PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

Estudos sobre a especificidade de antígenos e sua aplicabilidade para o imunodiagnóstico das angiostrongilíases.

Bianca Barbieri Cognato

TESE DE DOUTORADO

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
Av. Ipiranga 6681 - Caixa Postal 1429
Fone: (051) 320-3500
CEP 90619-900 Porto Alegre - RS
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Orientador: Dr. Carlos Graeff-Teixeira

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Sumário

Agradecimentos

Foram 4 anos imaginando como seria chegar ao final do doutorado. Parecia tão longe, mas passou tão rápido. Pensava em como seria sentar na frente do computador e escrever os agradecimentos. Pois então, chegou a hora.

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Resumo

Duas espécies de nematódeos pertencentes a superfamília Metastrongyloidea, são capazes de produzir doença em humanos: *Angiostrongylus cantonensis* e *Angiostrongylus costaricensis*. Ambos são parasitas próprios de roedores e a infecção humana é considerada acidental. A meningite eosinofílica, causada por *A. cantonensis*, tem gerado preocupação na comunidade científica pela expansão da área geográfica de ocorrência. Métodos moleculares e imunológicos para o diagnóstico desta infecção são cruciais para o diagnóstico, entretanto, após muitos estudos com diferentes moléculas antigênicas, até hoje não foi desenvolvido um teste de diagnóstico que seja específico e sensível o suficiente para discriminar as angiostrongilíases de outras parasitoses. A reatividade cruzada tem sido o principal problema encontrado nos estudos já desenvolvidos para este propósito. Com o objetivo de aprimorar o diagnóstico das angiostrongilíases, foi realizado neste estudo análise comparativa do reconhecimento de proteínas de diferentes extratos teciduais de *A. cantonensis, Toxocara canis, Schistosoma mansoni* e *Strongyloides stercoralis* contra soro positivo para *Angiostrongylus* spp Através de kit de extração, eletroforese unidimensional e bidimensional, western blot e espectrometria de massas foram identificadas 149 proteínas. Dentre estas, 34 foram exclusivas para *A. cantonensis*, sendo que *COI* estava presente apenas no extrato de *A. cantonensis* não possuindo similaridades com nenhum outro parasito comparado no NCBI (nr database) e WormBase Database. Estas proteínas podem ser consideradas promissoras como marcadores de reatividade humoral específica para o parasito. Todavia, outras nove proteínas foram reconhecidas por mais de um parasito em mais de um extrato testado, sendo importantes marcadores de reatividade cruzada em infecções parasitárias. Com os dados obtidos neste estudo, sugerimos como marcadores de reatividade cruzada o uso das proteínas: Galectin 1, HSPA-5 e Ifa1. Além disso, proteínas imunogênicas de *A. cantonensis* ES-7, Lec-5 e 14-3-3, foram expressas de forma recombinante em dois tipos celulares, CHO e HEK, e o potencial uso diagnóstico destas proteínas foi verificado através de eletroforese uni e bidimensional, *western e dot blot*, e tratamento por N-glycosidase F (PNGase F), utilizando soro positivo para *Angiostrongylus* spp., soro negativo e positivo para outras parasitoses. Proteína ES-7 expressa em células HEK e CHO, e Lec-5 expressa em CHO foram reconhecidas apenas pelo soro positivo para *Angiostrongylus* spp. e não pelo soro negativo e controles de especificidade em testes 2D e 1D, respectivamente. Já nas análises em 2D, Lec-5 apresentou fraco reconhecimento com soro negativo. Já a proteína 14-3-3 não apresentou nenhuma especificidade pelo soro de *A. cantonensis*, já que foi reconhecida por todos os soros testados. O reconhecimento antígeno-anticorpo se mostrou dependente das porções glicídicas, já que quando tratadas com N-glycosidase F (PNGase F), o reconhecimento das proteínas pelo soro desapareceu. A expressão heteróloga, utilizando células de mamíferos, assim como a identificação de moléculas compartilhadas e/ou específicas, podem representar uma promissora fonte de antígenos para o diagnóstico da meningite eosinofílica, requerendo aprimorados testes moleculares para seu diagnóstico.

Palavras-chave: *Angiostrongylus cantonensis*; reatividade cruzada; diagnóstico; proteínas recombinantes

Abstract

Study of antigens specificity and its application for the immunodiagnosis of angiostrongyliasis.

Two nematode species belonging to the Metastrongyloidea superfamily are capable to produce disease in humans: *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis*. Both are rodent parasites and human infection is considered accidental. Eosinophilic meningoencephalitis, caused by *A. cantonensis*, raises concern due to the expanding number of cases and geographical area of occurrence. Molecular and immunological methods for the diagnosis are crucial, however, after many studies with different antigenic molecules, has a specific and sensitive test to discriminate the angiostrongyliasis to others parasitoses is lacking. Cross-reactivity with other helminthes, which may cause similar symptoms, and eosinophilic meningitis, has been a problem for the satisfactory performance of specificity in serological tests. In order to improve the diagnosis of angiostrongyliasis, a comparative analysis of protein recognition from different extracts from *A. cantonensis*, *Toxocara canis*, *Schistosoma mansoni and Strongyloides stercoralis* against positive serum for *Angiostrongylus*.spp was performed. Through extraction kit, one-dimensional and two-dimensional electrophoresis, western blot and mass spectrometry, 149 proteins were identified. Among these, 34 were exclusive to *A. cantonensis*, *COI* being present only in the *A. cantonensis* extract, having no similarities with any other parasite compared in NCBI (nr database) and WormBase Database. Additionally, nine proteins were recognized by more than one parasite and extract, being important cross reactivity markers in parasitic infections. With the data obtained in this study, we suggest the use of the follow proteins as cross-reactivity markers: Galectin 1, HSPA-5 and Ifa1. In addition, immunogenic proteins of *A. cantonensis* ES-7, Lec-5 and 14-3-3, were recombinant expressed in two cell types, CHO and HEK. Their potential diagnostic values were verified by uni and bidimensional electrophoresis, western and dot blot, and N-glycosidase F (PNGase F) treatment using serum from patients infected with *A. cantonensis*, negative serum for parasites, and positive for other parasites. ES7 protein expressed in HEK and CHO cells and Lec-5 expressed in CHO were recognized only by *Angiostrongylus*-positive serum and not by negative control and specificity control in 2D and 1D tests, respectively. In the 2D analyzes Lec-5 showed a weak recognition with negative serum. However, the 14-3-3 protein didn´t show any specificity against *A. cantonensis* serum, since it was recognized by all tested sera. Antigen-antibody recognition was found to be dependent on the glycogenic portions, since when treated with N-glycosidase F (PNGase F), recognition between proteins and serum disappeared. The heterologous expression, using mammalian cells, as well as the identification of shared and/or specific molecules, may represent a promising source of antigens for the diagnosis of eosinophilic meningoencephalitis, and molecular diagnostic tests become necessary.

Keywords: *Angiostrongylus cantonensis*; cross-reactivity; diagnosis; recombinant proteins

Apresentação

A presente tese de doutorado intitulada *Estudos sobre a especificidade de antígenos e sua aplicabilidade para o imunodiagnóstico das angiostrongilíases* é composta por dois manuscritos independentes, onde serão descritos os principais resultados, apresentados nos seguintes capítulos:

Capítulo I: **Molecular markers for cross reactivity in the immunodiagnosis of angiostrongyliasis and other parasitosis**. Será submetido ao periódico *Experimental Parasitology*. Neste estudo foram investigadas moléculas antigênicas de diferentes parasitos, também causadores da meningite eosinofílica, e que podem causar reatividade cruzada no imunodiagnóstico das angiostrongilíases.

Capítulo II: **Recombinant expression of three antigenic proteins from *Angiostrongylus cantonensis*: ES-7, Lec-5 and 14-3-3 in mammalian cells**. Será submetido ao periódico *Experimental Parasitology*. Neste trabalho, proteínas imunogênicas de *A. cantonensis*, foram expressas de forma recombinante em células de mamíferos CHO e HEK, obtendo resultados satisfatórios se comparadas com expressões anteriores em células de organismos procarióticos.

Após a apresentação dos manuscritos, serão apresentadas as conclusões gerais, onde são compilados os principais resultados obtidos nos estudos citados, assim como discussões de perspectivas futuras.

Por fim, a presente tese é complementada por um apêndice onde se apresenta o estudo de **Análise comparativa entre as seguintes variáveis: componentes celulares, processos biológicos e função molecular de antígenos de *A. cantonensis, T.canis, S. stercoralis* e *S. mansoni* reconhecidos pelos soros controles positivos para *Angiostrongylus* spp.**

Capítulo I

Molecular markers for cross reactivity in the immunodiagnosis of angiostrongyliasis and other parasitosis.

Manuscrito formatado segundo as instruções do periódico *Experimental Parasitology*.

Molecular markers for cross reactivity in the immunodiagnosis of angiostrongyliasis and other parasitosis

Bianca B. Cognato[a], Sukwan Handali[b], Leandro de Mattos Pereira[a], José Ricardo Barradas[a], Alessandra L. Morassutti[a*], Alexandre Januário da Silva[c], Carlos Graeff-Teixeira[a]

[a] Laboratorio de Parasitologia Molecular, Instituto de Pesquisas Biomédicas and Laboratorio de Biologia Parasitaria, Faculdade de Biociências da Pontifícia Universidade Catolica do Rio Grande do Sul (PUCRS), Av Ipiranga 6690, 90690-900 Porto Alegre RS, Brazil

[b] Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention. 1600 Clifton Road, MS D-64, Bldg 23, Room 9-440 Atlanta, GA 30329, EUA.

[c] U.S. Food & Drug Administration, Center for Foods Safety and Applied Nutrition, Office of Applied Research and Safety Assessment, Division of Virulence Assessment.

*Corresponding author - Address: Laboratório de Biologia Parasitaria da Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga 6690, Prédio 12, Bloco C, Sala 241, 90690 900 Porto Alegre RS, Brasil. Phone: +55 51 3320 4144.

E-mail address: bicognato@hotmail.com

Abstract

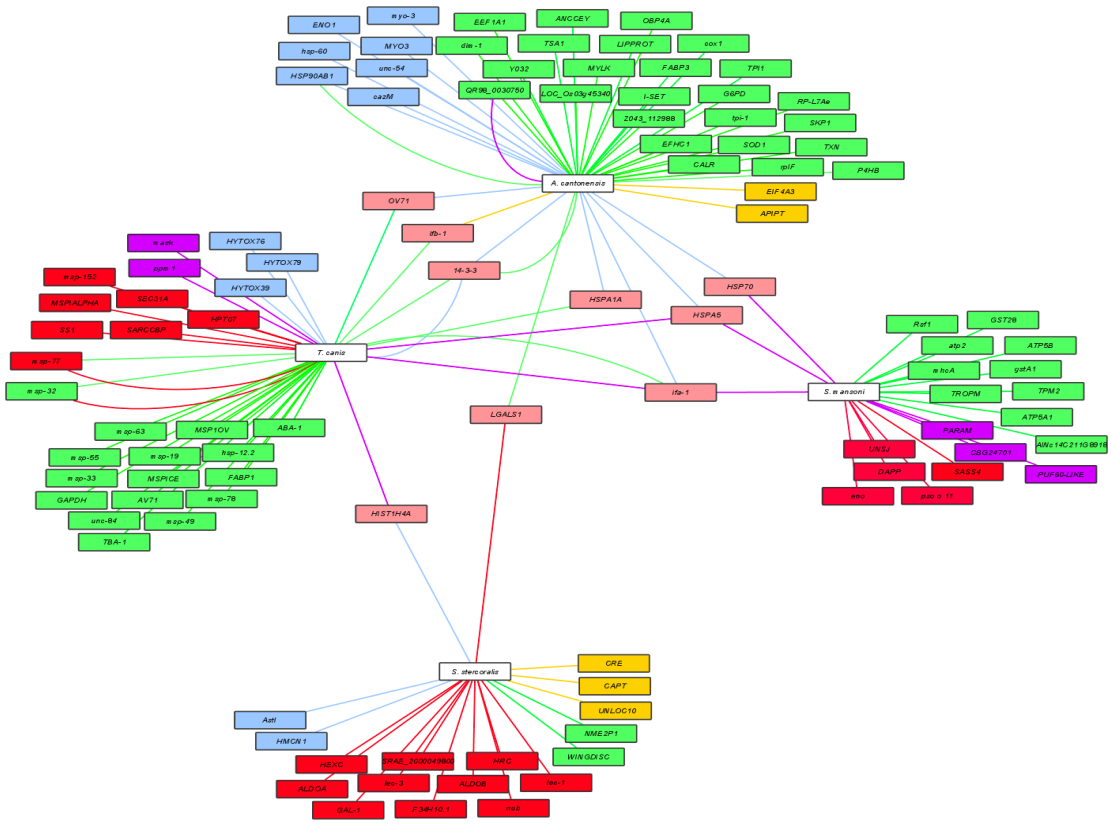
Angiostrongylus cantonensis is a parasitic nematode of rodents and the causative agent of eosinophilic meningoencephalitis (EoM) in humans. Molecular diagnostic methods are important since identification of larvae in CSF is extremely rare. Western blot and recognition of a 31kDa antigen has been the main immunodiagnostic method for EoM caused by *A.cantonensis* but cross-reactivity with other parasites has been demonstrated. The main goal of the present study was to investigate this cross-reactivity. Comparative analysis of the recognition by sera from individuals with angiostrongyliasis of proteins from different cellular sources of *A. cantonensis, Toxocara canis, Schistosoma mansoni* and *Strongyloides stercoralis* allowed the identification by mass spectrometry of 115 cross-reacting proteins. Three of them were considered possible markers for cross reactivity, a heat shock protein (HSP), an intermediate filament protein (Ifa) and Galectin 1. In addition, synthetic peptides from some of those proteins were tested against sera from individuals infected with several other parasites, allowing the identification of two other markers for cross reactivity. On the other hand, 34 proteins were found exclusively in the *Angiostrongylus* extracts, so standing as promising diagnostic molecules for a specific identification of *A.cantonensis* infection, as *COI* shown to have a great value in angiostrongyliasis immunodiagnostic.

