Universidade Federal do Rio Grande do Sul

Centro de Biotecnologia

Programa de Pós-Graduação em Biologia Celular e Molecular

Análise das proteínas constituintes do secretoma e dos potenciais mecanismos envolvidos na interação da forma larval patogênica de cestódeos do gênero *Echinococcus* com seu hospedeiro

Guilherme Brzoskowski dos Santos

Orientador: Prof. Dr. Arnaldo Zaha

Porto Alegre, Maio de 2016

Universidade Federal do Rio Grande do Sul

Centro de Biotecnologia

Programa de Pós-Graduação em Biologia Celular e Molecular

Análise das proteínas constituintes do secretoma e dos potenciais mecanismos envolvidos na interação da forma larval patogênica de cestódeos do gênero *Echinococcus* com seu hospedeiro

Guilherme Brzoskowski dos Santos

Tese submetida ao programa de Pós-graduação em Biologia Celular e Molecular da UFRGS como requisito para a obtenção do grau de Doutor em Ciências.

Orientador: Prof. Dr. Arnaldo Zaha

Porto Alegre, Maio de 2016

Este trabalho foi realizado no Laboratório de Biologia Molecular de Cestódeos do Centro de Biotecnologia da UFRGS, sendo financiado pela CAPES, CNPq e FAPERG.

Agradecimentos

Ao meu orientador Dr. Arnaldo Zaha pela confiança depositada em mim para a execução deste trabalho, pelos desafios impostos os quais me proporcionaram um aprendizado inestimável, além da amizade.

Ao Dr. Henrique Bunselmeyer Ferreira e a Dra. Karina Monteiro pela contribuição prestada no planejamento e desenvolvimento do presente trabalho;

Aos professores do PPGBCM, PPGBM, e do curso de Ciências Biológicas da UFRGS, em especial a Dra. Karen Haag pelos ensinamentos e incentivo dado no início de minha formação acadêmica;

Ao Laboratório Nacional de Biociências (LNBio, Campinas, Brasil, proposta MAS-15019) pelo suporte técnico nos experimentos de espectrometria de massas e ao Laboratório de Especial de Toxinologia Aplicada (LETA)/Instituto Butantan, em especial a Dra. Solange Maria de Toledo Serrano e ao estudante de Doutorado Eduardo Kitano pelo auxílio no planejamento e execução de experimentos de espectrometria de massas.

Ao Dr. Rogerio Margis pelo auxilio no planejamento e execução dos experimentos de *high-resolution melting*;

Ao Dr. Charley Staats pelas correções e sugestões importantes para o aperfeiçoamento do texto aqui apresentado;

Ao Centro de Microscopia Eletrônica da FURG (CEME-SUL) pelo suporte técnico nos experimentos de microscopia eletrônica de transmissão;

A CAPES, CNPq e FAPERGS pelo auxílio financeiro necessário para o cumprimento deste trabalho.

A todos os colegas dos laboratórios 204/206/210, em especial ao Dr. Martin Cancela, aos doutorandos Edileuza da Silva, Sergio Espínola, e Tatiana Basika, ao mestrando Helier Balbinotti e a estudante de graduação Eduarda Battistella pelas discussões, sugestões, colaborações em trabalhos realizados e em desenvolvimento no laboratório, além da amizade indispensável e fortalecida pela convivência dos últimos seis anos;

Aos funcionários da UFRGS e do Centro de Biotecnologia, em especial aos funcionários da limpeza e aos funcionários da secretaria, Sílvia Centeno e Luciano Saucedo.

A minha família, meus pais e irmã, e a Jana e Pinga pelo apoio e acima de tudo compreensão;

"**Imagine a world where parasites** control the minds of their hosts, sending them to destruction;

Imagine a world where parasites are masters of chemical warface and camouflage, able to cloak themselves with their hosts' own molecules;

Imagine a world where parasites steer the course of evolution, where the majority of species are parasites….

Welcome to earth!"

Parasite Rex, Carl Zimmer.

Índice

Lista de abreviaturas, símbolos e unidades

Ag5 – antígeno 5, do inglês *antigen 5*

AgB – antígeno B, do inglês *antigen B*

BLAST – ferramenta básica de alinhamento local, do inglês *Basic Local Alignment Search Tool*

Cox1 – citocromo c oxidase 1

DNA – ácido desoxirribonucleico, do inglês *deoxyribonucleic acid*

DTT – ditiotreitol, do inglês dithiothreitol

ES – excreção/secreção, do inglês *excretion*/*secretion*

G1 – *Echinococcus granulosus*

G5 – *Echinococcus ortleppi*

GL – camada germinativa, do inglês *germinal layer*

GO – ontologia gênica, do inglês *gene ontology*

HF – líquido hidático, do inglês *hidatid fluid*

HRM – desnaturação de alta resolução, do inglês *high-resolution melting*

HSP – proteína de choque térmico, do inglês *heat shock protein*

kDa – quilodalton (1000 daltons)

KCl – cloreto de potássio

LC-MS/MS – cromatografia líquida acoplada à espectrometria de massas em tandem, do inglês *liquid chromatography-tandem mass spectrometry*

LL – camada laminar, do inglês *laminar layer*

mtDNA - DNA mitocondrial, do inglês *mitochondrial DNA*

pb – pares de base

PCR – reação em cadeia da polimerase, do inglês *polymerase chain reaction*

PBS – solução salina tamponada com fosfato, do inglês *phosphate-buffered saline*

pH – potencial hidrogeniônico

PSC – protoescolex, do inglês *protoscolex*

rDNA - DNA ribossômico, do inglês *ribosomal DNA*

RFLP – polimorfismos no comprimento de fragmentos de restrição, do inglês *restriction fragment length polymorphism*

RNA – ácido ribonucleico, do inglês *ribonucleic acid*

SCX – troca catiônica forte, do inglês *strong cation exchange*

SDS – dodecilsulfato de sódio, do inglês *sodium dodecyl sulfate*

SDS-PAGE – eletroforese em gel de poliacrilamida na presença de SDS

SSCP - polimorfismo de conformação de fita única, do inglês *Single Strand Conformation Polymorphism*

TCA – ácido trifluoroacético, do inglês *trifluoroacetic acid*

TEM – microscopia eletrônica de transmissão, do inglês *transmission electron microscopy*

U – unidade de enzima

Resumo

O gênero *Echinococcus* consiste de parasitos que têm ciclo de vida com dois hospedeiros mamíferos. Sua fase larval, cisto hidático, desenvolve-se predominantemente no fígado e pulmões de hospedeiros intermediários, ungulados domésticos, como bovinos, ovinos, suínos, e do próprio ser humano. O cisto hidático é o agente causador da hidatidose, e no Brasil, *Echinococcus granulosus* (G1) e *Echinococcus ortleppi* (G5) são ambos responsáveis pela grande maioria dos casos de hidatidose em hospedeiros humanos (G1 e G5), bovinos (G1 e G5) e ovinos (G1). Visando à identificação mais rápida das espécies, a técnica de *high-resolution melting* utilizando o gene cox1 em sete espécies de tenídeos foi padronizada, resultando em uma metodologia que requer apenas um único par de iniciadores e proporciona diferenciação das espécies de forma rápida e eficiente. Em estudo de análise proteômica deste trabalho nós identificamos 498 proteínas, por cromatografia líquida acoplada a espectrometria de massas em tandem (LC-MS/MS), a partir de cistos férteis e inférteis. A análise funcional *in silico* permitiu destacar os seguintes aspectos: a existência de uma possível competição envolvendo parasito e hospedeiro; uma série de proteínas no líquido hidático sem anotação funcional e/ou com possíveis funções alternativas; a presença de vesículas extracelulares, como exossomos, no líquido hidático de *E. granulosus*. Também identificamos neste trabalho as proteínas compartilhadas entre G1 e G5, em uma tentativa de elucidar os mecanismos envolvidos na sobrevivência destes parasitos. As amostras de líquido hidático de seis isolados foram analisadas por LC-MS/MS e permitiu-nos identificar um total de 842 proteínas. A análise *in silico* destes dados permitiu a identificação de um conjunto de 162 proteínas presentes em, ao menos, cinco das seis amostras utilizadas, e identificar maior grau de especialização na infecção causada por G5 em cistos de pulmão de bovino.

Abstract

The *Echinococcus* genus consists of parasites that have life cycle with two mammalian hosts. Their larval stage, called hydatid cyst develops predominantly in the liver and lungs of intermediate hosts, domestic ungulates, such as cattle, sheep, pig, and the human being himself. The hydatid cyst is the causative agent of hydatid disease, and in Brazil *Echinococcus granulosus* (G1) and *Echinococcus ortleppi* (G5) are both responsible for the vast majority of hydatid disease cases in human hosts (G1 and G5), cattle (G1 and G5) and sheep (G1). In order to rapidly identify the species, we have standardized the high-resolution melting technique using the cox1 gene in seven Taeniidae species, resulting in a technique that only requires a single pair of primers and provide a quick, closed-tube and gel-free species differentiation. In a study of proteomic analysis we identified 498 proteins, by liquid chromatography-tandem mass spectrometry (LC-MS/MS) from fertile and infertile cysts. The functional *in silico* analysis allowed us to emphasize some important aspects: the existence of a possible competition involving parasite and host responses; a number of proteins in hydatid fluid without functional annotation and with possible alternative functions; the presence of extracellular vesicles, such as exosomes, in hydatid fluid from *E. granulosus.* We also identified in this work the proteins shared between G1 and G5, in an attempt to elucidate mechanisms involved in the survival of these parasites. The hydatid fluid samples from six isolates were analyzed by LC-MS/MS and allowed us to identify a total of 842 proteins. The *in silico* analysis of these data enabled us to set a core of 162 proteins present in at least five of the six samples, besides some degree of infection specialization from G5 for lung bovine cysts.

1. Introdução

1.1. O gênero *Echinococcus* e a hidatidose

As infecções parasitárias têm grande impacto na saúde pública devido às suas altas prevalências. Além dos efeitos patológicos destes parasitos, tais infecções exercem grande influência no contexto socioeconômico, e disseminam-se, principalmente, por más condições de saneamento básico e higiene (McManus *et al.*, 2003).

Helmintos da classe Cestoda são endoparasitos obrigatórios de grande importância no mundo todo. O gênero *Echinococcus* (Cestoda: Taeniidae) é um grupo evolutivamente jovem no qual a especiação e a disseminação global ocorreram rapidamente (Knapp *et al.*, 2011), havendo ainda controvérsias quanto à taxonomia do gênero, que vem sofrendo modificações ao longo do tempo. A revisão taxonômica foi facilitada recentemente com o advento de ferramentas moleculares, que forneceram novos dados biológicos. Classicamente, o gênero *Echinococcus* Rudolphi 1801 incluía quatro espécies: *E. granulosus*, causadora da hidatidose cística; *Echinococcus multilocularis*, causadora da hidatidose alveolar; e *Echinococcus oligarthra* e *Echinococcus vogeli*, causadoras da hidatidose policística (Thompson, 1995). Mais recentemente, contudo, a composição de espécies do gênero, antes baseada essencialmente em caracteres morfológicos, foi revisada com base em critérios genéticos, chegando-se à proposição de 9 a 11 espécies (Nakao *et al*., 2015; Lymbery *et al*., 2015).

Estudos moleculares baseados em sequências de marcadores mitocondriais e nucleares distinguiam dez genótipos (G1-G10), antes descritos como linhagens de *E.*

granulosus, os quais apresentam distribuição cosmopolita (McManus & Thompson, 2003) (Figura 1).

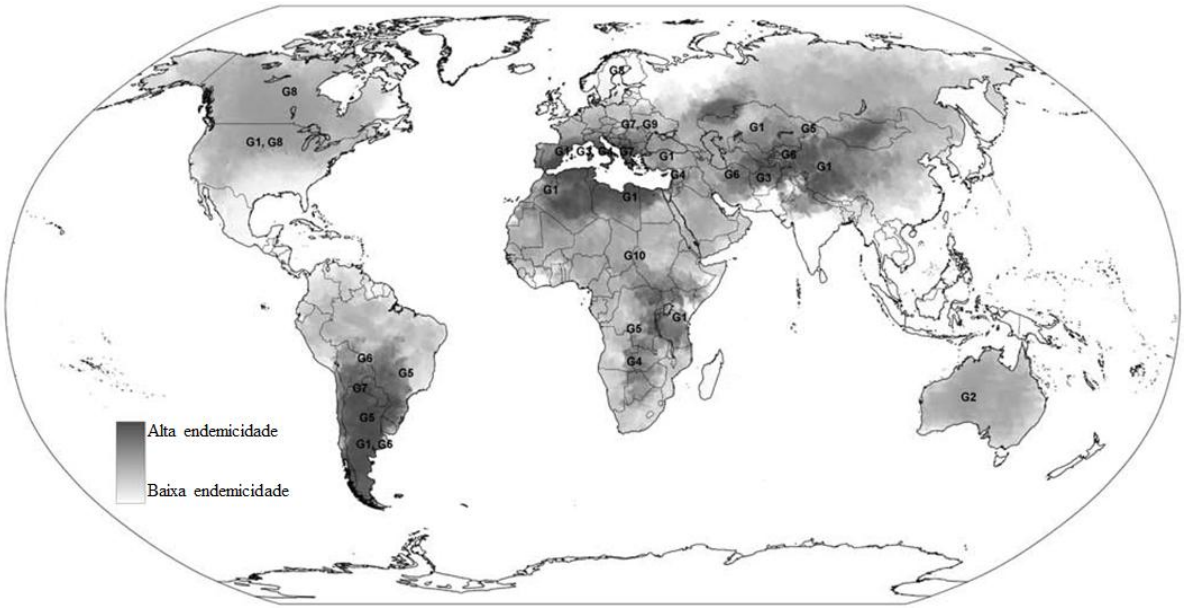


Figura 1. Distribuição geográfica mundial e endemicidade dos antigos genótipos de *Echinococcus granulosus*. Modificado de Grosso *et al.*, 2012.

Atualmente os genótipos G1, G2 e G3 são considerados todos como *E. granulosus sensu stricto* (Nakao *et al.*, 2007), já o genótipo G4, que parasita equinos, foi definido como *Echinococcus equinus* (Lavikainen *et al*., 2003; Romig *et al*., 2006; Nakao *et al*., 2007), e o haplótipo G5, encontrado comumente em bovinos, foi definido como *Echinococcus ortleppi* (Thompson & McManus, 2002; Nakao *et al*., 2007). Há ainda o genótipo de camelos (G6), dois genótipos de suínos (G7 e G9) e dois genótipos de cervídeos (G8 e G10) (Thompson & McManus, 2002; Lavikainen *et al*., 2003), sendo que Saarma *et al.*(2009) propõem as espécies *Echinococcus intermedius*, para o complexo de

genótipos G6, G7 e G9, e *Echinococcus canadensis*, para o complexo de genótipos G8 e G10. Para Nakao *et al.* (2013) o relacionamento extremamente próximo entre os genótipos G10 e G6/G7 invalida claramente o tratamento dos indivíduos que parasitam cervídeos silvestres (G8 e G10) como uma espécie distinta. Baseado em um conceito de espécie evolutiva, Lymbery *et al.*, (2015) publicaram o seu parecer sobre os genótipos G6/G7, G8, G10. Estes autores recomendam que *E. canadensis* deva ser dividido em três espécies: *Echinococcus intermedius* para G6/G7, *Echinococcus borealis* para G8, e *E. canadensis* para G10. Entretanto, a divisão ou manutenção do complexo de genótipos G6-G10 como *E. canadensis* permanece em constante discussão e dependente da aquisição de novos dados para o esclarecimento completo desta questão (Nakao *et al*., 2015; Lymbery *et al*., 2015). Acredita-se que o genótipo G9 identificado em pacientes humanos na Polônia seja provavelmente uma variante de G7 e, dessa forma, seu estado como genótipo permanece incerto (Scott *et al*.,1997). Há ainda a descrição das espécies *Echinococcus shiquicus* (Xiao *et al*., 2006a) e *Echinococcus felidis* (Hüttner *et al.*, 2008) das quais pouco ainda se sabe.

Assim como outras espécies do gênero *Echinococcus*, *E. granulosus* e *E. ortleppi,* amplamente disseminados no rebanho de bovinos do estado do Rio Grande do Sul (Balbinotti *et al.*, 2012), necessitam de dois hospedeiros mamíferos para completar seu ciclo de vida. Os humanos são hospedeiros intermediários acidentais, enquanto ovinos e bovinos são os hospedeiros intermediários mais frequentes para *E. granulosus* e *E. ortleppi,* respectivamente. O verme adulto, uma pequena tênia de 7 a 10 mm de comprimento e tipicamente com três proglótides, vive no intestino delgado dos hospedeiros definitivos, que são o cão doméstico e outros canídeos. Já a sua fase larval, chamada metacestódeo ou cisto hidático, desenvolve-se predominantemente no fígado e pulmões

dos hospedeiros intermediários, os quais podem ser ungulados domésticos, como bovinos, ovinos e suínos, e o próprio homem (Figura 2). O cisto hidático é o agente etiológico da hidatidose (ou equinococose) cística, uma parasitose crônica causada pelo crescimento do cisto e pelas lesões decorrentes no órgão infectado e tecidos adjacentes (Thompson, 2008).

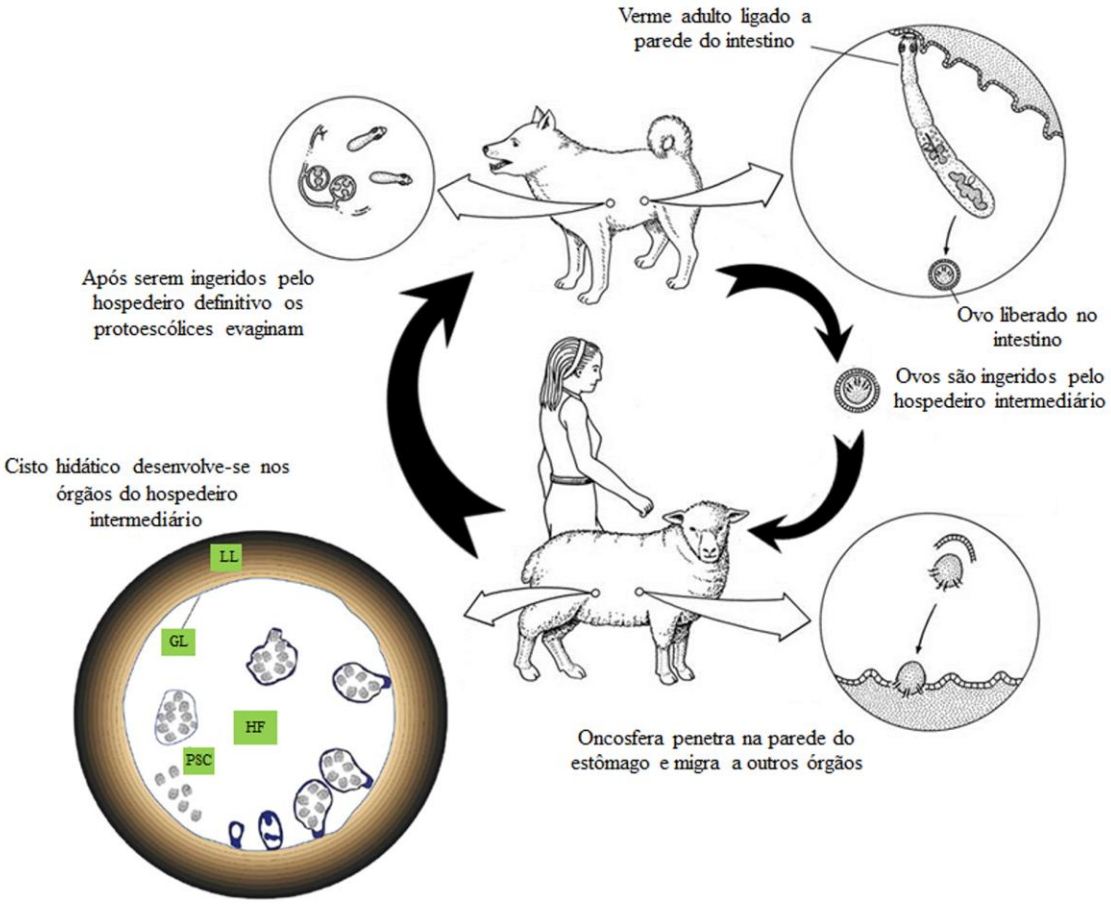


Figura 2. Ciclo de vida do *Echinococcus granulosus*. As setas escuras mostram a passagem do parasito entre os hospedeiros intermediário e definitivo. As setas em branco mostram em detalhe a estrutura assumida pelo parasito em cada etapa de seu ciclo de vida. LL,

camada laminar; GL, camada germinativa; PSC, protoescólices; HF, líquido hidático. Modificado de Rogan *et al.*, 2006.

A hidatidose cística representa um problema sério de saúde pública humana e animal em todo mundo, com significativo impacto socioeconômico, sendo considerada pela Organização Mundial da Saúde como uma doença negligenciada (http://www.who.int/neglected_diseases/diseases/en/), apesar do reconhecimento de seu caráter de zoonose cosmopolita emergente/reemergente em nível mundial (Moro & Schantz, 2009; Grosso *et al*., 2012; Mandal & Mandal, 2012). Levantamentos recentes demonstram que a hidatidose cística causa, mundialmente, prejuízos anuais da ordem de US$ 760 milhões em gastos no tratamento de pacientes humanos, e mais de US$ 2 bilhões associados à diminuição de rendimento de carne na pecuária (Budke *et al*., 2006; Battelli, 2009). Na América do Sul, a hidatidose é uma doença endêmica de grande relevância, principalmente em humanos, constituindo um sério problema de saúde pública, sendo que as regiões com pecuária intensa são as que possuem maiores taxas de infecção. Na Argentina existem cerca de 50 milhões de bovinos, 7% deles com hidatidose cística; 12 milhões de ovelhas, 12,5% infectadas; e dois milhões de porcos, 9,8% infectados, o que acarreta um número estimado de três milhões de argentinos vivendo em regiões de alto risco de contágio (Guarnera, 2001; Guarnera *et al.,* 2004). Já no Uruguai, assim como a Argentina um país de fronteira com o Rio Grande do Sul (RS), estima-se que 24,8% dos cães estejam infectados com a forma adulta do parasito *E. granulosus* (Cohen *et al.,* 1998). A hidatidose é um importante problema de saúde pública na região sul do Brasil, mais especificamente nas áreas que fazem fronteira com Argentina e Uruguai, as quais possuem

uma economia rural baseada na criação extensiva de bovinos e ovinos, animais estes que mantem intenso contato com cães (De La Rue *et al.,* 2006). Segundo dados do Programa Estadual de Vigilância da Hidatidose, divulgados pela Secretaria Estadual da Agricultura, Pecuária e Agronegócio do Rio Grande do Sul (SEAPA), a prevalência da hidatidose, em abatedouros, entre 2001 e 2009 foi de 25,20% em ovinos e 10,31% em bovinos. Em um claro reconhecimento à importância da doença para a saúde pública do RS, o governo estabeleceu a notificação compulsória de casos de hidatidose humana e a cobertura de seu tratamento pelo SUS em âmbito estadual a partir de março de 2010 (Portaria 203/2010 de 17/03/2010; disponível em http://www1.saude.rs.gov.br/dados/1273689474489Resolu%E7%E3o%2006-1-2010%20-%20pol%EDtica%20estadual%20de%20alimenta%E7%E3o%20e%20nutri%E7%E3o.doc.pdf).

