings for cerebral angiostrongyliasis involves the visualization of larvae in cerebrospinal fluid (CSF), which rarely results a positive diagnosis since only small volumes can be collected and the larvae concentrations in the CSF are low (Punyagupta et al., 1975).

Despite the identification of various antigens with diagnostic potential, reliable sources of these antigens are not available, making validation and generation of standardized tests impossible. Most of the antigens described in the literature are derived from crude extracts that vary greatly and require time consuming purification and accuracy for reproducibility. For these reasons, the goal of the present study was to identify antigenic proteins using mass spectrometry as an initial step towards development of recombinant protein based immunodiagnostic procedures.

Screening of proteins with immunodiagnostic potential was carried out in *A. cantonensis* ES preparations obtained following the *in vitro* cultivation of female worms. ES proteins subjected to 2DE analysis and Western blot analysis using serum from infected patients (but not by serum collected from uninfected controls) identified various novel protein targets.

ES products are constantly in contact with host immune cells. Parasites continuously release molecules necessary for tissue penetration, immune system evasion, oxidative stress and nutrient acquisition (Dzik, 2006). Each of these molecules are promising diagnostic targets due to their presence at the parasite–host interface and their accessibility to immune system components. Analysis of ES fractions may also contribute to a better understanding of the host–parasite relationship. Indeed ES products of *A. cantonensis* have been studied. The ES from the third stage larvae have shown to possess serine protease and metalloprotease activities likely associated with duodenal penetration (Lee and Yen, 2005). A recent study investigating the antioxidant enzyme profile of adult *A. cantonensis* worms demonstrated that superoxide dismutase and catalase were highly active ES products likely involved in mediating parasite survival against oxidative stresses generated by host immune responses (Morassutti et al., 2011).

Antioxidant proteins play an important role in parasite-mediated anti-cytotoxic and proinflammatory responses against reactive oxygen species (ROS) generated by the host immune response (Dzik, 2006). Peroxiredoxin is known to plays a central role in H_2O_2 detoxification. One of the proteins identified in this study was homologous to a *H. contortus* peroxiredoxin. This finding suggests that *A. cantonensis* adult worms release peroxiredoxin which acts as a protection mechanism against H_2O_2. In addition, helminth peroxiredoxin has been reported to be critical to immune modulation of Th2 type responses (Donnelly et al., 2008). Interestingly, local Th2 responses have been observed in CSF and have been implicated in development of CSF and peripheral eosinophilia in *A. cantonensis* infections (Sugaya et al., 1997). Possibly, peroxiredoxin released by *A. cantonensis* may be involved in both driving the Th2 response and in mediating protection by acting as an antioxidant. Peroxiredoxin could also serve as an immune target since antibodies present in the serum of infected patients recognized this target antigen.

Heat shock protein 70 has been identified as a ES protein component (Wu et al., 2009; Oladiran and Belosevic, 2009) and reported to activate macrophages in addition to inducing the production of pro-inflammatory cytokines during *in vitro Trypanosoma carassii* infection (Oladiran and Belosevic, 2009). In addition, Hsp70s have been recognized by sera from patients infected with either *Schistosoma mansoni*, *Echinococcus granulosus*, or *T. carassii* (Kanamura et al., 2002; Ortona et al., 2003; Oladiran and Belosevic, 2009), suggesting that the *A. cantonensis* Hsp70 might also be involved in immune stimulation, cytokine production and pathogenesis as reported for *T. carassii*.

Hemoglobinases are enzymes involved in blood degradation; a process fundamental to parasite nutrient acquisition, and in this report we demonstrated the presence of hemoglobinase and β-amylase enzymes in ES products, suggesting that these enzymes

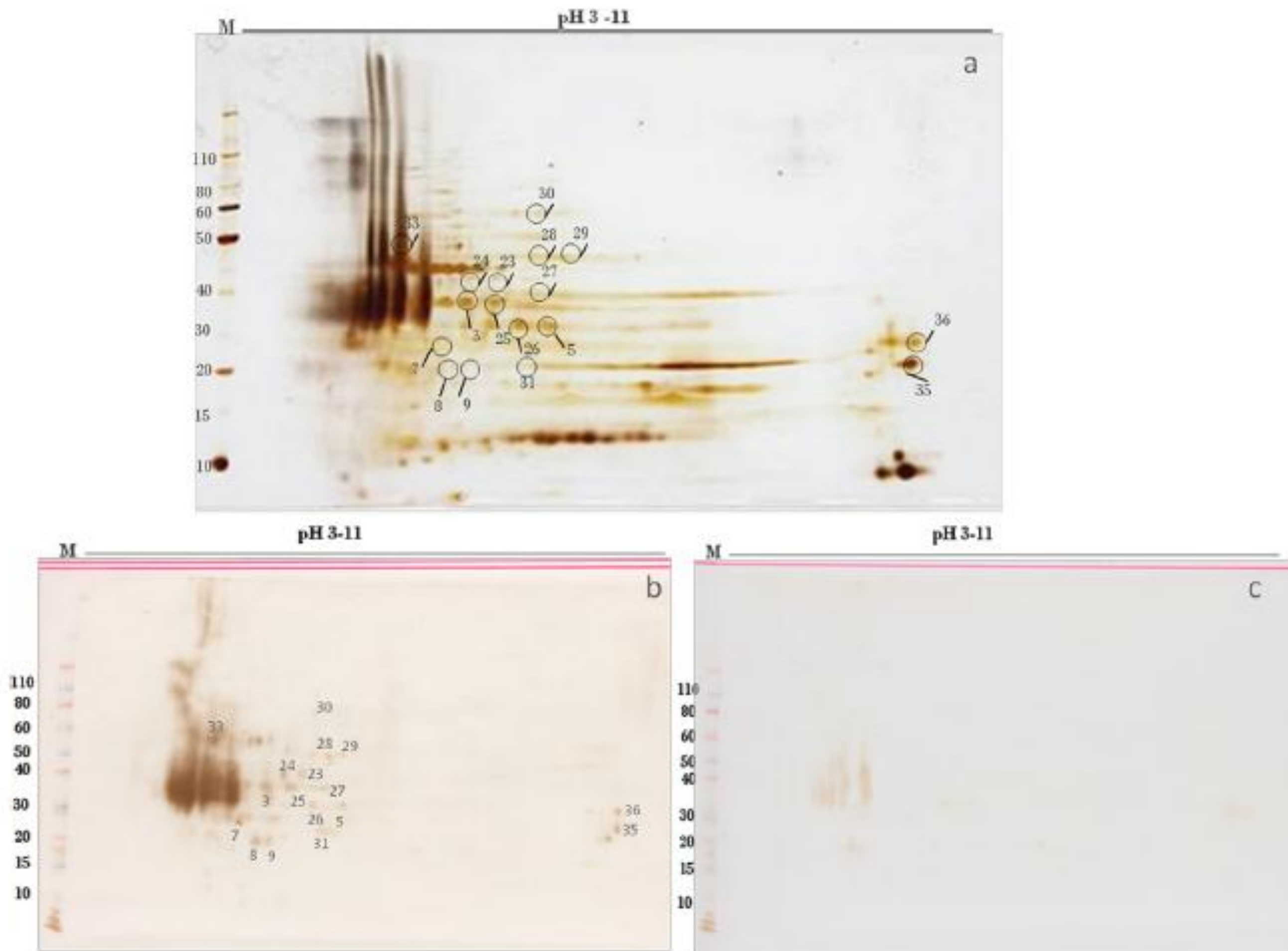

Fig. 1. 2DE gel electrophoresis of *A. cantonensis* ES proteins. Isoelectric focusing was carried out using 11-cm immobilized pH gradient strips pH 3–11NL. The second dimension was carried out on 12% SDS–polyacrylamide gels. Gels were subsequently silver stained (a) or electro-blotted and tested against angiostrongyliasis pooled serum (b) and from healthy individuals polled serum (c). Identified spots are shown circled. M, molecular mass marker.

might be secreted by the parasite. This is of interest since hemoglobinases have been suggested as potential vaccine targets for hookworm infections (Pearson et al., 2009). In addition, these enzymes may constitute therapeutic targets for disease treatment since Sijwali et al. (2006) demonstrated that disruption of the falsipain 2 protein (FP2; involved in hemoglobin degradation by *Plasmodium falciparum*) caused fitness injuries to early trophozoites.

It is interesting to note that our data supported observations resulting from a recent *in silica* study where *Signal-P* analyses were employed to predict *A. cantonensis* proteins likely to be secreted (Fang et al., 2010). These authors identified different types of proteases and proteases inhibitors, in addition to putative antigens and allergens, based on sequence similarities to cathepsin B-like cysteine proteinase; hemoglobinase-type cysteine proteinase, galectins, an aspartyl protease inhibitor and the antioxidant protein peroxiredoxin (Fang et al., 2010).

The peptide sequences corresponding to spot 27 were homologous to protein As37, which is a highly immunoreactive 37 kDa antigen of *A. suum*. Additional BLAST database searches demonstrated 100% homology between the peptides obtained from protein spot 27 and antigen-3 of *Baylisascaris schroederi* (BsAg3) and 99% homology to the disorganized muscle protein-1 of *Brugia malayi*. Those proteins have been described as vaccine candidates (Tsuji et al., 2002; Wang et al., 2008b) further suggesting that the *A. cantonensis* protein corresponding to spot 27 may also be considered an important antigen.

Aspartyl proteases inhibitors, ferritin and aldolase, have also been reported as potential antigens for the diagnosis of hookworm, *Paragonimus westermani*, and *Schistosoma japonicum* infections (Delaney et al., 2005; Kim et al., 2002; Peng et al., 2009) highlighting the importance of these proteins for additional study regarding angiostrongyliasis diagnostic.

In conclusion, several molecules were identified in ES products released by *A. cantonensis* that were specifically recognized by sera from *A. costaricensis*-infected patients, suggesting that these antigens could serve as potential candidates for the development of improved immunodiagnostic tests for the detection of abdominal angiostrongyliasis and eventually also for the diagnosis of *A. cantonensis* infections. Compared to similar peptide sequences from other parasites, these molecules may play important roles in modulating and evading the host's immune system. Generating recombinant proteins of the targets described in this report will be the necessary next step to providing a reliable source of abundant, well-defined molecules that can form the basis of further studies aimed at improving angiostrongyliasis immunodiagnostic procedures and provide a better understanding of *Angiostrongylus*–host interactions.

Table 1
ES protein identification.