Keywords: *Angiostrongylus cantonensis*; cross-reactivity; immunodiagnostic

Highlights

- 149 proteins were identified among five extracts from *A. cantonensis, T. canis, S. mansoni* and *S. stercoralis*;
- Nine proteins were classified as cross reactivity markers, since they were present in more than two parasites and extracts;
- Peptide 4 from 14-3-3 protein and peptide 12 from Lec-5 protein could serve as cross reactivity markers for immunodiagnosis.
- 34 proteins were exclusively found in *A. cantonensis* extracts;
- *COI* protein, from cytoplasmic extract, showed to be an exclusive protein from *A. cantonensis* and not found in any parasites sequences tested;

Graphical Abstract



1. Introduction

Cerebral angiostrongyliasis, and/or eosinophilic meningoencephalitis (EoM) caused by *A. cantonensis* are a neglected disease, (Eamsobhana, 2014) which has been causing concern due to the growing number of diagnosis and expanding geographical area of occurrence. More than 2800 cases of eosinophilic meningitis had been reported in many countries, especially in Asia and Pacific Islands (Wang et al, 2008). In Brazil, the first case of EoM caused by *A.cantonensis* was reported in 2007, and since then the parasite has been found in definitive, intermediate and accidental host widely distributed in Brazilian coastal areas (Caldeira et al., 2007; Maldonado Júnior et al., 2010; Simões et al., 2011; Carvalho et al., 2012; Cognato et al., 2013). Different organisms may cause EoM (Graeff-Teixeira et al., 2009), and in human infection parasitological diagnosis is almost never possible, requiring indirect methods for diagnosis. Almost 80 years after the discovery of the parasite and the disease, a sensitive and specific immunodiagnostic test is still not worldwide available. The most important antigen for the immunodiagnosis of EoM due to *Angiostrongylus* is the 31kDa band characterized by Eamsobhana et al, (1998). It was first shown to have 100% of specificity and sensitivity (Eamsobhana et al., 2001), however a larger study observed the 31kDa antigen with cross-reactivity to other helminth infection such as gnathostomiasis, toxocariasis, hydatidosis, and strongyloidiasis (Morassutti et al., 2016 accepted).

Cross-reactivity with other helminths may be observed due to molecules shared by neighboring taxonomic groups (Vitta et al., 2010; Dekumyoy et al., 2000). This is particularly important in those cases of EoM where similar clinical presentation prevents a clear distinction of different etiological agents (Wang et al., 2008). Although *A. cantonensis* infection is the main causative agent of EoM, the disease may have other less frequent causes such as fungus, viral infections, drugs and other parasitic infections, like strongyloidiasis, toxocariasis, cysticercosis, trichinellosis, schistosomiasis and gnathostomiasis (Graeff-Teixeira et al., 2009).

Here we present a study of immunological cross-reactivity between antigenic components from four different helminths that may cause EoM and their sub-components extracted from cytoplasm, membrane, nucleus, chromatin-bound and cytoskeleton.

2. Materials and Methods

2.1 Parasites

Adult worms of *Schistosoma mansoni, Angiostrongylus cantonensis,* and *Strongyloides stercoralis* were obtained from the life cycle maintained in the Parasite Biology Laboratory in the Pontifícia Universidade Católica do Rio Grande do Sul. Adult worms of *Toxocara canis* were kindly provided by veterinary clinics obtained from dogs naturally infected treated with anti-helminth drugs.

2.2 Ethical issues

Parasites were obtained by *in vivo* passages (rats and snails) for antigen extraction. The protocol for use of animals was approved by PUCRS ethics committee (CEUA 11/00279.). All clinical samples at CDC used in this study were collected in previous studies with specific permission for future use of stored samples (CDC Study Protocol Number 3580). Samples were anonymized and the study was performed in compliance with protocols approved by the ethical review boards of the CDC. Serum samples were stored in biobank of Immunochemistry laboratory of Division of Parasitic Diseases and Malaria at CDC and Laboratory of Molecular Parasitology at PUCRS, with collection protocols originally approved by ethics committee.

2.3 Sera samples

For immunological tests of cross reactivity *Angiostrongylus*-positive serum (5 individuals and a pool of 5 positive sera for angiostrongyliasis); normal human sera (NHS) (individuals with no symptoms) and 9 positive sera from other parasitosis (*Schistosoma mansoni, Schistosoma haematobium, Echinococcus granulosus, Echinococcus multilocularis, Toxocara canis, Trichinella spiralis, Strongyloides stercoralis, Wuchereria bancrofti*, and cysticercosis).

2.4 Subcellular protein fractionation of worms

Subcellular extracts were obtained by using Subcellular Protein Fractionation Kit for Tissues (Thermo Fisher Scientific), for protein purification, isolation and separation of cytoplasmic, membrane, nuclear soluble, chromatin-bound and cytoskeletal protein extracts, following manufacturer's' instructions.

2.5 Peptides

Synthetic peptides were produced from the main antigenic components comprising the 31kDa band: 14-3-3 (Morassutti et al., 2012) and other two proteins previously identified from ES products as specific antigenic targets for angiostrongyliasis, Lec-5 and ES-7 proteins (Morassutti et al., 2013). Peptides were tested against serum from those other EoM etiologic causes listed on serum samples section. Sequences of proteins were obtained from NCBI bank (Table1). A total of 18 peptides were randomly selected in order to cover all protein sequence and holding 30 amino acids in average (Table1). Peptides were chemically produced by Biotechnology Core Facility Branch at CDC (Centers for Disease Control, and Prevention). Composition of each peptide was confirmed by ESI mass spectrometry analysis.

Table 1

All 18 peptides amino acid sequences and their respective original protein.

Identification	Amino acid sequence	Original protein	Protein Acession number
Peptide 1	MTDNRGELVQRAKLAEQAERYDDMAQSMKKVTELGAELSNEERNLLSVAY	14-3-3	AEK98129.1
Peptide 2	EERNLLSVAYKNVVGARRSSWRVISSIEQKTEGSEKKQQMAKEYREKVEK	14-3-3	AEK98129.1
Peptide 3	AKEYREKVEKELRDICQDVLNLLDKFLIPKAGNPESKVFYLKMKGDYYRY	14-3-3	AEK98129.1
Peptide 4	LAEVACGEDRSSVVEKSQQSYQEAFDIAKD	14-3-3	AEK98129.1
Peptide 5	KMQPTHPIRLGLALNFSVFYYEILNAPDKACQLAKQAFDDAIAELDTLNE	14-3-3	AEK98129.1
Peptide 6	AIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDAAADDQDTGEQGEGAN	14-3-3	AEK98129.1
Peptide 7	MRMKVLLLLTIVCAFA	Lec-5	AEK98127.1
Peptide 8	DDSKDNSSEEAKEKNYRKYIGEKDYKLPFKTRVSEAFVVGQTIHAVGTLTEK	Lec-5	AEK98127.1
Peptide 9	IHAVGTLTEKPTRIDFNFHKGAAKDADLPLHFSIRFDEGFFSGKVVYNTFKD	Lec-5	AEK98127.1
Peptide 10	GKVVYNTFKDGNWSDNEQRISNPFKAGQEFDLRVRILEGKFQVFANRVEVGV	Lec-5	AEK98127.1
Peptide 11	VFANRVEVGVFEQRQPLDGIDHVSIRGDLEKLRLFHYGGRIFPNPYMAVAEL	Lec-5	AEK98127.1
Peptide 12	PNPYMAVAELKPGKRLDISALPTGKRVNINLYRENKEYALQVSIRFNEGAIV	Lec-5	AEK98127.1
Peptide 13	RNAMNNNVWGKEEREGNMPISKGEVFDLTIINEEFSFQIFFNGKRFASFSHRGSPTDIKTLEIDGDVEIHTV	Lec-5	AEK98127.1
Peptide 14	APPLMPDAASSGPCVDGVNNVFDLDNVGNESALYIRAKNVIAQ	ES-7	CAR63559.1
Peptide 15	VIAQTYDVNEKPSCYNGRASVQLPGMIKLVSGTLIVTKKFDLEK	ES-7	CAR63559.1
Peptide 16	FDLEKSGDLRLTVTKDSFFIGTVCMDGVSQTSLVPSSDCHRKIF	ES-7	CAR63559.1
Peptide 17	HRKIFPGLDKGFVDMISNPGTYDLQEIEKGAGRSNVVSLPAIPST	ES-7	CAR63559.1
Peptide 18	PAIPSTEAFIVTGDWEAQLTLISDGETVADIKAPSNTHWLYVS	ES-7	CAR63559.1

2.6 Unidimensional electrophoresis (1D)

All proteins extracts were submitted to unidimensional electrophoresis using Mini-PROTEAN® TGX™ Precast Gels 15% (Bio-Rad Laboratories) and a final concentration of 0.5ug. Sample buffer used was composed of 10% SDS, 6% glycerol and Tracking Dye (50mg bromophenol blue, 8ml glycerol, 1ml 0.5 M Tris HCL, pH 8.0, 1ml distilled water). The proteins were applied to the gel with a voltage of 75V for 25 min using a BIO-RAD MiniProtean electrophoresis system.

2.7 Bidimensional electrophoresis (2D)

The extracts detectable cross-reactivity in the 1D assay, were subjected to a bidimensional electrophoresis (2D). 1µg of each extract was used for Western Blot gels and 50ug were used for staining gels. Each extract was desalted using 2-D Clean-Up Kit (GE Healthcare, Piscataway, NJ), and also submitted by a resolubilization kit DeStreak Rehydration Solution (GE Healthcare), with 66mM DTT (Dithiothreitol, Sigma-Aldrich, Canada 0.05g) and 0.5% carrier ampholytes. The samples were in-gel rehydrated on 11cm pH 3-11 NL IPG strips (GE Healthcare) overnight and isofocalized with voltages increasing stepwise as follows: 500 V for 500 V h, a linear gradient from 500–8000 V for 6500 V h, followed by a hold at 6000 V for 22,000 V h. After isoelectric focusing, the strips were soaked for 15 min in fresh equilibration buffer (20% v/v glycerol, 6 M urea, 1% DTT, and 2% SDS). IPG strips were run in the second dimension on Criterion XT Precast Gels 4-12% Bis-Tris IPG (Bio-Rad Laboratories). The gels were then stained with colloidal Coomassie blue or transferred to nitrocellulose membranes for immunological analyses.

2.8 Dot blot and Western blot

Peptides were analyzed by Dot Blot and proteins extracts were analyzed by Western blot in order to verify the reactivity using *Angiostrongylus*-positive sera, non-infected sera and other helminth-infected sera as above described. Peptides were blotted onto nitrocellulose membrane with 1ul in a concentration of 1ug/ml, and proteins extracts were transferred from gel to nitrocellulose membrane. Membranes were incubated with phosphate buffered saline (PBS) with Tween (0.05%) and skim powdered milk 0,5% for 1h in room temperature and then incubated for 1h with positives or negative control serum (1:100 dilution). After three washes with PBS Tween (0.05%), the membranes were probed with a secondary peroxidase-conjugated anti-human IgG (diluted 1:8000) for 1h at room temperature. Diaminobenzidine (DAB) (Sigma-Aldrich; 0.05% DAB and 0.015% H2O2 in PBS, pH 7.4) was added as developer reagent. Proteins titration from different parasite´s extracts were

developed based on negative controls for *Angiostrongylus* infection in comparison with their respective SDS PAGE (50ul).

2.9 Shared protein identification

After 2D and WB analysis of each extract, spots from the four species of parasites that were recognized by *Angiostrongylus*-positive sera were compared by molecular weight and isoelectric point (IP) of the migrating gel. Those that matched the same molecular weight and IP were considered as cross-reactive spots and were recovered from the original 2D gel and excised for mass spectrometry analyses. All analysis were done in duplicate.

2.10 Mass spectrometry analysis

Immunoreactive spots were cut from 2D gels with a sterile scalpel blade and subjected to in-gel tryptic digestion (Promega, Madison, WI). Protein identification was made by electrospray ionization (ESI) mass spectrometric analysis using a Bruker model maXis ESI-Q-TOF instrument interfaced with an on-line nanospray source (Bruker Daltonics) to perform LC-MS/MS using a U3000 RSLCnano HPLC configured for nanoliter per minute flows. The Dionex U-3000 RSLCnano nanobore HPLC was configured with a binary nanoflow ultra-high pressure pump and a ternary high-pressure microbore pump. The system used a pulled-loop autosampler configured with a 20 ul sample loop. A desalting trap column (0.3 x 5 mm, 5 um C18 PepMap 120 A, Dionex) was used and the analytical column used was a C18 PepMap (0.075 x 250 mm, 2um, 120A, Dionex). The solvents used were 0.1% formic acid in water (A) and 80% acetonitrile/0.1% formic acid (B). The gradient was 2-55% B in 90 minutes. 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 1200V with a drying gas of nitrogen flowing at 6 liters per minute. The capillary temperature was set to 150 °C. The mass spectrometer was set to acquire spectra of m/z 50 to 2200. MS/MS data was acquired in an automated fashion using the three most intense ions from the MS scan with precursor active exclusion for 90 seconds after two spectra were acquired for each parent ion. MS data was acquired at a scan speed of 4 Hz and MS/MS data was acquired at a scan speed of 1-10 Hz depending on the intensity of the parent ion. MS internal calibration was achieved by the use of a lock mass (HP-1222, Agilent Technologies). The collected data was processed by DataAnalysis (Bruker Daltonics) to produce deconvoluted and internally calibrated data and saved as a xml peaklist which was uploaded to our Proteinscape database (Bruker Daltonics). Proteinscape automatically submitted the peaklist to the MASCOT server (http://www.matrixscience.com/search_form_select.html).

2.11 Protein Identification

All files with ".mgf" format obtained from mass spectrometry, were analyzed by Mascot Server and MetaproteomeAnalyser (Muth et al., 2015) program using the following parameters: MS/MS perform search, NCBInr database, trypsin as enzyme, other metazoan as taxonomic group, carbamidomethyl as fixed modifications, oxidation as variable modification, peptide 0.6 Da, data file: mascot generic, ESI-QUAD-TOF as instrument. The significance threshold required that each protein containing at least one peptide considered was a p-value < 0.05.