1.2. Aspectos clínicos, tratamento e diagnóstico da hidatidose

O espectro clínico da hidatidose em humanos é amplo, geralmente associado ao curso crônico da doença. A manifestação clínica, frequentemente, tende a se desenvolver em indivíduos adultos, sendo que os sinais e sintomas mais proeminentes incluem dor abdominal, fadiga, perda de peso, massa abdominal palpável, febre, náuseas e vômitos (Siqueira *et al.,* 2013). Ressalta-se, porém, que a manifestação clínica depende da localização e tamanho das lesões císticas, bem como das estruturas anatômicas comprometidas (Eckert, 2001).

O órgão predominantemente acometido pela doença é o fígado, no qual o crescimento dos cistos determina, por vezes, que as lesões sejam palpáveis como massas

ou nódulos na superfície hepática. Os sintomas também podem incluir icterícia (por compressão das vias biliares), colúria, sinais de hipertensão portal (esplenomegalia, varizes de esôfago e ascite), sendo que, ocasionalmente, as lesões císticas hepáticas ou mesentéricas podem evoluir para fístulas órgão cutâneas (Alvarez-Rojas *et al.*, 2014). Já na hidatidose pulmonar os sintomas incluem dor torácica, hemoptise e expectoração mucopurulenta. A detecção de casos assintomáticos é realizada por exames clínicos ou de imagem (Biava *et al.*, 2001).

O tratamento da hidatidose pode ser clínico e/ou cirúrgico, arbitrado de acordo com as características morfológicas e de localização dos cistos e do estado geral do paciente. O tratamento com albendazol (10 a 15 mg/Kg/dia) é preconizado atualmente, porém, a resposta à terapia tem se demonstrado variável, principalmente devido as diferenças encontradas na infecção causada por diferentes espécies e do curso da doença que pode ser recente ou avançado (Vuitton, 2009; Loos *et al.*, 2015). Embora a remoção cirúrgica das lesões e o tratamento farmacológico sejam o curso natural para o tratamento da hidatidose, relatos na literatura descrevem, inclusive, a necessidade de transplante hepático em pacientes com hidatidose policística e alveolar (Soares *et al.*, 2004). Um estudo de coorte realizado na Amazônia ocidental brasileira, em 60 pacientes com hidatidose policística causada por *E. vogeli,* demonstrou que a abordagem cirúrgica teria maior eficiência para cura desta helmintíase. Entretanto, a conduta cirúrgica deve ser avaliada com cautela, levando-se em consideração o número, características e localização dos cistos, bem como a existência de lesões secundárias (Siqueira *et al.*, 2010).

O diagnóstico diferencial da hidatidose é amplo e inclui doenças infecciosas e/ou não infecciosas, devendo-se levar em conta a presença de abscessos amebianos, piogênicos

e fúngicos. Em casos clínicos graves, com lesões hepáticas e/ou pulmonares de aspecto cístico-pseudotumorais, a hidatidose deve ser considerada no diagnóstico diferencial de tumores malignos (Kern *et al.,* 2006). No caso de pacientes que apresentam sinais de hipertensão portal, associados também à lesão compressiva no hilo hepático, pelas lesões císticas, a cirrose hepática deve ser considerada no diagnóstico diferencial (Eckert, 2001). O diagnóstico da hidatidose pode ser estabelecido com base na associação dos achados epidemiológicos, clínicos, laboratoriais e de imagem. Dentre as provas que auxiliam no diagnóstico destacam-se técnicas de sorologia, diagnóstico por imagem, parasitoscopia, histopatologia e biologia molecular.

A pesquisa de anticorpos para o gênero *Echinococcus* foi desenvolvida a partir de amostras de soro ou plasma. Entretanto, apesar de serem auxiliares no diagnóstico, os kits comerciais de ELISA disponíveis até o presente momento carecem de maior acurácia diagnóstica, uma vez que apresentam sensibilidade e especificidade variáveis. Em grande parte isto ocorre, pois estes exames disponíveis comercialmente são baseados em antígenos recombinantes de *E. granulosus*, o que limita o diagnóstico da hidatidose causada pelas demais espécies do gênero (Gottstein *et al.*, 1991; Sakai *et al.,* 1996). Reações cruzadas de falso-positivos também são observadas com outras parasitoses, como cisticercose e esquistossomose, fazendo com que a sorologia seja atualmente utilizada com prudência, nunca como critério único para confirmação da doença (Eckert, 2001).

As técnicas de radiografia simples e ultrassonografia são capazes de identificar lesões císticas causadas pela hidatidose, as quais, segundo a forma de apresentação da doença, uni ou policística, podem ser encontrados de modo isolado ou multifocal. Entretanto, as lesões policísticas podem apresentar-se de modo coalescente em estágios

avançados da doença, podendo mimetizar a aparência de cisto único, o que acarreta em interpretações errôneas sobre a espécie causadora da infecção. Exames de tomografia computadorizada podem auxiliar no diagnóstico por imagem, principalmente quando acompanhadas por contraste venoso, por permitirem a localização dos cistos, presença de calcificações e comprometimento de órgãos, estruturas vasculares e/ou biliares (Thompson *et al.*, 1995).

As formas larvais geralmente são encontradas na superfície do fígado, porém a proliferação do cestódeo pode levar também ao comprometimento do interior do parênquima hepático. É importante ressaltar que estes cistos hepáticos superficiais podem sofrer ruptura espontânea, disseminando o parasito a outras partes do corpo, como mesentério, pleura, pulmões e pericárdio. Os protoescólices apresentam ganchos (acúleos) rostelares, usados frequentemente no diagnóstico parasitoscópico direto da doença a partir das lesões císticas (Eckert & Deplazes, 2004). A caracterização morfológica e morfométrica dos acúleos permite a identificação de algumas das espécies do gênero *Echinococcus*, uma vez que existem diferenças de tamanho e forma entre as quatro principais espécies de maior importância médica (*E. granulosus*, *E. vogeli*, *E. oligarthra* e *E. multilocularis*). Os critérios de identificação taxonômica a partir da morfometria dos acúleos parasitários foram estabelecidos a partir das medidas de comprimento total, tamanho do cabo e tamanho da lâmina (Thompson & Lymbery, 1988; Xiao *et al.*, 2006a; De la Rue *et al.*, 2011). Entretanto, em mais da metade dos casos de hidatidose não há formação, por reprodução assexuada, de protoescólices dentro dos cistos, o que inviabiliza a confirmação da hidatidose por parasitoscopia.

As lesões císticas obtidas por remoção cirúrgica também podem ser encaminhadas para histopatologia para confirmação diagnóstica. Microscopicamente, os cistos hidáticos são formados externamente por uma camada laminada externa, de aspecto hialino, homogêneo e espessura variando entre 8 a 65 µm e, internamente, por uma membrana germinativa delgada (3 a 13 µm), que apresenta projeções para o interior do cisto, formando as cápsulas prolígeras, de aspecto ligeiramente circular, dentro dos quais são encontrados os protoescólices (Thompson & Lymbery, 1995). A reação tissular adjacente às lesões incluem infiltrado inflamatório composto predominantemente por eosinófilos e células mononucleares, reação granulomatosa, com macrófagos dispostos em paliçada podendo ser observadas lesões em estágio avançado de necrose, que apresentam degeneração de protoescólices (encontrados como corpúsculos calcificados) e da camada laminada (encontrada com aspecto cerebroide, formando dobras que preenchem a cavidade cística) (Thompson & Lymbery, 1995; Eckert, 2004).

O diagnóstico preciso das infecções causadas por parasitos do gênero *Echinococcus* é central para investigar a epidemiologia da hidatidose, além de sustentar o tratamento e programas de controle da doença (Jabbar & Gasser, 2013). Abordagens bioquímicas e moleculares tradicionais, tais como eletroforese *multilocus* e análise de RFLP, auxiliaram durante muito tempo na identificação genética e caracterização das diferentes variantes (Xiao *et al.*, 2006b; McManus & Rishi, 1989). Os métodos baseados na utilização de PCR (Bowles *et al.,* 1993; 1994) encontraram ampla aplicabilidade à investigações genéticas e epidemiológicas, principalmente por sua alta sensibilidade e por permitirem a análise de regiões nucleares e/ou mitocondriais a partir de quantidades muito pequenas de DNA

oriundo de amostras frescas, congeladas ou mesmo fixadas em etanol e paraformaldeído (Siles-Lucas, 1993; Sharbatkhori *et al*., 2009; McManus, 2006).

A técnica de PCR apresenta alta sensibilidade e especificidade no diagnóstico e, quando utilizada em conjunto com sequenciamento nucleotídico e análise filogenética, permite determinar, com precisão, a espécie envolvida. Marcadores nucleares, como genes de DNA ribossômicos (rDNA), forneceram inúmeros marcadores específicos e possibilitaram a identificação e caracterização genotípica de muitos eucariotos, incluindo espécies de cestódeos. Tais genes são formados por uma grande família multigênica, que consiste de sequências em *tandem* (dezenas a centenas de vezes), geralmente encontradas em cromossomos específicos. Tais sequências de rDNA exibem padrões de evolução em concerto, que acarreta em uma maior homogeneização das sequências envolvidas entre indivíduos que constituem uma espécie do que entre indivíduos de diferentes espécies. Tanto o gene rDNA 28S quanto os espaçadores intergênicos internos e externos (ITS e ETS, respectivamente) foram descritos como sendo úteis para a identificação de diferentes espécies de tenídeos e outros helmintos (Gasser & Chilton, 1995; Van Herwerden *et al.*, 2000). Embora em algumas poucas espécies de helmintos a variação nas sequências de rDNA seja baixa, nas espécies do gênero *Echinococcus* estas sequências apresentam heterogeneidade o suficiente para sua utilização em estudos populacionais e diferenciação entre as espécies do gênero (Van Herwerden *et al.*, 2000).

Os marcadores de DNA mitocondrial (mtDNA), por sua vez, são largamente utilizados para identificação e delimitação de espécies estreitamente relacionadas, devido à sua taxa relativamente rápida de evolução. Este tipo de DNA é haploide, simplificando assim a análise das sequências geradas. O gene mitocondrial que codifica a subunidade 1

da citocromo c oxidase (cox1) é frequentemente utilizado como sequência alvo de amplificação parcial para análise filogenética e caracterização taxonômica do gênero *Echinococcus* (Bowles *et al.*, 1992). A identificação taxonômica baseada na análise filogenética de sequências parciais do cox1 apresenta ampla consistência com resultados de exames parasitoscópicos e histopatológicos de amostras obtidas tanto de humanos quanto de animais.

O sequenciamento de DNA acrescentou grande valor aos estudos realizados com diferentes espécies, devido à disponibilidade de informação para uma vasta gama de genes de uma infinidade de organismos em bases de dados públicas, tais como o GenBank (http://www.ncbi.nlm.nih.gov). No entanto, em alguns casos, pode haver limitações no sequenciamento direto de algumas regiões mais "complexas" de DNA. Se houver heterogeneidade genética ou polimorfismo dentro de um isolado (por exemplo, protoescólices de um cisto), como descrito para espécies do gênero *Echinococcus*, nas sequências de ITS-1, ITS-2 e antígeno B (AgB) (Van Herwerden *et al.*, 2000; Chemale *et al.*, 2001), pode não ser possível determinar uma sequência de forma correta o suficiente a ponto de diferenciar entre as espécies do gênero. Além disso, outro agravante do sequenciamento de DNA é seu custo mais elevado e tempo dispendido tanto para a procura de novos marcadores como para a identificação e diferenciação de espécies. Para facilitar o processo de varredura de novos genótipos, agilizar a identificação dos já descritos e tonar mais barato o custo da identificação de espécies, diferentes técnicas de genotipagem vem sendo empregadas com este intuito.

Uma técnica de varredura de mutações muito disseminada é *Single Strand Conformation Polymorphism* (SSCP) (Orita *et al.*, 1989). Esta técnica baseia-se no

princípio de que a mobilidade electroforética de uma molécula de DNA fita simples em gel não desnaturante, e é altamente dependente de sua estrutura e tamanho, oferecendo a capacidade de detectar poucas bases mutadas a partir de produtos de PCR de até 400 pb. A alta capacidade de detecção e a relativa simplicidade da PCR-SSCP faz da mesma uma ferramenta poderosa para a identificação de espécies ou genótipos e para caracterização de variabilidade genética dentro e entre indivíduos e, sobretudo, para triagem de mutações desconhecidas (Haag *et al*., 1997; Badaraco *et al*., 2008; Santos *et al.*, 2012). Quando utilizado como uma ferramenta de triagem para avaliar a extensão de variações genéticas dentro de uma população, a técnica de SSCP diminui o custo e tempo associado com análises, devido à redução substancial (frequentemente 70-90%) no número de amostras que necessitam de sequenciamento. No entanto, uma desvantagem da PCR-SSCP é o custo de aquisição do equipamento exigido (US$ 200 mil dólares). Uma alternativa seria o uso de serviços de um fornecedor comercial, o que, entretanto pode ter impacto sobre o tempo de eficiência desta abordagem em relação às demais (Gasser *et al.*, 2006).

Métodos baseados na curva de dissociação de produtos de PCR desnaturados foram utilizados para diferenciar organismos procariotos e eucariotos, incluindo diferentes genótipos de *E. granulosus* (G1, G2, G3) Maurelli *et al*. (2009). A utilização apenas da Tm como ferramenta para genotipagem é restrita, devido à baixa especificidade, contudo, uma alternativa mais recente é a aplicação da PCR acoplada à *high-resolution melting-curve analysis* (HRM) como ferramenta de procura por novas mutações e identificação das já existentes, a qual vem sendo empregada em diferentes organismos. A procura de mutações por HRM baseia-se no comportamento de dissociação da molécula de DNA exposta ao aumento da temperatura. A transição a partir de uma cadeia dupla fita (dsDNA), na

presença de intercalantes fluorescentes, para uma cadeia de DNA fita simples leva à modificação de sinal emitido pelo desprendimento do intercalante. O perfil gerado pela desnaturação do dsDNA por HRM origina um padrão único e diretamente relacionado com a sequência, o qual permite a discriminação de uma única base mutada em fragmentos de até 400 pb (Wittwer, 2009).

Estudos, utilizando diferentes intercalantes, demonstraram que a desnaturação de amplicons de DNA é reprodutível e as informações coletadas vem sendo utilizadas no diagnóstico, fornecendo *fingerprints* únicos e distintos o suficiente para a diferenciação entre espécies e mesmo entre variantes genéticas (Pangasa *et al.*, 2009). Programas, como Poland (http: //www.biophys. uni-duesseldorf.de/local/POLAND/poland.html) e MeltSim (http:. //ftp.bioinformatics Org / pub / meltsim), permitem predições *in silico* dos pontos de fusão de moléculas de DNA, o que pode auxiliar na escolha de genes a serem utilizados em futuras análises de PCR-HRM. Nos últimos anos, o método tem sido adotado para a caracterização de genes e genotipagem de organismos parasitas, por exemplo, *Leishmania*, *Cryptosporidium*, *Giardia*, *Plasmodium*, *Toxoplasma* e *Fasciola* (Pangasa *et al.*, 2009; Tan *et al.*, 2015; Costa *et al.*, 2011; Cancela *et al.*, 2015; Chua *et al.*, 2015; Zampieri *et al.*, 2016).

1.3. Estudos proteômicos em *Echinococcus* spp.

A hidatidose cística é uma grave doença tropical negligenciada, havendo uma necessidade urgente de se conhecer melhor os mecanismos de evasão da resposta imune, interação parasito-hospedeiro e imunopatogênese, bem como encontrar novos biomarcadores para diagnóstico e caracterização da doença.

Neste sentido, estudos do proteoma em distintos estágios das espécies do gênero *Echinococcus* vêm provendo informações valiosas sobre a biologia e a interação do parasito com seus hospedeiros definitivos e/ou intermediário.

O processo de infecção dos helmintos é complexo e conta com diversos mecanismos adaptativos, vinculados à sobrevivência dos parasitos, que dependem de um diálogo molecular estabelecido entre parasito e hospedeiro. Para sobreviver após a infecção, o parasito deve evitar ativamente o sistema imunológico do hospedeiro, utilizando diferentes mecanismos moleculares como a excreção/secreção de diversas proteínas, que impossibilitem uma resposta contra o parasito (Siracusano *et al*., 2012a).

Nos últimos 30 anos, estudos experimentais sobre a biologia do *E. granulosus* identificaram várias etapas na história adaptativa da interação parasito-hospedeiro. Em termos gerais, as proteínas do parasito interagem ativamente com a imunidade inata e adaptativa do hospedeiro com o objetivo de diminuir a resposta imunológica e facilitar a sobrevivência do parasito. Na fase larval, os cistos hidáticos podem apresentar ou não protoescólices, podendo ser classificados como férteis ou inférteis, respectivamente. No cisto hidático, a presença ou ausência da forma pré-adulta depende da interação parasito-hospedeiro, embora os mecanismos moleculares da fertilidade/infertilidade sejam desconhecidos (Siracusano *et al*., 2012a).

A resposta imunológica do *E. granulosus* foi dividida em duas fases: uma em que o parasito está mais suscetível aos efetores do hospedeiro, e outra em que induz a doença crônica. Nesta última fase, o parasito é capaz de sobreviver por décadas no organismo do hospedeiro intermediário. Na hidatidose cística a relação parasito-hospedeiro é interativa e o desfecho da infecção é o resultado do balanço entre os mecanismos de defesa do

hospedeiro e as estratégias de sobrevivência do parasito (Siracusano *et al*., 2012b). O líquido hidático, que preenche o cisto, contém produtos de excreção-secreção do metacestódeo envolvidos na modulação da resposta imune inata e adaptativa do hospedeiro (Virginio *et al.*, 2007; Siracusano *et al.*, 2008), além de proteínas do hospedeiro. Por isso, o líquido hidático é um componente rico para a análise de moléculas relevantes na interação parasito-hospedeiro (Monteiro *et al*., 2010).

Os primeiros estudos dos componentes proteicos da fase larval foram realizados com a espécie *E. granulosus*, e identificaram principalmente proteínas que desempenham papéis na modulação da resposta imune do hospedeiro (Chemale *et al.*, 2003). A presença de proteínas do hospedeiro em associação com proteínas do cisto hidático sugerem que o parasito poderia adsorver moléculas do hospedeiro sobre a sua superfície, as quais teriam distintas funções como, por exemplo, despistar antígenos do hospedeiro, além de realizar a captura de nutrientes (Monteiro *et al.,* 2010). Estes estudos baseavam-se na utilização de géis bidimensionais seguidos da identificação dos *spots* por espectrometria de massas do tipo LC-MS (*liquid chromatography-mass spectrometry*), que em sua época foram muito importantes para a geração de informação preliminar sobre a expressão gênica das espécies responsáveis pela hidatidose. Estudos mais recentes, baseados em metodologias que garantem a geração de maior quantidade de dados, e que utilizaram espectrômetros mais precisos, do tipo Orbitrap Velos, têm possibilitado a identificação mais fiel do perfil de proteínas existentes durante a infecção causada pela hidatidose. Através de estudos de proteômica em larga escala foi possível identificar mais de 1500 proteínas no protoescólex. Além disso, identificaram-se importantes antígenos como, por exemplo, variantes de calnexina, principal antígeno encontrado na oncosfera do *E. granulosus*, e 6-

fosfofrutoquinase (PFK), que têm sido há muito tempo descrita como antígeno em *S. mansoni* (Cui *et al.,* 2013). Outras proteínas potencialmente antigênicas incluem duas variantes do antígeno TSES38 usado abundantemente em diagnóstico de tenídeos, a glutamato desidrogenase (GDH) em *Haemonchus contortus*, e a frutose-1,6-bisfosfatase (FBPase) usada em *Clonorchis sinensis* (Dicker *et al.,* 2014; Zheng *et al.,* 2011). Já no verme adulto foram encontradas abundantemente as proteínas, miosina, frutose bisfosfato-aldolase (FBA), enolase, fosfoglicerato-quinase e malato-desidrogenase (MDH) (Cui *et al.,* 2013). O verme adulto de *E. granulosus* expressa proteínas antigênicas como, por exemplo, o antígeno 5 (Ag5), antígeno encontrado abundantemente também na fase larval e utilizado para diagnóstico da hidatidose (Li *et al*., 2012). Outros antígenos identificados no verme adulto incluem a proteína antigênica EPC1, o antígeno diagnóstico P-29, além do antígeno EgTeg, que contém similaridade com ortólogos de *S. mansoni*, e é localizado nos ganchos de protoescólices e na membrana laminar do cisto hidático. De forma geral, o verme adulto expressa vários candidatos conhecidos à vacina, como a proteína 14-3-3, um alvo vacinal já testado em *E. multilocularis* e para a qual já se conhece as proteínas interatoras (Siles-Lucas *et al.,* 2003; Teichmann *et al*., 2015).

A incorporação de azidohomoalanina em proteínas recém-sintetizadas durante o processo de estrobilização *in vitro* de *E. granulosus*, possibilitou a análise detalhada das alterações moleculares que ocorrem no parasito durante seu desenvolvimento. Há evidências de um deslocamento do metabolismo citosólico ao mitocondrial, o que leva a uma maior produção de acetato e succinato, dois produtos finais com um consequente rendimento energético mais elevado do que o lactato (Debarba *et al.,* 2015). Desse modo, as alterações nas vias de produção de energia associados à maturação do verme deve ser

essencial para a correta progressão do ciclo de vida do parasito, bem como para a sua sobrevivência. Interessantemente, também foi identificada a proteína clatrina, a qual participa em várias vias de tráfego de membrana incluindo comunicação célula-célula, a qual tem sido relacionada com a diferenciação sexual e sobrevivência do parasito frente ao hospedeiro (Regev-rudzki *et al.*, 2013; Debarba *et al.*, 2015). Em um estudo pioneiro Lorenzatto *et al.* (2015), usando o método de proteômica *Top-down*, realizaram uma caracterização inicial de modificações pós-traducionais em proteínas intracelulares de protoescólices de *E. granulosus*. As proteoformas identificadas apoiam a existência de um processamento N-terminal conservado além de acetilações internas nas proteínas da forma pré-adulta de *E. granulosus*, o que abre caminho para estudos de proteômica focando nas ocorrências diferenciais, localização subcelular e funções específicas das proteínas e das modificações pós-traducionais encontradas.

Não apenas o proteoma do protoescólex e do verme adulto de *E. granulosus* estão disponíveis, como também dispõe-se de informações sobre as proteínas presentes no líquido hidático. Por meio de contagem espectral, um método semiquantitativo utilizado para registrar a abundância relativa de proteínas em uma mistura complexa, foi possível confirmar que o Ag5 e diferentes subunidades do AgB são as proteínas parasitárias mais abundantes neste meio (Aziz *et al.*, 2011; Virginio *et al.*, 2012). Estima-se que proteínas com função metabólica e contendo domínios de ligação (principalmente a lipídeos e outras proteínas) correspondem a mais de 70% das proteínas identificadas. Os dados disponíveis mostram que apenas 30% do proteoma secretado contém um motivo consenso com sinal de secreção na região N-terminal, abrindo caminho para estudos de identificação de rotas de excreção/secreção não convencionais dessas proteínas parasitárias (Aziz *et al.*, 2011).

Outras proteínas identificadas que chamam a atenção são a ciclofilina, que em humanos atua de modo a regular a resposta inflamatória através de interações com a ciclosporina e CD147 (Yurchenko *et al.*, 2010), bem como a ferritina, uma molécula de ligação ao ferro, que pode estar ligada ao processo de captação de ferro, e que tem mostrado ser uma candidata à vacina eficaz contra *E. granulosus* (Wang *et al.*, 2009). As proteínas contidas no líquido hidático de *E. granulosus* e oriundas do hospedeiro têm se tornado alvo de muitos estudos, não apenas pelo fato de estarem presentes em grande quantidade, como também por terem importantes funções. As mais abundantes são as proteínas séricas, tais como albumina e hemoglobina, mas destacam-se também as imunoglobulinas, como as IgG de cadeia pesada, IgM e IgA. Um total de 44 proteínas do parasito e 77 do hospedeiro foram identificadas até o presente momento no líquido hidático de *E. granulosus* (Monteiro *et al.*, 2010; Aziz *et al.*, 2011).