Spot #	Peptide sequence	Protein name (mass)	Organism	Score*
3	R.ICIASNEK.I	Cathepsin B-like cysteine proteinase 1 (44715)	*Angiostrongylus cantonensis*	147
	R.HDLYPTPK.C			
	K.DLDIDIPETFDAR.Q			
	R.GVDECGIESGVVGGIPK.S			
	K.GDNDPIDVIEIGSK.V	CBR-PYP-1 protein (38319)	*Caenorhabditis briggsae*	86
5	K.CVPSYK.E	Cathepsin B-like cysteine proteinase 1 (44715)	*Angiostrongylus cantonensis*	215
	K.NDVAAIQK.E			
	R.ICIASNEK.I			
	R.HDLYPTPK.C			
	R.QHWSNCQSIK.N			
	R.GVDECGIESGVVGGIPK.S			
	K.IQVTLSADDLLSCCR.T			
7	K.IFPGLDK.G	Hypothetical protein (23073)	*Angiostrongylus cantonensis*	171
	K.LVSGTLIVTK.K			
	R.ASVQLPGMIK.L			
	K.GFVDMISNPGTYDLQEIEK.G			
	R.WLDSNQTAEK.D	Heat shock protein 70s (71352)	*Perinereis aibuhitensis*	132
	R.WLDSNQTAEKDEFEHQQK.E			
	K.SAPEELVQQVLSAGWR.E	Beta-amylase (55058)	*Oryza sativa Japonica*	116
	R.NIEYLTLGVDDQPLFHGR.T			
8	R.DLTPSEIEELK.V	Aspartyl protease inhibitor (24943)	*Parelaphostrongylus tenuis*	68
	K.LVSGTLIVTK.K	Hypothetical protein (23073)	*Angiostrongylus cantonensis*	62
	R.ASVQLPGMIK.L			
9	R.MSQFEINILTR.D	Proteosome subunit beta type-1 (28655)	*Ascaris suum*	125
	K.GAVFSYDPIGCIER.			
	K.DDEGIAYR.G	Peroxiredoxin (21946)	*Haemonchus contortus*	65
23	K.IATEPVR.W	Hemoglobinase-type cysteine proteinase (49849)	*Angiostrongylus cantonensis*	99
	K.ALQEMHEK.K			
	K.NFLSVLQGK.S			
	R.HQADIAHAYHLMR.N			
	R.HDLYPTPK.C	Cathepsin B-like cysteine proteinase 1 (44715)	*Angiostrongylus cantonensis*	79
	R.GVDECGIESGVVGGIPK.S			
24	K.AGFAGDDAPR.A	Actin A3 (41865)	*Bombyx mori*	85
	R.VAPEEHPVLLTEAPLNPK.A			
	K.IATEPVR.W	Hemoglobinase-type cysteine proteinase (49849)	*Angiostrongylus cantonensis*	79
	K.NFLSVLQGK.S			
	R.HQADIAHAYHLMR.N			
25	K.CVPSYK.E	Cathepsin B-like cysteine proteinase 1 (44715)	*Angiostrongylus cantonensis*	258
	K.NDVAAIQK.E			
	R.HDLYPTPK.C			
	K.DLDIDIPETFDAR.Q			
	R.GVDECGIESGVVGGIPK.S			
	K.IQVTLSADDLLSCCR.T			
	R.YAYGHGIIDEK.T	Serine carboxypeptidase (53453)	*Ascaris suum*	78
26	K.IATEPVR.W	Hemoglobinase-type cysteine proteinase (49849)	*Angiostrongylus cantonensis*	74
	K.ALQEMHEK.K			
	K.NFLSVLQGK.S			
	R.HQADIAHAYHLMR.N			
	K.ITETVLSYCYR.A	Aldolase (39673)	*Haemonchus contortus*	69
27	K.GNANFNLK.L	As37 (35522)	*Ascaris suum*	130
	K.DAGQFVCTAK.N			
	K.APHFPQQPVAR.Q			
	R.DDGQVMVMEFR.A			
	K.FEVPQGAPTFTR.K			
	R.DDGQVMVMEFR.A			
28	K.YEELAEK.L	Protein disulfide isomerase 1 (54915)	*Ostertagia ostertagi*	129
	K.VHFAVSNK.E			
	K.NFLVHETVGFAGIR.T			
	K.FPMDDEFSVENLK.A			
	K.MDATANDVPPLFEVR.G			
29	K.QGIVPGIK.L	Fructose-bisphosphate aldolase 2 (38822)	*Caenorhabditis elegans*	140
	R.ALQASVLK.A			
	K.VTEQVLAFVYK.A			
	K.GILAADESTGTIGK.R			
30	R.ALQASVLK.A	Fructose-bisphosphate aldolase 2 (38822)	*Caenorhabditis elegans*	129
	K.VTEQVLAFVYK.A			
	K.GILAADESTGTIGK.R			
	K.ITETVLSYCYR.A	Aldolase (39673)	*Haemonchus contortus*	88

(continued on next page)

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Table 1 (*continued*)

Spot #	Peptide sequence	Protein name (mass)	Organism	Score*
31	K.DADLPLHFSIR.F R.ISNPFK.A K.FQVFANR.V R.LFHYGGR.I R.VNINLYR.E	Galectin (CBR-LEC-5) (35555)	*Caenorhabditis briggsae*	108
33	R.VGPGIGEYIFDK.E K.ASAANDPHMSDFLESK.F	Putative ferritin protein 2 (6893)	*Angiostrongylus cantonensis*	90
35	K.VTEQVLAFVYK.A	Hypothetical protein F01F1.12 (38822)	*Caenorhabditis elegans*	96

After 2DE analysis 28 proteins were excised from the gel for trypsin digestion. Mass spectrometry analyses were performed for protein identification.
* MASCOT score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event.

Role of the funding sources

Funding sources did not participate in the study design, data collection, analysis of the data, interpretation of data, writing of the report, nor in the decision to submit the paper for publication.

Acknowledgments

Financial support was provided by CNPq, CAPES, and FAPERGS. C. Graeff-Teixeira is a recipient of a CNPq PQ 1D fellowship and of Grants 300456/2007-7 and 477260/2007-1 (Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico do Brazil).

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Capítulo 3

Interface molecules of *Angiostrongylus cantonensis* – their role in parasite survival and modulation of host defenses.

Dear Mrs. Morassutti,

The Review Article titled "**Interface molecules of *Angiostrongylus cantonensis* – their role in parasite survival and modulation of host defenses**," by Alessandra Morassutti and Carlos Graeff-Teixeira has been received and assigned the number 512097.

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Interface molecules of *Angiostrongylus cantonensis* – their role in parasite survival and modulation of host defenses

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Abstract

Angiostrongylus cantonensis is a nematode parasite that causes eosinophilic meningoencephalitis in humans. Disease presents following the ingestion of third stage larvae residing in the intermediate mollusk host and disease manifests as an acute inflammation of the meninges characterized by eosinophil infiltrates which release a battery of pro-inflammatory and cytotoxic agents in response to the pathogen. As a mechanism of neutralizing these host defenses, *A. cantonensis* expresses different molecules with immunomodulatory properties that are excreted or secreted (ES). In this mini-review we discuss the role of ES proteins on disease exacerbation and their potential use as a therapeutic targets.

Keywords: angiostrongyliasis, ES molecules, host-parasite interface, immune evasion, pathogenesis

Introduction

Establishment of parasitic infections is dependent on a delicate and constant interaction between host and parasite; specifically, interactions between the host immune system and molecules released by the parasite or located at the parasite surface (Maizels et al., 1993; Henkle-Dürhsen and Kampkötter, 2001). Parasitic organisms have evolved the ability to survive in such hostile environments by evading or neutralizing host defense systems. This process is mediated in part by molecules released by parasites that consist of excretion and secretion products (ES) which may contain metabolites, enzymes, hormone-like factors, antioxidants and proteinase inhibitors among others (Dzik, 2006; Hewitson et al., 2009).

Eosinophilic meningitis, also known as cerebral angiostrongyliasis, is an acute inflammation caused mainly due the presence of *Angiostrongylus cantonensis* young in the meninges, parenchyma of the medulla, pons or cerebellum (Graeff-Teixeira et al., 2009). Humans get infected after ingestion of third stage larvae residing in raw mollusks, vegetables or contaminated water. To date, more than two thousand angiostrongyliasis cases have been reported, with most cases occurring in Southeast Asia and the Pacific Islands where the disease is endemic (Wang et al. 2008). However, angiostrongyliasis cases have now been reported in regions of the world where this disease has not previously been reported *i.e.*, Brazil, Caribe, Ecuador, Australia and the USA. This change in the epidemiology of angiostrongyliasis should serve as a warning to authorities that this disease is an emerging public health problem (Caldeira et al., 2007; Diaz 2008; Pincay et al., 2009; Maldonado et al., 2010).

The pathogenicity and pathophysiology of cerebral angiostrongyliasis, however, still remain poorly defined. The present review discusses the potential role of excreted and

63 secreted (ES) proteins in relation to *Angiostrongylus* infections in the context of developing

64 novel diagnostic and treatment modalities.

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Eosinophils and Meningoencephalitis

Eosinophils play a critical role in protection against helminthes and in mediating allergic responses. Eosinophils possess specialized granules containing a battery of pro-inflammatory and cytotoxic agents. In addition, various molecules, including interleukin (IL)-2, -4, -5, -10, -12, -13, -16, -18, TGF-α/β, leukotrienes, proteases, reactive oxygen species (ROS) and nitric oxide (NO) secreted by eosinophils can play important roles in mediating protective anti-helminthic responses (Behm and Ovington, 2000; Rothenberg and Hogan, 2006). However the cost of producing these molecules can damage cell membranes and tissues, ultimately contributing to the pathogenesis and pathophysiology associated with hypereosinophilic syndromes (Ackerman and Bochner 2007).

Cerebral angiostrongyliasis is characterized by eosinophil infiltrates that kill immature worms residing in the meninges (Yoshimura et al., 1984). Sasaki and coworkers (1993) demonstrated enhanced intracranial survival of *A. cantonensis* when eosinophilic responses were inhibited following treatment with anti-IL-5 antibodies (Sasaki et al. 1993). By contrast, mice over-expressing IL-5 killed worms faster and female worms were smaller than those developing in wild-type mice (Sugaya et al., 1997). The same results were observed with another *Angiostrongylus* species, *e.g.*, *A. costaricensis* that causes abdominal angiostrongyliasis, a disease also associated with eosinophililia (Sugaya et al., 2002).

IL-5 is an important cytokine associated with the progression of eosinophillia following an *A. cantonensis* infection (Sugaya et al., 1997). Specifically, IL-5 levels were significantly elevated in the CSF and peripheral blood of patients with eosinophilic meningoencephalitis due to infections with *A. cantonensis* (Intapan et al., 2008; Diao et al., 2009), corroborating previous data generated in mouse models of disease (Sugaya et al., 1997; Chen and Lai, 2007).

90 Several studies have focused on developing therapeutic strategies designed to prevent
91 eosinophil infiltrates by eliciting a switch from a Th-2 to a Th-1 type of response. Du et al.,
92 (2003) observed decreased IL-5 levels and elevated INF-γ levels in mice when an anti-
93 helminthic drug was administrated in combination with IL-12 in an experimental *A.*
94 *cantonensis* infection model (Du et al., 2003). Another study using anti-helminthic drugs in
95 combination with steroids (to avoid severe inflammation due to larval death in the meninges)
96 determined that patients receiving both drugs, the IL-5 levels and peripheral eosinophil
97 counts were reduced (Diao et al., 2009). Recently, Chuang et al. demonstrated that
98 administration of an anti-CCR3 monoclonal antibody that blocked the major receptor present
99 on eosinophils (CCR3) reduced eosinophil infiltrates and consequently reduced the severity
100 of neurological damage in mice (Chuang et al., 2010).

101 These data suggested that controlling the level of eosinophil infiltrates and the
102 polarization of Th-2 responses may reduce neurological damage resulting from *A.*
103 *cantonensis* infections. A better understanding of the host-parasite interplay would facilitate
104 the development of different approaches for disease treatment and reduction of disease-
105 associated sequelae.

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112 **Released *Angiostrongylus cantonensis* molecules and their potential roles in disease**

113 ES released by parasites are likely key to parasite survival since ES are continuously
114 released and may promote tissue penetration, nutrient acquisition and also immune system
115 and oxidative stress evasion (Dzik, 2006). Studies of ES products from third stage *A.*
116 *cantonensis* larvae have demonstrated serine protease and metalloprotease activity likely
117 associated with duodenal penetration (Lee and Yen, 2005). We previously demonstrated the
118 presence of high levels of antioxidant enzymatic activities in ES fractions of adult *A.*
119 *cantonensis* worms, including superoxide dismutase (SOD) and catalase (CAT), which may
120 be involved in parasite survival against oxidative stress generated by host immune responses
121 (Morassutti et al., 2011a). Another recent study investigating immunoreactive proteins from
122 adult ES preparations identified peroxiredoxin, serine proteases, heat-shock proteins, ferritin,
123 galectin, aldolase and proteases inhibitors (Morassutti et al., 2011b). The potential role of
124 these proteins on inflammatory processes and disease exacerbation is discussed below.