2.12 Clustering of shared protein

Clustering of shared proteins were performed by *heatmap.2* from *gplots* (version 3.0.1) (Warnes GR, et al 2016), package for *R environment* (R Core Team, 2016) and *Cytoscape* (version 3.2.1) (http://www.cytoscape.org/what_is_cytoscape.html) with yFiles Layout/Organic layout. With this visualization´s method, data were organized by different colors indicating presence and absence to *Angiostrongylus*-positive serum recognition, in *Heatmap* graphic. Using Cytoscape software, proteins extracts from the four different species of parasites were organized by connections of nodes by interconnections of edges, indicating shared proteins between *A. cantonensis*, *S. stercoralis*, *S.mansoni* and *T. canis* helminths.

3. Results

3.1 Shared proteins among different parasite extracts

A total of 106 proteins from *T. canis*, *S. stercoralis* and *S. mansoni* were identified as being recognized by *Angiostrongylus*-positive sera thus considered as cross reactivity markers. In the other hand 34 proteins recognized by the *Angiostrongylus*-positive sera were exclusively identified in *Angiostrongylus* extracts (Figure 1). Nine proteins were identified among the four parasite extracts as being recognized by *Angiostrongylus*-positive sera (Figure 2). Between *T. canis* and *A. cantonensis* were found OV71 (Muscle cell intermediate filament protein), ifb-1(intermediate filament protein), 14-3-3 and HSPA1A (Heat shock protein). While HIST1H4A (Histone) was found being shared only among *T. canis* and *S. stercoralis* extracts and HSP70 (Heat shock protein 70) being shared between *A.cantonensis* and *S. mansoni*. LGALS1 (Galectin 1) was found being shared only between *A.cantonensis* and *S. stercoralis.* Finally ifa-1(Intermediate filament protein) and HSPA5 (78kDa glucose-regulated protein) were found to be shared among the three different parasites *A.cantonensis*, *T. canis* and *S. mansoni* which were considered cross reactivity markers. All reactive proteins from the different extracts are presented at figure 3, where all identified proteins are connected with the correspondent organism.

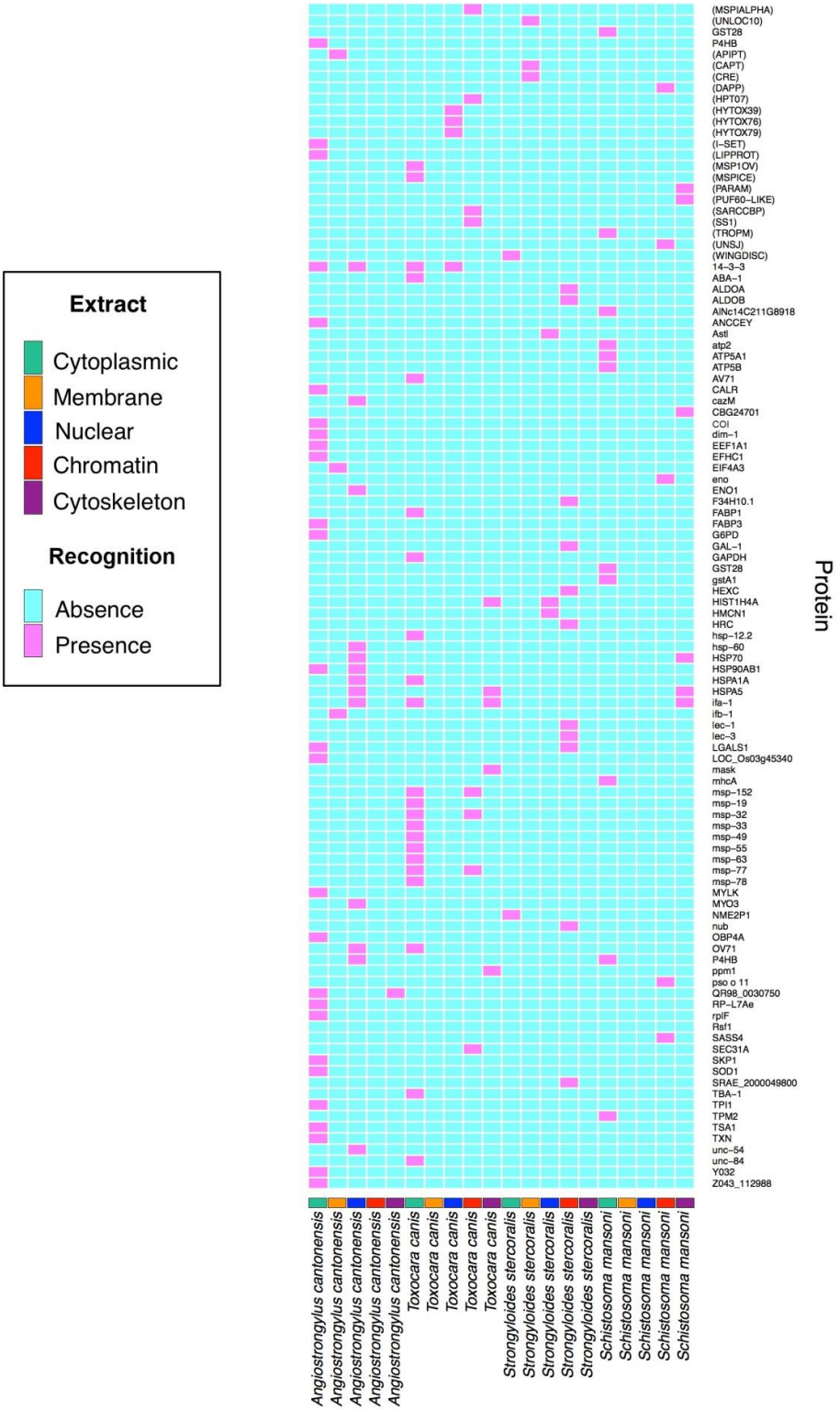


Fig. 1: Heat map diagram with all proteins recognized by *Angiostrongylus*-positive serum and the corresponding parasite and extract.

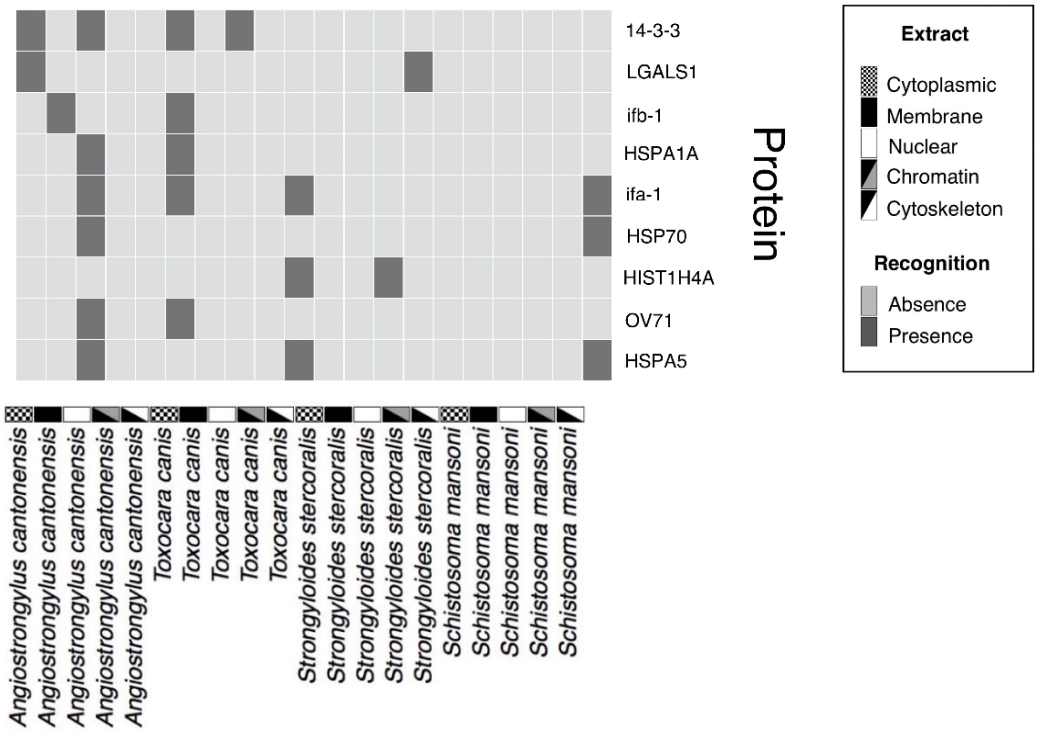


Fig. 2: Shared proteins for different parasite extracts recognized for *Angiostrongylus*-positive serum**.**

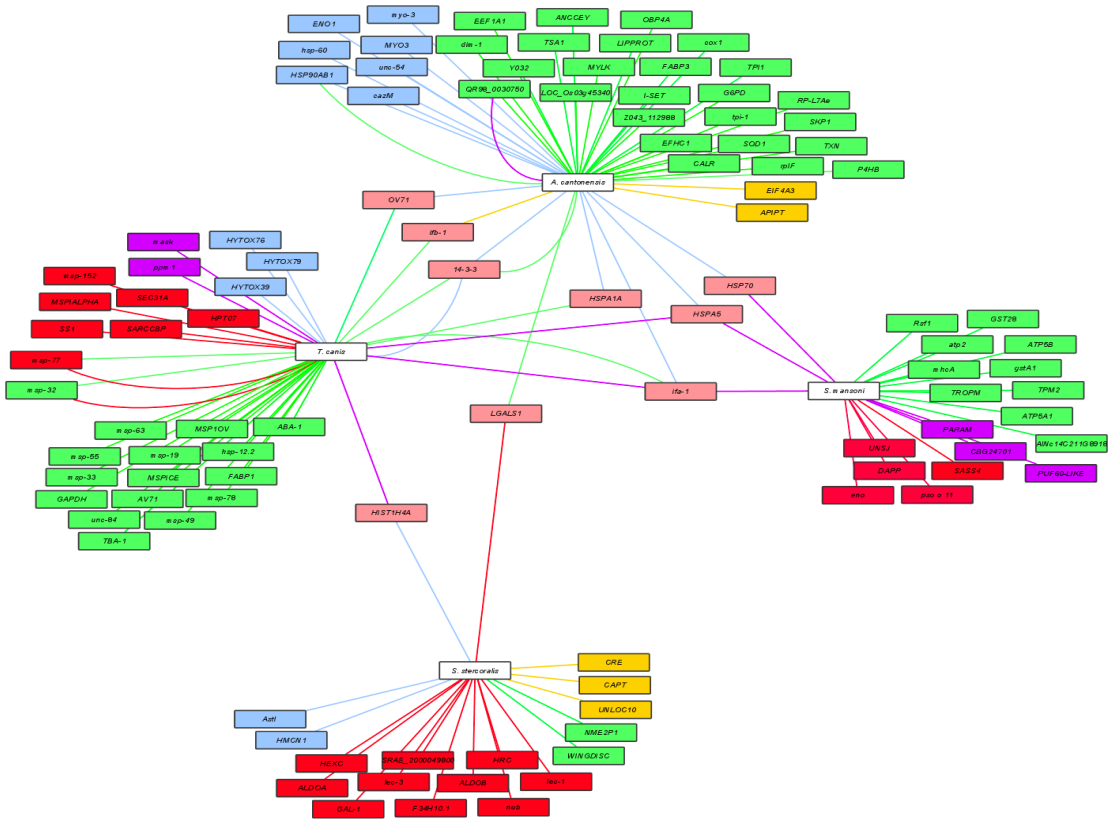


Fig. 3: Cross reactivity between *Angiostrongylus cantonensis*, *Toxocara canis*, *Strongyloides stercoralis* and *Schistosoma mansoni* antigenic proteins against *Angiostrongylus*-positive serum, represented by Cytoscape network**.** (Green: cytoplasmic extract – Yellow: membrane extract – Blue: nucleus extract – Red: chromatin extract – Purple: cytoskeleton extract – Pink: shared proteins)

3.2 Peptides

Peptide recognition to the several tested serum sample revealed that peptide number 1 was recognized only by *E. multilocularis* positive serum and not by any *Angiostrongylus*-positive serum. Peptide number 3 was recognized by *S. mansoni*, *E. multilocularis*, Cysticercosis *and T. spiralis* as well to the pool of NHS, and not for individual serum. Peptides 4 and 12 were recognized only by the positive sera for other parasites, but not for *Angiostrongylus*-positive serum and NHS. Peptides 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16, 17 and 18 were not recognized by neither of sera tested (Figure 4).

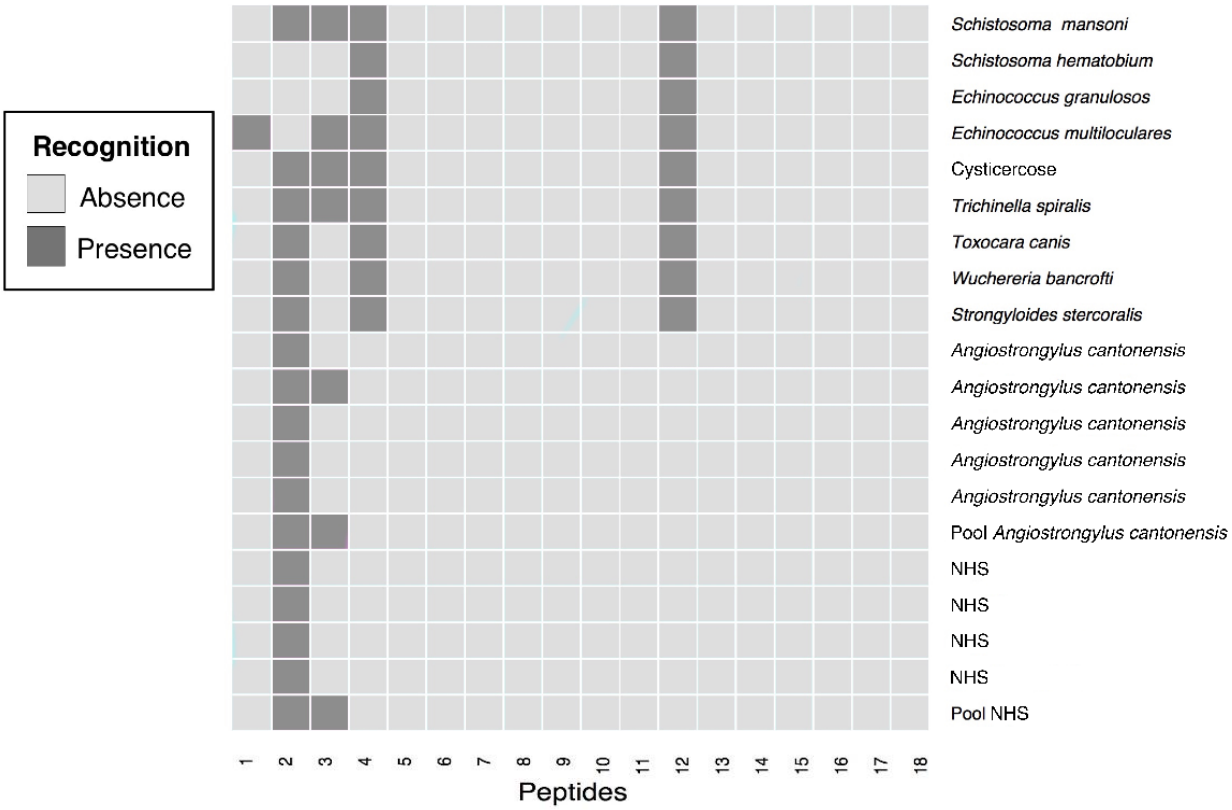


Fig. 4: Heat map diagram indicating recognition of peptides by different sera analyzed by dot blot.