Uma das dificuldades na análise dos produtos de excreção/secreção, presentes no líquido hidático da fase larval de *E. granulosus,* é a existência de uma grande quantidade de proteínas séricas oriundas do hospedeiro. Como ocorre em experimentos de proteômica de plasma, proteínas abundantes tendem a diminuir o desempenho da análise por espectrometria de massas, e muitas vezes acabam impedindo a identificação de proteínas presentes em concentrações mais baixas. Um método bem estabelecido e utilizado tanto em análises de plasma quanto para os proteomas de líquido hidático é a remoção das proteínas mais abundantes, proteínas séricas em especial. Entretanto, isto implica também na depleção não específica de outras proteínas, o que acaba limitando significativamente as análises quantitativas e interpretações fidedignas da real condição biológica do cisto.

2. Justificativas e objetivos

O sequenciamento direto de um grande número de amostras de DNA é demorado e dispendioso para muitos laboratórios de parasitologia, além de que frequentemente requer o uso da bioinformática em grande escala. Já a técnica de HRM-PCR é rápida, eficaz e de baixo custo financeiro, o que a torna uma ferramenta alternativa na identificação das espécies do gênero *Echinococcus* ou mesmo de seus genótipos em diferentes contextos da infecção. A técnica de HRM permitiria a identificação precisa e rápida das diferentes espécies do gênero, fazendo com que todo o processo de genotipagem pudesse ser realizado em até seis horas, ao invés das 48 h necessárias para a obtenção de resultados pelo método de sequenciamento, o que acarretaria na identificação e subsequente utilização de um grande número de amostras oriundas de diferentes espécies com maior precisão e rapidez.

Com o avanço das mais diversas tecnologias, os dados de sequências de genomas, transcritomas e proteomas completos de diferentes organismos, em distintos estágios de desenvolvimento e/ou sob diversas condições ambientais, vêm aumentando exponencialmente. As novas metodologias fornecem recursos poderosos para a realização de novos estudos, que, entretanto muitas vezes carecem de profundidade devido à ausência de foco ou a baixa aplicabilidade de seus resultados. Muitos destes trabalhos se preocupam apenas com a identificação de novos alvos para drogas e determinação de novos candidatos a vacinas. No entanto, não estariam as questões mais fundamentais da biologia sendo negligenciadas?

O desenvolvimento de novas plataformas para análises proteômicas deve servir não apenas para gerar grandes volumes de dados, mas também para esclarecer questões

relevantes a respeito dos mecanismos moleculares envolvidos nas interações parasito-hospedeiro, para assim melhorarmos nossa compreensão sobre a etiologia da hidatidose.

A presente tese de doutorado teve como objetivo a implantação e implementação de ferramenta de genotipagem rápida e específica, e que possibilite o uso otimizado de amostras biológicas; além da análise de proteínas presentes no líquido hidático de cistos de *E. granulosus* e *E. ortleppi*, relacionando tais proteínas a sobrevivência detes parasitos frente a resposta do hospedeiro bovino.

Sendo assim, este trabalho teve como objetivos específicos:

1. Tornar a identificação das diferentes espécies do gênero *Echinococcus* mais rápida, precisa e econômica utilizando, para isso, a técnica de HRM;

2. Identificar quais são as proteínas, presentes no líquido hidático de *E. granulosus*, e os possíveis mecanismos envolvidos na infertilidade do cisto hidático;

3. Identificar as proteínas e compreender os mecanismos que são compartilhados e os que distinguem a hidatidose causada pelas espécies *E. granulosus* e *E. ortleppi*, em bovinos.

Capítulo I

Manuscrito publicado na revista *Parasites & Vectors* em 2013

Rapid detection of *Echinococcus* species by a high-resolution melting (HRM) approach

Santos, G.B., Espínola, S. M; Ferreira, H. B., Margis, R.; Zaha, A.

Santos et al. Parasites & Vectors 2013, 6:327
http://www.parasitesandvectors.com/content/6/1/327

Parasites & Vectors

SHORT REPORT Open Access

Rapid detection of *Echinococcus* species by a high-resolution melting (HRM) approach

Guilherme Brzoskowski Santos[1], Sergio Martín Espínola[2], Henrique Bunselmeyer Ferreira[1,3], Rogerio Margis[1,3] and Arnaldo Zaha[1,2,3*]

Abstract

Background: High-resolution melting (HRM) provides a low-cost, fast and sensitive scanning method that allows the detection of DNA sequence variations in a single step, which makes it appropriate for application in parasite identification and genotyping. The aim of this work was to implement an HRM-PCR assay targeting part of the mitochondrial cox1 gene to achieve an accurate and fast method for *Echinococcus* spp. differentiation.

Findings: For melting analysis, a total of 107 samples from seven species were used in this study. The species analyzed included *Echinococcus granulosus* (n = 41) and *Echinococcus ortleppi* (n = 50) from bovine, *Echinococcus vogeli* (n = 2) from paca, *Echinococcus oligarthra* (n = 3) from agouti, *Echinococcus multilocularis* (n = 6) from monkey and *Echinococcus canadensis* (n = 2) and *Taenia hydatigena* (n = 3) from pig. DNA extraction was performed, and a 444-bp fragment of the cox1 gene was amplified. Two approaches were used, one based on HRM analysis, and a second using SYBR Green Tm-based. In the HRM analysis, a specific profile for each species was observed. Although some species exhibited almost the same melting temperature (Tm) value, the HRM profiles could be clearly discriminated. The SYBR Green Tm-based analysis showed differences between *E. granulosus* and *E. ortleppi* and between *E. vogeli* and *E. oligarthra*.

Conclusions: In this work, we report the implementation of HRM analysis to differentiate species of the genus *Echinococcus* using part of the mitochondrial gene cox1. This method may be also potentially applied to identify other species belonging to the Taeniidae family.

Keywords: *Echinococcus* species, High-resolution melting (HRM), Genotyping

Findings

The cyclophyllidean family Taeniidae is generally accepted to be composed of two valid genera, *Taenia* Linnaeus, 1758 and *Echinococcus* Rudolphi, 1801. Currently, there are nine recognized species within the genus *Echinococcus*, and four of them have medical significance: *Echinococcus multilocularis*, *Echinococcus granulosus*, *Echinococcus oligarthra* and *Echinococcus vogeli* [1]. The taxonomic position of *E. granulosus* has been recently revised, and species status was attributed to some of its 10 genotypes (G1-G10): *E. granulosus sensu stricto* (G1-G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6-G10) [2]. Another two species are now included in the *Echinococcus* genus, *E. shiquicus* and *E. felidis* [3,4]. Additionally, many of these species coexist in the same area, as is the case for *E. granulosus* and *E. ortleppi* and for *E. oligarthra* and *E. vogeli* [5,6]. The genus *Taenia* consists of approximately 50 species that are difficult to identify. Most of these are of medical and veterinary importance and lead to systemic (cysticercosis and coenurosis) and intestinal infections (taeniasis) [7,8].

As a genotyping tool, the high-resolution melting (HRM) method has been used for the rapid differentiation of influenza A subtypes, for identification of the *Cryptococcus neoformans-Cryptococcus gatti* complex, and for parasites of the phylum Platyhelminthes and Protozoa [9-13]. For *Trypanosoma cruzi*, the HRM method has been implemented based on the amplification of a 383-bp

* Correspondence: zaha@cbiot.ufrgs.br
[1]Programa de Pós-Graduação em Biologia Celular e Molecular, Universidade Federal do Rio Grande do Sul, Caixa Postal 15005, CEP 91501-970, Porto Alegre, RS, Brazil
[2]Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul, Caixa Postal 15053, CEP 91501-970, Porto Alegre, RS, Brazil
Full list of author information is available at the end of the article

DNA fragment of the cytochrome b gene (cyt b), allowing the effective differentiation of 14 genotypes and showing that the use of amplicons derived from mitochondrial genes is reliable and sufficiently robust for use in HRM [14].

The aim of this work was to develop an HRM-PCR assay targeting part of the mitochondrial cox1 gene to achieve a rapid, accurate, and low cost diagnostic method for detecting species belonging to the *Echinococcus* genus. In this work, we report the successful implementation of HRM analysis to differentiate species of the genus *Echinococcus* using part of the mitochondrial cox1 gene.

A total of seven species belonging to the Taeniidae family were used in this study (Table 1). The DNA extraction of *E. granulosus* and *E. ortleppi* was performed from protoscoleces, using proteinase K, and from germinal layer [5,15]. DNA samples from the remaining species were obtained using the PureLinkTM Genomic DNA Kit (Invitrogen, USA), following the manufacturer's instructions.

A 444-bp fragment of the cytochrome c oxidase subunit I (cox1) gene [16], was amplified using the primers 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ (forward) and 5′-TAAAGAAAG AACATAATGAAAATG-3′ (reverse) in the following reaction mixture: 50 ng of DNA template, 5 mM dNTP, 5 pmol of each primer, 1.2 mM $MgCl_2$, 1 U Taq polymerase (Invitrogen, USA), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, in a total volume of 20 μL. The PCR reactions were performed in a 7500 thermal cycler (Applied Biosystems) under primer annealing touchdown strategy [17]. All PCR reactions were carried out in technical triplicates with non-template controls (NTCs).

In an attempt to discriminate and accurately identify differences in all seven species, we used two approaches. SYBR Green Tm-based analysis, in which the dissociation step was obtained from the 7500 Applied Biosystems machine (with SDS software) using 1X SYBR green dye (Invitrogen, USA) and increasing the temperature from 60°C to 95°C at ramping increments of 0.2°C/s (Table 1). A second approach employed the HRM technique (Table 1), that was carried out with SYTO 13 (Life technologies), a DNA dye that does not preferentially bind to GC- or AT-rich sequences, does not increase the melting temperature, and has a minimal inhibitory effect on PCR [18,19], at a 2.5 μM final concentration, in a final volume of 10 μL, using the Eco Real-Time PCR System (Illumina ®). In this assay, melting was conducted by increasing the temperature from 60°C to 99°C at ramping increments from 0.1°C/s. The HRM analysis was carried out using the Eco software (version 3.0) with normalization regions between 79.1–79.4°C and 87.1–87.4°C. A difference melting curve was produced with *E. ortleppi* as baseline.

To validate HRM-cox 1 profiles, 58 isolates of *Echinococcus* spp. collected from hydatid cysts of bovine livers and lungs were analyzed. DNA from protoscoleces and germinal layer were used to evaluate the usefulness of this technique to differentiate *Echinococcus* species. The HRM was carried out using Rotor-gene Q 2plex System (Roche ®) as described above, in attempt to show that this method is reproducible with alternative systems. As reference curves of HRM, a known sample for *E. granulosus* and *E. ortleppi* was used.

One positive amplicon of each sample derived from PCR-HRM that displayed distinct curve shapes were sequenced. Sequence quality assessment and assembly was performed using DNAStar software (version 8.1.3). After PCR amplification, confirmation of the species was performed by a homology search against reference sequences using the Basic Local Alignment Search Tool (BLAST) program hosted by the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

A DNA fragment of 444 bp from the cox1 mitochondrial gene was amplified in all analyzed samples and no amplification was observed in the control reactions (NTCs). With SYBR Green Tm-based and HRM analyses, very low Tm variability was observed (Table 1). *E. granulosus* and *E. ortleppi* melting curves showed a difference in

Table 1 Species examined, the samples location, host and the number of isolates

Species	Location	Host	SYBR Green Tm-based			HRM analysis			Validation test		
			Number of isolates	Tm	SD	Number of isolates	Tm	SD	Number of isolates	Tm	SD
E. ortleppi	Southern Brazil	Bovine	18	82.70	0.08	6	83.35	0.02	32	81.70	0.03
E. granulosus	Southern Brazil	Bovine	15	84.04	0.13	4	83.72	0.07	26	82.30	0.05
E. oligarthrus	Northern Brazil	Agouti	3	82.56	0.05	3	82.70	0.07	-	-	-
E. vogeli	Northern Brazil	Paca	2	83.30	0.05	2	84.10	0.14	-	-	-
E. multilocularis	Europe	Monkey	-	-	-	6	82.70	0.07	-	-	-
E. canadensis (G7)	Southern Brazil	Pig	-	-	-	2	82.05	0.03	-	-	-
T. hydatigena	Southern Brazil	Pig	-	-	-	3	81.95	0.07	-	-	-

The melting temperatures (Tm) and standard deviations (SD) obtained in the SYBR Green Tm-based, HRM analyses and validation test are shown.

the Tm of 1.34°C (Table 1; Figure 1A). Likewise, *E. vogeli* and *E. oligarthra* showed a difference of 0.74°C (Table 1; Figure 1C). The differences in the Tm were at least nine and eight times higher than the respective standard deviation (SD), respectively (Table 1). These results refer to the total number of samples used, although in Figure 1, for comparison, only the curves of the isolates that were also used in the HRM approach are presented. Similarly, the Tm obtained from the HRM analysis, showed a difference of 0.37°C between *E. granulosus* and *E. ortleppi* and a difference of 1.40°C between *E. vogeli* and *E. oligarthra*. In this analysis, the SDs were five and ten times lower than the differences in Tm, respectively (Table 1; Figure 1B and D).

In the HRM analysis, a specific profile for each species analyzed was observed in both the standard and the difference curve (Figures 2A and B, respectively). Despite the close Tm value observed for some species, the HRM profiles provide a clear and undoubted discrimination among them. See, for example, the comparisons between *E. multilocularis* and *E. ortleppi* or between *E. canadensis* (G7) and *E. oligarthra* (Table 1; Figure 2A and B). In our validation test, from the HRM profiles of the cox1 gene, thirty-two samples were correctly identified as *E. ortleppi* and twenty-six of the samples obtained were identified as *E. granulosus sensu stricto* (G1) (Table 1; Figure 2C). The results obtained by PCR-HRM for all the tested samples

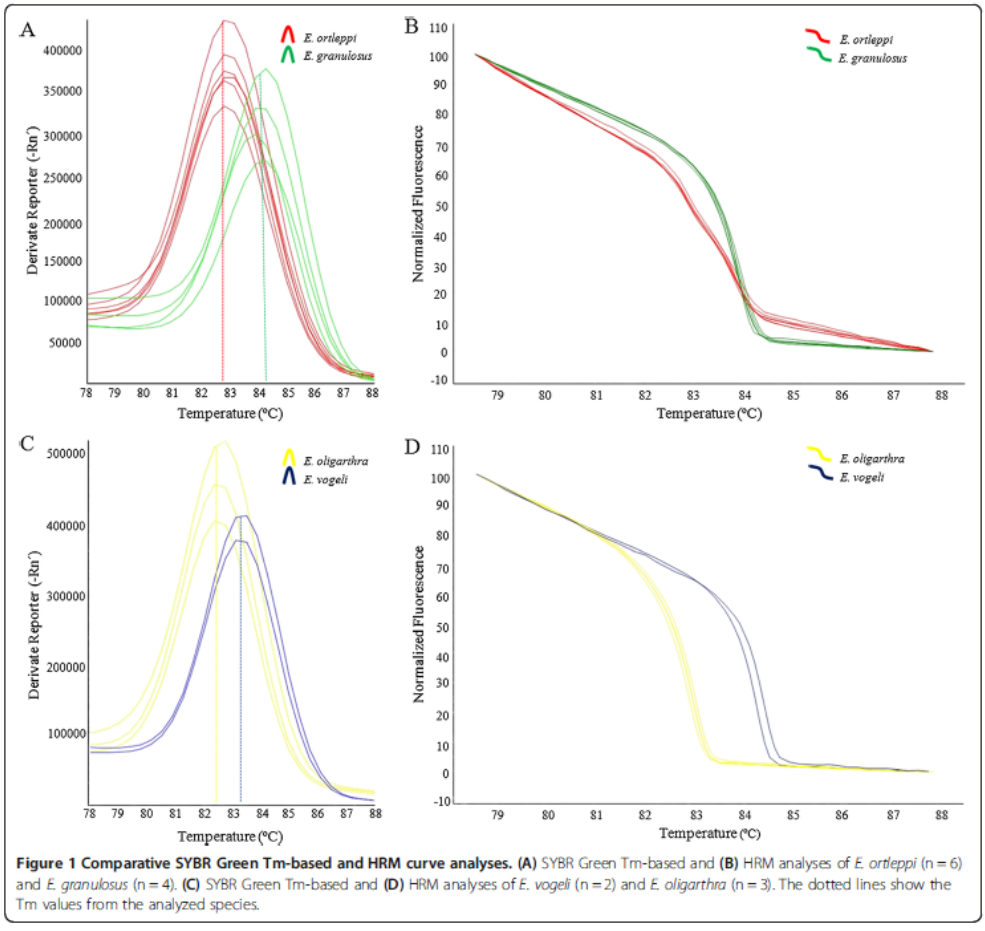


Figure 1 Comparative SYBR Green Tm-based and HRM curve analyses. (A) SYBR Green Tm-based and **(B)** HRM analyses of *E. ortleppi* (n = 6) and *E. granulosus* (n = 4). **(C)** SYBR Green Tm-based and **(D)** HRM analyses of *E. vogeli* (n = 2) and *E. oligarthra* (n = 3). The dotted lines show the Tm values from the analyzed species.

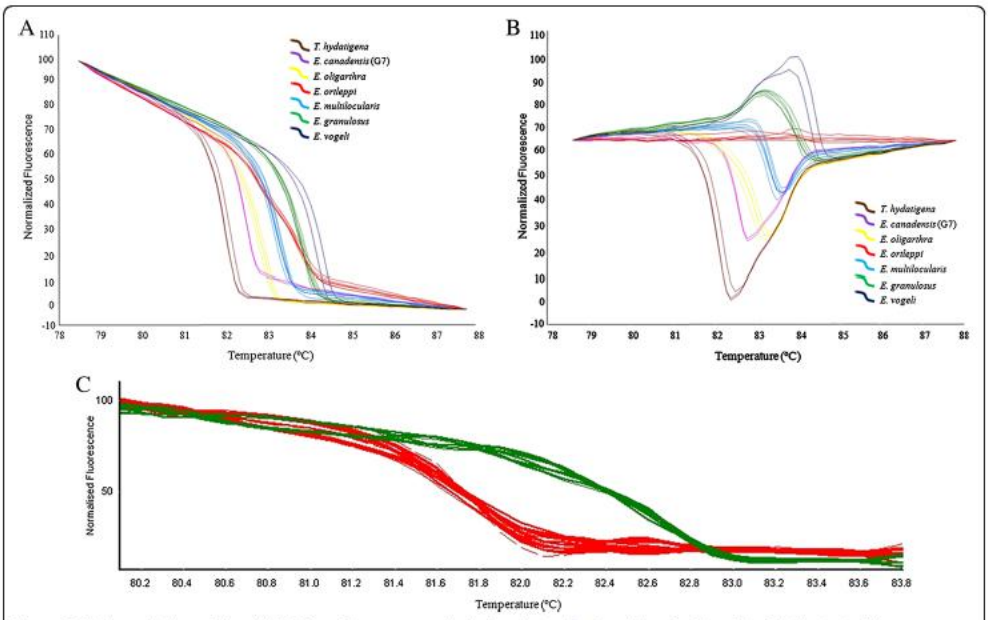


Figure 2 High-resolution melting (HRM) from the seven species belonging to the Taeniidae family, and validation test. (A) Normalization curves derived from the raw data plots. **(B)** Difference curves derived from the normalization data using *E. ortleppi* as baseline. **(C)** HRM profile obtained from *E. ortleppi* and *E. granulosus* samples in the validation test.

were confirmed by sequencing the amplicons. BLASTn analysis showed identity values greater than 99% for all samples.

We show, for the first time, that it is possible to distinguish *E. granulosus* from *E. ortleppi* as well as *E. vogeli* from *E. oligarthra* using the Tm obtained from the melting analysis of cox1. Furthermore, we compared this species with *E. multilocularis*, *E. canadensis* and *T. hydatigena*, and discriminated them by HRM, that is a useful and advantageous method that can be routinely employed in endemic regions of echinococcosis. This observation will be useful for additional HRM analyses of the Taeniidae family and, thus, will serve as a basis for subsequent interpretations.

The development of fast and effective tools for *Echinococcus* species identification has been the subject of numerous studies. For instance, morphological differences and the cut-off value for adult and larval hook were determined to distinguish *E. granulosus sensu lato* isolates [20,21]. However, the larval rostellar hook morphometry method is dependent on the presence of protoscoleces. In contrast, HRM analyses allow the use of DNA extracted from any parasite material as we showed in the validation test (Figure 2C).

In an attempt to implement an *Echinococcus* genotyping tool, a multiplex PCR (mPCR) test was used, using eleven pairs of primers, and it successfully differentiated the species of the *Echinococcus granulosus* complex [22]. Here, we present a technique that only requires a single pair of primers that amplify the cox1 gene, providing a quick, closed-tube and gel-free detection method for species belonging to the *Echinococcus* genus (Figure 2A and B). In our procedure, the identification of the species of the genus *Echinococcus* took approximately 6 hours. Recently, the application of HRM analysis was reported for genotyping *E. granulosus sensu lato* in Iran to distinguish G6 from the G1 and G3 genotypes, but it did not show good results in distinguishing G1 from G3 [23].

In conclusion, we propose the use of HRM of the cox1 gene as a routine method to distinguish *Echinococcus* species in epidemiological surveys or in basic research (Figure 2). This method may also be applied to identify other species belonging to the Taeniidae family. Finally, we believe that the HRM method could be implemented using a smaller amplicon product when the goal is to identify minor differences, such as in the case of closely related genotypes.

Abbreviations
HRM: High-resolution melting; NTCs: Non-template controls; BLAST: Basic local alignment search tool; Tm: Melting temperature; SD: Standard deviation.

Competing interests
There are no conflicts of interest.

Authors' contributions
GBS participated in the design of the study, and in the HRM experiments. Wrote the manuscript. SME participated in the SYBR Green Tm-based experiments, helped to draft the manuscript. HBF participated in the design of the study and helped to draft the manuscript. RM participated in HRM experiments and helped to draft the manuscript. AZ participated in the design of the study and helped to draft the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgments
This work was supported by CAPES and CNPq. G.S.B. and S.M.E. are recipient of fellowships from CAPES and CNPq, respectively. We would like to express our thanks to Dr. Karen Haag for providing access to her *Echinococcus* DNA database and Dr. Itabajara Vaz da Silva Junior and MSc. Daiani Oldiges for providing access to Rotor-gene Q 2plex.

Author details
[1]Programa de Pós-Graduação em Biologia Celular e Molecular, Universidade Federal do Rio Grande do Sul, Caixa Postal 15005, CEP 91501-970, Porto Alegre, RS, Brazil. [2]Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul, Caixa Postal 15053, CEP 91501-970, Porto Alegre, RS, Brazil. [3]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, CEP 91501-970, Porto Alegre, RS, Brazil.