125 Peroxiredoxin

126 Antioxidant proteins mediate important protective mechanism against ROS generated
127 by the host immune response (Dzik, 2006). Peroxiredoxin (Prx) is an enzyme reported to
128 exist in many parasites and known to play a central role in H_2O_2 detoxification. However
129 another function has been attributed to Prx *e.g.*, *Fasciola hepatica* ES products containing
130 Prx has been shown to down-regulate Th-1 type responses and to affect macrophage
131 activation following injection into mice (Donnelly et al., 2005). In another study,
132 neutralization of secreted Prx during the course of an *F. hepatica* infection significantly
133 reduced the Th-2 responses (Donnelly et al., 2008), indicating that Prx is a target for disease
134 treatment. Indeed, knocking down the *S. mansoni* Prx genes using RNA-i dramatically
135 increased oxidative damage to parasite proteins and lipids, which in turn reduced worm

136 survival (Sayed et al. 2006). Prx was found in ES products of adult *A. cantonensis* worms that
137 were recognized by immunoglobulins present in the serum of infected patients (Morassutti et
138 al., 2011b). Interestingly, as mentioned above, local Th-2 responses were implicated in the
139 development of CSF and peripheral eosinophilia associated with *A. cantonensis* infections
140 (Sugaya et al, 1997), and elimination of the worm combined with IL-12 administration
141 shifted the response from a Th-2 to a Th-1 type response (Du et al., 2003). These
142 observations raised the following hypothesis: blocking *A. cantonensis* Prx activity would
143 make the parasite vulnerable and weaken the Th-2 response, making this molecule a viable
144 treatment target.

145 Heat shock proteins

146 Heat shock proteins are a highly conserved group of proteins present in both
147 prokaryotic and eukaryotic organisms. They are grouped into different families based on their
148 molecular weights. HSPs function as chaperones, assisting in the proper folding of newly
149 synthesized proteins even though HSPs were first associated with stress-induced stimuli
150 (Hartl and Hayer-Hartl, 2002). HSP70 has been identified in ES preparations of many
151 parasites, including *A. cantonensis* (Morassutti et al., 2011b). HSP70 was implicated as an
152 adaptive response associated with the early stages of infection with the nematode *Trichinella*
153 *spiralis* (Zocevic et al., 2011) and HSPs have also been associated with drug resistance in
154 various *Leishmania spp.* protozoans (Vergnes et al., 2007). In addition, knocking down
155 HSP90 in adult *Caenorhabditis elegans* worms using RNA-i resulted in cessation of egg
156 production and in an embryonic lethal phenotype (Piano et al., 2000; Inoue et al., 2006).
157 Interestingly, inhibiting oviposition is of special interest as a new treatment alternative for
158 abdominal angiostrongiliasis because eggs play a central role in pathogenesis (Bender et al.,
159 2003), thereby making *Angiostrongylus* HSPs viable targets for disease treatment.

160 Administration of recombinant HSP from the protozoan *Trypanosoma carassii*
161 activated goldfish macrophages *in vitro* and stimulated the production of the proinflammatory
162 cytokines INFγ and TNFα (Oladiran and Belosevic, 2009). Indeed, secreted HSP forms have
163 been demonstrated to bind toll-like receptors 2 and 4 (TLR2 and TLR4) expressed on the
164 surface of antigen-presenting cells (APCs) in a similar manner as lipopolysaccharide (LPS),
165 resulting in the production of pro-inflammatory cytokines. Moreover, HSPs have been
166 considered to play a role in the development and pathogenesis of some rheumatic diseases
167 (Schultz and Arnold, 1993). Together, these data suggested that released *A. cantonensis* HSPs
168 may facilitate the inflammatory process, making further studies to better understand the role
169 of this protein in disease pathology crucial.

170 Galectin

171 Galectins are a family of sugar-binding proteins with affinity for N-acetyl
172 lactosamines, an interaction mediated via a conserved carbohydrate-recognition domain
173 (CRD). In mammals, these proteins possess the ability of inhibiting both Th-1 and Th-2-
174 mediated inflammation (Toscano et al., 2006). However, the function of helminth galectins
175 still remains unclear even though *Brugia malayi* and *Ochocerca volvulus* galectins have been
176 hypothesized to function as potential immune modulators (Hewitson et al., 2008; Klion and
177 Donelson 1994). One of the most important classes of antigens expressed by several
178 helminths is comprised of sugar molecules. Interestingly, helminths activate innate immune
179 cells via surface-expressed or -secreted products, including glycolipids and glycoproteins,
180 through lectin receptors (Linehan et al., 2003). This association may interfere with the
181 induction of effective immune responses that could contribute to the modulation of
182 inflammatory T cell responses (van Die and Cummings, 2010). In fact, the *Schistosoma* egg
183 glycan was shown to be recognized and internalized by immature dendritic cells (iDCs)

184 which in turn did not upregulate stimulatory molecules or produce cytokines, indicating that
185 conventional maturation was prevented (van Liempt et al., 2007). Moreover, galectins have
186 also been identified as targets for disease diagnosis *e.g*., diagnosis of *Trichostrongylus*
187 *colubriformis* (gastrointestinal nematode) infections in sheep (Kiel et al., 2007). In similar
188 fashion, an ES galectin from *A. cantonensis* was shown to be immunoreactive to antibodies
189 present in serum from angiostrongyliasis patients, further supporting the potential use of this
190 protein as a diagnostic antigen (Morassutti et al., 2011b).

191 <u>Proteases</u>

192 Proteases are enzymes that catalyze the cleavage of amide linkages in
193 macromolecular proteins and oligomeric peptides. Proteases are very import for parasite
194 survival because they facilitate tissue penetration and nutrient acquisition. For example,
195 hemoglobinases are proteases that degrade hemoglobin into peptides and amino acids; a
196 fundamental process for nutrient acquisition for many parasites. Hemoglobinases from
197 hookworms have been suggested as potential vaccine targets because of their
198 immunogenicity and because their inactivation would interfere with hookworm feeding
199 (Pearson et al., 2009). Our previous work demonstrating that hemoglobinases present in ES
200 products from adult worms were recognized by sera from angiostrongiliasis infected patients
201 supports these observations. In addition, these enzymes may constitute therapeutic targets as
202 observed by Sijwali et al. (2006) who demonstrated that disruption of falsipain-2 protein (an
203 enzyme involved in *Plasmodium falciparum* hemoglobin degradation) resulted in fitness
204 injuries to early stage trophozoites (Sijwali et al., 2006). It is reasonable therefore to
205 hypothesize that blocking hemoglobinase activity would interfere with nutrient uptake
206 resulting in death of the parasite. In fact, knocking down an *S. mansoni* hemoglobinase
207 resulted in significant growth retardation *in vitro* (Morales et al., 2008). A parallel approach

208 targeting enzymes responsible for sugar digestion (such as aldolase and beta-amylase) could
209 also result in parasite elimination.

210 Another protein identified in ES samples was a cathepsin B-like protein, which is a
211 cysteine protease. Cystein proteases from helminthes have been shown to be involved in
212 degrading host proteins, including immunoglobulins, complement components, kininogen,
213 hemoglobin, albumin and extracellular matrix proteins (Sajid and McKerrow 2002).
214 Interestingly, cysteine proteases from ES preparations of *Paragonimus westermani*, a tissue-
215 invasive parasite that causes either pulmonary or extrapulmonary paragonimiasis in humans,
216 were also implicated in human eosinophil degranulation *in vitro* (Shin et al., 2005). These
217 findings may help in the understanding of the mechanisms of tissue inflammation associated
218 with meningoencephalitis due to *A. cantonensis* infections since the cathepsin B-like protein
219 was secreted by the parasite.

220 <u>Proteases Inhibitors</u>

221 Besides secreted proteases, parasitic organisms also have the ability to produce and
222 release inhibitors for many types of proteases that may block host protease function, thereby
223 facilitating parasite survival. Three kinds of protease inhibitors are commonly described in
224 parasites: aspins, specific to aspartic proteases; cystatins, which block the activity of cysteine
225 proteases and serpins, that act on serine proteases.

226 A cystatin from *A. cantonensis* (AcCystatin) was identified from a cDNA library of
227 fourth stage larvae that was cloned and expressed in a prokaryotic system. The authors
228 observed that recombinant AcCystatin significantly inhibited cathepsin B and significantly
229 up-regulated nitric oxide production by IFNγ activated macrophages (Liu et al., 2010).
230 Interestingly, cystatins identified from parasitic nematodes have been implicated in blocking

231 cathepsin activity, however, they are also associated with stimulating the production of anti-
232 inflammatory cytokines (Hartmann and Lucius, 2003). These cystatin properties suggest that
233 they can inhibit cellular proliferation while concomitantly establishing an anti-inflammatory
234 environment favorable to parasite survival (Zavasnik-Bergant 2008). As therapeutic targets,
235 these inhibitors have been demonstrated to prevent allergic inflammation in both lung and
236 intestines of mice treated with a filarial cystatin that modulated macrophage-mediated colitis,
237 in addition of inhibiting eosinophil recruitment, down-regulating IL-4 production and
238 suppressing allergic airway hyper-reactivity (Schnoeller et al., 2008).

239 An aspartyl protease inhibitor secreted by *A. cantonensis* female adult worms was
240 identified in an *in vitro* study (Morassutti et al., 2011b), however, the role of aspins in
241 helminthes is not clear. Potentially, these proteins could block the activity of host aspartyl
242 proteases, however, the activity of porcine pepsin was not inhibited by a recombinant
243 hookworm aspartic proteinase inhibitor (Delaney et al., 2005). To date, only asipns have been
244 reported in *A. cantonensis* (Fang et al., 2010). However, molecular analysis of the *A.*
245 *cantonensis* genome revealed that only a small number of sequences have been deposited at
246 Genbank. As a consequence, protein identification by mass spectrometry is ineffective since
247 the lack of peptide sequence homology to related proteins from other organisms makes
248 identification difficult.

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254 **Conclusions**

255 The pathogenesis of eosinophilic meningitis is related to the development of 256 significant inflammatory reactions in response to *A. cantonensis* worms residing in the 257 nervous system. In response to the infection, eosinophills are recruited and several potent 258 cytotoxic agents are released in an attempt to eliminate the pathogen. This immune-mediated 259 attack frequently results in tissue damage and ultimately may exacerbate disease severity. In 260 this review, we discussed the putative diverse roles of released *A. cantonensis* molecules. 261 Many kinds of molecules may act as immunomodulators, but these molecules may also be 262 involved disease exacerbation. Further studies using recombinant forms of the target proteins 263 discussed above will be essential in evaluating and confirming the hypothesis presented here.

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452

Capítulo 4

The 31 kDa antigen of *Angiostrongylus cantonensis* comprises an antigenic glycoprotein complex

Elsevier Editorial System(tm) for Parasitology International
Manuscript Draft

Manuscript Number: PARINT-D-11-00245

Title: The 31 kDa antigen of Angiostrongylus cantonensis comprises distinct antigenic glycoproteins.

Article Type: Research Paper

Keywords: Angiostrongylus; eosinophilic meningitis; abdominal angiostrongyliasis; immunodiagnosis; 31kDa antigen;

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Abstract: Human angiostrongyliasis results from accidental infection with Angiostrongylus an intra-arterial nematode. Angiostrongylus cantonensis infections result in eosinophilic meningitis and A. costaricensis infections cause eosinophilic enteritis. Immunological methodologies are critical to the diagnosis of both infections since these parasites cannot be isolated from fecal matter and are rarely found in cerebrospinal fluid samples. A. costaricensis and A. cantonensis share common antigenic epitopes which elicit antibodies that recognize proteins present in either species. Detection of antibodies to a 31 kDa A. cantonensis protein, present in crude adult worms extracts is a sensitive and specific method for immunodiagnosis of cerebral angiostrongyliasis. The objective of the present work was to isolate and characterize the 31 kDa protein(s) using soluble protein extracts derived from adult female worms using both single (1DE) and two-dimensional (2DE) gel electrophoresis. Separated proteins were blotted onto nitrocellulose and probed using sera from infected and non-infected controls. The 31 kDa band present in 1DE gels and the 4 spots identified in 2DE gels were excised and analyzed by electrospray ionization mass spectrometry. Using the highest scores obtained following Mascot analysis, amino acid sequences were obtained that matched four unique proteins: tropomyosin, the 14-3-3 phosphoserine-binding protein, a protein containing a nascent polypeptide-associated complex domain, and the putative epsilon subunit of coatomer protein complex isoform 2. Oxidative cleavage of diols using sodium m-periodate demonstrated that carbohydrate moieties are essential for the antigenicity of all four spots of the 31 kDa antigen. In this paper we describe the identification of the 31kDa antigen and provide DNA sequence of the targets. In conclusion, these data suggest that reactivity to the 31 kDa proteins may represent antibody recognition of more than one protein and recombinant protein-based assays for cerebral angiostrongyliasis diagnosis may require eukaryotic expression systems to maintain antigenicity.