4. Discussion

Angiostrongylus cantonensis is the main causative agent of eosinophilic meningoencephalitis (EoM) in humans. Immunodiagnostic and molecular methods are useful and necessary tools for angiostrongyliasis diagnosis. Since the parasite and the disease was described 80 years ago, many studies have been done in order to develop a specific and sensitive diagnostic test and cross- reactivity has always been a challenge (Vitta et al., 2010; Dekumyoy et al., 2000; Nuamtanong, 1996; Eamsobhana et al., 1998).

The present study allowed an extensive analysis of cross reactivity between proteins from different extracts of *A. cantonensis* and other parasites. From the 149 proteins here identified (supplementary material Table 1), being recognized by *Angiostrongylus*-positive sera, nine were found within all parasites extracts (Figure 2). Among these nine proteins, highlights the 14-3-3, which was identified previously as the main antigenic component of the 31kDa band (Morassutti et al., 2013). A recent study showed cross-reactivity within the 31kDa band with many parasitic infections causing EoM, including toxocariasis, strongyloidiasis and hydatidosis (Morassutti et al., 2016). Our result corroborate with this finding since 14-3-3 of *T. canis* was found in two different extracts being recognized by *Angiostrongylus*-positive sera.

In addition, we evaluated the 14-3-3 protein fragmented into six peptides, which presented cross-reactivity among toxocariasis, hydatidosis strongyloidiasis, and others (Figure 4). Particularly, peptide 4 of 14-3-3 was only recognized by the serum of other parasite infection and not by the *Angiostrongylus*-positive sera. Protein folding may not allow the complete exposition of some sites of the protein that could be recognized by immunoglobulins. This has been observed for immunodiagnostic using short peptides for strongyloidiasis (Feliciano et al., 2016). Other studies have demonstrated the efficacy of using peptides for diagnosis in hydatidosis, schistosomiasis and leishmaniasis (Chamekh,M., 1992; Oliveira, E.J., 2006; Menezes-Souza, D. 2014). Angiostrongyliasis may present as an acute disease and the antigenic fragments of 14-3-3 may not be released in host's tissues in sufficient quantity for a recognizable immunoglobulin production. Otherwise, hydatidosis, schistosomiasis, and strongyloidiasis are mostly chronic infections, leading to a higher production of antibodies.

Moreover, peptide sequences alignments demonstrate similarities with high scores (≥100) and identity above 80% to many other parasites such as *S. mansoni, E. multilocularis,* and *T. spiralis*. Most organisms that shared the same 14-3-3 sequence with *A. cantonensis* were insects and other arthropods, showing 14-3-3 is a conserved protein and may not be a specific antigen. In the other hand it is been proposed as useful diagnostic antigen or vaccine candidates for *T. spiralis*, *E. multilocularis* and *S. mansoni*, (Schechtman et al., 2001; Siles-Lucas et al., 2008; Wang et al., 2009; Yang et al., 2015).

Another protein identified here among those nine proteins within all parasites extracts (Figure 2) belongs to a family of lectins formerly known as S-type or S-Lac lectins, galectins. Galectin 1 was found here in *A. cantonensis* and *S. stercoralis* cytoplasm and chromatin extracts respectively. Galectins have been proposed for angiostrongyliasis immunodiagnosis, but extensive evaluation of specificity is required (Hao et al., 2007; Morassutti et al., 2012). In the present study, peptide 12 derived from Lec-5 protein (a previous identified galectin) was recognized by all infected sera and high scores of sequence identity was also found within *T. canis* (81%) and three other species of filaria, *Brugia malayi* (71%) *Loa Loa* (73%), and *W. bancrofti* (69%).

In addition, in this study we were able to identify 34 proteins exclusively found into *Angiostrongylus* extracts mainly from the cytoplasmic fraction (supplementary material Table 2). From these, only *COI* (cytochrome oxidase subunit I) with the sequence of VTMQNLNDRLASYLENVRDAEAWFNE presented no similarities among sequences of other parasites using NCBI (nr database) and WormBase Database. Interestingly, *COI* has been used for molecular taxonomic identification of *Angiostrongylus* spp. (Caldeira et al., 2003), being able to differentiate between *Trichuris skrjabini, Thelazia callipaeda* and *Necator americanus*.

Proteins that confer evolutionary success are conserved, become widely distributed and this explains cross-reactivity. Moreover, protein structure is conserved during evolution more than protein sequence (Illergård et al., 2009). This conservation of proteins may explain the high scores of sequence identity between helminthes proteins found in this study.

Cross-reactivity appears to be more common than specific reactivity and immunodiagnostic systems are seldom 100% specific, especially for diagnosing helminthic infections (Nuamtanong, 1996; Eamsobhana et al., 1998; Dekumyoy et al., 2000; Vitta et al., 2010; Morassutti et al., 2016). The selection of highly specific antigens usually results in significant decreases in sensitivity (Geiger et al., 2001). We still face many challenges to improve immunodiagnostic methods and it is important to search for new ideas and to try several strategies to overcome cross-reactivity and to reveal the specific reactivity. The alternative would be to have markers of a dominant cross-reacting response. Here we suggest the use of the protein Galectin 1, peptide number 12, protein HSPA-5, and Ifa1 as molecular markers for cross reactivity in immunodiagnostic tests, as protein *COI* appears to be a useful addition as a specific marker for angiostrongyliasis diagnostic.

5. Conclusion

We identified 34 proteins exclusively present in *Angiostrongylus* spp. extracts, and especially *COI* protein as a promising antigen for highly specific immunodiagnosis. Peptides 4 and 12 as well as the proteins HSPA-5, Galectin 1 and Ifa1 had their cross-reactivity well demonstrated. They are conserved antigens and should not be used in immunodiagnosis. Data about antigens linked to inespecific recognition are usually discarded or even not published. We propose the establishment of data bank of cross-reacting antigens, what is important as a short cut avoiding extensive evaluations of specificity in parasite extracts. Also, if you find these cross-reacting proteins as a dominant component in a given type of extract, one may avoid laborious search for diagnostic antigens. The study of shared molecules may aid in the development of specific diagnostic tests by subtracting those identified shared antigens.

Supplementary material Table 1

149 proteins identified in all four parasites tested among five extracts.

Proteins	*A. cantonensis*	*T. canis*	*S. stercoralis*	*S. mansoni*
protein disulfide isomerase (55091) (P4HB)	1			
Thioredoxin domain containing protein (55323) (TXN)	1			
calreticulin (46695) (CALR)	1			
disorganized muscle protein 1 (35825) (dim-1)	1			
immunoglobulin I-set domain protein (34606) (I-SET)	1			
14-3-3 protein (28475) (14-3-3)	1 3	1 3		
14-3-3 zeta (28279) (14-3-3)	1 3	1		
hypothetical protein Y032_0340g2972 (42128) (Y032)	1			
EF hand (26056) (EFHC1)	1			
SKP1 component domain containing protein (19414) (SKP1)	1			
EF hand (18817) (EFHC1)	1			
EF hand (20302) (EFHC1)	1			
hypothetical protein Y032_0253g261 (17015) (Y032)	1			
Myosin, essential light chain family protein (18744) (MYLK)	1			
galectin 1 (32720) (LGALS1)	1			
antioxidant, AhpC/TSA family (22116) (TSA1)	1			
antioxidant, AhpC/TSA family, partial (18229) (TSA1)	1			
ribosomal protein L7Ae (15545) (RP-L7Ae)	1			
Hsp90 protein (74610) (HSP90AB1)	1			
MSP domain protein (11562) (MSP)	1			
elongation factor-1 alpha, partial (59720) (EEF1A1)	1			
PREDICTED: 4-aminobutyrate aminotransferase, mitochondrial-like (51769) (QR98_0030750)	1 5			
Hsp20/alpha crystallin family protein (24145) (LOC_Os03g45340)	1			
lipocalin / cytosolic fatty-acid binding protein (15936) (FABP3)	1			
glucose-6-phosphate dehydrogenase, partial (59720) (G6PD)	1			
cytochrome oxidase subunit I (50284) (cox1)	1			
PREDICTED: poly(A) RNA polymerase, mitochondrial-like (51769) (Z043_112988)	1			
triose-phosphate isomerase (18802) (TPI1)	1			
C. briggsae CBR-TPI-1 protein (26696) (tpi-1)	1			
ribosomal protein L6 (21480) (rplF)	1			
copper/zinc superoxide dismutase (16641) (SOD1)	1			
odorant binding protein 4 precursor (65696) (OBP4A)	1			
putative Lipid Binding Protein (19042) (LIPPROT)	1			
hypothetical protein ANCCEY_12179 (18682) (ANCCEY)	1			
putative nucleoside diphosphate kinase (23325) (NME2P1)			1	
abnormal wing discs 2, partial (5434) (WINGDISC)			1	
hypothetical protein CRE_18007 (41642) (CRE)			2	
uncharacterized protein LOC101463398 (168275) (UNLOC10)			2	
hypothetical protein CAPTEDRAFT_189322 (40613) (CAPT)			2	
Hemicentin-1 (548278) (HMCN1)			3	
Astacin-like metalloendopeptidase (48268) (Astl)			3	
EF-hand domain and Zinc finger, C2H2 domain and EF-hand domain pair and Zinc finger, C2H2-like domain-containing protein (190884) (SRAE_2000049800)			4	
chitooligosaccharidolytic beta-N-acetylglucosaminidase (89339) (HEXC)			4	
Fructose-bisphosphate aldolase, class-I family and Aldolase-type TIM barrel domain-containing protein (39768) (ALDOB)			4	
fructose-bisphosphate aldolase class-I (41537) (ALDOB)			4	
Galectin, carbohydrate recognition domain and Concanavalin A-like lectin/glucanases superfamily domain and Concanavalin A-like lectin/glucanase, subgroup domain-containing protein (32076) (LGALS1)			4	
Full=Sarcoplasmic calcium-binding protein, partial (842)			4	
TBA-1 precursor (16314) (TBA-1)		1		
ABA-1 allergen, partial (31201) (ABA-1)		1		
Heat shock protein Hsp-12.2 (13586) (hsp-12.2)		1		
hypothetical protein Tcan_04469 (30339) (HYTOX39)		3		
hypothetical protein Tcan_04455 (33779) (HYTOX79)		3		
hypothetical protein Tcan_11546 (36076) (HYTOX76)		3		
PREDICTED: protein transport protein Sec31A (151023) (SEC31A)		4		
RecName: Full=Major sperm protein 1; Short=MSP1 (14447) (SS1)		4		
hypothetical protein T07_7528 (61919) (HPT07)		4		
RecName: Full=Sarcoplasmic calcium-binding protein, partial (842) (SARCCBP)		4		
RecName: Full=Tropomyosin-2; AltName: Full=Tropomyosin II; Short=SmTMII; Short=TMII (TROPM)				1
putative tropomyosin (32571) (TPM2)				1
protein disulfide-isomerase,putative (54127) (P4HB)				1
unnamed protein product (50219) (AlNc14C211G8918)				1
myosin heavy chain (211141) (mhcA)				1
putative atp synthase beta subunit (55916) (atp2)				1
ATP synthase beta chain, partial (34222) (ATP5B)				1
ATP synthase beta subunit, partial (24134) (ATP5B)				1
Chain A, Crystal Structure Of Glutathione S-Tranferase From Schistosoma Mansoni (gstA1)				1
RecName: Full=Glutathione S-transferase class-mu 28 kDa isozyme; Short=GST 28; AltName: Full=GSH transferase S.m. 1-1; AltName: Full=Protective 28 kDa antigen; AltName: Full=Sm28 antigen; AltName: Full=Sm28GST; AltName: Full=Smp28 (GST28)				1
PREDICTED: RNA-binding protein Rsf1 (21256) (Rsf1)				1
putative paramyosin (84183) (pso o 11)				4
enolase, partial (21301) (eno)				4
hypothetical protein DAPPUDRAFT_219389 (102004) (DAPP)				4
unknown Schistosoma japonicum (13232) (UNSJ)				4
RecName: Full=Paramyosin (100383) (PARAM)				5