Received: 11 October 2013 Accepted: 5 November 2013
Published: 14 November 2013

References

1. Nakao M, McManus DP, Schantz PM, Craig PS, Ito A: **A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes.** *Parasitology* 2007, **134**:713–722.
2. Nakao M, Lavikainen A, Yanagida T, Ito A: **Phylogenetic systematics of the genus *Echinococcus* (Cestoda: Taeniidae).** *Int J Parasitol* 2013, **43**:1017–1029.
3. Xiao N, Qiu J, Nakao M, Li T, Yang W, Chen X, Schantz PM, Craig PS, Ito A: ***Echinococcus shiquicus*, a new species from the Qinghai-Tibet plateau region of China: discovery and epidemiological implications.** *Parasitol Int* 2006, **55**:233–236.
4. Hüttner M, Nakao M, Wassermann T, Siefert L, Boomker JDF, Dinkel A, Sako Y, Mackenstedt U, Romig T, Ito A: **Genetic characterization and phylogenetic position of *Echinococcus felidis* (Cestoda: Taeniidae) from the African lion.** *Int J Parasitol* 2008, **38**:861–868.
5. Balbinotti H, Santos GB, Badaraco J, Arend AC, Graichen DA, Haag KL, Zaha A: ***Echinococcus ortleppi* (G5) and *Echinococcus granulosus sensu stricto* (G1) loads in cattle from Southern Brazil.** *Vet Parasitol* 2012, **188**:255–260.
6. D'Alessandro A, Rausch RL: **New aspects of neotropical polycystic (*Echinococcus vogeli*) and unicystic (*Echinococcus oligarthrus*) echinococcosis.** *Clin Microbiol Rev* 2008, **21**:380–401.
7. Loos-Frank B: **An up-date of Verster's (1969) Taxonomic revision of the genus *Taenia* Linnaeus (Cestoda) in table format.** *Syst Parasitol* 2000, **45**:155–183.
8. Nakao M, Lavikainen A, Iwaki T: **Molecular phylogeny of the genus *Taenia* (Cestoda: Taeniidae): proposals for the resurrection of Hydatigera Lamarck, 1816 and the creation of a new genus *Versteria*.** *Int J Parasitol* 2013, **43**:427–437.
9. Lin JH, Tseng CP, Chen YJ, Lin CY, Chang SS, Wu HS, Cheng JC: **Rapid differentiation of influenza A virus subtypes and genetic screening for virus variants by high-resolution melting analysis.** *J Clin Microbiol* 2008, **46**:1090–1097.
10. Gago S, Zaragoza Ó, Cuesta I, Rodríguez-Tudela JL, Cuenca-Estrella M, Buitrago MJ: **High resolution melting analysis for identification of the *Cryptococcus neoformans-Cryptococcus gattii* complex.** *J Clin Microbiol* 2011, **49**:3663–3666.
11. Nasereddin A, Jaffe CL: **Rapid diagnosis of Old World Leishmaniasis by high-resolution melting analysis of the 7SL RNA gene.** *J Clin Microbiol* 2010, **48**:2240–2242.
12. Wongkamchai S, Monkong N, Mahannol P, Taweethavonsawat P, Loymak S, Foongladda S: **Rapid detection and identification of *Brugia malayi*, *B. pahangi*, and *Dirofilaria immitis* by high-resolution melting assay.** *Vector Borne Zoonotic Dis* 2013, **13**:31–36.
13. Higuera SL, Guhl F, Ramírez JD: **Identification of *Trypanosoma cruzi* Discrete Typing Units (DTUs) through the implementation of a High-Resolution Melting (HRM) genotyping assay.** *Parasit Vectors* 2013, **6**:112.
14. Peña VH, Fernández GJ, Gómez-Palacio AM, Mejía-Jaramillo AM, Cantillo O, Triana-Chávez O: **High-resolution melting (HRM) of the cytochrome B gene: a powerful approach to identify blood-meal sources in Chagas disease Vectors.** *PLoS Negl Trop Dis* 2012, **6**:e1530.
15. Haag KL, Alves-Junior L, Zaha A, Ayala FJ: **Contingent, non-neutral evolution in a multicellular parasite: natural selection and gene conversion in the *Echinococcus granulosus* antigen B gene family.** *Gene* 2004, **333**:157–167.
16. Bowles J, Blair D, McManus DP: **Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing.** *Mol Biochem Parasitol* 1992, **54**:165–173.
17. Haag KL, Gottstein B, Ayala FJ: **The EG95 antigen of *Echinococcus* spp. contains positively selected amino acids, which may influence host specificity and vaccine efficacy.** *PLoS ONE* 2009, **4**:e5362.
18. Gudnason H, Dufva M, Bang DD, Wolff A: **Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature.** *Nucleic Acids Res* 2007, **35**:e127.
19. Eischeid AC: **SYTO dyes and EvaGreen outperform SYBR Green in real-time PCR.** *BMC Res Notes* 2011, **4**:263.
20. De ML, Takano K, Brochado JF, Costa CV, Soares AG, Yamano K, Yagi K, Katoh Y, Takahashi K: **Infection of humans and animals with Echinococcus granulosus (G1 and G3 strains) and E. ortleppi in Southern Brazil.** *Vet Parasitol* 2011, **177**:97–103.
21. Soriano SV, Pierangeli NB, Pianciola LA, Mazzeo M, Lazzarini LE, Debiaggi MF, Bergagna HF, Basualdi JA: **The optimum cut-off value to differentiate *Echinococcus granulosus sensu stricto* from other species of *E. granulosus sensu lato* using larval rostellar hook morphometry.** *J Helminthol* 2013, **10**:1–8.
22. Boubaker G, Macchiaroli N, Prada L, Cucher MA, Rosenzvit MC, Ziadinov I, Deplazes P, Saarma U, Babba H, Gottstein B, Spilliotis M: **A multiplex PCR for the simultaneous detection and genotyping of the *Echinococcus granulosus* complex.** *PLoS Negl Trop Dis* 2013, **7**:e2017.
23. Rostami S, Talebi S, Babaei Z, Sharbatkhoti M, Ziaali N, Rostami H, Harandi MF: **High resolution melting technique for molecular epidemiological studies of cystic echinococcosis: differentiating G1, G3, and G6 genotypes of *Echinococcus granulosus sensu lato*.** *Parasitol Res* 2013, **112**:3441–3447.

doi:10.1186/1756-3305-6-327
Cite this article as: Santos *et al.*: Rapid detection of *Echinococcus* species by a high-resolution melting (HRM) approach. *Parasites & Vectors* 2013 **6**:327.

Capítulo II

Manuscrito submetido à publicação na revista *International Journal for Parasitology*

Excretory/secretory products in *Echinococcus granulosus* metacestode: is the intermediate host complacent with infection caused by the parasite's larval form?

Santos, G.B., Monteiro, K. M., Silva, E. D., Battistella, M. E., Ferreira, H. B., Zaha, A.

Excretory/secretory products in *Echinococcus granulosus* metacestode: is the intermediate host complacent with infection caused by the parasite's larval form?

Guilherme B. dos Santos[1], Karina M. Monteiro[1,2], Edileuza Danieli da Silva[1], Maria Eduarda Battistella[3], Henrique B. Ferreira[1,2], Arnaldo Zaha[1,2]*

[1] Programa de Pós-Graduação em Biologia Celular e Molecular, Universidade Federal do Rio Grande do Sul, Caixa Postal 15005, CEP 91501-970, Porto Alegre, RS, Brazil

[2] Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, CEP 91501-970, Porto Alegre, RS, Brazil

[3] Graduação em Biotecnologia, Universidade Federal do Rio Grande do Sul, Caixa Postal 15005, CEP 91501-970, Porto Alegre, RS, Brazil.

* **Corresponding author**

E-mail address: zaha@cbiot.ufrgs.br

Abstract

The genus *Echinococcus* consists of parasites that have a life cycle with two mammalian hosts. Their larval stage, called hydatid cyst, develops predominantly in the liver and lungs of intermediate hosts. The hydatid cyst is the causative agent of cystic hydatid disease, and the species *Echinococcus granulosus*, G1 haplotype, is responsible for the vast majority of cases in humans, cattle, and sheep. Protein characterization in hydatid cysts is essential for better understanding of the host–parasite relationship and the fertility process of *Echinococcus*. The aims of this work were the identification and quantitative comparison of proteins found in hydatid fluid from fertile and infertile cysts from *E. granulosus*, in order to highlight possible mechanisms involved in cyst fertility or infertility. Hydatid fluid samples containing proteins from both *E. granulosus* and *Bos taurus* were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Our proteomic analysis of fertile and infertile cysts allowed identified a total of 498 proteins, of which 153 proteins were exclusively identified in the fertile cyst, 271 in the infertile cyst, and 74 in both. Functional *in silico* analysis allowed to highlight some important aspects: (1) clues about the possible existence of an "arms race" involving parasite and host responses in fertile and infertile cysts; (2) a number of proteins in hydatid fluid without functional annotation or with possible alternative functions; (3) the presence of extracellular vesicles (EVs) such as exosomes, which was confirmed by transmission electron microscopy.

Keywords: Host-parasite relationship; proteomics; arms race; extracellular vesicles; infertility.

Abbreviations: GL, germinal layer; GO, gene ontology; HF, hydatid fluid; HRM, high-resolution melting; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LL, laminar layer; PSC, protoscolex; SCX, strong cation exchange;

Highlights:

- Proteomic analysis reveals 498 proteins in extracellular environment involved with parasite surveillance and death.
- Existence of an “arms race” involving parasite and host responses;
- Presence of extracellular vesicles such as exosomes in hydatid fluid from *E. granulosus*.

1. Introduction

Echinococcosis is one of the major human and animal zoonoses and is caused by the larval form (metacestode) of parasites from the genus *Echinococcus*(Cucher et al., 2015). *Echinococcus granulosus* sensu stricto (*E. granulosus* s.s., haplotypes G1, G2, and G3) and *Echinococcus multilocularis* are responsible for most of the infections in humans and livestock. Cystic echinococcosis or cystic hydatid disease (CHD) caused by *E. granulosus* (G1) is recognized as an emergent or re-emergent human zoonosis in several countries worldwide and has been included by the World Health Organization in its list of serious "neglected tropical diseases", necessitating greater insight into the host–parasite interactions (Nakao et al., 2013; Otero-Abad and Torgerson, 2013).

Transmission of CHD can occur through domestic and sylvatic cycles, and creates severe global burdens due to costs associated with human treatment and losses in livestock production (Budke et al., 2006). Ingestion of parasite eggs by suitable intermediate hosts (humans, sheep, or goats) allows the hatching of oncospheres and the subsequent development of metacestodes (or hydatid cysts) in the viscera (mostly the liver and lungs). The *E. granulosus* metacestode is unilocular and grows slowly, often remaining unnoticed for several years until they are large enough to cause symptoms. The hydatid cyst is delimited by a laminar layer (LL), which is internally lined by the germinal layer (GL), which gives rise to pre-adults (protoscoleces, PSCs) by asexual reproduction. Upon ingestion by a definite host (e.g., a dog or a wolf), PSCs mature into adult worms within the gut (Mcmanus et al., 2003).

Although easily infected, for unknown reasons, bovines generally produce infertile *E. granulosus* lung cysts, which do not produce PSCs by proliferation of brood capsules from

GL, thus ending the parasite life cycle (Balbinotti et al., 2012). Proteins present in the hydatid fluid (HF) that fills the hydatid cysts result from host–parasite cross talk and are therefore likely to reflect the parasite's physiological state, including cyst fertility or infertility. Efforts to characterize proteins from HF were hindered by its composition, in which the overabundance of serum proteins interfered in the identification of less abundant proteins.

Some studies attempted to assess parasite proteins in HF, but the large amount of host serum proteins, especially albumin and globulins, led to resolution problems and prevented proper identification of parasite proteins (Frayha, 1980; Chemale et al., 2003). Using immobilized anti-PSC antibodies, a LC-MS/MS analysis of immunopurified samples allowed a total identification of 20 parasite proteins in HF (Monteiro et al., 2010). A combination of albumin depletion, 1D SDS-PAGE gel fractionation, and peptide Off-gel electrophoresis enables the identification of 117 proteins (44 from *E. granulosus* and 73 from three distinct hosts) (Aziz et al., 2011). Using a distinct experimental approach, a LC–MS/MS proteomic analysis of the excretory/secretory products obtained from the first 48 h of *in vitro* culture of *E. granulosus* PSCs, allowed an identification of 32 parasite proteins (Virginio et al., 2012). HF contains host and parasite products of excretion and secretion, as well as intracellular and membrane proteins that are present in HF due to parasite damage or death and shedding of cells from GL and the tegument of PSCs (Monteiro et al., 2010; Aziz et al., 2011).

The knowledge on the parasite and host protein repertoires in HF is still limited. It is important to compare HF samples from fertile and infertile cysts obtained from the same host and infected organ to understand the interaction between the parasite and host

molecules, which may end in the survival or death of the parasite. Molecular studies using the “omics” tools are necessary to improve our general understanding of the proximate mechanisms involved in the molecular manipulative strategies used in parasitic lifestyles (Thompson and Lymbery, 2013).

We describe the proteomic analysis and comparison of HF samples from *E. granulosus* fertile and infertile cysts from *Bos taurus* lung. Our analyses allowed the identification of several proteins, including both parasite and host ones. Comparison of the identified protein repertoire from fertile and infertile cysts allowed qualitative and quantitative differences between them. The biological significance of these differences is discussed.

2. Material and Methods

2.1. Parasite material and species determination

E. granulosus cysts were obtained from cattle slaughtered at a commercial abattoir in the metropolitan region of Porto Alegre, RS (Brazil). A total of 85 lung viscera samples were dissected and HF was aseptically aspired from cysts and centrifuged at 10.000 g for 15 min at 4°C to sediment PSCs and debris (Monteiro et al., 2010). HF samples were kept frozen at -80ºC prior to use.

PSCs from fertile cysts and GL from infertile cysts were collected and used for species identification, which was performed by high-resolution melting PCR (Santos et al., 2013). DNA extraction from PSCs and GLs was performed using proteinase K and Trizol® reagent, respectively [6]. A 444-bp fragment of the cytochrome c oxidase subunit I (cox1) gene was amplified using the primers 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ (forward) and 5′-TAAAGAAAG AACATAATGAAAATG-3′ (reverse) in the following

reaction mixture: 50 ng of DNA template, 5 mM of dNTP, 5 μM of each primer, 1.2 mM of $MgCl_2$, 1U of Taq DNA polymerase (Invitrogen, USA), 20 mM of Tris–HCl (pH 8.4), 50 mM of KCl, and 5 μM of SYTO 13 in a total volume of 20 μL.

The PCR reactions were performed in a 7500 Fast thermal cycler (Applied Biosystems) under primer annealing touchdown strategy starting with 55°C and decreasing 1°C every two cycles during the first 20 cycles, followed by 15 more cycles at 45°C for 30 sec each. The first denaturing step (95ºC) lasted 5 min, and the remaining cycles lasted 30 sec. Extension was performed at 72°C for 60 sec in the first 34 cycles and for 5 min in the last cycle. All PCR reactions were carried out in technical duplicates with non-template control. Melting was conducted by increasing the temperature from 60°C to 99°C at a ramp rate of 0.1ºC/s. The high-resolution melting analysis was carried out using the software from Applied Biosystems (version 3.0.1) with normalization regions between 79.1–79.4ºC and 87.1–87.4ºC.

2.2. *E. granulosus* hydatid fluid protein samples

The overall protein concentration of each *E. granulosus* HF sample was determined using Qubit™ and qualitatively evaluated by 12% SDS-PAGE using 50 μg of protein sample. HF samples were classified according to the correlation between bovine albumin band intensity in SDS-PAGE and the volume of the hydatid cyst from which the sample was collected. We use ImageJ (https://imagej.nih.gov/ij/) to quantify band intensity and Spearman's rank correlation test to estimate the correlation between cyst volume and albumin band intensity. We selected three individual *E. granulosus* (G1) lung cysts under

two conditions (fertile and infertile) and pooled samples from the same condition for further proteomics analysis.

2.3. Sample processing for LC-MS/MS analysis

One hundred and fifty micrograms of fertile and infertile pooled HF samples were lyophilized and resuspended in 100 µl of denaturing buffer (25 mM NH_4HCO_3/8 M urea, pH 8.0), reduced by adding DTT (0.02 µg/µg protein), and incubated for 30 minutes at 37°C. Samples were then carboxyamidomethylated with iodoacetamide (0.1 µg/µg protein) for 30 minutes at room temperature. For protein digestion, samples were diluted to 1 M urea using 25 mM NH_4HCO_3 (pH 8.0), and trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) was added (0.02 µg/µg protein). After 4 h of digestion at 37°C, an additional aliquot of enzyme was added, and samples were incubated for 16 h at 37°C.

The tryptic peptide mixture was desalted using OASISs HLB Cartridge (Waters, USA). Each peptide mixture was then fractionated into four subgroups by strong cation exchange (SCX). First, the SCX columns were conditioned using 100 µl of methanol by centrifugation at 110 x g for 1 minute. Afterward, the columns were flushed with two volumes of water (by centrifugation at 110 x g for 1 minute) and conditioning with 0.2 M monosodium phosphate + 0.3 M sodium acetate (pH ~5.7) for 60 min (adding 100 µl of conditioning buffer, spinning for 10 sec, and letting stand in the column). Next, the columns were equilibrated by flushing two times at 110 x g for 45 sec with 100 µl of 5 mM of potassium phosphate + 25% acetonitrile (pH 3.0 adjusted with phosphoric acid).

The samples (in 100 µl of 5mM phosphate buffer at pH 3.0) were added to the columns and centrifuged at 110x g by 45 sec. An additional 50 µl of phosphate buffer was

added to wash out any trace of impurities. Phosphate buffer was added to the columns to release the sample with a salt gradient as follows: fraction A (phosphate buffer + 75 mM of KCl), fraction B (phosphate buffer + 125 mM of KCl), fraction C (phosphate buffer + 200 mM of KCl), and fraction D (phosphate buffer + 400 mM of KCl). All these steps were performed with centrifugations at 110 x g for 30 sec. Each SCX fraction was lyophilized using a vacuum lyophilizer and stored at -80°C.

Finally, each fraction was desalted using C18 columns. First, the columns were conditioned with 100 µl of 100% acetonitrile by centrifugation at 110 x g for 90 sec and then flushed two times with TFA 0.1% at 110 x g for 60 sec. The samples (in TFA 0.1%) were added to the columns and centrifuged at 110 x g for 90 sec. An additional 200 µl of TFA 0.1% was added to the columns to wash out any trace of salts. To release the samples, 50 µl of 80% acetonitrile + 0.1% TFA was added to each column. Each resulting fraction was lyophilized using a vacuum lyophilizer and stored at -80°C until LC-MS/MS analysis.

2.4. Mass spectrometry analysis of SCX fractions

The mass spectrometric analysis of each SCX sample was performed in technical triplicates using a nanoflow liquid chromatography-tandem mass spectrometry system (nLC−MS/MS) on an EASY-nLC system (Proxeon Biosystems, USA), which was connected to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, USA) through a Proxeon nanoelectrospray ion source. Two micrograms of peptides were separated with a 2–90% acetonitrile gradient in 0.1% formic acid using the analytical PicoFrit Column (C18, 20 cm × ID 75 µm, 5-µm particle size, New Objective, USA) at a flow rate of 300 nl/min for 65 min. The nanoelectrospray voltage was set to 2.2 kV, and

the source temperature was 275°C. The full-scan MS spectra (m/z 300−1600) were acquired in an Orbitrap analyzer after accumulation to a target value of 1×10^6. The resolution in the Orbitrap was set to r = 60,000, and the 20 most intense peptide ions with charge states ≥ 2 were sequentially isolated to a target value of 20,000 and fragmented in the linear ion trap using low-energy CID (normalized collision energy of 35%). The signal threshold for triggering an MS/MS event was set to 1000 counts. Dynamic exclusion was enabled with an exclusion size list of 500 with duration of 60 sec and a repeat count of 1. An activation of q = 0.25 and activation time of 10 ms were used.

2.5. Mass spectrometry data analysis for parasite and host protein identifications

The raw files were processed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.3.0.339) to generate mgf files. For protein identification, MS/MS data were searched using a local *E. granulosus* and *B. taurus* concatenated database containing deduced amino acid sequences (20.173) from genome annotation. The search parameters consisted of carbamidomethylation as a fixed modification, oxidation of methionine as a variable modification, one trypsin missed cleavage, and a tolerance of 10 ppm for precursor and 1 Da for fragment ions. Ion type was set as monoisotopic, and peptide charges 2+, 3+, and 4+ were taken into account.

Scaffold version 4.3.4 was used to validate the peptide and protein identifications. The peptide identifications were accepted if they could be established with >99% probability, as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 99% probability and contained two unique identified peptides in at least two technical replicates. The identified proteins were grouped in three

subsets: shared proteins, exclusively from fertile cyst, and exclusively from infertile cyst. These subsets were further grouped into two sets: Fertile, which corresponds to proteins found in shared and fertile subsets, and Infertile, which corresponds to proteins found in shared and infertile subsets. We used normalized spectral abundance factor (NSAF) and total spectral count parameters, both acquired using Scaffold, to quantify the differences in protein abundance between samples. To determine statistical differences between shared proteins, we performed a Fisher's exact test using NSAF values and plotted this data in Matrix2png 1.2.1 software to generate a heat map (Pavlidis, 2003).

Gene ontology (GO) terms were searched for each protein using Blast2GO with the default parameters and following the second and third level hierarchy of GO (Gotz et al., 2011). For all proteins, Blastp was applied on the GenBank non-redundant protein database. Gene ontology term enrichment was performed using DAVID (Huang et al., 2009). Identified proteins from parasite and host were used to investigate the presence of signal peptide using SignalP 4.1 (Nielsen et al., 1997) and the presence of a non-classical signal of exportation using SecretomeP 2.0 (Bendtsen et al., 2004). A protein was considered to contain a signal peptide if the D-score was significant and to be non-classically secreted if the NN score was higher than 0.6 but at the same time not predicted to contain a signal peptide. We used Phobius to determine protein transmembrane topology and signal peptides from the amino acid sequence of a protein (Käll et al., 2007).

2.6. Isolation, morphological characterization, *in silico* search, and immunoblotting of extracellular vesicles from hydatid fluid of *E. granulosus*

After the HF collection and selection as described in section 2.2, the HFs from fertile and infertile cyst were immediately subjected to successive centrifugations (300 x g for 10 min; 2,000 x g for 10 min; 10,000 x g for 30 min) and finally two rounds of ultracentrifugation at 100,000 x g for 70 min. The vesicles were resuspended in PBS and stored at -80°C (Théry et al., 2006). EVs were characterized by transmission electron microscopy. Samples were fixed in 2% paraformaldehyde, and then formvar/carbon grids were placed on drops of 20 µL solution containing the purified EVs for 15 min. The grids were washed three times with milli-Q water and further fixed in 2.5% glutaraldehyde. After five more washes with milli-Q water, the grids were negatively stained in 2% uranyl acetate for 5 min. The images were recorded on a JEOL microscope (Jeol, JEM-1400 model).

Our *in silico* search was performed using EVpedia database (Kim et al., 2015), which contains proteins, mRNA, miRNA, lipid and metabolite already found in EVs from Prokaryotes and Eukaryotes. Each of the 498 proteins identified in the present work was used as a decoy to search for publications/data that already identified them as constituents of EVs.

The immunoblotting was performed using protein samples (20 □g) from purified EVs, hydatid fluid and protoscoleces that were resolved by 12% SDS-PAGE and transferred onto PVDF membranes (Hybond, GE Healthcare). The membranes were blocked for 16 h with 5% nonfat dry milk in PBS-T (PBS containing 0.1% Tween-20). Rabbit polyclonal anti-rEg14-3-3.1/3,diluted 1:20,000 v/v (Teichmann et al., 2015), or anti-rEgEno ,diluted

1:5,000 v/v (Lorenzatto et al., 2012), were used as primary antibodies and horseradish peroxidase (HRP)-labeled anti-rabbit IgG (ECL, GE Healthcare) was used as secondary antibody (1:7,000 v/v dilution). Antigen−antibody complexes were detected with the ECL Plus Western Blotting Substrate (Pierce) and imaged using the VersaDoc imaging system (Bio-Rad).