The 31 kDa antigen of *Angiostrongylus cantonensis* comprises distinct antigenic glycoproteins.

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20 **Abstract**

21 Human angiostrongyliasis results from accidental infection with *Angiostrongylus* an
22 intra-arterial nematode. *Angiostrongylus cantonensis* infections result in eosinophilic
23 meningitis and *A. costaricensis* infections cause eosinophilic enteritis. Immunological
24 methodologies are critical to the diagnosis of both infections since these parasites
25 cannot be isolated from fecal matter and are rarely found in cerebrospinal fluid
26 samples. *A. costaricensis* and *A. cantonensis* share common antigenic epitopes
27 which elicit antibodies that recognize proteins present in either species. Detection of
28 antibodies to a 31 kDa *A. cantonensis* protein, present in crude adult worms extracts
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30 angiostrongyliasis. The objective of the present work was to isolate and characterize
31 the 31 kDa protein(s) using soluble protein extracts derived from adult female worms
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43 antigen and provide DNA sequence of the targets. In conclusion, these data suggest

44 that reactivity to the 31 kDa proteins may represent antibody recognition of more
45 than one protein and recombinant protein-based assays for cerebral
46 angiostrongyliasis diagnosis may require eukaryotic expression systems to maintain
47 antigenicity.

48

49 **Keywords:** *Angiostrongylus*; eosinophilic meningitis; abdominal angiostrongyliasis;
50 immunodiagnosis; 31kDa antigen.

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63 ## 1. Introduction

64 The nematode *Angiostrongylus cantonensis* is the most common causative
65 agent of eosinophilic meningoencephalitis (Graeff-Teixeira et al., 2009). Completion
66 of its lifecycle requires two hosts: an intermediate mollusk host and a definitive
67 rodent host, typically *Rattus norvegicus*. The first stage larva (L1) is released in rat
68 feces and mollusks become infected by ingesting organic debris contaminated with
69 L1 larvae. Inside mollusk tissues, L1 larvae develop into the infective third stage L3
70 larvae. Rats may ingest L3 larvae that penetrate the mucosa, invade blood vessels
71 and migrate to the meninges. In the central nervous system (CNS) the larvae mature
72 into young adults (5th stage larvae) that complete their maturation inside the
73 pulmonary arteries and right cardiac cavities. Humans can be accidentally infected
74 by ingesting L3 larvae present in contaminated water, or food that is raw or
75 undercooked. In humans, L3 larvae are incapable of completing the lifecycle and die
76 in the CNS resulting in disease.

77 Cerebral angiostrongyliasis has been reported in Southeast Asia, Africa,
78 Australia, America (Wang et al., 2008) and recently a transmission foci have been
79 identified in Brazil (Caldeira et al., 2007; Maldonado et al., 2010) and Ecuador
80 (Pincay et al., 2009). In addition, angiostrongyliasis is considered an emerging public
81 health problem in the United States (Diaz, 2008).

82 Confirmed diagnosis of cerebral angiostrongyliasis is seldom possible since
83 larvae are typically not found in cerebrospinal fluid (CSF) (Yii, 1976). Several
84 molecular targets have been identified as potential antigens for angiostrongyliasis
85 immunodiagnosis (Eamsobhana and Yong, 2009). However, these targets are not
86 widely available for independent evaluation or testing in either clinical or

87 epidemiological investigations. Standardization of immunological tests requires their
88 validation using various geographical isolates and sera collected from patients with
89 different co-infections to rule out potentially cross-reactive responses. Preparation of
90 large quantities of respective target antigens is a complicated and laborious process.
91 Molecular cloning and expression of recombinant proteins represent a reliable
92 alternative for generating sufficient amounts of well-defined antigens for use in
93 immunodiagnostic assays.

94 Immuoblotting studies have identified an immunoreactive band with an
95 estimated molecular weight of 31 kDa that has been considered a target for a highly
96 sensitive and specific antibody detection assay for *A. cantonensis* infections
97 (Nuamtanong, 1996; Kirsch et al., 2008). Eamsobhana *et al*. demonstrated that the
98 31 kDa glycoprotein possessed sugar residues that did not affect antibody
99 recognition (Eamsobhana et al., 1998); furthermore, this protein was purified and
100 employed in ELISA and dot-blot assays resulting in 100% sensitivity and specificity
101 (Eamsobhana et al., 2001; Eamsobhana and Yong, 2009). Nevertheless the identity
102 of this 31kDa antigen is unknown.

103 Heterologous antigens have been used in various immunodiagnositic assays
104 taking into account the various shared epitopes present between different helminth
105 species. This approach has also been utilized in the diagnosis of angiostrongyliasis
106 since *A. cantonensis* and *A. costaricensis* possess cross-reactive antigens that can
107 be used to diagnose infections with either pathogen (Dekumyoy et al., 2000; Ben et
108 al., 2010). Since *A. cantonensis* is more easily maintained parasite in the laboratory,
109 proteins from this nematode may be used to identify antigenic targets with the
110 potential of being used in the diagnosis of infections with either pathogen.

The present study characterized the makeup of the 31 kDa *A. cantonensis* antigen complex using 1DE and 2DE gel electrophoresis that allowed the identification of various targets which can be used in the development of recombinant antigens for immunodiagnostic purposes.

2. Materials and Methods

2.1 Biological Materials

2.1.1 Worms

Adult *A. cantonensis* worms were recovered from experimentally infected rats. *A. cantonensis* were originally obtained from the Department of Parasitology, Akita Medical School, Japan and have been maintained in our laboratory since 1997. Wistar rats served as definitive hosts and *Biomphalaria glabrata* as intermediate hosts. Rats were infected with 104 larvae by gavage inoculation and 42 days post infection animals were sacrificed and worms collected.

2.1.2 Antigen preparation

Total extract (TE) was obtained from harvested female worms that were macerated in liquid nitrogen and homogenized in phosphate buffer saline (PBS, pH 7.4). The suspension was centrifuged at 12,000 x g for 1 h at 4°C and the supernatants used to derive the TE. Protein concentrations were determined by the Bradford assay using bovine serum albumin as a standard.

2.2 Two-dimensional Electrophoresis (2DE)

An aliquot of TE that contained 60 µg of total protein was desalted using a 2-D Clean-Up Kit (GE Healthcare, Piscataway, NJ) followed by resolubilization in

133 DeStreak Rehydration Solution (GE Healthcare) with 66 mM DTT and 0.5% carrier
134 ampholytes (v/v). Samples were in-gel rehydrated on 11 cm pH 3-11NL or 3-6NL
135 IPG strips (GE Healthcare) and isoeletric focusing was performed using an IPGphor
136 Isoelectric Focusing System (GE Healthcare) with voltages increasing stepwise as
137 follows: 500 V for 500 V h, a linear gradient from 500-8000 V for 6500 V h followed
138 by a hold at 6000 V for 22000 V h.

139 After isoeletric focusing, strips were soaked for 15 min in a fresh equilibration
140 buffer (20% v/v glycerol, 6 M urea, 1% DTT, 2% SDS). IPG strips were run in the
141 second dimension on 4-12% polyacrylamide Bis-Tris gels with sodium dodecyl
142 sulfate (SDS-PAGE) (Bio-Rad, Hercules, CA). Gels were then stained with Colloidal
143 Coomassie blue or mass spectrometry compatible silver stain (Mortz et al., 2001) or
144 transferred to nitrocellulose membranes for immunological analyses.

145

146 **2.3 Western Blot Analysis**

147 Resolved proteins were electro-transferred onto nitrocellulose membranes
148 using a semi-dry trans-blot apparatus (Bio-Rad, Hercules, CA). The membrane was
149 washed 3 times with PBS-T (0.05% Tween) and blocked with 5% skim-milk for 1 h at
150 room temperature. Membranes were then incubated for 2 h with a pool of sera
151 (1:200 dilution) prepared from either 20 patients histopathologically diagnosed with
152 abdominal angiostrongyliasis or with 20 patients positives for meningoencephalitis
153 eosinophilic or 20 pooled serum from uninfected controls. After three washes,
154 membranes were probed with a secondary peroxidase-conjugated anti-human IgG
155 (Sigma, St. Louis, MO) (diluted 1:8000) for 1 h at room temperature.

156 Diaminobenzidine (DAB) (Sigma, St. Louis, MO) (0.05% DAB - 0.015% H_2O_2 in PBS,
157 pH 7.4) was added as developer reagent.

158

159 **2.4 MS/MS analysis**

160 Immunoreactive spots were manually excised from 2DE gels and subjected to
161 in-gel tryptic digestion (Promega, Madison, WI) and mass spectrometric analysis.
162 Electrospray ionization (ESI) mass spectrometric analysis was performed using a
163 Bruker model maXis ESI-Q-TOF instrument interfaced with an on-line nanospray
164 source (Bruker Daltonics, Billerica, MA) to perform LC-MS/MS using a U3000 HPLC
165 configured for nanoliter per minute flows. The Dionex U-3000 nanobore HPLC was
166 configured with dual ternary pumps with the flow output of one pump split using a
167 calibrated 1:1000 splitter with an active flow control. This system used a pulled-loop
168 auto sampler configured with a 20 µl sample loop. A desalting trap column (0.3 x 5
169 mm, 5 µm C18 PepMap 120 A, Dionex, Sunnyvale, CA) and C18 PepMap (0.075 x
170 150 mm, 3 µm, 120 A, Dionex, Sunnyvale, CA) were used. The solvents used were
171 0.1% formic acid in water and 80% acetonitrile/0.1% formic acid. The gradient was 2-
172 55% in 90 min. The eluent from the analytical column was introduced into the maXis
173 using the Bruker on-line nanospray source. The source was operated at a spray
174 voltage of 900 V with a drying gas of nitrogen flowing at 6 L/min. The capillary
175 temperature was set to 150°C. The mass spectrometer was set to acquire line
176 spectra of m/z 50-1900. MS/MS data was acquired in an automated fashion using
177 the 3 most intense ions from the MS scan with precursor active exclusion for 90 s
178 after 3 spectra were acquired for each parent ion. MS data were acquired at a scan
179 speed of 3 Hz and MS/MS data acquired at a scan speed of 1-1.5 Hz depending on

180 the intensity of the parent ion. MS internal calibration was achieved by the use of a
181 lock mass (HP-1222, Agilent Technologies, Santa Clara, CA).

182 Collected data were processed by Data Analysis (Bruker Daltonics, Billerica,
183 MA) to produce deconvoluted and internally calibrated data and saved as a xml
184 peaklist which was searched against the NCBInr database with the Mascot on line
185 program (http://www.matrixscience.com). The data were acquired in data-dependent
186 mode (DDA), and multiple charged peptide ions (+2 and +3) were automatically
187 mass selected and dissociated in MS/MS experiments. Mascot search parameters
188 allowed a maximum of one missed cleavage, carbamidomethylation of cysteine as
189 fixed modifications, methionine oxidation as a variable modification, peptide
190 tolerance of 0.2 Da and MS/MS tolerance of 0.2 Da. The significance threshold was
191 set at $p < 0.05$, and identification required that each protein contain at least one
192 peptide with an expected value < 0.05.