putative heat shock protein 70 (hsp70) (71479) (HSP70)				5
PREDICTED: poly(U)-binding-splicing factor PUF60-like (55558) (PUF60-LIKE)				5
IFEB_ASCSU Intermediate filament protein B OS=Ascaris suum PE=1 SV=1 (ifb-1)	2			
IF4A_CAEEL Eukaryotic initiation factor 4A OS=Caenorhabditis elegans GN=inf-1 PE=2 SV=1 (EIF4A3)	2			
API_PARTN Aspartyl protease inhibitor OS=Parelaphostrongylus tenuis PE=2 SV=1 (APIPT)	2			
PDI2_CAEEL Protein disulfide-isomerase 2 OS=Caenorhabditis elegans GN=pdi-2 PE=1 SV=1 (P4HB)	3			
CH60_CAEEL Chaperonin homolog Hsp-60, mitochondrial OS=Caenorhabditis elegans GN=hsp-60 PE=2 SV=2 (hsp-60)	3			
HSP90_CAEBR Heat shock protein 90 OS=Caenorhabditis briggsae GN=daf-21 PE=3 SV=1 (HSP90AB1)	3			
MYO4_CAEEL Myosin-4 OS=Caenorhabditis elegans GN=unc-54 PE=1 SV=2 (unc-54)	3			
CAZM_CHAGB Iterative polyketide synthase CazM OS=Chaetomium globosum (strain ATCC 6205 / CBS 148.51 / DSM 1962 / NBRC 6347 / NRRL 1970) GN=cazM PE=1 SV=1 (cazM)	3			
HSP7A_CAEEL Heat shock 70 kDa protein A OS=Caenorhabditis elegans GN=hsp-1 PE=1 SV=2 (HSPA1A)	3			
HSP70_SCHMA Heat shock 70 kDa protein homolog OS=Schistosoma mansoni PE=2 SV=2 (HSPA1A)	3	1		
MYO3_CAEEL Myosin-3 OS=Caenorhabditis elegans GN=myo-3 PE=2 SV=1 (myo-3)	3			
GRP78_ECHMU 78 kDa glucose-regulated protein OS=Echinococcus multilocularis GN=GRP78 PE=2 SV=1 (HSPA5)	3			
HSP70_BRUMA Heat shock 70 kDa protein OS=Brugia malayi GN=HSP70 PE=3 SV=1 (HSPA1A)	3			
OV71_ONCVO Muscle cell intermediate filament protein OV71 (Fragment) OS=Onchocerca volvulus GN=OV71 PE=2 SV=1 (OV71)	3			
HSP7C_CAEBR Heat shock 70 kDa protein C OS=Caenorhabditis briggsae GN=hsp-3 PE=3 SV=2 (HSPA1A)	3			
IFA1_CAEEL Intermediate filament protein ifa-1 OS=Caenorhabditis elegans GN=ifa-1 PE=1 SV=2 (ifa-1)	3			
IFB1_CAEEL Intermediate filament protein ifb-1 OS=Caenorhabditis elegans GN=ifb-1 PE=1 SV=1 (ifa-1)	3			
ENO_CAEEL Enolase OS=Caenorhabditis elegans GN=enol-1 PE=1 SV=3 (ENO1)	3			
MYO3_CAEBR Myosin-3 OS=Caenorhabditis briggsae GN=myo-3 PE=3 SV=1 (MYO3)	3			
GRP78_ECHGR 78 kDa glucose-regulated protein OS=Echinococcus granulosus GN=GRP78 PE=2 SV=1 (HSPA5)	3			
HSP70_ECHGR Heat shock cognate 70 kDa protein OS=Echinococcus granulosus GN=HSP70 PE=2 SV=1 (HSP70)	3			
ALF1_CAEEL Fructose-bisphosphate aldolase 1 OS=Caenorhabditis elegans GN=aldo-1 PE=1 SV=1 (ALDOA)			4	
SCP_CHIOP Sarcoplasmic calcium-binding protein (Fragment) OS=Chionoecetes opilio PE=1 SV=1 (HRC)			4	
LEC1_CAEEL 32 kDa beta-galactoside-binding lectin OS=Caenorhabditis elegans GN=lec-1 PE=1 SV=1 (lec-1)			4	
LEG1_HAECO 32 kDa beta-galactoside-binding lectin OS=Haemonchus contortus GN=GAL-1 PE=2 SV=1 (GAL-1)			4	
PDM1_DROME Protein nubbin OS=Drosophila melanogaster GN=nub PE=2 SV=1 (nub)			4	
YQX1_CAEEL Probable ribosomal protein F34H10.1 OS=Caenorhabditis elegans GN=F34H10.1 PE=3 SV=1 (F34H10.1)			4	
LEC3_CAEEL 32 kDa beta-galactoside-binding lectin lec-3 OS=Caenorhabditis elegans GN=lec-3 PE=2 SV=3 (lec-3)			4	
H4_ASCSU Histone H4 OS=Ascaris suum PE=3 SV=2 (HIST1H4A)			3	
MSP1_ASCSU Major sperm protein isoform alpha OS=Ascaris suum PE=1 SV=3 (MSPIALPHA)		4		
MSP77_CAEEL Major sperm protein 77/79 OS=Caenorhabditis elegans GN=msp-77 PE=2 SV=3 (msp-77)		4		
MS152_CAEEL Major sperm protein 152 OS=Caenorhabditis elegans GN=msp-152 PE=2 SV=3 (msp-152)		4		
MSP32_CAEEL Major sperm protein 32 OS=Caenorhabditis elegans GN=msp-32 PE=2 SV=1 (msp-32)		4		
ABA1_ASCSU Polyprotein ABA-1 (Fragment) OS=Ascaris suum GN=ABA-1 PE=1 SV=2 (ABA-1)		1		
IFA1_CAEEL Intermediate filament protein ifa-1 OS=Caenorhabditis elegans GN=ifa-1 PE=1 SV=2 (ifa-1)		1		
IFEB_ASCSU Intermediate filament protein B OS=Ascaris suum PE=1 SV=1 (ifb-1)		1		
MSP78_CAEEL Major sperm protein 78 OS=Caenorhabditis elegans GN=msp-78 PE=2 SV=3 (msp-78)		1		
IFB1_CAEEL Intermediate filament protein ifb-1 OS=Caenorhabditis elegans GN=ifb-1 PE=1 SV=1 (ifb-1)		1		
AV71_ACAVI Muscle cell intermediate filament protein AV71 (Fragment) OS=Acanthocheilonema viteae GN=AV71 PE=2 SV=1 (AV71)		1		
IFEA_ASCSU Intermediate filament protein A (Fragment) OS=Ascaris suum PE=1 SV=1 (ifa-1)		1		
OV71_ONCVO Muscle cell intermediate filament protein OV71 (Fragment) OS=Onchocerca volvulus GN=OV71 PE=2 SV=1 (OV71)		1		
ABA1_ASCSU Polyprotein ABA-1 (Fragment) OS=Ascaris suum GN=ABA-1 PE=1 SV=2 (ABA-1)		1		
IFEB_ASCSU Intermediate filament protein B OS=Ascaris suum PE=1 SV=1 (ifb-1)		1		
IFB1_CAEEL Intermediate filament protein ifb-1 OS=Caenorhabditis elegans GN=ifb-1 PE=1 SV=1 (ifb-1)		1		
UNC84_CAEEL Nuclear migration and anchoring protein unc-84 OS=Caenorhabditis elegans GN=unc-84 PE=1 SV=2 (unc-84)		1		
ABA1_ASCSU Polyprotein ABA-1 (Fragment) OS=Ascaris suum GN=ABA-1 PE=1 SV=2 (ABA-1)		1		
MSP49_CAEEL Major sperm protein 49 OS=Caenorhabditis elegans GN=msp-49 PE=2 SV=3 (msp-49)		1		
MSP19_CAEEL Major sperm protein 19/31/40/45/50/51/53/59/61/65/81/113/142 OS=Caenorhabditis elegans GN=msp-19 PE=1 SV=2 (msp-19)		1		
MS152_CAEEL Major sperm protein 152 OS=Caenorhabditis elegans GN=msp-152 PE=2 SV=3 (msp-152)		1		
MSP1_ONCVO Major sperm protein 1 OS=Onchocerca volvulus PE=2 SV=3 (MSP1OV)		1		
MSP32_CAEEL Major sperm protein 32 OS=Caenorhabditis elegans GN=msp-32 PE=2 SV=1 (msp-32)		1		
MSP77_CAEEL Major sperm protein 77/79 OS=Caenorhabditis elegans GN=msp-77 PE=2 SV=3 (msp-77)		1		
MSP33_CAEEL Major sperm protein 33 OS=Caenorhabditis elegans GN=msp-33 PE=2 SV=2 (msp-33)		1		
MSP3_CAEEL Major sperm protein 3 OS=Caenorhabditis elegans GN=msp-3 PE=2 SV=3 (MSPICE)		1		
MSP78_CAEEL Major sperm protein 78 OS=Caenorhabditis elegans GN=msp-78 PE=2 SV=3 (msp-78)		1		
MSP55_CAEEL Major sperm protein 55/57 OS=Caenorhabditis elegans GN=msp-55 PE=2 SV=3 (msp-55)		1		
MSP63_CAEEL Major sperm protein 63 OS=Caenorhabditis elegans GN=msp-63 PE=2 SV=3 (msp-63)		1		
G3P_ONCVO Glyceraldehyde-3-phosphate dehydrogenase OS=Onchocerca volvulus PE=2 SV=1 (GAPDH)		1		
FABH_ASCSU Fatty acid-binding protein homolog OS=Ascaris suum PE=2 SV=1 (FABP1)		1		
ANKHM_CAEBR Ankyrin repeat and KH domain-containing protein CBG24701 OS=Caenorhabditis briggsae GN=CBG24701 PE=3 SV=3 (mask)		5		
GRP78_ECHMU 78 kDa glucose-regulated protein OS=Echinococcus multilocularis GN=GRP78 PE=2 SV=1 (HSPA5)		5		
PPMNT_MYCBP Bifunctional apolipoprotein N-acyltransferase/polyprenol monophosphomannose synthase OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=lnt PE=1 SV=1 (ppm1)		5		
IFA1_CAEEL Intermediate filament protein ifa-1 OS=Caenorhabditis elegans GN=ifa-1 PE=1 SV=2 (ifa-1)		5		
H4_CAEEL Histone H4 OS=Caenorhabditis elegans GN=his-1 PE=1 SV=2 (HIST1H4A)		5		
SAS4_CAEEL Spindle assembly abnormal protein 4 OS=Caenorhabditis elegans GN=sas-4 PE=2 SV=2 (SASS4)				4
ATPB_CAEEL ATP synthase subunit beta, mitochondrial OS=Caenorhabditis elegans GN=atp-2 PE=1 SV=2 (ATP5A1)				1
GST28_SCHMA Glutathione S-transferase class-mu 28 kDa isozyme OS=Schistosoma mansoni GN=GST28 PE=1 SV=1 (GST28)				1
GST28_SCHHA Glutathione S-transferase class-mu 28 kDa isozyme OS=Schistosoma haematobium PE=1 SV=1 (GST28)				1
GST28_SCHBO Glutathione S-transferase class-mu 28 kDa isozyme OS=Schistosoma bovis PE=1 SV=2 (GST28)				1
ANKHM_CAEBR Ankyrin repeat and KH domain-containing protein CBG24701 OS=Caenorhabditis briggsae GN=CBG24701 PE=3 SV=3 (CBG24701)				5
GRP78_ECHMU 78 kDa glucose-regulated protein OS=Echinococcus multilocularis GN=GRP78 PE=2 SV=1 (HSPA5)				5
IFA1_CAEEL Intermediate filament protein ifa-1 OS=Caenorhabditis elegans GN=ifa-1 PE=1 SV=2 (ifa-1)				5

1-Cytoplasmic extract; 2-Membrane extract; 3-Nuclear extract; 4-Chromatin extract; 5-Cytoskeleton extract

Supplementary material Table 2

34 proteins found exclusively in *A. cantonensis* extracts

***Angiostrongylus cantonensis* proteins**

1 protein disulfide isomerase (55091) (P4HB)
2 Thioredoxin domain containing protein (55323) (TXN)
3 calreticulin (46695) (CALR)
4 disorganized muscle protein 1 (35825) (dim-1)
5 immunoglobulin I-set domain protein (34606) (I-SET)
6 EF hand (26056) (EFHC1)
7 SKP1 component domain containing protein (19414) (SKP1)
8 hypothetical protein Y032_0253g261 (17015) (Y032)
9 Myosin, essential light chain family protein (18744) (MYLK)
10 galectin 1 (32720) (LGALS1)
11 antioxidant, AhpC/TSA family (22116) (TSA1)
12 ribosomal protein L7Ae (15545) (RP-L7Ae)
13 elongation 28arbo-1 alpha, partial (59720) (EEF1A1) N
14 Hsp20/alpha 28arbor28tis28 family protein (24145) (LOC_Os03g45340)
15 lipocalin / cytosolic fatty-acid binding protein (15936) (FABP3)
16 glucose-6-phosphate dehydrogenase, partial (59720) (G6PD)
17 cytochrome oxidase subunit I (50284) (COI)
18 PREDICTED: poly(A) RNA polymerase, mitochondrial-like (51769) (Z043_112988)
19 triose-phosphate isomerase (18802) (TPI1)
20 C. briggsae CBR-TPI-1 protein (26696) (tpi-1)
21 ribosomal protein L6 (21480) (rplF)
22 copper/zinc superoxide dismutase (16641) (SOD1)
23 odorant binding protein 4 precursor (65696) (OBP4A)
24 putative Lipid Binding Protein (19042) (LIPPROT)
25 hypothetical protein ANCCEY_12179 (18682) (ANCCEY)
26 ENO_CAEEL Enolase OS=Caenorhabditis elegans GN=enol-1 PE=1 SV=3 (ENO1)
27 MYO3_CAEBR Myosin-3 OS=Caenorhabditis briggsae GN=myo-3 PE=3 SV=1 (MYO3)
28 CH60_CAEEL Chaperonin homolog Hsp-60, mitochondrial OS=Caenorhabditis elegans GN=hsp-60 PE=2 SV=2 (hsp-60)
29 HSP90_CAEBR Heat shock protein 90 OS=Caenorhabditis briggsae GN=daf-21 PE=3 SV=1 (HSP90AB1)
30 MYO4_CAEEL Myosin-4 OS=Caenorhabditis elegans GN=unc-54 PE=1 SV=2 (unc-54)
31 CAZM_CHAGB Iterative polyketide synthase CazM OS=Chaetomium globosum (strain ATCC 6205 / CBS 148.51 / DSM 1962 / NBRC 6347 / NRRL 1970) GN=cazM PE=1 SV=1 (cazM)
32 GRP78_ECHMU 78 kDa glucose-regulated protein OS=Echinococcus multilocularis GN=GRP78 PE=2 SV=1 (HSPA5)
33 IF4A_CAEEL Eukaryotic initiation factor 4A OS=Caenorhabditis elegans GN=inf-1 PE=2 SV=1 (EIF4A3)
34 API_PARTN Aspartyl protease inhibitor OS=Parelaphostrongylus tenuis PE=2 SV=1 (APIPT)

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Capítulo II

Recombinant expression of three antigenic proteins from *Angiostrongylus cantonensis*: ES-7, Lec-5 and 14-3-3 in mammalian cells.