3. Results

3.1. Hydatid cyst genotyping and selection of hydatid fluid samples from fertile and infertile cysts for LC-MS/MS analysis

Our HRM genotyping analysis revealed that from 85 lung cysts collected, 42 were identified as *E. granulosus* (G1) comprising 11 from infertile cysts, and 31 from fertile ones. The six HF samples used for this study were identified as *E. granulosus* haplotype G1. The HFs from the 42 collected *E. granulosus* samples showed volumes ranging from 6 to 350 mL and an estimated content of bovine albumin protein ranging from 3 to 95% of the total protein content from each sample. We identified a positive correlation between cyst volume and proportion of albumin ($r = 0.74$). The three fertile and infertile cysts selected were those with the lowest content of host serum proteins (3 to 13% of albumin) and had between 6 and 14 mL of HF (Figures 1 A, B).

3.2 LC-MS/MS analysis of hydatid fluid samples from fertile and infertile cysts

To shed some light on HF protein components and to verify possible differences in the protein repertoires of two physiologically different states of *Echinococcus*, we performed a proteomic survey of *E. granulosus* HF samples from fertile and infertile cysts. The analysis

of ES products from parasitic Platyhelminthes is limited by the very low amount of protein usually recovered from *in vivo* samples. For this analysis, we pooled and concentrated the total HF recovered from three independent samples of fertile and infertile cysts from *E. granulosus* (G1) and with low possible albumin content (Figure 1 A, C).

A total of 498 proteins were identified by LC-MS/MS comprising 204 from *E. granulosus* and 294 from *B. taurus*, that were validated by Scaffold, which reported a protein FDR of 0.98% (Supplementary table S1). A total of 153 proteins were exclusively identified in the fertile condition, with 141 from parasitic origin and only 12 from the host. Remarkably, the opposite occurred in infertile state, with 271 proteins exclusively identified comprising 250 from the host and 21 from the parasite. Only 74 proteins were qualitatively shared between fertile and infertile samples (Figures 1 D, E, F; Supplementary table S2). In order to statistically distinguish the shared proteins found in fertile and infertile cysts, we quantified their significant abundance by performing Fisher's exact test using the NSAF parameter. Of the 42 shared proteins of *E. granulosus*, 18 and five of them are statistically more abundant in fertile and infertile cysts, respectively (Figure 2). The opposite occurs with the 32 shared proteins of *B. taurus*, 14 and one of them are statistically more abundant in infertile and fertile cyst, respectively (Figure 2).

3.3 *In silico* functional categorization of identified hydatid fluid proteins

We used the Blast2GO tool to illuminate the different functions and processes in an attempt to summarize and explore the functional categories of the *E. granulosus* and *B. taurus* ES products. A total of 149 (73%) *E. granulosus* and 287 (98%) *B. taurus* protein sequences were annotated with GO terms in three independent categories: biological

processes, molecular functions, and cellular components (Supplementary table S3). The analysis of the biological processes from *E. granulosus* HF proteins (level 2) using the individual subsets (fertile, infertile, and shared) indicated that the three most enriched categories were metabolic process (GO:0008152), cellular process (GO:0009987), and single-organism process (GO:0044699). Interestingly, biological adhesion (GO:0022610), response to stimulus (GO:0050896), signaling (GO:0023046), developmental process (GO:0032502) and localization (GO: 0051179) are absent in the infertile subset (Figure 3A). Considering all individual subsets at level 3, cell adhesion (GO:0007155) is exclusively present in the fertile subset (Supplementary figure S1A).

In the molecular function analysis from *E. granulosus* HF proteins (level 2), binding (GO:0005488) and catalytic activity (GO:0003824) were the two most enriched categories (Figure 3B). Considering all individual subsets at level 3, ion binding (GO:0043167) and hydrolase activity (GO:0016787) were the major categories, which have at least three times more proteins in the fertile subset than in the shared and infertile subsets. Extracellular constituent matrix (GO:0005201) is exclusively present in the fertile subset (Supplementary figure S1B). In the cellular component analysis of *E. granulosus* HF proteins (level 2), cell (GO:0005623), membrane (GO:0016020), and macromolecular complex (GO:0032991) were the three most enriched categories (Figure 3C). Organelle (GO:0043226) and the extracellular region (GO:0005576) were present in the fertile and shared subsets but absent in the infertile one. Considering all individual subsets at level 3, intracellular (GO:0005622) and protein complex (GO:0043234) were the major categories. Intrinsic component of the membrane (GO:0031224) and cell periphery (GO:0071944) were found only in the fertile subset (Supplementary figure S1C).

Of the 42 shared proteins (fertile and infertile cysts) of *E. granulosus,* 18 of them are statistically more abundant in fertile cyst (Figure 2). The GO analyses of this set of proteins supports hydrolase activity (GO:0016787), membrane component (GO:0016020) as the most prominent GO terms of this group of proteins (Supplementary figure S2A,B,C).

Analysis of the biological process of *B. taurus* HF proteins (level 2) using individual subsets (fertile, infertile, and shared) showed that metabolic process (GO:0008152) and cellular process (GO:0009987) were the two most enriched categories (Figure 3D). At level 3, cellular response to stimulus (GO:0051716) and response to stress (GO:0006950) had at least three times more proteins in the infertile subset than in the shared and fertile subsets (Supplementary figure S1D). The molecular function analysis of *B. taurus* HF proteins (level 2) indicated that binding (GO:0005488) and catalytic activity (GO:0003824) were the two most enriched categories (Figure 3E). Considering all individual subsets at level 3, ion binding (GO:0043167) and protein binding (GO:0005515) were the two major categories.

Interestingly, hydrolase activity (GO:0016787) and lipid binding (GO:0008289) had at least four times more proteins in the infertile subset than in the shared subset and were absent in the fertile subset (Supplementary figure S1E). In the cellular component analysis from *B. taurus* HF proteins (level 2), intracellular (GO:0005622), organelle (GO:0043226), and macromolecular complex (GO:0032991) were the three most enriched categories (Figure 3F). Considering all individual subsets at level 3, cell part (GO:0044464) and membrane-bound organelle (GO:0043227) were the major categories. Interestingly, cell periphery (GO:0071944), extracellular vesicle (GO:1903561), and vesicle (GO:0031982)

had at least three times more proteins in the infertile subset than in the shared and fertile subsets (Supplementary figure S1F).

Of the 32 shared proteins (fertile and infertile cysts) of *B. taurus*, 14 of them are statistically more abundant in infertile cyst (Figure 2). The GO analyses of this set of proteins supports response to stress (GO:0006950), protein binding (GO:0005515) and vesicle (GO:0031982) as the most prominent GO terms of this group of proteins (Supplementary figure S2D,E,F).

3.4. *In silico* prediction of pathways used for the secretion of proteins identified in hydatid fluid samples

The analysis of fertile cyst data showed that 51% (n=93) and 14% (n=25) of parasite proteins are possibly secreted through a signal peptide sequence and non-classical secretory pathways, respectively (Supplementary figure S3A). In contrast, 30% (n=19) of parasite proteins from infertile cysts may be secreted through a signal peptide. In contrast, most of the *B. taurus* proteins found in infertile cyst are predicted to be secreted by a non-classical secretory pathway (32%; n=91), followed by signal peptide sequence (11%; n=32) (Supplementary figure S3B). Intriguingly, we found a larger number of proteins with an unidentified pattern of secretion in *E. granulosus* and *B. taurus* in fertile and infertile cysts, respectively (Supplementary figure S3A,B). Using Phobius to identify the location of the different domains of a specific protein we found that the majority of *Echinococcus* proteins are non-cytoplasmic in fertile (62%; n=114) and infertile cyst (90%; n=57). Interestingly, we found a large amount of proteins involved in membrane composition or attached to it in fertile cyst (37%; n=68) (Supplementary figure S3C). The

vast majority of host proteins were identified as non-cytoplasmic in both fertile (98%; n=43) and infertile cyst (96%; n=270) (Supplementary figure S3D; Supplementary table S4).

3.5. Protein excretion/secretion in *E. granulosus* hydatid fluid involves the release of extracellular vesicles

Based on the obtained LC-MS/MS data we hypothesized that many of the proteins found in HF from *E. granulosus* could be secreted through EVs. In order to confirm or discard this hypothesis, we analyzed the secreted vesicles from fertile and infertile cysts by transmission electron microscopy. We showed that HF from *E. granulosus* contains EVs ranging from 50 to 200 nm in diameter (Figure 4A, B). We highlight two aspects: the presence of spherical structures with 50 to 100-nm diameter, which probably correspond to exosomes in fertile and infertile cysts, and the presence of these structures in HF from infertile cyst, in which the amount of parasite proteins is lower than host proteins (Figure 1D).

By using EVpedia, we found that Enolase and 14-3-3 are two of the most frequent proteins usually found in EVs, each one with more than a hundred of confirmations (Supplementary table 4). For this reason anti-rEg14-3-3 and anti-rEgEno sera were used in immunoblot experiments to further investigate the Eg14-3-3 and EgEnolase presence in the *E. granulosus* HF and EVs. The two proteins were detected in both samples from fertile parasite cysts, confirming them as EVs components (Figure 4C).

4. Discussion

The available *E. granulosus* and *E. multilocularis* genomes, which contain >10,000 genes (Tsai et al., 2013; Zheng et al., 2013), have motivate some bioinformatics studies based on the prediction of potential secreted proteins (Pan et al., 2014; Wang et al., 2015). Although such studies are relevant, it is important to consider that not all predicted secreted protein will be exported from an organism during its whole lifetime. Proteomic data have shown a surprisingly large proportion of excretion/secretion proteins that are not encoded by a signal peptide or even predicted to be secreted (Tirloni et al., 2014; Wen et al., 2014; Cao et al., 2016), which is why empirical proteomic studies remain essential. While proteomic techniques allow unbiased identification of the more abundant ES proteins, they may still miss those expressed at low but bioactive levels.

We avoided such bias by careful collection, screening, sample selection. Using an off-line MudPIT to fractionate the selected samples, we were able to identify 204 *E. granulosus* HF proteins, which represents at least four times more identifications than the previous HF proteomics studies (Monteiro et al., 2010; Aziz et al., 2011). Our study highlights the occurrence of a large number of host proteins mainly in infertile cysts. *E. granulosus* HF is a parasitic interface that contains both parasite and host proteins that play important roles in parasite survival and development. In this scenario, HF can be considered as a reservoir of proteins that are produced and at least to a certain extent exchanged by the host and parasite, which must be the determinant for the outcome of the host–parasite relationship.

The GO functions highlight some interesting aspects of proteins from HF. We identified the heat shock protein (HSP) 70, which is commonly associated as a chaperone

and is critical to the maintenance of cellular proteostasis through its role in the folding, refolding, aggregation suppression, translocation, and degradation of proteins (Bukau et al., 2006). More recently, HSP70 has been described in exosomes, suggesting a specialized function in protein trafficking or protein folding, which can improve the protein-folding environment in neighbor cells and even at the organismal level (Takeuchi et al., 2015). In the same way, we identified the major egg antigen described in *S. mansoni* as a homolog of heat shock proteins (Nene et al., 1986), as well as Syndecan, a protein involved in the regulation of wound healing, cell adhesion (GO:0022610), migration (GO:0051179), signaling (GO:0023046), and exosome formation (Friand et al., 2015; Stepp et al., 2015) (Figure 3A).

The G protein-coupled receptor superfamily constitutes the most expansive family of membrane proteins in the metazoan, and we identified two frizzled proteins that are members of this family. These proteins play a central role in eukaryotic signal transduction and are crucial in developmental processes, including cell fate determination, cell motility, and synaptic organization. They are also important for mediating cellular responses to a diverse range of extracellular stimuli (Zamanian et al., 2011). Together, these observations may indicate that the parasite of an infertile hydatid cyst has lost the capacity for detecting and reacting to stimulus from the host. They are thus unable to maintain their protein integrity, which can be considered crucial to parasite communication (GO:0023046), development (GO:0032502), and survival, finally leading to infertility and even to protoscoleces death (Figure 3A).

The opposite was found in infertile cyst, in which prominent members of the HSP 90 family, HSP 90α and β from *B. taurus*, were found in high abundance, and are both known

to be essential for the viability of eukaryotic cells (Sreedhar et al., 2004). Briefly, extracellular HSPs can be detected as membrane bound or membrane free, depending on their origin. It is likely that these two variants of extracellular HSPs could play alternative roles in the modulation of the immune system or other systems. Interestingly, some studies have pointed out that parasite recognition by the host's innate immune system is based on the recruitment of a multimeric receptor complex, including HSP 90 within lipid rafts, which can provide danger signals for the host's immune system (Matzinger, 2002; Trianta and Trianta, 2004). On the other hand, extracellular membrane-free HSP 90 has been shown to transport antigens from outside the cell to the cytosol in an "HSP-antigen protein" complex, resulting in antigen proteasomal degradation (Oura et al., 2011). Yet exosomes have been discussed as potent export vehicles for HSPs from the endosomal compartment into the extracellular space as a form of communication during injury, infection, and cell damage (De Maio, 2011). These may indicate that the host in the case of infertile cyst is reacting to an external stimulus (GO:0051716; GO:0006950) supplied by the parasite (Figure 3D).

Chemical and mechanical stress can damage basement membranes of the cells, which can modify their extracellular matrix and basement membrane constituents in response to signals or stress. Specific proteases act in the extracellular environment when cells have a reason to move or change their surroundings. We identified three *E. granulosus* cathepsins in this work. In a recent study, cathepsin D was described as being responsible for extracellular matrix remodeling (Margaryan et al., 2010), which was found, in the present study, to be more abundant in fertile cyst than in infertile one (GO:0003824; GO: 0005198). Interestingly, the large amount of basement-membrane-specific heparan sulfate

protein identified in fertile cyst can be considered as a signal to the constant matrix remodeling in response to external damage (Figure 3B).

Two other identified cathepsins belong to the cysteine peptidase family and are involved in evasion of the host immune response by degrading immune effectors, including immunoglobulin, complement components, hemoglobin, and albumin. This has been documented in *Fasciola hepatica*, *Fasciola gigantica*, *Taenia crassiceps, Taenia cruzi*, *Taenia solium*, and *E. multilocularis* cathepsins (Sajid and McKerrow, 2002; Sako et al., 2011). Not surprisingly, we found low abundance of *B. taurus* albumin, immunoglobulin, and hemoglobin in fertile cyst. Looking at *B. taurus* cysteine proteases, we highlight two calpain proteins (GO: 0003824) present only in infertile subset (Figure 3E). Despite their presence in the infertile subset, we did not find any host cysteine protease in fertile cysts, which we believe to be due to a high abundance of proteinase inhibitor I25 cystatin from *E. granulosus*. This inhibitor was already described in *T. cruzi* to specifically inhibit host cysteine proteases like calpain, which are important in host defense (Ljunggren et al., 2007). Based on these data and the vast literature about host–parasite relationships, we illustrate the existence of a counterbalance in host–parasite responses, which has already been demonstrated in some studies using ^{1}H MRS and immunoproteome profiles from *Echinococccus* spp. (Renema et al., 2007; Paredes et al., 2011; Ahn et al., 2015).

We identified 12 protocadherins and one cadherin in fertile cyst and none in infertile one. These proteins have signal peptides and are transmembrane (GO:0016020) and calcium binding (GO:0043167) that were shown to be regulated by proteolysis (Figure 3B,C), modulating cell adhesion through matrix remodeling (Yagi, 2008). This family of

protein was not found in previous HF studies but has already been identified as an exosome constituent (Reinhardt et al., 2013). We also identified 10 collagen proteins that are constituents of the extracellular matrix (GO: 0031012), all of which were found exclusively in fertile cysts in high abundance (Figure 3C).

In counterpart, metalloproteinase-9, a matrix metalloprotease from *B. taurus*, was detected only in infertile subset. It performs multiple roles in the host immune response to *Echinococcus* infection, facilitating the leucocyte recruitment, cytokine, and chemokine processing (Basika et al., 2012). This family of proteases is an important group of zinc-dependent proteolytic enzymes responsible for the degradation of extracellular matrix and basement membranes. These enzymes are secreted in a latent form and become activated in the pericellular environment, with matrix metalloproteinase-9 specifically involved in host defense against bacterial infection (Renckens et al., 2006; Hong et al., 2016). Together, these may indicate that *E. granulosus* has lost the capacity for cell adhesion (GO:0022610) in the infertile cyst due to host protease action (GO: 0003824; GO:0043167), which probably interferes in cell-cell communication and, more importantly, in germinal layer-protoscoleces communication (Figure 3A,E).

We found important proteins in our proteomic analyses that have already been identified in HF, such as 14-3-3 proteins from the parasite and host. Recent studies showed that Eg14-3-3 isoforms from *E. granulosus* are involved in matrix-mediated cell adhesion and signaling events in the host–parasite interface, and they are considered as promising vaccine candidates to prevent cystic echinococcosis (Teichmann et al., 2015). We found three Antigen B subunits that were described to be responsible for fatty acid uptake, protease inhibitor, and immunomodulation (Silva-Álvarez et al., 2015), as well as

lipoprotein antigen 5. These proteins are recognized as the most relevant diagnostic target for hydatidosis, and are present both, in fertile and infertile cysts (Figure 2; Supplementary figure S2A,B,C) (Zhang et al., 2003).

Enolase was identified in higher abundance in fertile cyst than in infertile (Figure 2), and has been described in *F. hepatica*, *Echinostoma caproni*, and *Schistosoma bovis* as having the ability to bind host macromolecules, such as plasminogen (Supplementary figure S2A,B,C) (Bernal et al., 2004; Marcilla et al., 2007), promoting degradation of extracellular matrix components and consequently favoring the pathogen spread in host tissues, as well as preventing clot formation around the parasite (Ramajo-Hernández et al., 2007). In helminths, the fibrinogen-binding ability appears to contribute to parasite establishment in the host. Interestingly we identified fibrinogen α and β from *B. taurus* only in infertile cyst, which had less *E. granulosus* enolase. In contrast, we found a high level of *B. Taurus* enolase in infertile cyst, although absent in fertile one. This protein is considered a key EVs biomarker (Duijvesz et al., 2013).

Among the proteins that could not be functionally annotated by using Blast2GO, we highlight 14 expressed conserved proteins and eight hypothetical proteins, particularly the expressed protein EgrG_001061900.1 found in high abundance in fertile cyst and absent in infertile one. We identified host proteins usually found in HF, like albumin, alpha-2-HS-glycoprotein, apolipoprotein A-I, and hemoglobin α/β, which are considered to be normal constituents of host plasma. Even these proteins should be investigated, considering the possibility of performing specific functions in the host–parasite relationship. For example, the protein alpha-2-HS-glycoprotein (known as fetuin-A) was already described as a constituent of EVs. This protein binds various ions such as calcium, which is abundant in

the LL and HF (Díaz et al., 2015). It has been described in tumor cells as being involved in a mechanism of extracting histones from the nucleus or elsewhere in the cytosol/membrane and loading them on exosomes, which then mediate adhesion by interacting with cell surface heparan sulfate proteoglycans via bound histones (Supplementary figure S2D,E,F). (Watson et al., 2012; Nangami et al., 2014). Not surprisingly, we found histones from *B. taurus* in the HF of both fertile and infertile cysts (Figure 2), with some of them being exclusive to the infertile subset. The origin and evolution of new genes or old genes with new functions can provide new molecular and cellular functions. Understanding these is important for providing insight into their roles in driving phenotypic diversity and in host–parasite relationship (Long et al., 2003; Chen et al., 2013).

The LL is widely regarded to be a crucial element of the host–parasite interplay in the hydatid cyst, including shielding the parasite from direct attack by host immune cells, and it probably down-regulates local inflammation (Díaz et al., 2011). However, neither the LL nor GL are considered as impermeable, allowing the diffusion of macromolecules up to at least 150 kDa (Díaz et al., 2011). The intracellular and membrane proteins identified in the *E. granulosus* HF by both Blast2GO and Phobius analyses have been previously described in the ES products of *Echinococcus* spp., as well as other parasites (Craig et al., 2006; Moreno and Geary, 2008). The presence of these proteins in external fluids has been explained by parasite tissue turnover and death (Monteiro et al., 2010), which we believe to be in response to the host immune attack (GO: 0002376) (Figure 3D).

More recently, it has been suggested that most *E. granulosus* ES proteins are secreted by a novel mechanism; however no alternative mechanism was identified or proposed (Aziz et al., 2011). Previously, host proteins identified in the HF samples of *Echinococcus*

and other helminths were described as being adsorbed and/or specifically bonded by the parasite for nutrient requirements or to actively interfere with host responses. Based on our data and the literature, we hypothesized that many of the intracellular and membrane proteins from *Echinococcus* spp. and *B. taurus* found in HF are actually deliberately secreted through extracellular space to play alternative functions. We also hypothesized that most proteins without signal peptide for exportation, from parasite and host, are in fact secreted through EVs, such as exosomes (Coakley et al., 2015).

The EVs we identified appeared as typical spherical structures released in the extracellular environment and previously described in many parasitic helminths, such as *E. caproni* and *F. hepatica* (Marcilla et al., 2012). However, we still do not know the origin of these vesicles, which may be from *E. granulosus* and/or *B. taurus*. Based in our immunoblotting experiment we can assume that the EVs present in fertile cyst are, at least at some part, from *E. granulosus*. We are conducting studies to identify the components and origins of the EVs from HF. Some studies have shown that EVs play a fundamental role in the host–parasite relationship by acting in molecule transfer (proteins, mRNAs, microRNAs, metabolites) between the host and parasite (Marcilla et al., 2012; Nowacki et al., 2015).

Full understanding of the interaction between *Echinococcus* and its host and the mechanisms mediated by them depends on the identification of the parasite and host proteins that coexist in the HF. Many of these proteins may be exclusively or predominantly expressed by certain isolates and species, which would make them interesting molecular markers for certain biological features. In conclusion, using LC-MS/MS and by comparing fertile and infertile cysts from *E. granulosus*, we showed three

important aspects: (1) clues about the possible existence of an “arms race” involving parasite and host responses; (2) a number of proteins in HF without functional annotation, including secreted conserved and hypothetical proteins from *E. granulosus*, and the possibility that many proteins identified in the HF have possible alternative functions, which warrant investigation; and (3) the presence of EVs vesicles like exosomes in HF fluid from *E. granulosus*. Overall, our results provide a better picture of the HF protein composition and also highlight larger differences that occur in the HF protein profiles between fertile and infertile cysts from *E. granulosus*. We encourage future studies to investigate the proteomic profile of hydatid cyst in different organs, physiological conditions, and species (of both parasite and host).

Competing interests

There are no conflicts of interest.

Authors' contributions

GBS participated in the design of the study, in the experiments, and wrote the manuscript. MEB and EDS participated in the transmission electron microscopy experiments, helped to draft the manuscript. KMM and HBF participated in the design of the study and helped to draft the manuscript. AZ participated in the design of the study and helped to draft the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgments

This work was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–Brazil), FAPERGS (Fundação de Amparo à Pesquisa do Rio Grande do Sul–Brazil), and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico–Brazil). GSB is the recipient of a fellowship from CAPES. MEB and EDS are recipients of CNPq fellowships. We would like to express our thanks to the Brazilian Biosciences National Laboratory (LNBio, Campinas, Brazil under proposal no. MAS-15019) for LC−MS/MS analyses, and to CEME-SUL (Electron Microscopy Center) at Universidade Federal de Rio Grande (FURG - Brazil) for MET analyses.