193

194 **2.5 Oxidation of the carbohydrates**

195 Carbohydrate moieties were oxidized using sodium periodate to investigate
196 their antigenicity. Proteins were electro-transferred onto nitrocellulose membranes,
197 washed three times with PBS-T and incubated for 30 min with 100 mM NaOAc pH
198 5.0. The membranes were incubated with a sodium *m*-periodate solution (20 mM
199 NaIO4 diluted in 100 mM NaOAc) and kept at 37ºC for 1h, in the dark. After washing
200 with 100 mM NaOAc the membranes were incubated with 50 mM $NaBH_4$ in PBS-T
201 for 30 min at room temperature and developed as described above (Western Blot
202 Analysis).

203 **3. Results and Discussion**

204 Immunodiagnostic targets were identified in crude female worm extracts using
205 1DE gel electrophoresis. This analysis identified a 31 kDa band (Figure 1), which is
206 consistent with previously published data (Eamsobhana et al., 1998). However, 2DE
207 resolved the 31 kDa band into four distinct antigenic spots in the acidic region that
208 appeared elongated and diffuse in shape. These spots were recognized by sera from
209 angiostrongyliasis patients but not by sera from uninfected controls (Figure 2). A
210 better separation of immunoreactive spots distributed between the 4-5 pH range was
211 obtained when a 3-6 pH-NL strip was employed (Figure 2).

212 The 31 kDa band detected on 1DE gels and the four antigenic spots identified
213 on 2DE gels were excised and digested with trypsin for further analysis using MS/MS
214 ESI-Q-TOF. The Mascot score was used to determine the probability that the
215 observed matches between the experimental data and the database sequences
216 were not random. Amino acid sequences of several peptides were obtained from
217 material excised from both 1DE (Table 1) and from 3 of 4 spots excised from the
218 2DE gels (Table 2). None of the identified peptides present in the 1DE band
219 corresponded to peptides identified in the 2DE gel spots. One explanation for this
220 result may be the relatively low concentration of the antigenic 31 kDa components
221 present in the 1DE gel. This was apparent when proteins were separated by 2DE gel
222 analysis over a 3-11 pH range and silver stained, which showed numerous proteins
223 visible in the 31 kDa molecular mass range (Figure 2a). One of peptides obtained by
224 1DE showed the highest Mascot score to the 33 kDa tropomyosin from
225 *Heligmosomoides polygyrus*, a rodent nematode. Tropomyosins are a highly
226 conserved muscle protein with potent allergenic potential. This protein is known to

227 induce IgE production in parasitic nematode infections such as anisakiasis, and
228 onchocerciasis (Sereda et al., 2008) but due to similarity between invertebrate
229 tropomyosins, IgE antibodies cross-react with tropomyosins from other species and
230 therefore tropomyosins are not useful for diagnostic purposes (Sereda et al., 2008).
231 However specificity may be further tested by epitope mapping of this protein.

232 Analysis of mass spectrometry data for three of the proteins (spots 2, 3 and 4)
233 identified amino acid sequences that matched several unique proteins or protein
234 domains in the database. Since there is little *Angiostrongylus* sequence available
235 most protein identifications rely on homologous sequences from closely related
236 organisms within the database. No peptide matches were obtained from spot 1.
237 Amino acid sequences of two proteins, the 14-3-3 protein and the NAC domain
238 containing protein, were obtained from all three of the spots in which protein
239 identifications were made. The highest Mascot scores for the 14-3-3 protein were
240 detected with database sequences derived from the 14-3-3 proteins of *Ancylostoma*
241 *caninum* and a 28 kDa protein of *Meloidogyne incognita*, a plant nematode. Peptide
242 sequences obtained from spot 4 matched sequences from the *A. cantonensis*
243 putative epsilon subunit of the coatomer protein complex isoform 2 (33 kDa). Amino
244 acid sequences from spots 2 and 3 matched heat shock proteins of *Loa loa* and
245 *Haemonchus contortus*. Peptide sequences also matched sequences from 2 other *A.*
246 *cantonensis* proteins (Table 2). The 14-3-3 proteins are dimeric phosphoserine-
247 binding proteins, which are members of a family of acidic regulatory molecules that
248 participate in signal transduction, transport and regulation of several eukaryotic
249 biochemical processes (Obsilova et al., 2008; Mrowiec & Schwappach, 2006). In
250 some parasites, such as *Echinococcus multilocularis* and *Schistosoma mansoni*, 14-

251 3-3 proteins have been described to be immunogenic and therefore have been
252 promoted as potential vaccine targets (Schechtman et al., 2001; Siles-Lucas et al.,
253 2008; Wang et al., 2009). In addition, the 14-3-3 protein has been identified as a
254 prominent product in the *S. mansoni* female worm reproductive system (Schechtman
255 et al., 2001). This may explain previous findings showing the female reproductive
256 system as the main source of antigenic targets useful for the diagnosis of abdominal
257 angiostrongyliasis caused by *A. costarecensis* (Bender, 2003). Moreover these
258 proteins might directly interact with immune system components since these
259 interactions have been modulated by 14-3-3 proteins secreted by *Toxoplasma gondii*
260 and *E. granulosus* (Assossou et al., 2004; Siles-Lucas et al., 2008).

261 Coatomer proteins (COP) form a coat protein complex which mediates protein
262 transport between the Golgi compartment (COPI), endoplasmic reticulum (COPII),
263 and the plasma membrane (clathrin/adaptin) (Lee & Goldberg, 2010). COPI from rat
264 liver peroxisomes contains stoichiometric amounts of seven subunits: including
265 alpha-COP (160 kDa), beta-COP (107 kDa), beta-prime-COP (102 kDa), delta-COP
266 (57 kDa), epsilon-COP (36 kDa), gamma-COP (97 kDa) and zeta-COP (20 kDa)
267 (Lay et al., 2006). To date, there is no evidence that these proteins can induce
268 immune responses. However, a crystallographic analysis showed that the epsilon-
269 COP and alpha-COP complex were exposed on COPI vesicles thereby facilitating
270 their extracellular targeting (Hsia & Hoelz, 2010), suggesting that the complex might
271 be attached to the Golgi membrane while transporting proteins that are eventually
272 exposed to the host's immune system.

273 The nascent polypeptide associated complex (NAC) is associated with
274 ribosomes and involved in nascent polypeptide chain folding (Hayashi et al., 2011).

275 NAC is also implicated in the targeting of ribosomes to the ER membrane
276 (Wiedmann and Prehn, 1999).

277 *Angiostrongylus* 31 kDa antigen was first described as a glycoprotein and its
278 antigenicity was considered independent of carbohydrate moieties (Eamsobhana
279 et.al, 1998). In this study, the proteins were better separated by 2DE and we were
280 able to distinguish the specific antigenic spots corresponding to the previously
281 described 31 kDa antigen. Periodate treatment eliminated the recognition by sera
282 from infected individuals (Figure 2f) demonstrating that carbohydrate moieties are
283 essential for antibody recognition of the 31 kDa protein. This finding has strong
284 implications for the choice of appropriate vectors to express such recombinant
285 targets for the development of diagnostic tests.

286 In order to achieve the complete DNA sequence of each identified protein for
287 further recombinant protein studies we sequenced the DNA in a randomly way using
288 the parallel sequencing approach (Morassutti et al., in preparation). NAC domain
289 revealed to be composed of 185 amino acids while 14-3-3 with 249 amino acids. The
290 sequences were published in Genebank under the numbers: GI: 341864443 for NAC
291 domain containing protein and GI: 341864441 for 14-3-3.

292 Analysis of the data presented in this report raises the question of whether the
293 reactivity observed with the native parasite 31 kDa molecules is due to reactivity with
294 one or more of the putative proteins identified by MS/MS. Interestingly the NAC
295 domain containing protein, epsilon-COPI and 14-3-3 protein all play putative
296 biological roles in protein translocation. Therefore, we hypothesize that they may
297 form a cell membrane complex, which may have led to co-isolation of these proteins

in the original TE preparation and may ultimately explain how all 3 could be antigenic.

In conclusion, the set of proteins with an estimated molecular weight of 31 kDa identified by 2DE consisted of several potential antigens. Cloning of corresponding cDNAs and expression of these proteins is the next critical step to further define their roles as diagnostic targets, as well as providing tools to better understand host-*Angiostrongylus* interactions.

Acknowledgements

Financial support was provided by CNPq, CAPES, FAPERGS and APHL-USA. C. Graeff-Teixeira is a recipient of a CNPq PQ 1D fellowship and of grants 300456/2007-7 and 477260/2007-1 (Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico do Brasil).

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411 **Legends**:

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413 Figure 1. Identification of 31 kDa molecules on 1DE. Female worm total protein
414 extract (TE) was resolved in 1DE gel and probed on Western bloting with: 1- pool of
415 positive controls for angiostrongyliasis ; 2- pool of normal human sera. Square
416 represents the band excised from the gel for MS analyses.

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419 Figure 2. Identification of the 31 kDa protein complex on 2DE. a - TE pH range 3-11,
420 silver staining; b- TE pH range 3-11 Western Blot using sera derived from pooled *A.*
421 *costaricensis* and *A. cantonensis* infection; c- TE pH range 3-6 sera derived from *A.*
422 *costaricensis* infection; d -TE pH range 3-6 sera derived from *A. cantonensis*
423 infection; e- normal human sera; f- carbohydrate oxidation; g - Coomassie blue
424 staining; The four spots of the 31 kDa proteins are indicated on pH 3-11 strip; circles
425 represent the excised spots for MS analyses.

Figure 1

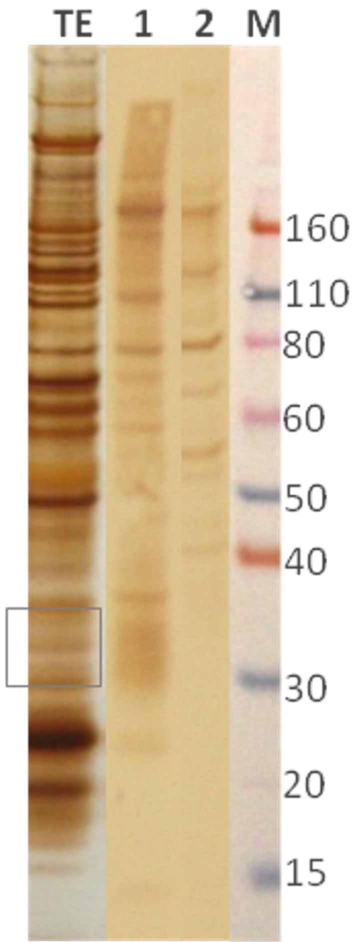

Figure 2

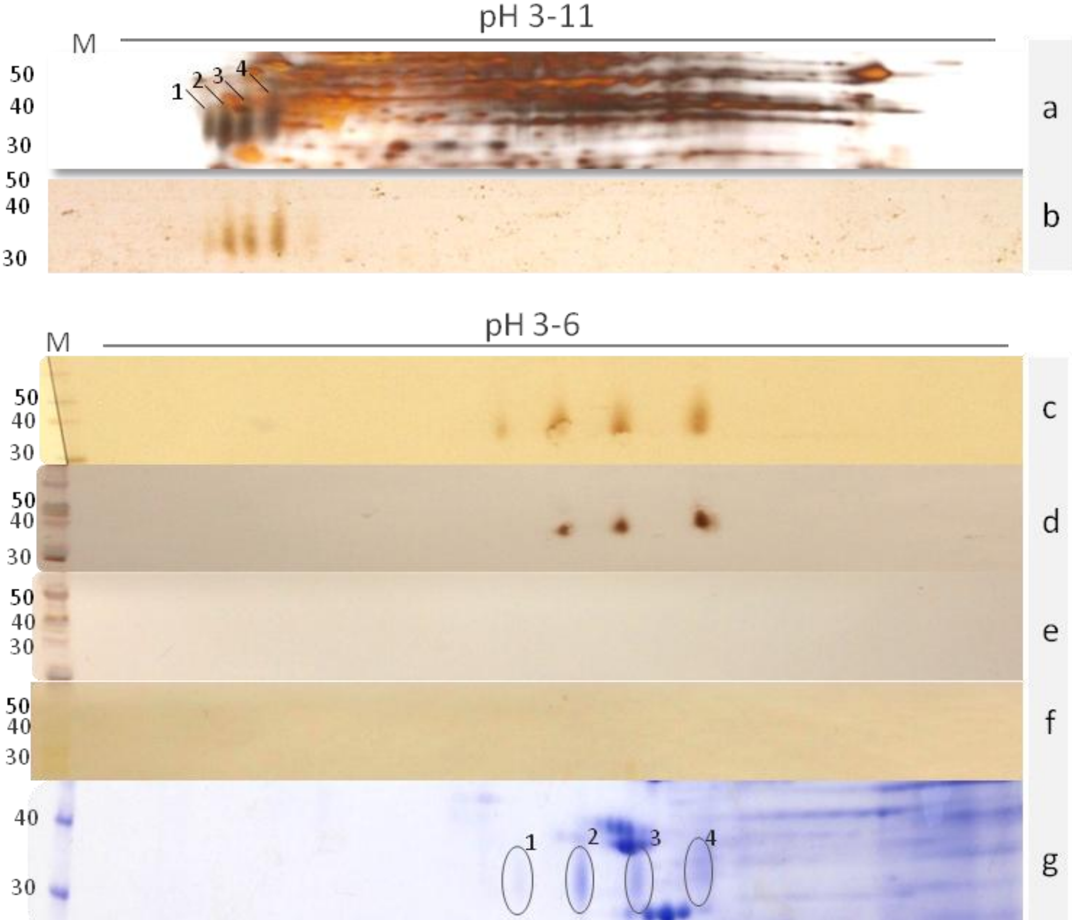

Table 1. Proteins identified following one-dimensional gel electrophoresis.