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Recombinant expression of three antigenic proteins from *Angiostrongylus cantonensis*: ES-7, Lec-5 and 14-3-3 in mammalian cells

Bianca B. Cognato[a], Sukwan Handali[b], Alessandra L. Morassutti[a*], Alexandre Januário da Silva[c], Carlos Graeff-Teixeira[a.]

[a]Laboratorio de Parasitologia Molecular, Instituto de Pesquisas Biomédicas and Laboratorio de Biologia Parasitaria, Faculdade de Biociências da Pontifícia Universidade Catolica do Rio Grande do Sul (PUCRS), Av Ipiranga 6690, 90690-900 Porto Alegre RS, Brazil

[b]Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention. 1600 Clifton Road, MS D-64, Bldg 23, Room 9-440 Atlanta, GA 30329, EUA.

[c]The Food and Drug Administration, Center for Foods Safety and Applied Nutrition, Office of Applied Research and Safety Assessment, Division of Virulence Assessment.

*Corresponding author – Address: Laboratorio de Biologia Parasitaria da Pontificia Universidade Catolica do Rio Grande do Sul, Avenida Ipiranga 6690, Predio 12, Bloco C, Sala 241, 90690 900 Porto Alegre RS, Brazil. Phone: +55 51 3320 4144.

E-mail address: bicognato@hotmail.com

Abstract

Angiostrongylus cantonensis is a parasitic nematode, and the main causative agent of eosinophilic meningitis in humans. Definitive diagnosis usually requires serology or molecular methods. The 31 kDa antigen is currently used for immunological tests however may present cross-reactivity with other helminthic infections. Angiostrongyliasis is in a worldwide expansion and improved molecular diagnostic tests are necessary. This study aimed to produce three recombinant glycoproteins from *A. cantonensis* antigen. ES-7, Lec-5, and 14-3-3, were expressed in cell lines (CHO and HEK) and their potential diagnostic value was verified through one- and two-dimensional electrophoresis, western and dot blot analysis, N-glycosidase F (PNGase F) treatment, using *Angiostrongylus*-positive sera, normal human sera (NHS), and other helminthic infected sera as specificity control. ES7 protein expressed in HEK and CHO cells and Lec-5 expressed in CHO cells were both recognized only by *A. cantonensis*-positive sera and not by NHS (normal human sera) or specificity controls in 2D analysis. The recognition was shown to be dependent on glycidic portions and denaturing conditions. The heterologous expression using mammalian cells may represent a promising source of diagnostic antigens for angiostrongyliasis.

Keywords: *Angiostrongylus cantonensis;* recombinant proteins; CHO HEK cells; glycoproteins

Highlights

- *A. cantonensis* proteins ES-7, Lec-5 and 14-3-3 expressed in mammalian cells CHO and HEK showed better specificity than proteins expressed in prokaryotic cells, since they were specifically recognized by sera from *Angiostrongylus*-infected humans.
- ES-7 expressed in HEK and CHO, submitted to 2D, was recognized only by *Angiostrongylus*-positive sera.
- Lec-5 expressed in CHO, submitted to 1D, was recognized only by *Angiostrongylus*-positive sera. In 2D, was recognized only by the pool of *Angiostronylus*-positive sera and with a very weak recognition by the pool of NHS.

Graphical abstract

	POOL SERUM				INDIVIDUAL SERUM	
	Angiostrongylus serum		NHS		*Angiostrongylus serum*	NHS
Protein	1D	2D	1D	2D	1D	1D
ES-7 HEK	+	+	+	-	0/20	1/20
ES-7 CHO	+	+	+	-	20/20	17/20
Lec-5 CHO	+	+	-	-	11/20	9/20
14 3 3 CHO	+	+	+	+	20/20	20/20

1. Introduction

Angiostrongylus cantonensis is a parasitic nematode most common causative agent of eosinophilic meningoencephalitis (EoM) or cerebral angiostrongyliasis (CA). Human is an accidental host, and becomes infected by ingestion of raw/undercooked snails or slugs, paratenic hosts such as prawns, or contaminated vegetables containing the infective larvae (L3). 2827 cases have been reported in the literature with a wide distribution in many continents, but especially from Asia and Pacific Islands (Wang et al., 2008). In Brazil, a first confirmed diagnosis of CA was reported from the southeastern State of Espírito Santo, in 2007, and since then the parasite has been found in definitive, intermediate hosts and causing human disease (Caldeira et al., 2007; Maldonado Júnior et al., 2010; Simões et al., 2011; Carvalho et al., 2012; Cognato et al., 2013; Morassutti et al., 2014).

Parasitological diagnosis is a challenge for this disease since larvae in the CSF are seldom found, thus requiring the use of indirect methods for diagnosis. The most important antigen for the immunodiagnostic of angiostrongyliasis is the 31kDa band (Eamsobhana et al., 1998). Morassutti and collaborators (2012) through bidimensional electrophoresis analysis identified and characterized several protein components present in the 31kDa band, also demonstrating the important contribution of glycidic moieties for immunogenicity.

With the purpose of developing diagnostic kits for human angiostrongyliasis, Morassutti and collaborators (2013) expressed in prokaryotic cells, one of those identified components of 31 kDa, the 14-3-3 protein and two other proteins from excretion and secretion products (ES), the Lec-5 and ES-7 proteins identified in another study (Morassutti et al., 2011). All three recombinant proteins failed as specific targets, since NHS indiscriminately recognized them. Carbohydrate incorporation during protein expression may be crucial for proper protein antigenicity (Morassutti et al., 2013). In addition, protein folding in prokaryotic expression system may occur in a different way of eukaryotic organism, since important organelles for protein synthesis are lacking (Dell et al., 2010). It probably had changed either the native protein-antibody binding sites or the correct glycan incorporation. This could be the reason of expression in prokaryotic cells did not reproduce well responsive proteins.

Thus, the aim of this study was the expression of three diagnostic targets proteins from *A. cantonensis*, ES-7, Lec-5, 14-3-3 in two widely used mammalian expression systems, CHO (Chinese Hamster Ovary) and HEK (Human Embryonic Kidney) (Ka Fai Suen et al., 2010) and the evaluation against an immunological panel of sensitivity- and specificity-control serum collection.

2. Materials and Methods

2.1 Recombinant proteins

All protocols for recombinant expression were performed by GenScript, Piscataway, NJ, EUA.

The proteins were expressed in CHO cells by the following protocol: transfection grade plasmid (pUC57 by EcoRI) was prepared for transient expression in CHO-3E7 cells. CHO-3E7 cells were grown in serum-free FreeStyle TM CHO Expression Medium (Life Technologies, Carlsbad, CA, USA). The cells were maintained in Erlenmeyer flasks (Corning Inc., Acton, MA) at 37° C with 5% CO_2 on an orbital shaker (VWR Scientific, Chester, PA). One day of transaction, DNA and PEI (Polysciences, Eppelheim, Germany) were mixed at an optimal ratio and then added into the flask with cells ready for transfection. The recombinant plasmids encoding ES7, 14-3-3 and Lec-5 were transiently transfected into 100ml suspension CHO-3E7 cell cultures. The cell culture supernatants collected on day 6 were used for purification.

The cell culture supernatants were collected on day six followed by centrifugation and filtration. Filtered supernatant was incubated with Glutathione Sepharose 4 HP 5ml (GE, Cat.No. 17-5279-01) for about 4 hours. After washing and elution with appropriate buffer, the eluted fractions were analyzed by SDS-PAGE and Western Blot. The eluted fractions were pooled according to analysis and buffer exchanged to PBS, ph 7.2. The purified protein was analyzed by SDS-PAGE and Western Blot by using standard protocols for molecular weight, yield and purify measurements. To confirm recombinant expression, Anti-GST-HRP primary antibody was used for Western blot analyses (GenScript, Cat. No. A00866).

ES7 was also expressed in HEK cells, due to higher potential as diagnostic target as indicated by data previously reported (Morassutti et al., 2012). The transient expression of ES7 in suspension HEK 293-6E cells was performed using serum free medium, followed by one-step purification. HEK 293-6E cells were grown in serum-free Freestyle 293 expression medium (Invitrogen, Carlsbad, CA, USA). The cells were maintained in Erlenmeyer flaks at 37° C with 5% CO_2 (Corning Inc., Acton, MA) on an orbital shaker (VWR Scientific, Chester, PA). One day before transfection, the cells were seeded at an appropriate density in Corning Erlenmeyer Flasks. On the day of transfection, DNA and PEI (Polysciences, Eppelheim, Germany) were mixed at an optimal ratio and then added into the flask with cells ready for transaction. The supernatant collected on day 6 was used for purification. Cell culture broth was centrifuged and followed by filtration. Filtered supernatant was loaded onto 3ml Glutathione Sepharose 4 HP resin (GE Healthcare, Uppsala, Sweden) at 1ml/min. After washing and elution with appropriate buffer, the fractions were collected. The purified protein was analyzed by SDS-PAGE and Western Blot by using standard protocols for molecular weight, yield and purity measurements. The recombinant plasmid encoding ES7 was transiently transfected into 100 ml suspension HEK 293-6E cell cultures. The target protein was captured from the cell culture

supernatant by 3 ml Glutathione Sepharose 4HP resin and followed by buffer exchange. The purified protein was analyzed by SDS-PAGE and Western Blot. The primary antibody for Western Blot was Mouse Anti-GST-HRP (GenScript, Cat.No.A00866).

2.2 Serum samples

To test antigenicity of the recombinant proteins a Western blot was performed using *Angiostrongylus*-positive sera (20 individuals sera and a pool of 5 positive sera); 20 Normal Human sera (NHS) and a pool of *Echinococcus granulosus*-positive sera as specificity control. Sera were diluted 1:100 in all perfomed testing. Serum from *Angiostrongylus*-infected patients were originates from biobanks from PUCRS, originally constituted in which the donors of the samples agreed through protocols approved by the ethics committeed, and the specificity controls were obtained in the serum bank of Immunochemistry laboratory of Division of Parasitic Diseases and Malaria at CDC.

2.3 Unidimensional electrophoresis (1D)

All recombinant proteins were submitted to Unidimensional Electrophoresis using Mini-PROTEAN® TGX™ Precast Gels 15% (Bio-Rad Laboratories). Sample buffer used was composed of 10% SDS, 6% glycerol and Tracking Dye (50mg bromophenol blue, 8ml glycerol, 1ml 0.5 M Tris HCL, pH 8.0, 1ml distilled water) with or without DTT. The proteins were applied to the gel with a voltage of 75V for 25 min using a BIO-RAD MiniProtean electrophoresis system. The amount of protein used for 1D was optimized for 0.12ug of ES-7 produced in HEK cells, 0.15ug of ES-7 produced in CHO, 0.07ug of Lec-5 and 0.1ug of 14-3-3 and IgG4 as peroxidase.

2.4 Bidimensional electrophoresis (2D)

Each recombinant protein was desalted using 2-D Clean-Up Kit (GE Healthcare, Piscataway, NJ), and also submitted by a resolubilization kit DeStreak Rehydration Solution (GE Healthcare), with 66mM DTT (Dithiothreitol, Sigma-Aldrich, Canada 0,05g) and 0.5% carrier ampholytes. The samples were in-gel rehydrated on 11cm pH 3-11 NL IPG strips (GE Healthcare) overnight and isofocalized with voltages increasing stepwise as follows: 500 V for 500 V h, a linear gradient from 500–8000 V for 6500 V h, followed by a hold at 6000 V for 22,000 V h. After isoelectric focusing, the strips were soaked for 15 min in fresh equilibration buffer (20% v/v glycerol, 6 M urea, 1% DTT, and 2% SDS). IPG strips were run in the second dimension on Criterion XT Precast Gels 4-12% Bis-Tris IPG (Bio-Rad Laboratories). The gels were then stained with colloidal Coomassie blue or transferred to nitrocellulose membranes for immunological analyses. The amount of protein used for 2D was optimized for 20ug of ES-7 produced in HEK cells, 0.5ug of ES-7 produced in CHO, 14ug of Lec-5 and 1.1ug of 14-3-3 and IgG4 as peroxidase conjugate.

2.5 Western blot analysis

Resolved proteins were transferred onto nitrocellulose membranes using a semi-dry trans-blot apparatus (Bio-Rad) in order to test their specificity against *Angiostrongylus*-positive sera, NHS and specificity control. The membrane was blocked with 5% skim milk for 1h at room temperature and then was incubated for 2h with a pool of positive or negative serum or specificity (1:100 dilution). After three washes with PBS Tween (0,05%), the membranes were probed with a secondary peroxidase-conjugated anti-human IgG (diluted 1:8000) and IgG4 (diluted 1:1000) for 1h at room temperature. Diaminobenzidine (DAB) (Sigma-Aldrich; 0.05% DAB and 0.015% H2O2 in PBS, pH 7.4) was added as developer reagent.

2.6 N-glycosidase F (PNGase F) treatment for deglycosilation

PNGase F treatment was performed according to the manufacturer's instructions (recombinant PNGase F, 500,000 U/mL; BioLabs, UK). To investigate proteins antigenicity without carbohydrate, PNGase F was mixed with the four different recombinant proteins (Lec-5, 14-3-3, ES7-HEK, ES7-CHO) at concentrations listed in 2.4, and the samples were incubated overnight at 37°C with 500 units of the enzyme for each (0.28mg/ml, 0,1mg/ml, 0,4mg/ml and 0,5mg/ml respectively) of the protein. PBS was used for negative control. The recognition of the carbohydrate moieties by positive or negative sera was tested by dot blot.

2.7 Dot blot

Recombinant proteins were blotted onto nitrocellulose membrane in a concentration of 1.4ug, 5.5ug, 0.5ug and 2.5ug for Lec-5, 14-3-3, ES7-HEK and ES7-CHO respectively. The membrane was incubated with PBS Tween milk 0,5% for 1h in room temperature and then incubated for 1h with positive or negative serum (1:100 dilution). After three washes with PBS Tween (0,05%), the membranes were probed with a secondary peroxidase-conjugated anti-human IgG (diluted 1:8000) for 1h at room temperature. Diaminobenzidine (DAB) (Sigma-Aldrich; 0.05% DAB and 0.015% H2O2 in PBS, pH 7.4) was added as developer reagent.