References

Ahn, C.-S., Han, X., Bae, Y.-A., Ma, X., Kim, J.-T., Cai, H., Yang, H.-J., Kang, I., Wang, H., Kong, Y., 2015. Alteration of immunoproteome profile of *Echinococcus granulosus* hydatid fluid with progression of cystic echinococcosis. Parasit. Vectors. 8, 10. doi:10.1186/s13071-014-0610-7.

Aziz, A., Zhang, W., Li, J., Loukas, A., McManus, D.P., Mulvenna, J., 2011. Proteomic characterisation of *Echinococcus granulosus* hydatid cyst fluid from sheep, cattle and humans. J. Proteomics. 74, 1560–72. doi:10.1016/j.jprot.2011.02.021.

Balbinotti, H., Santos, G.B., Badaraco, J., Arend, A.C., Graichen, D.Â.S., Haag, K.L., Zaha, A., 2012. *Echinococcus ortleppi* (G5) and *Echinococcus granulosus* sensu stricto (G1) loads in cattle from Southern Brazil. Vet. Parasitol. 188, 255–60. doi:10.1016/j.vetpar.2012.04.007.

Basika, T., Muñoz, N., Casaravilla, C., Irigoín, F., Batthyány, C., Bonilla, M., Salinas, G., Pacheco, J.P., Roth, J., Durán, R., Díaz, A., 2012. Phagocyte-specific S100 proteins in the local response to the *Echinococcus granulosus* larva. Parasitology 139, 271–83. doi:10.1017/S003118201100179X.

Bendtsen, J.D., Jensen, L.J., Blom, N., Von Heijne, G., Brunak, S., 2004. Feature-based prediction of non-classical and leaderless protein secretion. Protein Eng. Des. Sel. 17, 349–356. doi:10.1093/protein/gzh037.

Bernal, D., De La Rubia, J.E., Carrasco-Abad, A.M., Toledo, R., Mas-Coma, S., Marcilla, A., 2004. Identification of enolase as a plasminogen-binding protein in excretory-secretory products of *Fasciola hepatica*. FEBS Lett. 563, 203–206.

doi:10.1016/S0014-5793(04)00306-0.

Budke, C.M., Deplazes, P., Torgerson, P.R., 2006. Global socioeconomic impact of cystic echinococcosis. Vet. Parasitol. 12, 296–303.

Bukau, B., Weissman, J., Horwich, A., 2006. Molecular chaperones and protein quality control. Cell. 125, 443–451. doi:10.1016/j.cell.2006.04.014.

Cao, X., Fu, Z., Zhang, M., Han, Y., Han, Q., Lu, K., Li, H., Zhu, C., Hong, Y., Lin, J., 2016. Excretory/secretory proteome of 14-day schistosomula, *Schistosoma japonicum*. J. Proteomics 130, 221–230. doi:10.1016/j.jprot.2015.10.001.

Chemale, G., Van Rossum, A.J., Jefferies, J.R., Barrett, J., Brophy, P.M., Ferreira, H.B., Zaha, A., 2003. Proteomic analysis of the larval stage of the parasite *Echinococcus granulosus*: causative agent of cystic hydatid disease. Proteomics 3, 1633–6. doi:10.1002/pmic.200300487.

Chen, S., Krinsky, B.H., Long, M., 2013. New genes as drivers of phenotypic evolution. Nat. Rev. Genet. 14(9), 645-660. doi:10.1038/nrg3521.

Coakley, G., Maizels, R.M., Buck, A.H., 2015. Exosomes and other extracellular vesicles: the new communicators in parasite infections. Trends Parasitol. 31, 477–489. doi:10.1016/j.pt.2015.06.009.

Craig, H., Wastling, J.M., Knox, D.P., 2006. A preliminary proteomic survey of the in vitro excretory/secretory products of fourth-stage larval and adult *Teladorsagia circumcincta*. Parasitology 132, 535–543. doi:10.1017/S0031182005009510.

Cucher, M.A., Macchiaroli, N., Baldi, G., Camicia, F., Prada, L., Maldonado, L., Avila, H.G., Fox, A., Gutiérrez, A., Negro, P., López, R., Jensen, O., Rosenzvit, M., Kamenetzky, L., 2015. Cystic echinococcosis in South America: systematic review of

species and genotypes of *Echinococcus granulosus* sensu lato in humans and natural domestic hosts. Trop. Med. Int. Heal. 21, 166–175. doi:10.1111/tmi.12647

De Maio, A., 2011. Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: A form of communication during injury, infection, and cell damage: It is never known how far a controversial finding will go! Dedicated to Ferruccio Ritossa. Cell Stress Chaperones 16, 235–249. doi:10.1007/s12192-010-0236-4.

Díaz, A., Casaravilla, C., Allen, J.E., Sim, R.B., Ferreira, A.M., 2011. Understanding the laminated layer of larval *Echinococcus* II: immunology. Trends Parasitol. 27, 264–273. doi:10.1016/j.pt.2011.01.008.

Díaz, Álvaro, Fernández, C., Pittini, Álvaro, Seoane, P.I., Allen, J.E., Casaravilla, C., 2015. The laminated layer: recent advances and insights into *Echinococcus* biology and evolution. Exp. Parasitol. 158, 23–30. doi:10.1016/j.exppara.2015.03.019.

Duijvesz, D., Burnum-Johnson, K.E., Gritsenko, M.A., Hoogland, A.M., Vredenbregt-van Den Berg, M.S., Willemsen, R., Luider, T., Paša-Tolić, L., Jenster, G., 2013. Proteomic profiling of exosomes leads to the identification of novel biomarkers for prostate cancer. PLoS One 8, 1–10. doi:10.1371/journal.pone.0082589.

Frayha, G.J., 1980. Comparative chemical composition of protoscolices and hydatid cyst fluid of (Cestoda). Int. J. Parasitol. 10, 359–364.

Friand, V., David, G., Zimmermann, P., 2015. Syntenin and syndecan in the biogenesis of exosomes. Biol. Cell. 107, 331–341. doi:10.1111/boc.201500010.

Gotz, S., Arnold, R., Sebastián-León, P., Martín-Rodríguez, S., Tischler, P., Jehl, M.A., Dopazo, J., Rattei, T., Conesa, A., 2011. B2G-FAR, a species-centered GO annotation

repository. Bioinformatics 27, 919–924. doi:10.1093/bioinformatics/btr059.

Hong, J., Greenlee, K.J., Pitchumani, R., Lee, S., Song, L., Shan, M., Hee, S., Park, P.W., Dong, C., Werb, Z., Corry, D.B., Kheradmand, F., 2016. Dual protective mechanisms of matrix metalloproteinases 2 and 9 in immune defense against *Streptococcus pneumoniae*. J. immunol. 186(11), 6427-6436. doi:10.4049/jimmunol.1003449.

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Bioinformatics enrichment tools : paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37, 1–13. doi:10.1093/nar/gkn923.

Käll, L., Krogh, A., Sonnhammer, E.L.L., 2007. Advantages of combined transmembrane topology and signal peptide prediction-the Phobius web server. Nucleic Acids Res. 35, 429–432. doi:10.1093/nar/gkm256.

Kim, D., Lee, J., Kim, S.R., Choi, D., Yoon, Y.J., Kim, J.H., Go, G., Nhung, D., Hong, K., Jang, S.C., Kim, S., Park, K., Kim, O.Y., Park, H.T., Seo, J.H., Aikawa, E., Baj-krzyworzeka, M., Balkom, B.W.M. Van, Belting, M., Blanc, L., Bond, V., Bongiovanni, A., Falcon-perez, J.M., Gardiner, C., Giebel, B., 2015. EVpedia : a community web portal for extracellular vesicles research. Bioinformatics 31, 933–939. doi:10.1093/bioinformatics/btu741.

Ljunggren, A., Redzynia, I., Alvarez-Fernandez, M., Abrahamson, M., Mort, J.S., Krupa, J.C., Jaskolski, M., Bujacz, G., 2007. Crystal structure of the parasite protease inhibitor chagasin in complex with a host target cysteine protease. J. Mol. Biol. 371, 137–153. doi:10.1016/j.jmb.2007.05.005.

Long, M., Betran, E., Thornton, K., Wang, W., 2003. The origin of new genes: glimpses from the young and old. Nat. Rev. Genet. 4, 865–875. doi:10.1038/nrg1204.

Lorenzatto, K.R., Monteiro, K.M., Paredes, R., Paludo, G.P., da Fonsêca, M.M., Galanti, N., Zaha, A., Ferreira, H.B., 2012. Fructose-bisphosphate aldolase and enolase from *Echinococcus granulosus* : genes , expression patterns and protein interactions of two potential moonlighting proteins. Gene 506, 76–84. doi:10.1016/j.gene.2012.06.046.

Marcilla, A., Pérez-García, A., Espert, A., Bernal, D., Muñoz-Antolí, C., Esteban, J.G., Toledo, R., 2007. *Echinostoma caproni*: identification of enolase in excretory/secretory products, molecular cloning, and functional expression. Exp. Parasitol. 117, 57–64. doi:10.1016/j.exppara.2007.03.011.

Marcilla, A., Trelis, M., Cortés, A., Sotillo, J., Cantalapiedra, F., Minguez, M.T., Valero, M.L., Sánchez del Pino, M.M., Munoz-Antoli, C., Toledo, R., Bernal, D., 2012. Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. PLoS One 7. doi:10.1371/journal.pone.0045974.

Margaryan, N.V, Kirschmann, D.A., Lipavsky, A., Bailey, C.M., Hendrix, M.J.C., Khalkhali-Ellis, Z., 2010. New insights into cathepsin D in mammary tissue development and remodeling. Cancer Biol. Ther. 10, 457–466. doi:10.4161/cbt.10.5.12534.

Matzinger, P., 2002. The danger model: a renewed sense of self. Science 296, 301–305. doi:10.1126/science.1071059.

Mcmanus, D.P., Zhang, W., Li, J., Bartley, P.B., 2003. Echinococcosis 362, 1295–1304.

Monteiro, K.M., de Carvalho, M.O., Zaha, A., Ferreira, H.B., 2010. Proteomic analysis of the *Echinococcus granulosus* metacestode during infection of its intermediate host. Proteomics 10, 1985–99. doi:10.1002/pmic.200900506.

Moreno, Y., Geary, T.G., 2008. Stage and gender-specific proteomic analysis of *Brugia malayi* excretory-secretory products. PLoS Negl. Trop. Dis. 2, e326. doi:10.1371/journal.pntd.0000326.

Nakao, M., Lavikainen, A., Yanagida, T., Ito, A., 2013. Phylogenetic systematics of the genus *Echinococcus* (Cestoda: Taeniidae). Int. J. Parasitol. 43, 1017–1029. doi:10.1016/j.ijpara.2013.06.002.

Nangami, G., Koumangoye, R., Goodwin, J.S., Sakwe, A.M., Marshall, D., Higginbotham, J., Ochieng, J., 2014. Fetuin-A associates with histones intracellularly and shuttles them to exosomes to promote focal adhesion assembly resulting in rapid adhesion and spreading in breast carcinoma cells. Exp. Cell. Res. 328, 388–400. doi:10.1016/j.yexcr.2014.08.037.

Nene, V., Dunne, D.W., Johnson, K.S., Taylor, D.W., Cordingley, J.S., 1986. Sequence and expression of a major egg antigen from *Schistosoma mansoni*. Homologies to heat shock proteins and alpha-crystallins. Mol. Biochem. Parasitol. 21, 179–188. doi:10.1016/0166-6851(86)90021-6.

Nielsen, H., Engelbrecht, J., Brunak, S., Von Heijne, G., 1997. Identication of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1–6.

Nowacki, F.C., Swain, M.T., Klychnikov, O.I., Niazi, U., Ivens, A., Quintana, J.F., Hensbergen, P.J., Hokke, C.H., Buck, A.H., Hoffmann, K.F., 2015. Protein and small non-coding RNA-enriched extracellular vesicles are released by the pathogenic blood fluke *Schistosoma mansoni*. J. Extracell. Vesicles. 4, 28665. doi: 10.3402/jev.v4.28665.

Otero-Abad, B., Torgerson, P.R., 2013. A systematic review of the epidemiology of echinococcosis in domestic and wild animals. PLoS Negl. Trop. Dis. 7, e2249. doi:10.1371/journal.pntd.0002249.

Oura, J., Tamura, Y., Kamiguchi, K., Kutomi, G., Sahara, H., Torigoe, T., Himi, T., Sato, N., 2011. Extracellular heat shock protein 90 plays a role in translocating chaperoned antigen from endosome to proteasome for generating antigenic peptide to be cross-presented by dendritic cells. Int. Immunol. 23, 223–237. doi:10.1093/intimm/dxq475.

Pan, W., Shen, Y., Han, X., Wang, Y., Liu, H., Jiang, Y., Zhang, Y., Wang, Y., Xu, Y., Cao, J., 2014. Transcriptome profiles of the protoscoleces of *Echinococcus granulosus* reveal that excretory-secretory products are essential to metabolic adaptation. PLoS Negl. Trop. Dis. 8, e3392. doi:10.1371/journal.pntd.0003392.

Paredes, R., Godoy, P., Rodríguez, B., García, M.P., Cabezón, C., Cabrera, G., Jiménez, V., Hellman, U., Sáenz, L., Ferreira, A., Galanti, N., 2011. Bovine (*Bos taurus*) humoral immune response against *Echinococcus granulosus* and hydatid cyst infertility. J. Cell. Biochem. 112, 189–99. doi:10.1002/jcb.22916.

Pavlidis, P., Noble, W.S., 2003. Matrix2png: a utility for visualizing matrix data. Bioinformatics. 19, 295-296.

Ramajo-Hernández, A., Pérez-Sánchez, R., Ramajo-Martín, V., Oleaga, A., 2007. *Schistosoma bovis*: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument. Exp. Parasitol. 115, 83–91. doi:10.1016/j.exppara.2006.07.003.

Reinhardt, T.A., Sacco, R.E., Nonnecke, B.J., Lippolis, J.D., 2013. Bovine milk proteome: quantitative changes in normal milk exosomes, milk fat globule membranes and whey

proteomes resulting from *Staphylococcus aureus* mastitis. J. Proteomics 82, 141–154. doi:10.1016/j.jprot.2013.02.013.

Renckens, R., Roelofs, J.J.T.H., Florquin, S., de Vos, A.F., Lijnen, H.R., van't Veer, C., van der Poll, T., 2006. Matrix metalloproteinase-9 deficiency impairs host defense against abdominal sepsis. J. Immunol. 176, 3735–3741. doi:10.4049/jimmunol.176.6.3735.

Renema, W.K.J., Kan, H.E., Wieringa, B., Heerschap, A., 2007. Metabolic viability assessment of cystic echinococcosis using high-field 1H MRS of cyst contents. NMR Biomed. 20, 448–467. doi:10.1002/nbm.

Sajid, M., McKerrow, J.H., 2002. Cysteine proteases of parasitic organisms. Mol. Biochem. Parasitol. 120, 1–21. doi:10.1016/S0166-6851(02)00043-9.

Sako, Y., Nakaya, K., Ito, A., 2011. *Echinococcus multilocularis*: identification and functional characterization of cathepsin B-like peptidases from metacestode. Exp. Parasitol. 127, 693–701. doi:10.1016/j.exppara.2010.11.005.

Santos, G., Espínola, S., Ferreira, H., Margis, R., Zaha, A., 2013. Rapid detection of *Echinococcus* species by a high-resolution melting (HRM) approach. Parasit. Vectors 6, 327. doi:10.1186/1756-3305-6-327.

Silva-Álvarez, V., Folle, A.M., Ramos, A.L., Zamarreño, F., Costabel, M.D., García-Zepeda, E., Salinas, G., Córsico, B., Ferreira, A.M., 2015. *Echinococcus granulosus* antigen B: a hydrophobic ligand binding protein at the host-parasite interface. Prostaglandins Leukot. Essent. Fat. Acids 93, 17–23. doi:10.1016/j.plefa.2014.09.008.

Sreedhar, A.S., Kalmár, E., Csermely, P., Shen, Y.F., 2004. Hsp90 isoforms: functions, expression and clinical importance. FEBS Lett. 562, 11–15. doi:10.1016/S0014-

5793(04)00229-7.

Stepp, M.A., Pal-Ghosh, S., Tadvalkar, G., Pajoohesh-Ganji, A., 2015. Syndecan-1 and its expanding list of contacts. Adv. wound care 4, 235–249. doi:10.1089/wound.2014.0555.

Takeuchi, T., Suzuki, M., Fujikake, N., Popiel, H.A., Kikuchi, H., Futaki, S., Wada, K., Nagai, Y., 2015. Intercellular chaperone transmission via exosomes contributes to maintenance of protein homeostasis at the organismal level. Proc. Natl. Acad. Sci. U. S. A. 112, e2497–506. doi:10.1073/pnas.1412651112.

Teichmann, A., Vargas, D.M., Monteiro, K.M., Meneghetti, B. V., Dutra, C.S., Paredes, R., Galanti, N., Zaha, A., Ferreira, H.B., 2015. Characterization of 14-3-3 isoforms expressed in the *Echinococcus granulosus* pathogenic larval stage. J. Proteome Res. 14, 1700–1715. doi:10.1021/pr5010136.

Théry, C., Amigorena, S., Raposo, G., Clayton, A., 2006. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr. Protoc. Cell. Biol. Chapter 3, Unit 3.22. doi:10.1002/0471143030.cb0322s30.

Thompson, R.C.A., Lymbery, A.J., 2013. Let's not forget the thinkers. Trends Parasitol. 29, 581–584. doi:10.1016/j.pt.2013.10.006.

Tirloni, L., Reck, J., Terra, R.M.S., Martins, J.R., Mulenga, A., Sherman, N.E., Fox, J.W., Yates, J.R., Termignoni, C., Pinto, A.F.M., Vaz, I.D.S., 2014. Proteomic analysis of cattle tick *Rhipicephalus* (Boophilus) *microplus* saliva: a comparison between partially and fully engorged females. PLoS One 9, e94831. doi:10.1371/journal.pone.0094831.

Trianta, M., Trianta, K., 2004. Heat-shock protein 70 and heat-shock protein 90 associate

with Toll-like receptor 4 in response to bacterial lipopolysaccharide. Biochem. Soc. Trans. 32, 636–639.

Tsai, I.J., Zarowiecki, M., Holroyd, N., Garciarrubio, A., Sanchez-Flores, A., Brooks, K.L., Tracey, A., Bobes, R.J., Fragoso, G., Sciutto, E., Aslett, M., Beasley, H., Bennett, H.M., Cai, J., Camicia, F., Clark, R., Cucher, M., De Silva, N., Day, T. a., Deplazes, P., Estrada, K., Fernández, C., Holland, P.W.H., Hou, J., Hu, S., Huckvale, T., Hung, S.S., Kamenetzky, L., Keane, J. a., Kiss, F., Koziol, U., Lambert, O., Liu, K., Luo, X., Luo, Y., Macchiaroli, N., Nichol, S., Paps, J., Parkinson, J., Pouchkina-Stantcheva, N., Riddiford, N., Rosenzvit, M., Salinas, G., Wasmuth, J.D., Zamanian, M., Zheng, Y., Sánchez-Flores, A., Cevallos, M. a., Morett, E., González, V., Portillo, T., Ochoa-Leyva, A., José, M. V., Landa, A., Jiménez, L., Valdés, V., Carrero, J.C., Larralde, C., Morales-Montor, J., Limón-Lason, J., Soberón, X., Laclette, J.P., Cai, X., Olson, P.D., Brehm, K., Berriman, M., 2013. The genomes of four tapeworm species reveal adaptations to parasitism. Nature 496, 57-63. doi:10.1038/nature12031.

Virginio, V.G., Monteiro, K.M., Drumond, F., de Carvalho, M.O., Vargas, D.M., Zaha, A., Ferreira, H.B., 2012. Excretory/secretory products from in vitro-cultured *Echinococcus granulosus* protoscoleces. Mol. Biochem. Parasitol. 183, 15–22. doi:10.1016/j.molbiopara.2012.01.001.

Wang, S., Wei, W., Cai, X., 2015. Genome-wide analysis of excretory/secretory proteins in *Echinococcus multilocularis*: insights into functional characteristics of the tapeworm secretome. Parasit. Vectors 8, 666. doi:10.1186/s13071-015-1282-7.

Watson, K., Koumangoye, R., Thompson, P., Sakwe, A.M., Patel, T., Pratap, S., Ochieng, J., 2012. Fetuin-A triggers the secretion of a novel set of exosomes in detached tumor

cells that mediate their adhesion and spreading. FEBS Lett. 586, 3458–3463. doi:10.1016/j.febslet.2012.07.071.

Wen, Y.T., Wang, J.S., Tsai, S.H., Chuan, C.N., Wu, J.J., Liao, P.C., 2014. Label-free proteomic analysis of environmental acidification-influenced *Streptococcus pyogenes* secretome reveals a novel acid-induced protein histidine triad protein A (HtpA) involved in necrotizing fasciitis. J. Proteomics 109, 90–103. doi:10.1016/j.jprot.2014.06.026.

Yagi, T., 2008. Clustered protocadherin family. Dev. Growth Differ. 1, 131-140.doi:10.1111/j.1440-169X.2008.00991.x.

Zamanian, M., Kimber, M.J., Mcveigh, P., Carlson, S.A., Maule, A.G., Day, T.A., 2011. The repertoire of G protein-coupled receptors in the human parasite *Schistosoma mansoni* and the model organism *Schmidtea mediterranea*. BMC Genomics 12, 596. doi: 10.1186/1471-2164-12-596.

Zhang, W., Li, J., Mcmanus, D.P., 2003. Concepts in immunology and diagnosis of hydatid disease 16, 18–36. doi:10.1128/CMR.16.1.18.

Zheng, H., Zhang, W., Zhang, L., Zhang, Z., Li, J., Lu, G., Zhu, Y., Wang, Y., Huang, Y., Liu, J., Kang, H., Chen, J., Wang, L., Chen, A., Yu, S., Gao, Z., Jin, L., Gu, W., Wang, Z., Zhao, L., Shi, B., Wen, H., Lin, R., Jones, M.K., Brejova, B., Vinar, T., Zhao, G., McManus, D.P., Chen, Z., Zhou, Y., Wang, S., 2013. The genome of the hydatid tapeworm *Echinococcus granulosus*. Nat. Genet. 45, 1168–75. doi:10.1038/ng.2757.