Peptide Sequence	Protein Name (Da)	Organism with homologous target	Score*	Coverage %
R.ANTIEAQLK.E R.LEDELVHEK.E K.IVELEEELR.V K.LAMVEADLER.A K.EAQMLAEEADR.K R.MTLLEEELER.A K.VQEAEAEVAALNR.R K.EVDRLEDELVHEK.E K.AQEDLATATSQLEEK.D	Tropomyosin (33)	*Heligmosomoides polygyrus*	262	36
R.ALQASCLAK.W K.GILAADESTGSMEK.R	Hypothetical protein CBG15316 (39)	*Caenorhabditis briggsae*	172	9
R.GAAQNIIPAATGAAK.A R.VPTPDVSVVDLTCR.L	glyceraldehyde-3-phosphate dehydrogenase (36)	*Dictyocaulus viviparus*	131	10
K.AGFAGDDAPR.A K.DSYVGDEAQSK.R K.QEYDESGPSIVHR.K R.VAPEEHPVLLTEAPLNPK.A	Actin-2 (41)	*Ascaris suum*	134	15
K.ITETVLSYCYR.A K.KPWALTFSYGR.A	Aldolase (39)	*Haemonchus contortus*	88	6
K.EPDWVQSER.E R.HLVGIADDNKDGK.L R.DWIMPVGFDHAEAEAR.H	CALUmenin (calcium-binding protein) homolog family member (36)	*Caenorhabditis elegans*	87	12
R.LLLEQMSQDPGAVR.E K.LMEFQR.A	TPR Domain (31)	*Brugia malayi*	78	7
R.DYGVLKEDDGIAYR.G R.LVQAFQFVDK.H R.QITVNDLPVGR.S	Peroxiredoxin (21)	*Ascaris suum*	75	17

Table 2. Identification of proteins excised from *A. cantonensis* preparations subjected to 2DE gel electrophoresis. Spots were excised from respective gels and subjected trypsin digestion and then analyzed by mass spectrometry for protein identification.

Spot #	Peptide Sequence	Protein Identified (kDa)	Organism with homologous target	Score*	Coverage (%)
2	K.ADLVNNLGTIAK.S K.EDQTEVLEER.R R.ELISNSSDALDK.I K.TLTIMDTGIGMTK.A R.YQALTEPAELESGK.E	heat shock protein 90 (80) heat shock protein 90 (81)	*Loa loa* *Haemonchus contortus*	201 144	7 5
	R.VLSSIEQK.T K.DSTLIMQLLR.D K.SQQSYQEAFDIAK.D	14-3-3 product (29)	*Meloidogyne incognita*	111	14
	K.SPGSDTYIVFGEAK.I K.NILFVINKPDVYK.S	NAC domain containing protein (24)	*Brugia malayi*	136	12
	K.AGIVFTGK.G K.YMNQFTK.A K.LEVGLFDTYR.C	PCNA (Proliferating Cell Nuclear Antigen) (29)	*Caenorhabditis briggsae*	98	11
3	R.YDDMAQSMK.K K.DSTLIMQLLR.D R.DICQDVLNLLDK.F K.VTELGAELSNEER.N K.SQQSYQEAFDIAK.D K.MQPTHPIR.L	14-3-3 protein isoform 2 (28)	*Ancylostoma caninum*	234	34
	K.SPGSDTYIVFGEAK.I K.NILFVINKPDVYK.S	NAC domain containing protein (24)	*Brugia malayi*	171	12
	K.EDQTEVLEER.R R.ELISNSSDALDK.I K.TLTIMDTGIGMTK.A R.YQALTEPAELESGK.E	heat shock protein 90 (80)	*Loa loa*	128	7
4	R.LAEYQNATDK.Q K.ASLVLNEISER.T K.AKENLFDELVAA K.DAEALLHEAQLR.D R.DINPNHPWVIDLK.A	putative epsilon subunit of coatomer protein complex isoform 2 (33)	*Angiostrongylus cantonensis*	289	30
	R.VISSIEQK.T K.DSTLIMQLLR.D K.VTELGAELSNEER.N K.SQQSYQEAFDIAK.D	14-3-3b protein (28)	*Meloidogyne incognita*	277	29
	R.VGPGIGEYIFDK.E K.FLDEQVESIAEIAK.M K.ASAANDPHMSDFLES K.F	putative Ferritin protein 2 (6.8)	*Angiostrongylus cantonensis*	155	67
	K.LAQIISQFER.A R.ALTSVNSLIEGVVQK. M	putative Uncoordinated protein 23 (11)	*Angiostrongylus cantonensis*	150	39
	K.SPGSDTYIVFGEAK.I	NAC domain containing protein (24)	*Brugia malayi*	92	6

*MASCOT score is -10 x log (P), where P is the probability that the observed match is a random event. (Mass) - molecular weight kDa.

Conclusões gerais

1. Foram identificadas diferentes moléculas com potencial uso no diagnóstico das angiostrongilíases a partir de duas fontes de antígeno ES e TE.
2. Nas amostras de ES:
    - foram verificadas atividade das enzimas superóxido dismutase (SOD) e catalase (CAT).
    - 17 proteínas alvo para o diagnóstico e tratamento foram identificadas.
3. Em amostras de TE:
    - além de SOD e CAT, glutationa peroxidase e glutationa transferases (GSTs) também foram identificadas.
    - As GSTs apresentaram massa molecular entre 20 e 25 kDa e com 9 pontos isoelétricos diferentes numa faixa de pH entre 4 e 8.
    - O antígeno de 31kDa quando submetido a eletroforese bidimensional apresenta-se composto por 4 pontos isoelétricos diferentes numa faixa de pH entre 4 e 5.
    - Duas proteínas diferentes estavam presentes em três dos 4 *spots*: 14-3-3 e proteína com domínio–NAC, além de uma proteína presente no *spot* 4, subunidade épsilon do coatamero.
    - A antigenicidade dos 4 *spots* é dependente de resíduos de açúcar.
    - As sequências codificadoras das últimas três proteínas foram acessadas por sequeciamento aleatório do DNA e depositadas no Genbank.

Com isso, os antígenos identificados neste trabalho contribuem para o desenvolvimento de um teste sorológico para o diagnóstico das angiostrongíliases podendo ser amplamente distribuído para validações independentes.

Apêndices

Apêndice 1

Avaliação do potencial diagnóstico das GSTs de *A. cantonensis*

Objetivo: Analisar o potencial de GSTs no diagnóstico das angiostrongilíases.

Método:

As enzimas foram purificadas por cromatografia de afinidade confome descrito no capítulo 1. Foram separadas por eletroforese unidimensional e transferidas a uma membrana de nitrocelulose para o ensaio de Western blot (conforme descrito no capítulo 2 e 4).

Resultado e discussão:

Durante os estudos realizados com as GSTs de *A. cantonensis*, também foi investigado o potencial antigênico destas proteínas, já que Abrahan e colaboradores haviam identificado tais enzimas como sendo um antígeno promissor no reconhecimento da angiostrongíliase abdominal (Abrahan et al., 2004). No entanto, as GSTs purificadas neste trabalho (Capítulo 1) não foram reconhecidas pelos soros de pacientes com angiostrongilíases.

No estudo de Abrahan et al. (2004) empregaram análises por eletroforese unidimensional. Identificaram GST em uma banda de 20kDa após ensaios de seqüenciamento protéico por degradação de Edman. Estes resultados não necessariamente poderiam corresponder à proteína que foi reconhecida pelo soro de pacientes. Isso fica claro, quando examinamos os géis bidimensionais dos *spots* de 31kDa (Capítulo 3), que são compostos por várias proteínas que possuem o mesmo perfil de migração em géis de uma dimensão, sendo possível sua separação apenas em géis bidimensionais. Além disso, a técnica de degradação de Edman é capaz de detectar apenas proteínas mais abundantes contidas em uma determinada amostra, uma vez que possui sensibilidade de cerca de 2 a 5 pmol, o que é muito inferior se comparado a técnica de MS que pode chegar a 100 fmol de detecção (Deutzmann, 2004).

Portanto, os resultados apresentados aqui descartam o uso das GSTs de *A. cantonensis* como alvos para o diagnóstico das angiostrongilíases.

Referencias:

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Apêndice 2

Expressão de proteínas recombinantes de *A. cantonensis*

A partir dos resultados obtidos da identificação de diversos alvos para o diagnóstico das angiostrongilíases, foram selecionadas duas proteínas pertencentes ao complexo 31kDa (capítulo 4). Subunidade epsilon do coatâmero e 14-3-3 e duas proteínas identificadas em ES (capítulo 2) galectina 5 e hipotética protena de *A. cantonensis* spot 7 (denominada ES7).

Objetivo: produzir antígenos recombinantes de *Angiostrongylus cantonensis*.

1. Material e Métodos

1.1 Sequenciamento de DNA (vide apêndice 3)

1.2 Obtenção de cDNA:

Para a obtenção do DNA complementar (cDNA) foi utilizado o kit de extração *NucleoSpin RNA II* (Machery-Nagel, Inc., Bethlehem, PA, USA). O RNA total foi extraído a partir de vermes fêmeas preservados em RNAlater® (Ambion). Cerca de 30 mg de material foi homogeneizado em tampão de lise (RA1) com homogenizador de tecidos T8 (IKA WORKS, Inc., Willington, NC, USA). Cerca de 5 ug de RNA foi

convertido a cDNA de fita simples usando o óligo (dT) kit *SuperScript III First-Strand Synthesis SuperMix* (Invitrogen, Carlsbad, CA, USA). O cDNA foi aliquotado e mantido a -20°C até o uso.

1.3 Clonagem: A amplificação das sequências codificadoras dos genes de interesse foi feita pela técnica de PCR utilizando-se cDNA como molde e enzima Platinum® Pfx DNA polymerase (Invitrogen) de alta fidelidade para garantir a correta inserção dos nucleotídeos. A estratégia de clonagem utilizou o vetor de expressão procariótico Champion Pet200® (Invitrogen), que permite a purificação da proteína recombinante através de uma cauda de histidina.