3. Results

3.1 ES7

Recombinant protein ES7 was expressed in both HEK (ES7-HEK) and CHO (ES7-CHO) cells, with a concentration of 0.4mg/ml and 0.5mg/ml respectively and purity around 90%. In both cells it was expressed at molecular weight around 47.83 and 50kDa (23kDa + 26kDa GST) seen at figure 1 with theoretical isoelectric point approximately 5 in 2D. However, in ES-7 HEK 6 spots were identified and isoelectric point varying between 3 and 7, in ES-7 CHO 5 spots were identified with IP varying between 3 and 7.

ES7-HEK and ES7-CHO was recognized by pool of positive sera of *A. cantonensis*, and not by NHS after 2D analysis (Figure 1). However, when the same protein was submitted to 1D using 0.12ug, the recognition occurred either with positive, negative and specificity control (Table 1).

3.2 Lec-5

Recombinant protein Lec-5 was expressed in CHO cells, with a concentration of 0.28mg/ml and purity around 85%. It was expressed varying between 50 and 75kDa, around 66kDa (36kDa + 26kDa GST), seen in figure 2 with a theoretical isoelectric point of 7.0. However, 11 spots were identified and isoelectric point varying between 3 and 7. Lec-5 was recognized only by the pool of *A. cantonensis* infected sera and with a very weak recognition by the pool of NHS in 2D (Figure 2) recognized by 11 of the 20 individuals *Angiostrongylus*-infected serum and by seven of NHS (Table 1).

3.3 14-3-3

Recombinant protein 14-3-3 was expressed in CHO cells; concentration was 0.11mg/ml and purity around 90%. It was expressed at the expected molecular weight, 54Kda (28kDa + 26kDa GST), seen at 54kDa in both 1D and 2D and isoelectric point of 4.83. 14-3-3 didn´t show any specificity to positive sera of *A. cantonensis* since the protein was recognized by all 20 positive serums for *A. cantonensis* and all 20 NHS.

After PNGase treatment, it was possible to see the loss of all three protein´s antigenicity against *Angiostrongylus*-positive control (Table 2). The identity of the three proteins was confirmed by mass spectrometry analysis.

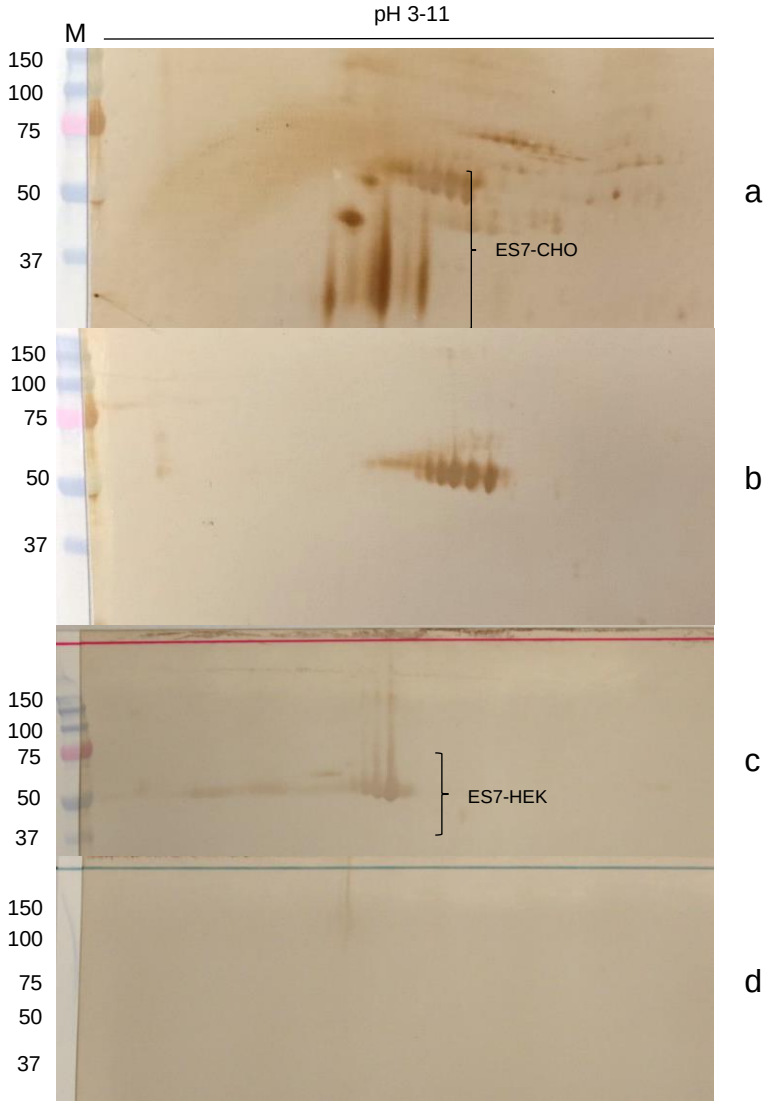

Fig.1 – Visualization of ES-7 CHO and HEK protein on 2D. **(a)** ES-7 CHO protein, on a range of 3-11 pH in a Western blot against *Angiostrongylus*-positive serum. **(b)** ES-7 CHO protein, range of 3-11 Western blot against NHS. **(c)** ES-7 HEK protein, on a range of 3-11 pH in a Western blot against *Angiostrongylus*-infected serum. **(d)** ES-7 HEK protein, range of 3-11 Western blot against NHS.

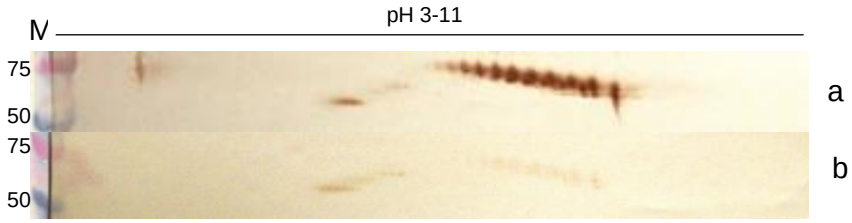

Fig.2 – (a) Visualization of Lec-5 protein on 2D against *Angiostrongylus*-positive serum. **(b)** Visualization of Lec-5 protein on 2D against NHS.

Table 1 – 1D and 2D assay from all proteins against pool and individual serum

	POOL SERUM				INDIVIDUAL SERUM	
	Angiostrongylus serum		NHS		*Angiostrongylus serum*	NHS
Protein	1D	2D	1D	2D	1D	1D
ES-7 HEK	+	+	+	-	0/20	1/20
ES-7 CHO	+	+	+	-	20/20	17/20
Lec-5 CHO	+	+	-	-	11/20	9/20
14 3 3 CHO	+	+	+	+	20/20	20/20

Table 2 – PNGase F treatment

	With PNGase F treatment	Without PNGase F treatment
Protein	Recognition	
Lec-5	-	+
14 3 3	-	+
ES7 CHO	-	+
ES7 HEK	-	+

4. Discussion

The main diagnostic antigen for angiostrongyliasis is a complex of glycoproteins with average molecular weight of 31kDa isolated from female worms of *A. cantonensis* (Eamsobhana et al., 1998, Morassutti et al., 2012). Several studies have demonstrated the importance of glycans for diagnosis as dominant antigens based on the ability to interact with the host immune system, especially those located on the surface of the parasite (Nyame et al., 2004; van Die and Cummings, 2006).

Glycosylation can affect protein folding, stability, solubility and specificity binding, what may influence immune recognition (Goettig, 2016). Veríssimo and collaborators (2016) have shown that antigenicity both of the 31kDa component and the total soluble extract of *A. cantonensis* is dependent on the presence of carbohydrate structures. This explains the lack of recognition when using heterologous expression in prokaryotic cells (Morassutti et al., 2013).

Here we demonstrate the importance of the use of eukaryotic cell systems for recombinant protein expression in order to obtain glycosylated forms. Indeed, immunogenicity of these recombinant proteins were dependent of glycan moieties, since recognition by *Angiostrongylu*-positive sera was depleted when carbohydrate was enzymatically removed (Table 2), as previously reported by Morassutti et al. (2012) and Veríssimo et al. (2016).

Besides of HEK and CHO are both mammalian cells, differences among the expression profile of the protein observed in 2D analysis were evident since ES-7 expressed in HEK presented six well defined spots while ES-7 expressed in CHO presented more elongated and blurred spots nearest to acidic IP (Figure 1a). Many studies have reported differences in the glycosylation patterns of proteins when produced in CHO/HEK cells. CHO cells produce proteins with higher molecular weights, and indeed MW varied from 48 kDa to 50 kDa, respectively when expressed in ES-7-CHO cells or HEK. This can be directly attributed to differences in the protein glycosylation in these cells, e.g., CHO cells consistently contain higher sialic acid (Croset et al., 2012), what can produce differences in isoform patterns in proteins expressed in CHO (Shinkawa et al., 2003; Ye et al., 2009; Zeck et al., 2011; Go et al., 2013).

Another interesting observation was the ES-7 specific recognition by *Angiostrongylus*-positive serum only after the use of reducing and denaturing agents. Breaking ES-7 disulfide bonds, leading to unfolding of the tridimensional structure are probably essential for antibody interaction with the protein. Interestingly, *Angiostrongylus*-positive sera did not recognize any synthetic peptides derived from this protein, reported elsewhere (Cognato et al., 2017). Taking together, possible differences on HEK and CHO cells, and lack of antigenicity both in isolated non-glycosilated peptides and ES-7 expressed in prokaryotic systems (Morassutti et al., 2012), we here demonstrate that HEK system is a good alternative for *Angiostrongylus*-antigens heterologous protein expression.

Exclusive recognition of recombinant Lec-5 protein by *Angiostrongylus*-positive serum, but not by NHS and specificity controls in 1D-WB and with only a very weak recognition by NHS in 2D-WB, clearly shows its usefulness for angiostrongyliasis immunodiagnosis, at 0.28µg/µL for 1D-WB. Interestingly, one Lec-5 peptide was proposed as a marker for cross-reactivity (Cognato et al., 2017). Lec-5 was expressed in a MW of 62kDa, varying between 50 and 75kDa probably due to the same reasons as ES-7 expressed in CHO.

An inespecific recognition by *Angiostrongylus*-positive sera and NHS was demonstrated for one 14-3-3 synthetic peptide: number 3. In addition, 14-3-3 protein was non-specifically recognized by all serum tested, demonstrating that even a peptide or whole protein are not specific targets.

Bidimensional analysis revealed ES7-HEK, ES7-CHO and Lec-5 proteins as several spots (Figure 1). Proteins generally present more than one isoform, having the same function, but encoded by a different gene and presenting small differences in their peptide sequences. However, this diversity seen in naturally synthetized proteins does not explain the number of spots in Figure 1, since the recombinant construction results in clones containing only one sequence of each protein. Another explanation could be related to the fusion protein, GST, which has isoforms and naturally occurs in mammalian cells. A contamination with cellular contents is excluded because of the purification procedure (see Methods), but a molecular contamination is possible. Actually, the MWs estimated from the spots localization in the 2D-WB are compatible with recombinant proteins fused with GST (Figure 1 and 2). In addition, the recognition of these spots by *Angiostrongylus*-positive sera indicates the presence of ES7, Lec-5 and 14-3-3.

5. Conclusion

Glycosylation is a very important posttranslational modification, especially for obtaining recombinant proteins. We have here demonstrated the importance of eukaryotic cell systems, specifically CHO and HEK mammalian cells, for the expression of three antigenic proteins from *Angiostrongylus cantonensis*: ES-7, Lec-5 and 14-3-3. Those proteins were correctly expressed and recognized by *Angiostrongylus*-positive serum, resulting in a more specific reaction than proteins expressed in prokaryotic cells. Glycan moieties are essential for correct antibody recognition. While 14-3-3 failed to produce a specific antibody-detecting system, ES7 CHO/HEK and Lec-5 were demonstrated to be very promising antigens for angiostrongyliasis immunodiagnosis.

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Conclusões Gerais

Capítulo I: No estudo de reatividade cruzada, foram identificadas moléculas antigênicas e de importante valor no diagnóstico das angiostrongilíases:

1) 149 proteínas compartilhadas foram identificadas entre os cinco extratos testados de *Angiostrongylus cantonensis*, *Toxocara canis*, *Schistosoma mansoni* e *Strongyloides stercoralis*;

2) 34 proteínas foram identificadas como exclusivas dos extratos de *A. cantonensis*;

3) A proteína *COI*, do extrato citoplasmático, mostrou ser exclusiva de *A. cantonensis*, e não foi encontrada em nenhuma sequência dos outros parasitos testados;

4) Nove proteínas foram classificadas como marcadores de reatividade cruzada, já que foram identificadas em mais de dois parasitos testados;

5) Peptídeo número 4, pertencente da proteína 14-3-3 e o peptídeo número 12, pertencente da proteína Lec-5, podem ser considerados marcadores de reatividade cruzada no imunodiagnóstico parasitológico.

Capítulo II: Neste capítulo, três proteínas antigênicas de *A. cantonensis* ES-7, 14-3-3 e Lec-5, foram expressas de forma recombinante em células de mamíferos obtendo resultados satisfatórios, tais como:

1) As proteínas ES-7, Lec-5 e 14-3-3 expressas em células de mamíferos CHO e HEK mostraram mais especificidade contra o soro específico de *Angiostrongylus* do que em células procarióticas;

2) Proteína ES-7 expressa em células HEK e CHO, submetidas a eletroforese bidimensional foram reconhecidas apenas por soro positivo para angiostrongilíase;

3) Pelos resultados obtidos, é possível afirmar que o reconhecimento das proteínas ES7 contra o soro positivo para *Angiostrongylus* spp., seu peso molecular, seu ponto isoelétrico e suas isoformas, são dependentes da célula escolhida para a expressão recombinante, CHO ou HEK;

4) Peptídeos sintéticos de *A. cantonensis*, produzidos a partir das proteínas ES-7, Lec-5 e 14-3-3, compartilham algumas sequencias de aminoácidos com outros nematódeos parasitos e de vida livre.