Figures

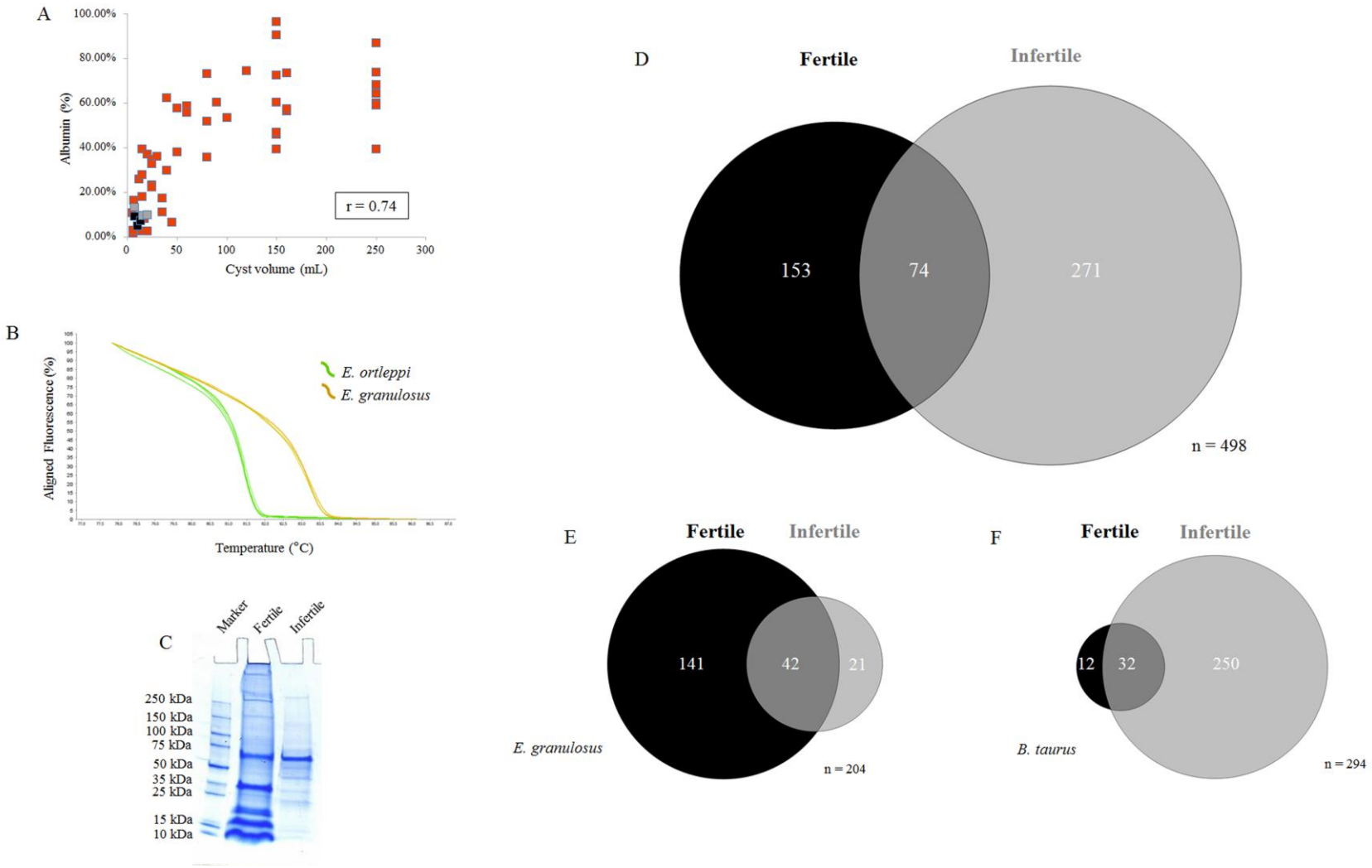

A
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Albumin (%)
r = 0.74
0
50
100
150
200
250
300
Cyst volume (mL)
B
Aligned Fluorescence (%)
E. ortleppi
E. granulosus
Temperature (°C)
C
Marker
Fertile
Infertile
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
35 kDa
25 kDa
15 kDa
10 kDa
D
Fertile
Infertile
153
74
271
n = 498
E
Fertile
Infertile
141
42
21
E. granulosus
n = 204
F
Fertile
Infertile
12
32
250
B. taurus
n = 294

Figure 1. Comparison of parasite and host proteins identified in fertile and infertile cysts. (A) Correlation between cyst volume and intensity of bovine serum albumin SDS-PAGE band. We used IMAGEJ to quantify band intensity and Spearman's rank correlation test to estimate correlation. The three fertile and infertile selected samples are represented by black and gray squares, respectively. (B) HRM of cox1 fragment gene of PSCs and GL from fertile and infertile cysts, respectively. We used *E. ortleppi* samples (green) to distinguish from *E. granulosus* samples (yellow). (C) A total of 50 μg of protein sample was evaluated by 12% SDS-PAGE gel of hydatid fluid from individual pools of three fertile and infertile cysts. In each sample, stained proteins from 10 kDa to 250 kDa could be identified. Marker is indicated on the left. (D) Comparison of protein identifications from fertile and infertile hydatid cyst fluid. In total, 498 proteins were identified: 153 exclusively in fertile cyst, 271 in infertile one, and 74 in both. (E) Venn diagram of the 204 proteins from *E. granulosus* identified in fertile and infertile cysts. (F) Venn diagram of the 294 proteins from *B. taurus* identified in fertile and infertile cysts.


0
1.5e+02
Fertile
Infertile
*** Basement membrane specific heparan sulfate
*** Antigen 5
*** Proteinase inhibitor I25 cystatin
*** Antigen B1
*** Citrate synthase
*** Lysosomal alpha mannosidase
*** Neurogenic locus notch protein
Hemicentin 1
** Efg domain protein
* Phosphoenolpyruvate carboxykinase
** Ornitine aminotransferase
*** Peroxidasin
*** Laminin
* Basement membrane
*** Cathepsin d (lysosomal aspartyl protease)
*** Enolase
* Laminin subunit gamma
* Fructose 1,6 bisphosphate aldolase
*** NADP dependent isocitrate dehydrogenase
** Lysosomal alpha glucosidase
Laminin subunit beta
Citosolic malate dehydrogenase
SPONdin extracellular matrix glycoprotein
** Malate dehydrogenase
** Expressed protein (EgrG_000596300.1)
Calsyntenin
*** EGF region
Thioredoxin peroxidase
Lactate dehydrogenase a
Prosaposin a preproprotein
Cathepsin b
14-3-3 protein
Glucose 6 phosphate isomerase
Heat shock 70 kDa protein
Fibulin
Glyceradehylde 3 phosphate dehydrogenase
** 14-3-3 protein beta/alpha
Heat shock 70 kDa protein
Tubulin beta 2C chain
Adenosylhomocysteinase
Heat shock protein 90 alpha
Tubulin alpha 1C chain
*** Albumin [Bos taurus]
*** Actin, cytoplasmic 2 [Bos taurus]
*** Immunoglobulin lambda-like polypeptide 1 precursor [Bos taurus]
***Alpha-2-HS-glycoprotein precursor [Bos taurus]
*Hemoglobin subunit beta [Bos taurus]
*** Phosphoglycerate mutase 1 [Bos taurus]
*** Vimentin [Bos taurus]
* Hemoglobin subunit alpha [Bos taurus]
*** Heat shock cognate 71 kDa protein [Bos taurus]
14-3-3 protein zeta/delta [Bos taurus]
*** Heat shock protein beta-1 [Bos taurus]
*** Tubulin beta-4B chain [Bos taurus]
Tubulin alpha-1D chain [Bos taurus]
Germinal histone H4-like [Bos taurus]
* TPA: profilin 1-like [Bos taurus]
14-3-3 protein epsilon [Bos taurus]
* 14-3-3 protein gamma [Bos taurus]
* keratin, type I cytoskeletal 14 [Bos taurus]
14-3-3 protein beta/alpha [Bos taurus]
Hypoxanthine-guanine phosphoribosyltransferase [Bos taurus]
14-3-3 protein theta [Bos taurus]
*78 kDa glucose-regulated protein precursor [Bos taurus]
Alpha-1B-glycoprotein precursor [Bos taurus]
Heat shock 70 kDa protein 1A [Bos taurus]
TPA: kininogen 1 isoform I [Bos taurus]
Elongation factor 1-beta [Bos taurus]
Ubiquitin-60S ribosomal protein L40 [Bos taurus]
Histone H2A type 3 [Bos taurus]
Proteasome activator complex subunit 1 [Bos taurus]
14-3-3 protein eta [Bos taurus]
Heat shock 70 kDa protein 1-like [Bos taurus]
Apolipoprotein A-I preproprotein [Bos taurus]

Figure 2. Heat map from shared proteins between fertile and infertile cysts.

Abundance of 74 shared proteins (dark red: high abundance; light red: low abundance). To quantify relative differences in protein abundance, we applied Fisher's exact test using NSAF values to determine statistical differences between shared proteins. The results were plotted in Matrix2png 1.2.1 software to generate the heat map. Statistical significance: *p< 0.05; **p< 0.001; ***p< 0.0001.

E granulosus

B. taurus

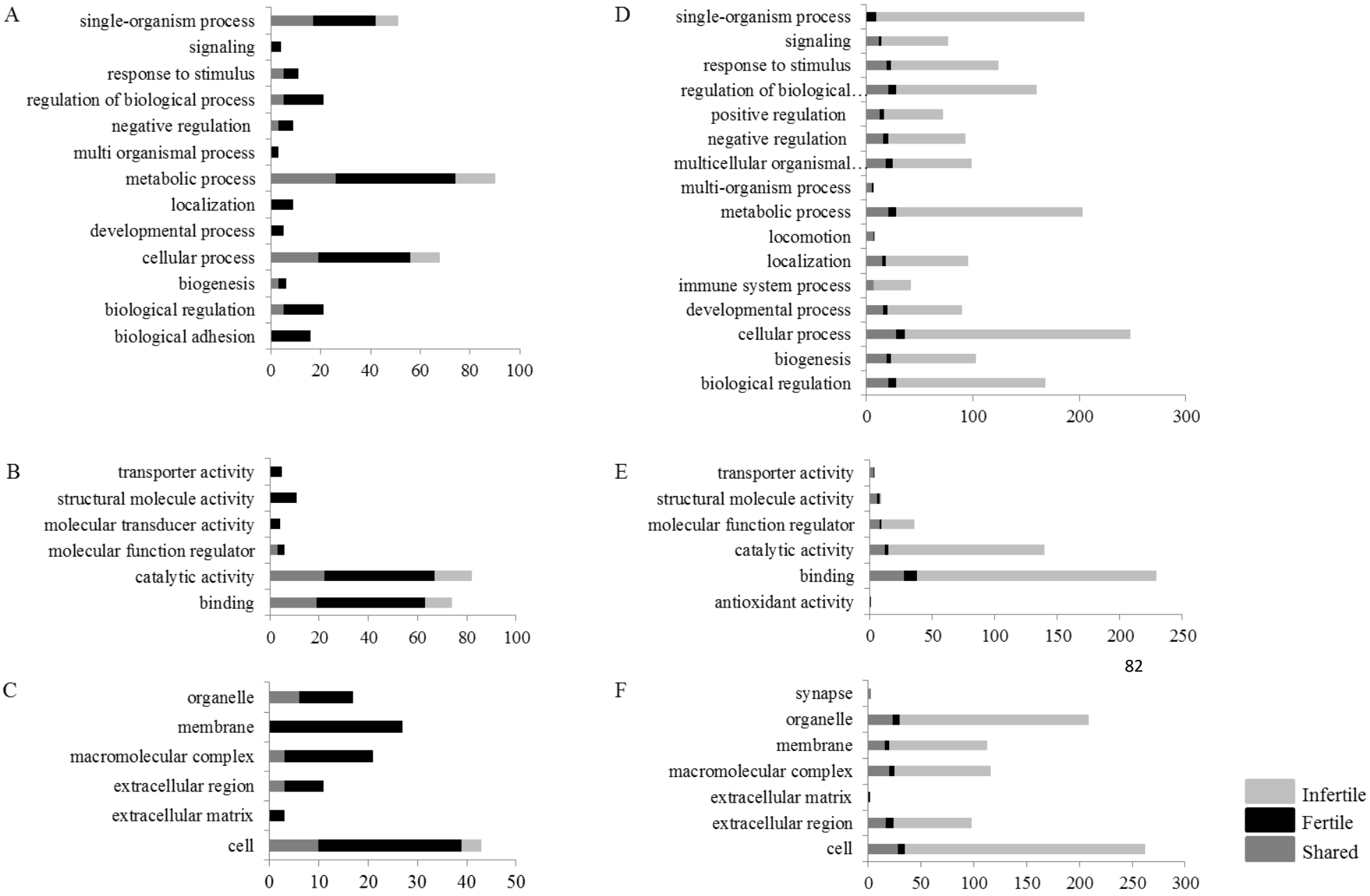


Figure 3. Gene ontology terms associated with *E. granulosus* and *B. taurus* proteins.

The identified proteins in shared, fertile, and infertile subsets were assigned using Blast2GO. Gene ontology terms from the biological process (A, D), molecular function (B, E), and cellular component (C, F) at second level.

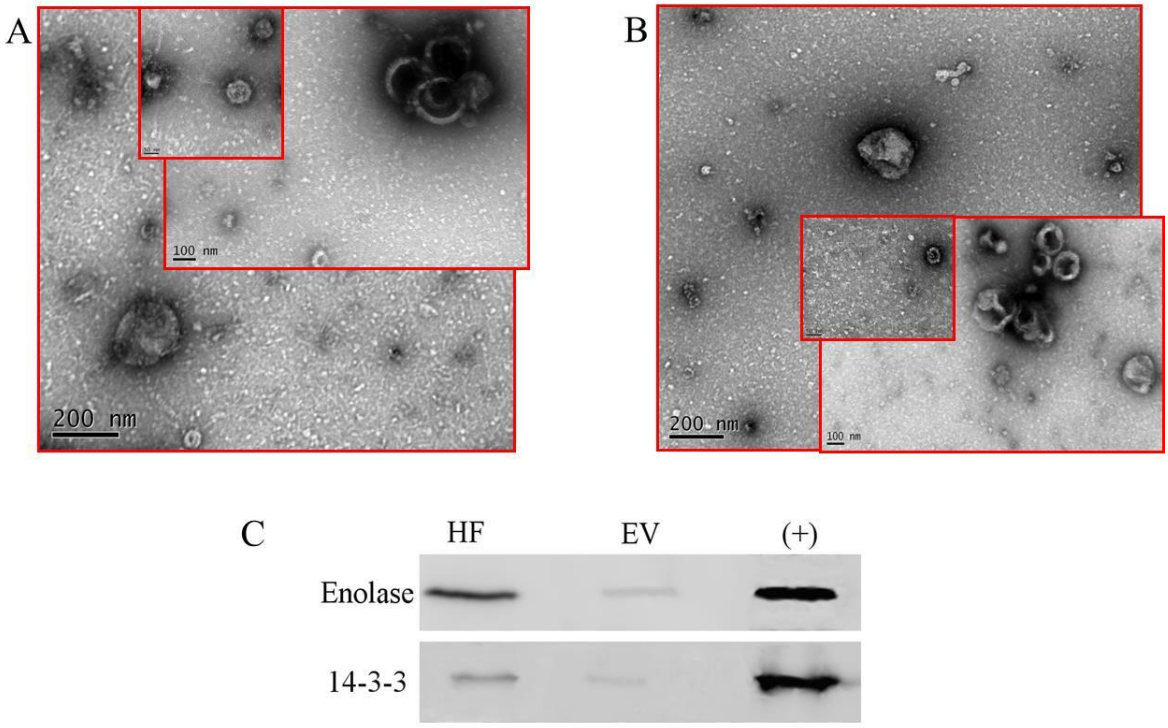


Figure 4. Extracellular vesicles obtained from *E. granulosus* hydatid fluid. EVs (50–200 nm) from *E. granulosus* hydatid fluid in fertile (A) and infertile cysts (B) achieved by ultracentrifugation and displayed as a result of transmission electron microscopy analyses. (C) Immunoblot using rabbit polyclonal anti-rEg14-3-3.1/3 and anti-rEgEno as primary antibodies against three extracts of proteins: HF, total hydatid fluid; EVs, extracellular vesicles obtained by ultracentrifugation; +, positive control-protoscoleces. All extracts were obtained from fertile cyst.

Tables

Table 1. Top hundred proteins exclusively identified in fertile cyst.

Identified proteins	AN	NSAF	SC	EUPC	Co
Hypothetical protein	EgrG_000534900.1	0.433	17	3	64
Expressed conserved protein	EgrG_000412600.1	0.284	8	2	19
Expressed conserved protein	EgrG_000088000.1	0.253	13	3	17
Expressed conserved protein	EgrG_001061900.1	0.222	104	18	35
Expressed protein	EgrG_000920600.1	0.139	32	3	27
Gynecophoral canal protein	EgrG_000824400.1	0.138	80	19	36
Expressed conserved protein	EgrG_000412400.1	0.125	18	4	33
Collagen alpha 2I chain	EgrG_000823800.1	0.122	119	26	33
Vesicular amine transporter	EgrG_000317300.1	0.117	69	12	42
Expressed conserved protein	EgrG_000523100.1	0.116	86	6	12
Collagen alpha 1(V) chain	EgrG_000203400.1	0.11	158	36	31
Protein l isoaspartate	EgrG_001133400.1	0.102	9	4	13
Amiloride sensitive amine oxidase	EgrG_000530400.1	0.099	14	7	8
Fibrillar collagen chain FAp1 alpha	EgrG_001060700.1	0.096	113	27	42
Iron:zinc purple acid phosphatase	EgrG_001169400.1	0.096	44	7	20
40S ribosomal protein S4	gi|78369408|ref|	0.095	8	5	22
Peptidase inhibitor 16	EgrG_000766600.1	0.095	29	4	15
Expressed conserved protein	EgrG_001051900.1	0.09	14	3	20
Expressed protein	EgrG_000550400.1	0.089	7	2	45
Collagen Alpha (V) chain	EgrG_001190600.1	0.069	75	19	27
Dynein light chain	EgrG_000990900.1	0.068	9	4	50
Dynein light chain type 1 2	EgrG_000991300.1	0.064	7	2	25

Syndecan	EgrG_000968100.1	0.06	15	4	17
Beta-casein precursor	gi|30794310|ref	0.06	8	2	12
Collagen alpha 1V chain	EgrG_000144300.1	0.049	81	18	20
Papilin	EgrG_001181950.1	0.048	32	8	17
Cathepsin b	EgrG_000790300.1	0.047	14	7	23
Collagen alpha 1(IV) chain	EgrG_000144350.1	0.046	62	22	24
Estrogen regulated protein EP45	EgrG_000824000.1	0.045	11	4	10
Expressed protein	EgrG_000302900.1	0.044	18	10	22
Gynecophoral canal protein	EgrG_000712600.1	0.041	27	13	19
Fatty acid binding protein FABP2	EgrG_000549850.1	0.039	10	3	14
Immunoglobulin	EgrG_000937300.1	0.039	43	13	18
Lipid transport protein, N terminal	EgrG_000684200.1	0.037	102	43	17
Expressed conserved protein	EgrG_000097200.1	0.035	7	3	17
Collagen alpha 1 (IV) chain	EgrG_000417600.1	0.034	59	19	17
Glypican	EgrG_000241300.1	0.032	16	8	9
Collagen alpha 1(XV) chain	EgrG_000729300.1	0.029	35	12	8
Poly(u) specific endoribonuclease	EgrG_001132700.1	0.028	10	4	25
Collagen type XI alpha 2	EgrG_000524200.1	0.027	46	17	20
Abnormal EMBroygenesis (emb 9)	EgrG_000144400.1	0.025	35	13	10
Type II collagen B	EgrG_001060600.1	0.025	35	21	22
Mastin	EgrG_000085400.1	0.025	14	5	12
Sj ts4 protein	EgrG_000393000.1	0.025	10	6	20
Secreted frizzled protein 5	EgrG_001023000.1	0.025	9	4	15
Protocadherin gamma a8	EgrG_000122500.1	0.025	25	11	13
Fgfr protein	EgrG_000842900.1	0.025	25	9	17
Major egg antigen	EgrG_000236500.1	0.023	6	4	16
Tyrosine protein kinase otk	EgrG_000212300.1	0.022	29	9	13

Thioredoxin fold	EgrG_000359800.1	0.022	15	3	11
Lysyl oxidase	EgrG_000217900.1	0.022	11	6	16
Neuroendocrine protein 7b2	EgrG_000119200.1	0.021	8	3	9
Expressed conserved protein	EgrG_000471400.1	0.019	8	3	10
Beta D xylosidase 2	EgrG_000879900.1	0.018	19	10	14
Lysosomal protective protein	EgrG_000170200.1	0.018	8	4	8
Neurexin 1 alpha	EgrG_000236200.1	0.017	17	7	10
Cadherin	EgrG_000117200.1	0.017	18	8	12
Alpha N acetylgalactosaminidase	EgrG_000340500.1	0.016	7	4	12
Glycogen phosphorylase	EgrG_000501500.1	0.015	20	9	14
NADP dependent malic enzyme	EgrG_001145700.1	0.015	17	5	11
Fras1 related extracellular matrix	EgrG_000176400.1	0.014	39	15	8
SLIT and NTRK protein 5	EgrG_000485000.1	0.013	6	5	16
Neuroendocrine convertase 2	EgrG_000412100.1	0.013	11	6	11
Tolloid protein 1	EgrG_000640700.1	0.012	25	9	8
Hypothetical protein	EgrG_000236300.1	0.012	14	4	7
Ndr	EgrG_001065500.1	0.012	6	4	15
Hypothetical protein	EgrG_000264700.1	0.012	12	6	8
Actin modulator protein	EgrG_000882500.1	0.012	6	2	6
Cathepsin I cysteine peptidase	EgrG_000989200.1	0.012	7	2	10
UDP glucose 4 epimerase	EgrG_000984800.1	0.011	6	3	12
Protocadherin 1	EgrG_000945000.1	0.011	18	7	7
Agrin	EgrG_000061400.1	0.011	12	7	6
Gamma glutamyltranspeptidase 1	EgrG_000806300.1	0.011	8	4	8
Discoidin containing receptor 2	EgrG_000549500.1	0.011	12	5	6
Beta hexosaminidase subunit alpha	EgrG_000901900.1	0.011	7	2	4
Roundabout 2	EgrG_000992700.1	0.01	13	7	7

Cell adhesion molecule	EgrG_000644850.1	0.01	18	9	12
Immunoglobulin subtype	EgrG_000570100.1	0.01	7	2	6
Fucosidase alpha L 1 tissue	EgrG_000618900.1	0.01	7	2	6
AdamTS protein 3	EgrG_000969100.1	0.009	11	4	8
Protocadherin 11	EgrG_000846700.1	0.009	7	6	6
Apple	EgrG_000601800.1	0.009	7	2	3
Mannosyl oligosaccharide 12 alpha	EgrG_000605100.1	0.008	7	4	10
Protocadherin 1	EgrG_000389000.1	0.008	14	8	9
Protocadherin 1	EgrG_000570400.1	0.008	18	8	9
Laminin	EgrG_000068100.1	0.008	17	11	16
Desert hedgehog protein	EgrG_001200300.1	0.008	7	2	6
Notch	EgrG_000373600.1	0.007	13	4	3
Pfam-B and DUF4381	EgrG_000591200.1	0.007	7	3	9
Protocadherin 9	EgrG_001045100.1	0.007	15	3	4
Cell adhesion molecule	EgrG_000363400.1	0.007	13	6	7
Disintegrin and metalloproteinase	EgrG_001069300.1	0.007	7	6	7
Discoidin containing receptor 2	EgrG_000769500.1	0.006	10	4	5
Protein disulfide isomerase A3	EgrG_001022300.1	0.006	7	2	3
Hypothetical protein	EgrG_001058700.1	0.006	8	5	10
Nephrin	EgrG_000239500.1	0.006	10	6	6
Protocadherin 11	EgrG_000583700.1	0.005	8	5	7
Receptor type tyrosine phosphatase	EgrG_001133800.1	0.004	16	8	5
GPI anchored surface glycoprotein	EgrG_000606700.1	0.004	8	5	4
Tissue type plasminogen activator	EgrG_000096600.1	0.003	9	4	3

AN, accession number; NSAF, normalized spectral abundance factor; SC, spectral account; EUPC, exclusive unique peptide count; Co, coverage.

Table 2. Top hundred proteins exclusively identified in infertile cyst.