1.4 Óligos e condições de amplificação:

Proteína/ bp	Óligos	TM °C	condições de amplificação	Reação de PCR
Epsilon coatomer/ 877	F: CACCATGTCTGGGGTTGATCGTTTGT R: TTAAGCGGCAACAAGTTCATCA	66	95°C 5min, 30x ciclo: 95°C 30s, 66°C 30s, 72°C 1min. extensao final de 72°C, 7 min.	-DNA polimerase *Pfx* 1u -DNTPs: 5mM -Tampão da enzima 1x -Oligos 10pmol, cada -$MgSO_4$ 1,5mM -cDNA: 50ng
ES7/648	F: CACCATGCGGTCAATTCTGATCTATT R: TTAACTGACGTAGAGCCAGTGA	61	95°C 5min, 30x ciclo: 95°C 30s, 61°C 30s, 72°C 1min. extensao final de 72°C, 7 min	
Lec 5/764	F:CACCATGAGGATGAAGGTGTTGCT R:CTTCATACTCCTGGAGCATCGTTG	59	95°C 5min, 30x ciclo: 95°C 30s, 59°C 30s, 72°C 1min. extensao final de 72°C, 7 min	
Proteina 14-3-3/ 750	F:CACCATGACGGACAACAGGGGCGA R:TCAGTTGGCACCCTCTCCTTGTTC	71	95°C 5min, 30x ciclo: 95°C 30s, 71°C 30s, 72°C 1min. extensao final de 72°C, 7 min	

1.5 Expressão e purificação dos recombinantes:

Para a expressão das proteínas recombinantes foram utilizadas bactérias *Escherichia coli* da linhagem DE3 BL21. Sendo multiplicadas em meio de cultura Luria-Bertani (LB) suplementando com o antibiótico canamicina (100mg/mL) a 37ºC em agitação (250 rpm) e ao atingir a fase logarítmica foram induzidas com IPTG (Isopropyl-β-D-thio-galactoside) (1 mM) por três horas. As bactérias foram

centrifugadas por 10min a 3000 x *g* e o sedimento foi ressuspendido em tampão de lise suplementado com inibidores de proteases (PBS, pefablocSC 1:100, leupeptina 1:1000 e pepstatina A 1:100, 0,1% de Triton X-100, pH 7,4) e submetidos a sonicação três pulsos de 30s cada em 15% de amplitude. As células lisadas foram centrifugadas a 20.000 x *g* por 1 hora para obtenção das proteínas na fração solúvel. Os recombinantes foram então purificados por cromatografia de afinidade através de colunas de níquel, sendo eluídas com 250mM de imidazol.

2. Resultados:

2.1 Expressão e purificação da proteína Epsilon coatomer (Ep31):

A proteína Ep31 foi expressa em sistema procariótico e purificada por cromatografia de afinidade ao níquel. A massa esperada de 36kDa (33kDa + 3kDa provenientes da construção do recombinante) foi confirmada por gel de poliacrilamida 12% (Figura 1) .Após a purificação da proteína por cromatografia, o recombinante apresentou um rendimento de cerca de 4mg/ml.

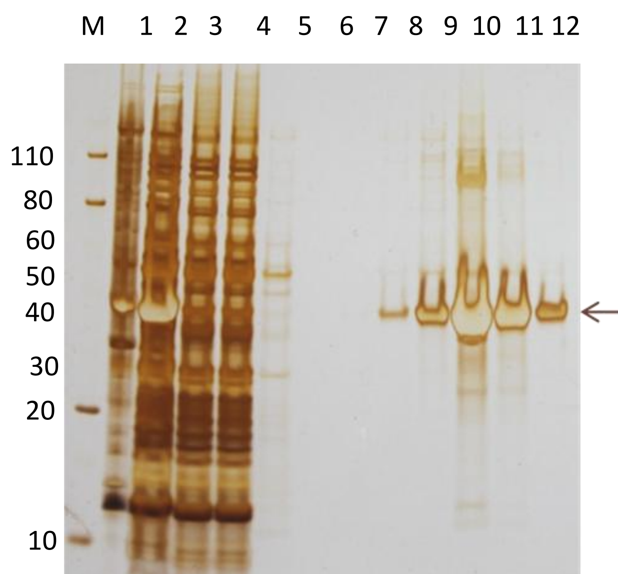

Figura 1: SDS-PAGE 12% corado com nitrato de prata; Ep31 é indicada na flecha. Legenda: M –marcador de massa molecular em kDa; 1 – extrato total da cultura bacteriana não induzido; 2 – extrato total de cultura bacteriana induzida com IPTG; 3 – primeira passagem na coluna; 4 - segunda passagem na coluna; 5 – lavagem; 6 – lavagem; 7 – primeira eluição; 8 – segunda eluição; 9 – terceira eluição; 10 – quarta eluição; 11 – quinta eluição; 12 – sexta eluição

2.1.2 Análises por Western Blot (WB)

A primeira fração da eluição da proteína (Figura 1 linha 8) foi usada para os testes de reconhecimento pelo anticorpo de soro de pacientes com angiostrongilíases. O ensaio demonstrou o reconhecimento principal de duas bandas tanto nos soros A e B quanto pelo C (Figura 2). O que indica que o reconhecimento da proteína recombinante não é capaz de discernir não infectados dos infectados, tornando o recombinante inviável para uso em diagnóstico.

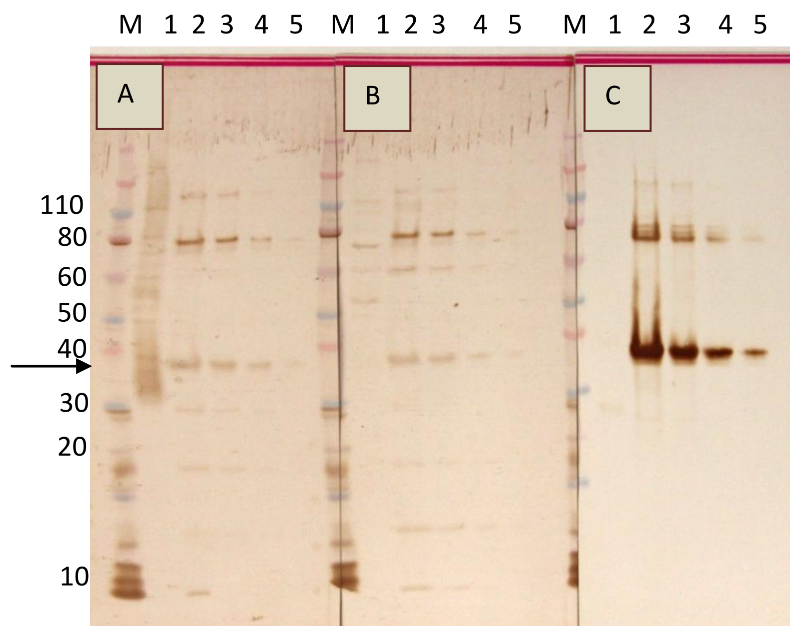

Figura 2: Western blot com anticorpo de pacientes infectados com *Angiostrongylus* spp. (A) não infectados com *Angiostrongylus* spp (B) e anti-histidina (Invitrogen) (C).

Legenda:

M –marcador de massa molecular (kDa)

1 – 0,8 ug de TE (antígeno bruto)

2 – 1 ug Ep31

3 – 0,75 ug Ep31

4 – 0, 5 ug Ep31

5 – 0,25 ug Ep31

2.2 – Expressão da proteína ES7 e análise por WB

A proteína ES7 foi expressa em sistema procariótico e purificada por cromatografia de afinidade ao níquel. A massa esperada de 26kDa (23kDa + 3kDa provenientes da construção do recombinante) foi confirmada por gel de poliacrilamida 12% (Figura 3) .Após a purificação da proteína por cromatografia, o recombinante apresentou um rendimento de cerca de 0,8 mg/ml. As frações 1 e 2 da proteína recombinante foram analisadas frente ao reconhecimento do soro de pessoas infectadas ou não com *Angiostrongylus* (Figura 3). A proteína recombinante apresentou reconhecimento fraco em C e quase imperceptível em D. Estes resultados indicam que ES7 pode ter valor diagnóstico, porém deverão ser realizados testes com diferentes soros para o estudo de reatividade cruzada entre outras infecções.

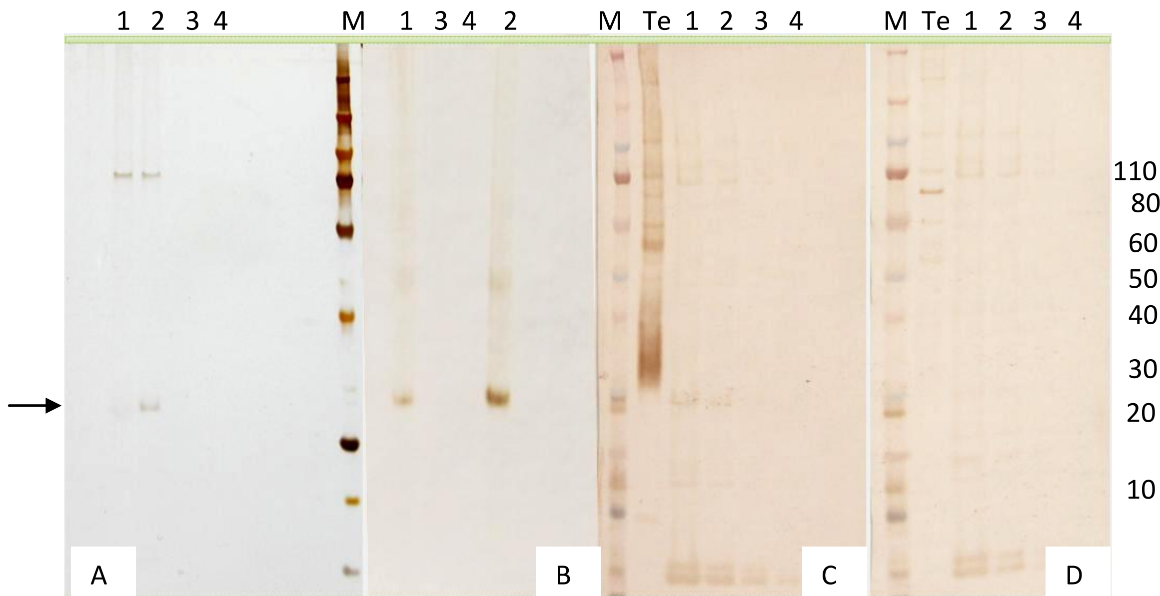

Figura 3 Análise do recombinante ES7- Purificação e WB. Legenda: A- purificação de ES7 por cromatografia. Gel de acrilamida 12% corado com nitrato de prata. B- WB com anticorpo anti-histidina; C- WB com soro de pacientes infectados com *Angiostrongylus*; D- WB com soro de pessoas não infectadas com *Angiostrongylus.* M –marcador de massa molecular (kDa); 1 – primeira eluição , 2- segunda eluição, 3- terceira eluição, 4 quarta eluição. Te (antigeno bruto) . A flecha indica ES7.

2.3 Expressão e análise por WB das proteínas 14-3-3 e Lec 5

As proteínas 14-3-3 e Lec-5 foram expressas em sistema procariótico e purificadas por cromatografia de afinidade ao cobalto. A massa esperada para 14-3-3 foi de 31kDa (28kDa + 3kDa provenientes da construção do recombinante) e para Lec-5 foi de 36kDa (33 kDa + 3kDa provenientes da construção do recombinante) foram confirmadas por gel de poliacrilamida 12% (Figura 4). Após a purificação das proteínas por cromatografia, os recombinantes foram analisados por WB para o reconhecimento pelo soro de pessoas infectadas ou não com *Angiostrongylus* e anticorpo específico anti-histidina (Figura 5). O recombinante de 14-3-3 não apresentou diferença de reconhecimento quando submetido aos soros de pacientes infectados e não. Já o recombinante de Lec-5 apresentou reconhecimento diferencial, porém fraco em B o que poderia indicar certo valor diagnóstico para esta

proteína. No entanto, ainda deverão ser feitos ensaios para a titulação do antígeno e também o estudo da reatividade cruzada entre outras infecções.