Perspectivas

1) Aprofundar os estudos sobre reatividade cruzada e buscar estratégias inovadoras para o aprimoramento do diagnóstico da angiostrongilíase cerebral;

2) Produzir de forma recombinante e testar a especificidade contra outras parasitoses as 34 proteínas exclusivas de *A. cantonensis*, a fim de aprimorar o diagnóstico das angiostrongilíases;

3) Ampliar os testes de especificidade das 9 proteínas de menor compartilhamento;

4) Produzir peptídeos sintéticos a partir das proteínas identificadas como exclusivas e/ou compartilhadas, e testar antigenicidade e especificidade para detecção de anticorpos anti-*Angiostrongylus* spp;

5) Padronizar imunodiagnóstico empregando as proteínas recombinantes ES7, Lec-5 e 14-3-3 em células CHO e HEK;

6) Estudar as variações de proteínas recombinantes Lec-5 e 14-3-3, quando expressas em células CHO e HEK;

Apêndices

Apêndice 1

Rascunho de potencial manuscrito:

Análise comparativa entre as variáveis: componentes celulares, processos biológicos e função molecular de antígenos de *A. cantonensis, T.canis, S. stercoralis* e *S. mansoni* reconhecidos pelos soros controles positivos para *Angiostrongylus* spp.

Introdução

Além dos conteúdos abordados nos dois manuscritos prontos para publicação, outro conjunto de experimentos produziu resultados interessantes, que vão ser descritos aqui. O objetivo destes experimentos foi comparar as variáveis (i) componentes celulares, (ii) processos biológicos e (iii) função molecular entre as proteínas reconhecidas pelos soros positivos para *Angiostrongylus* spp.

Metodologia

Através do programa MetaproteomeAnalyser (Muth et al., 2015), dados referentes a componentes celulares, processos biológicos e função molecular foram gerados. Utilizando o *R environment* (R Core Team, 2016), gráficos foram desenvolvidos para melhor visualização e interpretação dos dados.

Resultados

Com base nos dados obtidos, na Figura 1 podemos destacar que as proteínas de *T. canis* reconhecidas pelo soro positivo de *Angiostrongylus* spp. se diferenciaram com maior nitidez em relação aos seus processos biológicos, comparadas com as proteínas dos outros parasitos. Aproximadamente 15% das proteínas deste parasito são relacionadas à quimiotaxia e a biossíntese de pirimidina e homeostase. Aproximadamente 5% das proteínas estão relacionadas aos processos de RNA, biossíntese de aminoácidos, metabolismo de lipídeos e autofagia. Já *A. cantonensis* e *S. mansoni* tiveram 25% de suas proteínas relacionadas a virulência, síntese de ATP e ciclo celular aproximadamente, e 5% relacionadas a apoptose. Todos os parasitos testados, apresentaram média entre 20 e 50% de suas proteínas relacionadas a resposta ao estresse.

Já na Figura 2, referente aos componentes celulares, todos os parasitos apresentaram porcentagens similares relacionadas a proteínas de citoplasma, citoesqueleto, filamento intermediário, queratina e membrana. Porém, *T. canis* mostrou um aumento de proteínas relacionadas à projeção de células e secreção se comparado aos outros helmintos.

Por fim, na Figura 3, relacionada às funções moleculares, nota-se um aumento de proteínas desempenhando papel de metiltransferase, nuclease e hidrolase em proteínas de *T.canis* assim como chaperonas em *A. cantonensis*.

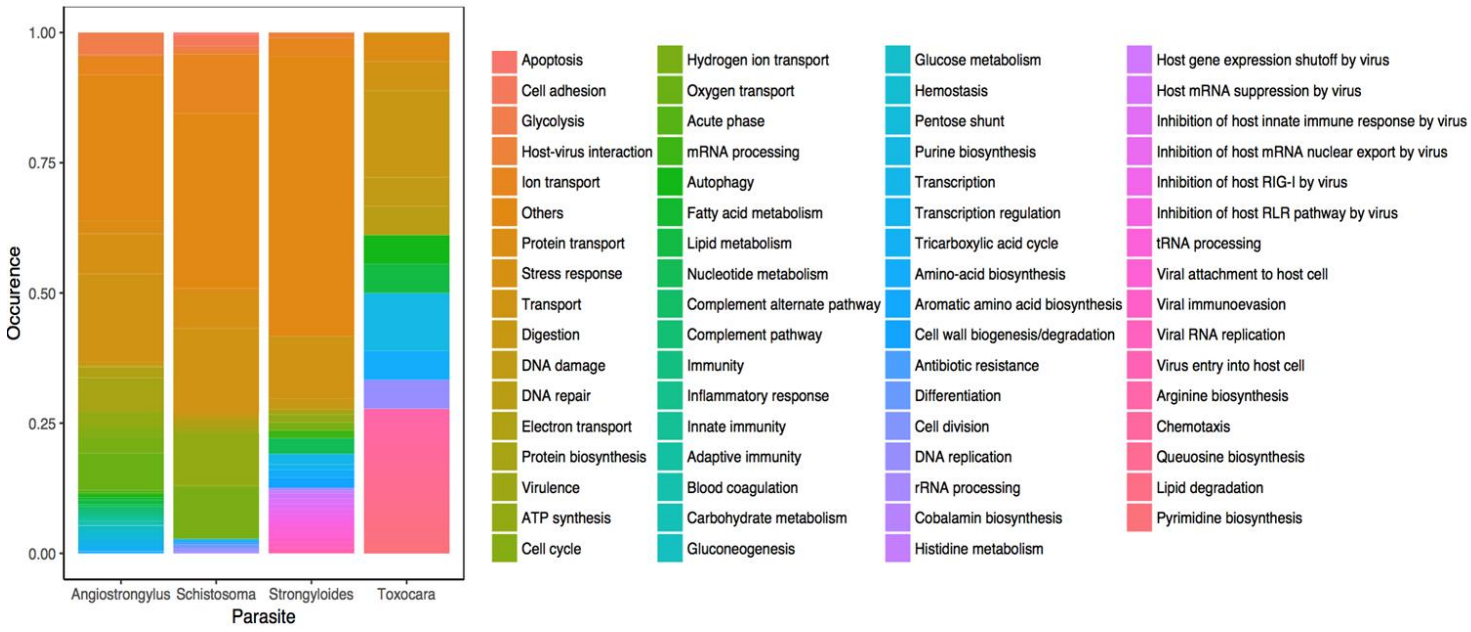

Figura 1: Processos biológicos das proteínas de *Angiostrongylus cantonensis*, *Schistosoma mansoni*, *Strongyloides stercoralis* e *Toxocara canis*, reconhecidas pelos soros positivos de *Angiostrongylus* spp.

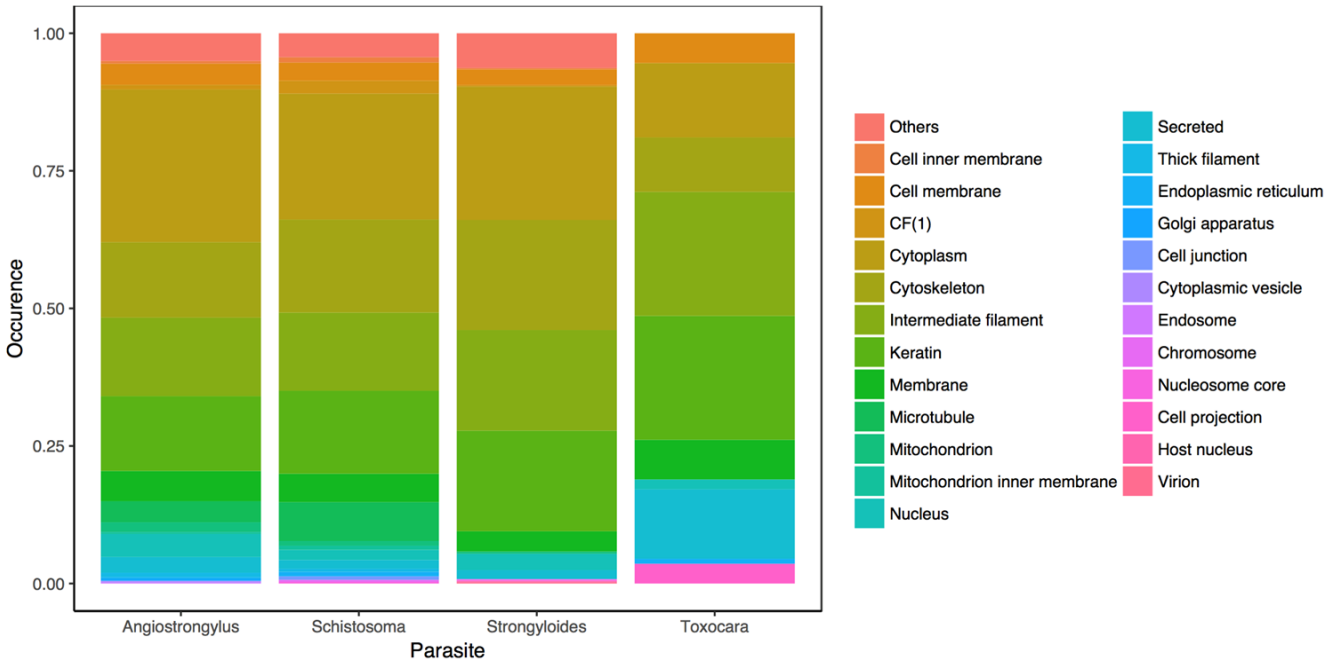

Figura 2: Componentes celulares das proteínas de *Angiostrongylus cantonensis*, *Schistosoma mansoni*, *Strongyloides stercoralis* e *Toxocara canis*, reconhecidas pelos soros positivos de *Angiostrongylus* spp.

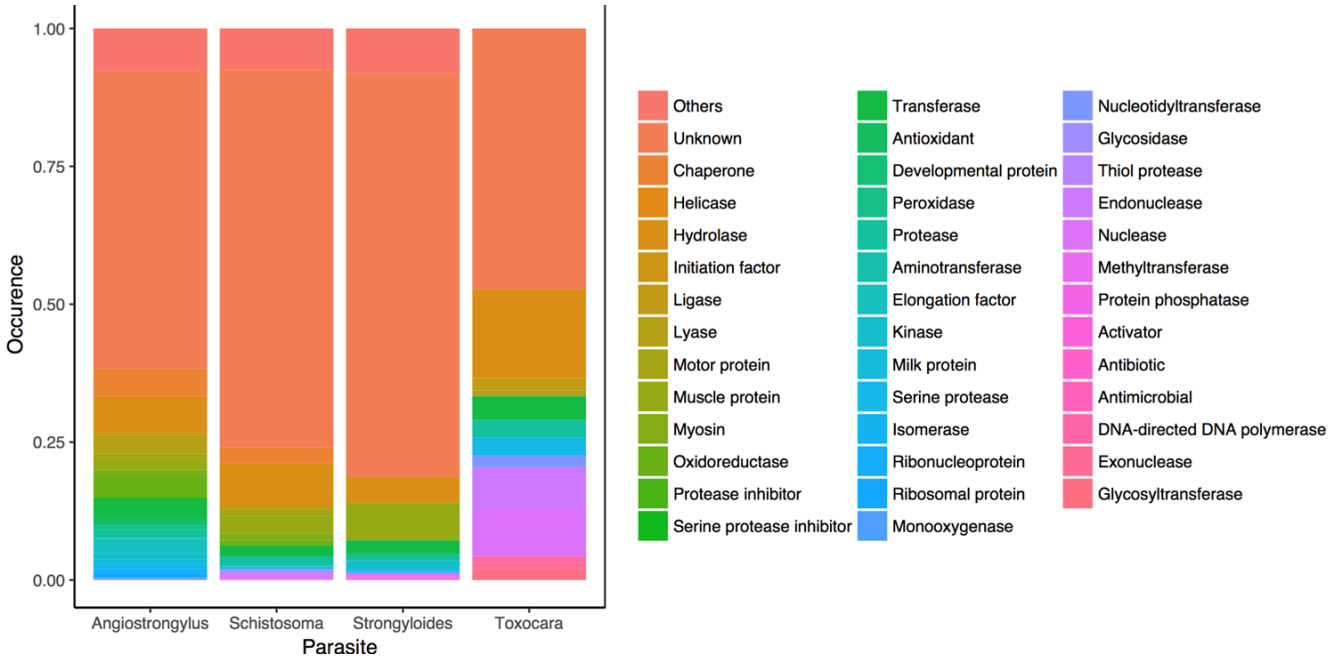

Figura 3: Funções moleculares das proteínas de *Angiostrongylus cantonensis*, *Schistosoma mansoni*, *Strongyloides stercoralis* e *Toxocara canis*, reconhecidas pelos soros positivos de *Angiostrongylus* spp.

Discussão

Muitos helmintos parasitas desenvolveram ao longo da evolução, mecanismos adaptativos de invasão e modulação do sistema imune do hospedeiro, secretando moléculas de superfície, auxiliando na sua entrada e estabelecimento. Algumas destas moléculas estão envolvidas na ativação de vias de sinalização, que são fundamentais para a sobrevivência do parasito. A apoptose, morte celular programada, é um exemplo de processo adaptativo de alguns parasitos, e tem papel significativo na diferenciação, desenvolvimento e homeostase (Kerr et al., 1972). Corroborando com o trabalho de Shao e colaboradores (2003), neste trabalho, o trematódeo *Schistosoma mansoni* apresentou proteínas indutoras de apoptose, assim como o nematódeo *A. cantonensis.* Ainda, Nguyen e colaboradores (2016), demonstraram a importância de proteínas responsáveis pela autofagia em protozoários parasitos, como *Toxoplasma gondii*, auxiliando no prolongamento de suas vidas dentro do hospedeiro, e contribuindo com o aumento de virulência, já que essas proteínas auxiliam em processos catabólicos envolvidos na reciclagem de componentes celulares não essenciais em resposta ao estresse. Podemos observar na Figura 1, o processo de autofagia presente em todos os parasitos testados, porém com mais destaque em *T. canis*. Ainda na mesma figura, nota-se uma grande porcentagem de proteínas relacionadas a resposta ao estresse em todos os helmintos testados. Sabe-se que existem classes de proteínas expressas apenas em condições estressantes para os organismos, como por exemplo, HSPs. Porém, foi demonstrado que essa classe de proteínas também estaria envolvida no auxílio do dobramento adequado de proteínas recém sintetizadas, desenpenhando o papel de chaperonas, proteínas presentes na Figura 3, com maior evidência em *A. cantonensis*. (Lindquist & Craig, 1988).

Por fim, pode-se afirmar, que os processos relacionados às proteínas de *T. canis* se diferem com mais nitidez aos processos dos outros helmintos testados. Apesar de pertencerem ao mesmo grupo dos nematódeos de *A. cantonensis e S. stercoralis*, *T. canis* se diferenciou dos demais grupos.

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