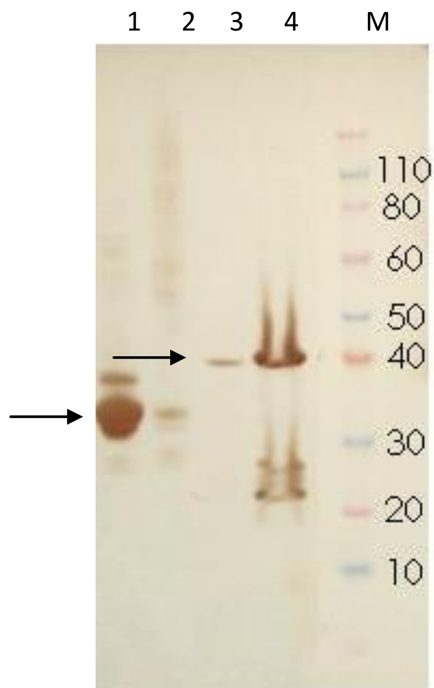

Figura.4: Expressão de 14-3-3 e Lec-5. Western blot com anticorpo anti-histidina: Legenda: M- marcador de massa molecular em kDa; 1 - 14-3-3 clone 39 – fração solúvel ; 2 - 14-3-3 clone 39 fração insolúvel; 3 - Lec5 - fração solúvel; 4 - Lec5 fração insolúvel. As flechas indicam as proteínas recombinantes.

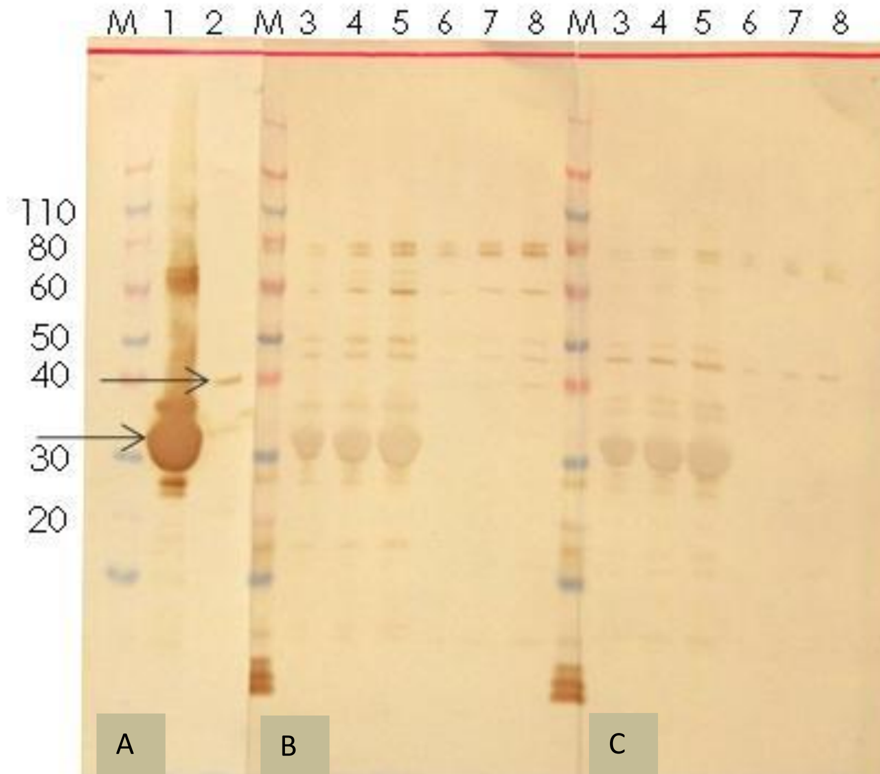

Figura 5: Análise das recombinantes por WB. Anticorpo anti-histidina (A) de pacientes infectados com *Angiostrongylus* spp. (B) não infectados com *Angiostrongylus* spp (C) Legenda: M- marcador de massa molecular (kDa); 1 - 14-3-3 após purificação; 2 – Lec5 após purificação; 3 – 1ul 14-3-3; 4 - 2ul 14-3-3; 5 – 3ul 14-3-3; 6 – 1ul Lec5; 7 - 2ul Lec5; 8 - 3ul Lec5 .

Conclusão

Quatro proteínas foram expressas em sistema de expressão procariótico. Duas provenientes de amostras de ES e duas identificadas em TE. Lec-5 e ES7 apresentaram melhor reconhecimento pelos anticorpos de pessoas infectadas com *Angiostrongylus* quando comparadas às 14-3-3 e Ep31 as quais não foram diferentemente reconhecidas. No entanto, os testes pilotos realizados para as duas primeiras apresentaram um reconhecimento específico pouco satisfatório para que

se desse prosseguimento com novos testes. Além disso os resultados apresentados no capítulo 4 mostram que a antigenicidade do antígeno de 31kDa é dependente da glicosilação das proteínas que o compõem. Portanto deverão ser utilizados sistemas de expressão eucarióticos para permitir que haja a adição de resíduos de açúcar na cadeia polipeptídica.

Apêndice 3

***Angiostrongylus cantonensis* – The Dirty Genome Approach**

Artigo em preparação.

Title suggestion:

***Angiostrongylus cantonensis* - Dirty Genome approach**

Angiostrongyliasis OR eosinophilic meningoencephalitis diagnosis.

***Angiostrongylus cantonensis* – Dirty Genome Approach – New targets for meningoencephalitis diagnosis.**

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Introduction

Eosinophilic meningitis also called cerebral angiostrongyliasis is an acute inflammation caused mainly by the infection of the nematode *Angiostrongylus cantonensis*. To date more than two thousands cases of the disease were reported most of them in Southeast Asia which is an endemic region. Nevertheless cases of the disease have been noticed around the world including areas never reported before such as Brazil, Caribe, Ecuador, Australia, and USA warning authorities for an emerging public health problem (Caldeira et al., 2007; Wang et al., 2008; Diaz JH 2008; Pincay et al., 2009; Maldonado et al., 2010).

To complete its life cycle the worm requires a definitive host such as rats and an intermediary host which are mollusks. First stage larvae (L1) are released in rat's feces which infect the mollusks and develop into infective third stage larvae (L3). Humans become infected by ingestion of the L3 contained in vegetables, water of raw and undercooked mollusks. In humans the worm is unable to complete the life cycle and dies in the meninges which cause the disease. As consequence L1 are not visualized in feces which difficult the parasitological diagnosis. Although rarely found fourth stage larvae in the cerebrospinal fluid (CSF) is considered the gold standard for diagnosis (Yii, 1976; Graeff-Teixeira et al., 2009).

The diagnosis based on the symptoms is not able to distinguish between other parasites infections such as gnathostomiasis that can also cause eosinophilic meningitis. Numerous studies have been performed to identify specific and sensitive targets for immunodiagnosis of the disease. However most of the investigations are achieved using crude extracts preparations of the nematode and do not provide the identity of those antigens which ultimately makes difficult recombinant production.

Currently the challenge facing by researchers is the lack of molecular information of many parasites. Indeed *Angiostrongylus* spp. has less than 700 nucleotides sequences, 2631

ESTs and 648 protein sequences deposited at the Genbank. Most of *Angiostrongylus* proteomics studies so far have been done using sequences of related organisms for peptide mass comparison (Morassutti el al., 2011; Rebello et al., 2011). It could elicit misinterpreted data since exclusive *Angiostrongylus* proteins or peptides would be missing observation without appropriated data bank. Finally it would imply directly on antigen identification where specific proteins are needed.

Recently, Greub et al. (2009) applied a combined strategy of genome sequencing and proteomics to identify immunogenic proteins of the emerging pathogen *Parachlamydia acanthamoebae* and named it "Dirty Genome approach". This technique allows the use of unfinished genome to identify proteins avoiding time-consuming cloning steps and gap closure.

At this work we present combined random high throughput sequencing of *Angiostrongylus cantonensis* genome together with proteomics tools to identify unknown immunoreactive proteins.

Results

DNA Sequencing

The genomic DNA of *Angiostrongylus cantonensis* was randomly sequenced by pyrosequencing using 454 (Roche) technology. Two million reads were obtained and assembled into 141,351 contigs. The average length of these contigs was 0.8 kb.

Gene identification

28,080 putative genes were identified and annotated according with their homology to known protein from Genbank which is summarized in the table 1. Annotated sequences were about 6863 as follows: 426 sequences with homology to *Angiostrongylus cantonensis*; 171

75 sequences homologous to *Neisseria* spp. 1216 sequeces homologous to *Caenorhabditis* spp.
76 and 21,209 sequeces did not matched with anything from Genbank. The identified genes
77 were clustered in appropriated categories due to their predicted functionality.

78

79 **Immuno-reactive protein identification**

80 Two different sources of antigen have been employed in our lab to investigate
81 potential antigens targets: excretion and secretion products (ES) and female crude extract
82 preparation (TE). Western Blot experiments using patient infected sera demonstrate at least
83 39 recognized spots (Morassutti et al., 2011). From these experiments the proteins were
84 identified using NCBInr data bank for peptide comparison, where several peptides did not
85 matched with any protein from that data bank. Those peptides were then searched against our
86 *A. cantonensis* genome sequences and most of peptides corresponded to the putative proteins
87 predicted by Augustus software. 206 genomic sequences were represented into protein
88 sources and that 156 were identified only at *A. cantonensis* data bank. The results are
89 summarized in table 2.

90

91 **Discussion**

92 To identify proteins by mass spectrometry a data bank is needed…

93 As expected several unique peptides were identified…

94 At least % of earlier mass spectrometry non-identified proteins were identified when
95 using *A. cantonensis* dirty genome even though there is no annotation clue of the identity or
96 function of numerous proteins. This data confirms predicted genes were present in protein
97 preparation which makes our algorithm suitable.

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99

100 **Methods**

101 DNA Extraction

102 Total DNA of *A. cantonensis* was extracted from female worms using Gentra
103 Puregene Tissue kit (Qiagen, Valencia, CA, USA) according to the Qiagen supplementary
104 protocol for purification of archive-quality DNA from nematodes
105 (www.qiagen.com/literature/render.aspx?id=103616). Briefly, approximately 100 mg of
106 female worms were incubated overnight in 3 ml Cell Lysis Solution at 55°C. Next morning,
107 the lysate was treated with RNase A at 37°C for 1 h and chilled on ice for 5 min. 1 ml Protein
108 Precipitation Solution was added to the lysate, vortexed vigorously for 20 s at high speed,
109 incubated on ice for 5 min and centrifuged at 2000 x g for 10 min. Supernatant was
110 transferred to a clean tube, mixed with 3 ml isopropanol, and DNA was precipitated by
111 centrifugation at 2000 x g for 5 min. DNA pellet was washed with 3 ml of 70% ethanol, dried
112 for 10 min at room temperature and solubilized in 200 µl of DNA Hydration Solution.

113

114

115 DNA Sequencing

116 *Angiostrongylus* DNA was sequenced using the platform, GS-FLX 454 Titanium
117 (Roche Applied Science). Two Titanium runs were performed using standard protocols from
118 the manufacturer. A Rapid Library was constructed utilizing nebulization, Ampure (Beckman
119 Coulter, Inc.) size selection, subjected to emulsion PCR and enrichment and then sequenced
120 utilizing Titanium chemistry. Data was compiled and assembled into a hybrid draft sequence
121 using CLC Workbench which utilized the long GS-FLX reads for contigs formation and
122 correction of homopolymer errors. Data reads were length trimmed (>100 bp) and assembled
123 *de novo*.

124

Sequence analysis

Contigs were examined by the program Augustus to verify sequences of putative genes using *C. elegans* sequences as a model.

Protein identification

Proteins data from MS/MS experiments were assembled against genome data by Myrimatch software. Final annotation of each peptide, score ranking and artifacts exclusion were done using IDPicker software. The scheme 1 shows the algorithm used to analyze the data.

Data access

Acknowledgments

Author Contributions

Figure legends

Figures

Tables

References