Identified proteins	AN	NSAF	SC	EUPC	Co
Antigen B2	EgrG_000381100.1	0.4714	47	3	37
Antigen B4	EgrG_000381400.1	0.2204	19	6	50
Fructose-1,6-bisphosphatase 1	gi|77735849	0.2179	81	5	58
Galectin-1	gi|28461189	0.1897	28	4	32
Phosphoglycerate kinase 1	gi|77735551	0.1638	76	18	52
Thioredoxin	gi|27806783	0.1592	24	7	51
Fructose-bisphosphate aldolase A	gi|156120479	0.1376	61	15	43
SH3 domain-binding glutamic acid 3	gi|77736471	0.1361	13	5	47
Rho GDP-dissociation inhibitor 1	gi|28603774	0.131	29	6	33
Alpha-enolase	gi|87196501	0.1292	79	18	50
Triosephosphate isomerase	gi|61888856	0.1292	31	10	61
Creatine kinase B-type	gi|62751863	0.1183	66	13	50
Histone H1.2	gi|134085671	0.117	19	4	15
Plastin-2	gi|77736385	0.1154	91	25	52
Elongation factor 1-alpha 2	gi|82697357	0.1141	39	6	12
Glucose-6-phosphate isomerase	gi|94966765	0.1102	81	13	31
GAPDH	gi|77404273	0.109	39	15	48
Alcohol dehydrogenase [NADP(+)]	gi|115495641	0.0994	42	10	28
Macrophage-capping protein	gi|30466254	0.0985	40	8	25
Myosin light polypeptide 6	gi|28461187	0.0946	19	8	56
Tryptophan--tRNA ligase,	gi|27806353	0.0928	45	12	32
Pyruvate kinase isozymes M1/M2	gi|329664500	0.0886	59	17	38
ADP-ribosylation factor 1	gi|28603778	0.0866	21	6	50
L-lactate dehydrogenase B chain	gi|27806561	0.0827	31	10	32

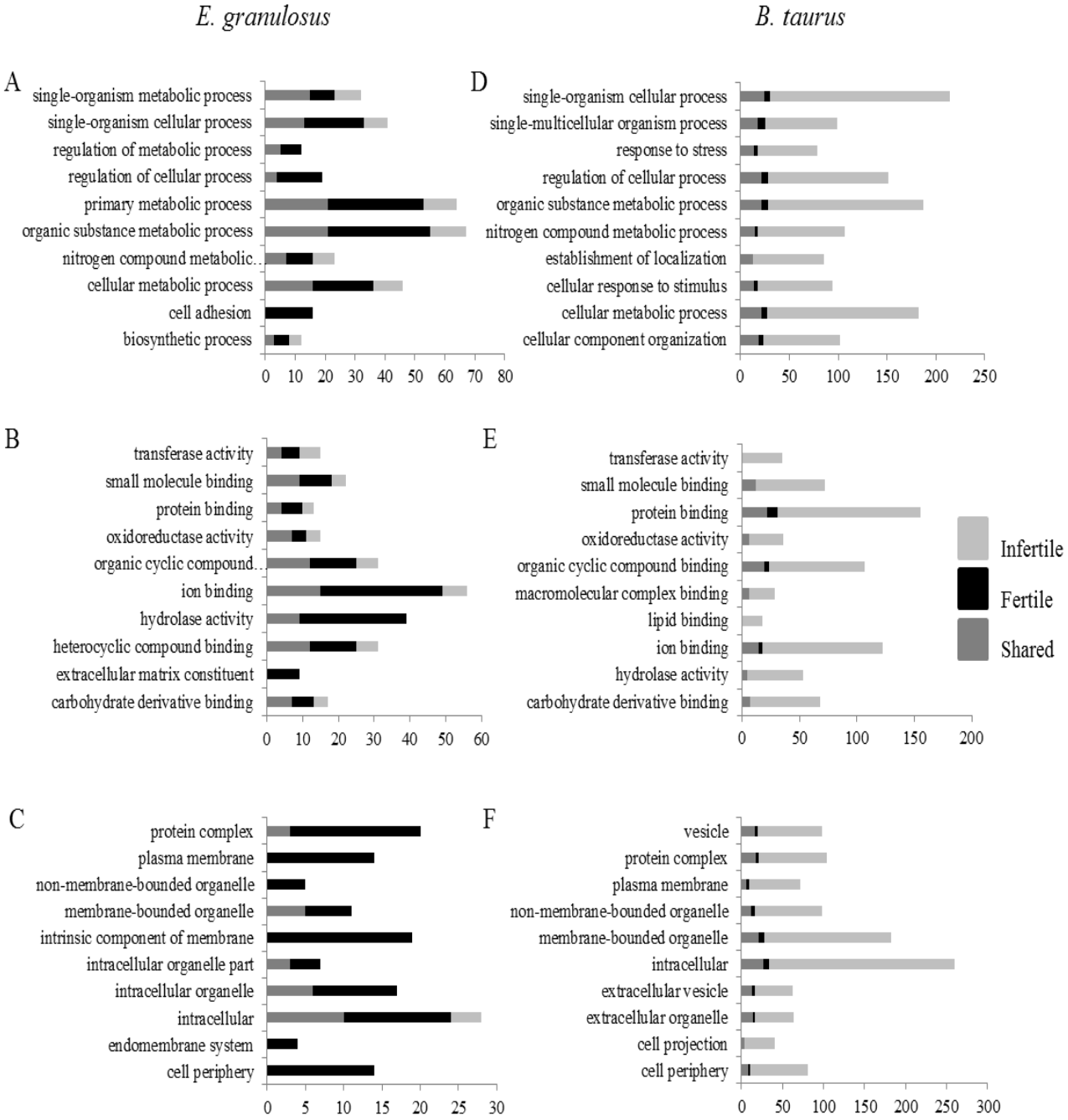
Peroxiredoxin-5	gi|27807445	0.0822	24	8	34
Isoaspartyl peptidase/L-asparaginase	gi|116004289	0.0803	31	10	44
Fatty acid-binding protein,	gi|28461261	0.0786	17	4	40
Glutamate dehydrogenase	EgrG_000589100.1	0.076	45	15	35
Phosphatidylethanolamine-binding 1	gi|75812940	0.0747	18	5	36
Rho GDP-dissociation inhibitor 2	gi|28461215	0.0719	16	5	35
Alpha-1-acid glycoprotein precursor	gi|94966811	0.0677	15	7	30
Peroxiredoxin-1	gi|27806081	0.0635	14	7	39
Purine nucleoside phosphorylase	gi|56119114	0.0622	21	8	34
Transgelin-2	gi|61888874	0.062	17	9	47
glutamyltransferase 2 [Bos taurus]	gi|29135313	0.0596	52	15	29
Purine nucleoside phosphorylase	EgrG_002001800.1	0.059	15	4	20
Brain acid soluble protein 1	gi|27807507	0.0578	16	8	63
Heat shock protein HSP 90-alpha	gi|60592792	0.056	49	20	28
Adenylyl cyclase-associated 1	gi|78042488	0.0534	35	7	24
Transgelin	gi|114051586	0.0487	14	7	40
Adenosylhomocysteinase	gi|77735583	0.0472	26	11	29
Cofilin-1	gi|62751777	0.0461	12	5	40
Dihydropyrimidinase-related 2	gi|115496400	0.045	38	9	21
Carbonic anhydrase 3	gi|77735829	0.0444	17	4	22
Tropomyosin alpha-4 chain	gi|155372051	0.044	16	6	13
TPA: ceruloplasmin (ferroxidase)	gi|296491101	0.0427	54	17	21
Protein DJ-1	gi|62751849	0.0427	13	6	43
Isocitrate dehydrogenase [NADP]	gi|75832090	0.0426	23	10	29
Vasodilator-stimulated	gi|84000193	0.0424	19	8	32
Glutathione S-transferase P	gi|29135329	0.0408	13	3	25
Alpha-1-antiproteinase precursor	gi|27806941	0.0382	20	11	25

Peroxiredoxin-6	gi|27807167	0.0381	13	5	28
Cytosol aminopeptidase	gi|165905571	0.0373	25	14	33
Pigment epithelium-derived factor	gi|27806487	0.0362	21	8	22
Ras-related protein Rab-5C	gi|77736431	0.0357	12	5	24
Transaldolase	gi|164420731	0.0349	17	4	12
Synaptic vesicle membrane VAT-1	gi|300795742	0.0348	22	9	31
Aconitate hydratase mitochondrial	EgrG_000158240.1	0.0342	33	14	24
Annexin A1	gi|73853762	0.0335	15	7	29
Serpin A3-3	gi|84000377	0.0326	18	6	18
Protein FAM49B	gi|114051381	0.0325	16	6	25
Uridine phosphorylase 1	gi|149773558	0.0319	13	4	20
Ezrin	gi|27806351	0.0318	24	11	22
Rab GDP inhibitor alpha	gi|27806617	0.0316	22	11	32
Serpin A3-7	gi|75832097	0.0315	18	6	18
Prostaglandin reductase 1	gi|78369426	0.0296	13	4	14
Transitional endoplasmic reticulum	gi|77735541	0.028	33	14	25
WD repeat-containing protein 1	gi|114052350	0.028	20	8	23
Fibrinogen beta chain	gi|218931172	0.0279	13	8	23
Adseverin	gi|27806415	0.0277	24	10	20
Threonine--tRNA ligase,	gi|77735697	0.0276	30	12	20
F-actin-capping subunit alpha-1	gi|134085807	0.0275	13	4	20
6-phosphogluconate dehydrogenase,	gi|219522066	0.0273	14	9	22
Pyridoxal kinase	gi|115497140	0.0248	12	5	22
Chain B, Arp2/3 Complex	gi|449802088	0.024	12	6	20
Hsc70-interacting protein	gi|156120501	0.0233	13	6	20
Galactokinase	gi|150247075	0.0227	15	6	20
Transketolase	gi|51491841	0.0218	19	11	20

Serine--tRNA ligase, cytoplasmic	gi|27806419	0.0216	15	9	22
Cytosolic non-specific dipeptidase	gi|78042564	0.0216	13	6	20
UTP-phosphate uridylyltransferase	gi|41386780	0.0215	16	7	16
Major vault protein	gi|78369428	0.0205	26	12	17
Alpha-actinin-4	gi|148238040	0.0202	27	12	17
Succinyl coenzyme A ligase	EgrG_000519200.1	0.0202	14	7	19
Glycine--tRNA ligase	gi|147902079	0.0189	18	9	19
Fermitin family homolog 3	gi|83035071	0.0187	19	10	18
V-type ATPase catalytic subunit A	gi|27806225	0.0187	15	7	19
Glycogen phosphorylase	gi|115498012	0.0186	23	14	21
ATP synthase subunit beta	gi|28461221	0.0183	13	7	18
D3-phosphoglyceratedehydrogenase	gi|78042498	0.0174	12	6	18
Heat shock protein HSP 90-beta	gi|118601868	0.0172	22	8	20
Fructose-bisphosphate aldolase C	gi|148238309	0.0144	16	3	16
Matrix metalloproteinase-9	gi|27807437	0.0138	13	9	15
Coagulation factor XIII A chain	gi|268607679	0.0136	17	7	12
6-phosphofructokinase	gi|122692503	0.0131	16	4	8
Rab GDP dissociation inhibitor beta	gi|76253900	0.0123	14	4	21
Ubiquitin-like modifier-activating 1	gi|156523068	0.0104	20	10	13
Microtubule-associated protein 4	gi|27806553	0.0096	18	9	12
Lactate dehydrogenase protein	EgrG_000661000.1	0.0069	13	2	31
Clathrin heavy chain 1	gi|27806689	0.0064	13	7	5
Dihydropyrimidinase-related 3	gi|155371867	0.006	18	3	9
Alpha-actinin-1	gi|78369242	0.0048	14	4	13
Fibronectin precursor	gi|255003702	0.0047	16	9	6

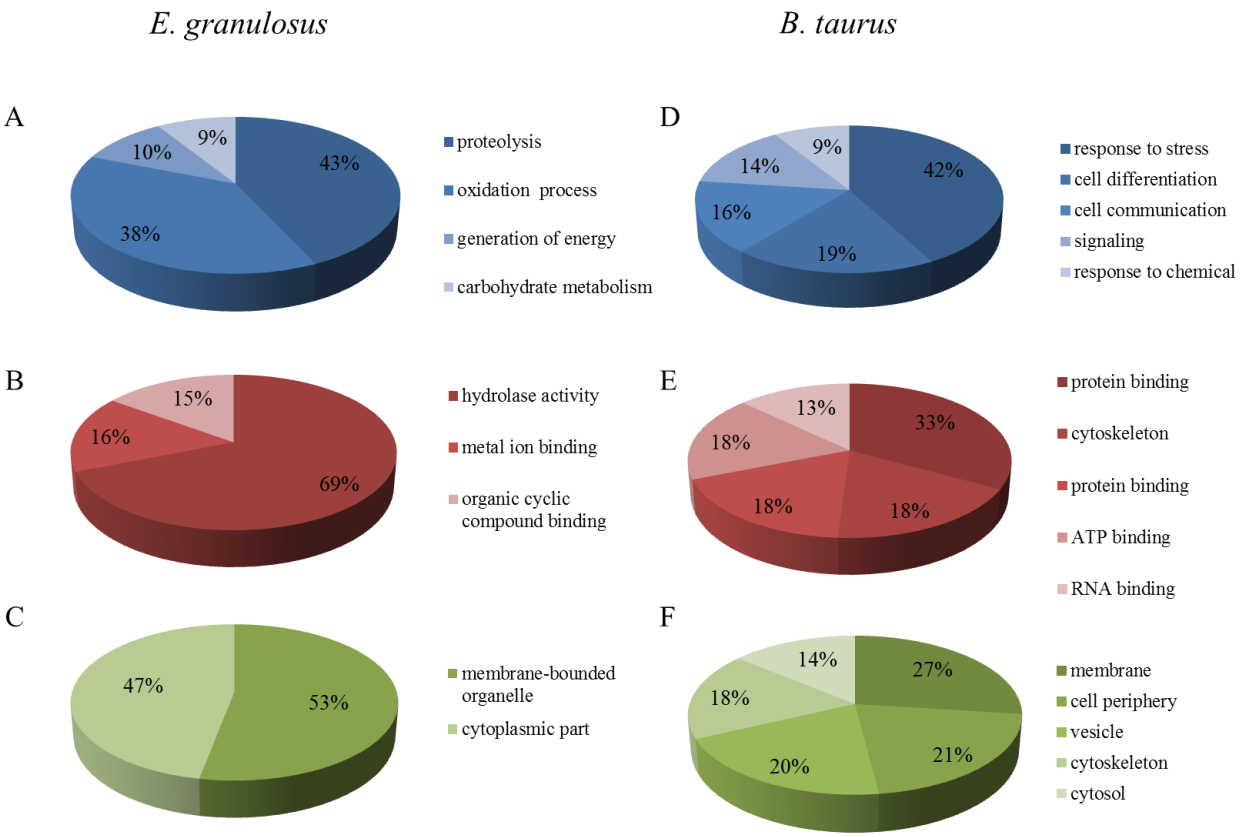
AN, accession number; NSAF, normalized spectral abundance factor; SC, spectral account; EUPC, exclusive unique peptide count; Co, coverage.

Supplementary figures

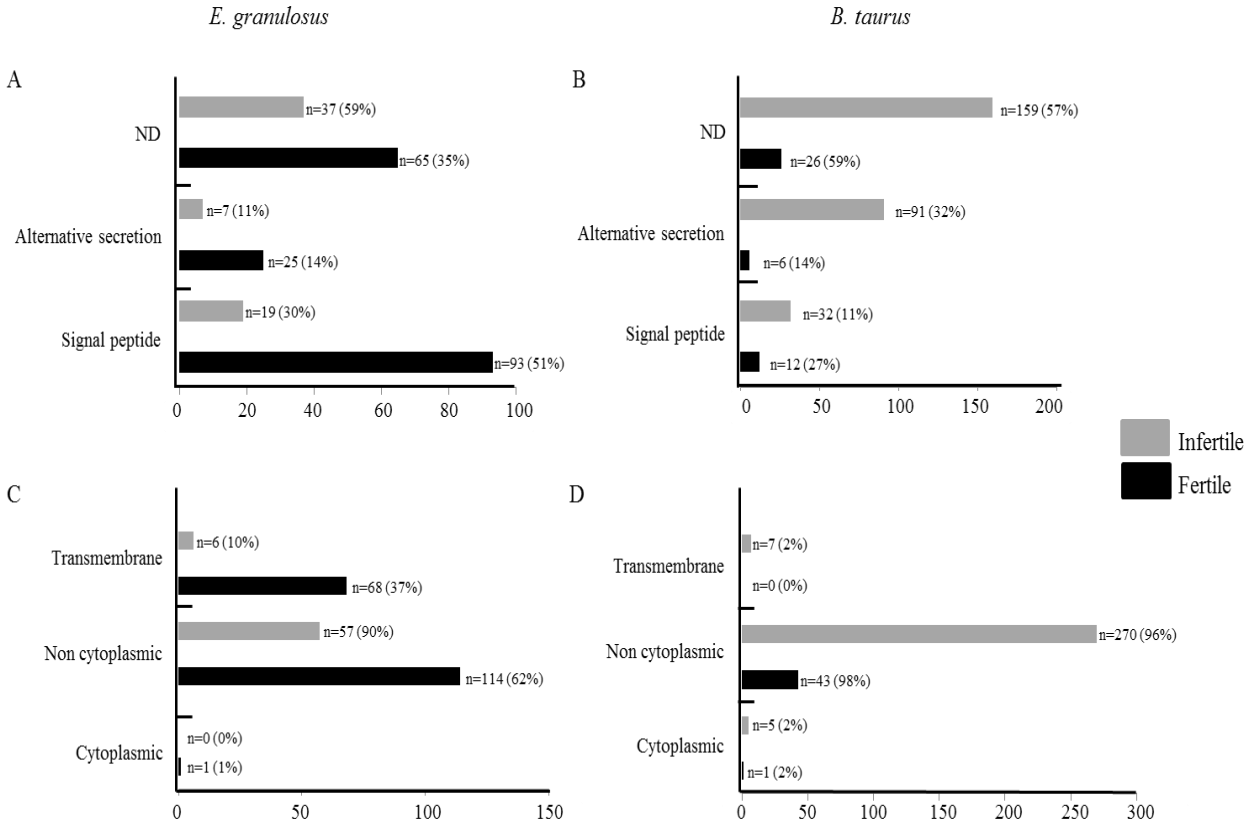


Supplementary figure 1. Gene ontology terms associated with *E. granulosus* and *B. taurus* proteins assigned using Blast2GO. Top ten gene ontology terms from the

Supplementary figure S1. Gene ontology terms associated with *E. granulosus* and *B. taurus* proteins assigned using Blast2GO. Top ten gene ontology terms from the biological process (A, D), molecular function (B, E), and cellular component (C, F) at third level.



Supplementary figure S2. Gene ontology terms associated with upregulated *E. granulosus* and *B. taurus* proteins identified in shared subset. Top five gene ontology terms, assigned using Blast2GO, from the biological process (A, D), molecular function (B, E), and cellular component (C, F).



Supplementary figure S3. Parasite and host protein associated with their secretion pathway. Proteins from *E. granulosus* (A) and *B. taurus* (B) secreted by signal peptide and alternative secretion assigned using SignalP and SecretomeP, respectively. Sublocalization of proteins from *E. granulosus* (C) and *B. taurus* (D) assigned using Phobius. ND, non-determined. The proteins used in these analyses were those identified in fertile and infertile cysts.

Capítulo III

Manuscrito a ser submetido à publicação na revista *Proteomics* como “brief report”

The secreted protein signature of hydatid fluid from pulmonary hydatidosis

Santos, G.B., Kitano, E., Battistella, M. E., Monteiro, K. M., Ferreira, H. B., Serrano, S. M. T., Zaha, A.

“Brief report”

The secreted protein signature of hydatid fluid from pulmonary hydatidosis

Guilherme B. dos Santos[1], Eduardo Kitano[2], Maria Eduarda Battistella[3], Karina M. Monteiro[1,3], Henrique B. Ferreira[1,3], Solange Maria de Toledo Serrano[2], Arnaldo Zaha[1,3g] g d h*

[1] Programa de Pós-Graduação em Biologia Celular e Molecular, Universidade Federal do Rio Grande do Sul, Caixa Postal 15005, CEP 91501-970, Porto Alegre, RS, Brazil

[2] Laboratório Especial de Toxinologia Aplicada, Center of Toxins, Immune -Response and Cell Signaling (CeTICS), Instituto Butantan, São Paulo, Brazil

[3] Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, CEP 91501-970, Porto Alegre, RS, Brazil

* **Corresponding author**

E-mail address: zaha@cbiot.ufrgs.br

Abstract

The species *Echinococcus granulosus* (G1) and *Echinococcus ortleppi* (G5) are both responsible for the vast majority of hydatid disease cases in southern Brazil. The infection occurs predominantly in the liver and lungs of humans and cattle. Comparative proteomic studies of helminths, including analyses of different species infecting a common host, have increased the knowledge about the molecular survival strategies adopted by parasites. The aim of this work was identify the proteins shared between *E. granulosus* and *E. ortleppi*, in an attempt to elucidate mechanisms involved in the survival of these parasites. The hydatid fluid samples from six isolates were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). We identified a total of 842 proteins by proteomic analysis, and the *in silico* analysis of these data allowed us to identify a core of 162 proteins present in at least five of the six samples, besides some degree of infection specialization from *E. ortleppi* in lung bovine cysts.

Keywords: *Echinococcus granulosus*, *Echinococcus ortleppi*, proteomics, protein core, species-specific.

Abbreviations: ES, excretion/secretion; GO, gene ontology; HF, hydatid fluid; HRM, high-resolution melting; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PSC, protoscolex; SCX, strong cation exchange;

Echinococcus granulosus and *E. ortleppi* are parasitic flatworms with heteroxenous life cycle go through distinct metamorphic events during their development. These events are mostly evident in the transitional steps from the larval stage to the adult worm [1]. Humans and ungulates animals - intermediate hosts - are affected by the formation of hydatid cyst, the larval stage of the parasite, predominantly in liver and lungs. This cyst is externally formed by a laminar layer and internally by a germinal layer that gives rise to protoscoleces (PSC) by asexual reproduction. When PSCs are ingested by a definitive host, such as wolves and domestic dogs, the PSCs matures into adult worms within the gut [2]. Ingestion of parasite eggs, which release oncospheres, by intermediate hosts allows the development of metacestode (hydatid cyst) in their viscera. Hydatid cyst is able to establish a chronic infection and can survive for decades in the host, being able to remain fertile, in most cases, with full capacity to generate PSCs [3]. To achieve this goal, the parasites adopt a wide repertoire of molecular strategies to both evade host defense mechanisms and capture nutrients necessary for their development. Thus, the hydatid cysts are interesting to investigate the molecular mechanisms involved in host-parasite relationship, which allow the cyst survive and develop despite the chronic exposure to hostile environment provided by the host responses against infection [4].

The host-parasite relationship in cystic hydatid disease is interactive and the outcome of the infection is the result of the balance between the defense mechanisms of the host and the parasite survival strategies. The hydatid fluid (HF), liquid that fills the hydatid cyst, contains the metacestode excretory-secretory products and host proteins, making it a rich component for analysis of relevant molecules involved in host-parasite interactions [5, 6]. Molecular characterization of HF is essential for a better understanding

of the infection caused by *Echinococcus* spp., as well as for the discovery of new molecules with potential use in hydatid disease diagnosis and treatment. Proteomic studies of helminth products of excretion/secretion (ES), have been particularly valuable in the identification of proteins involved in host-parasite relationship [7, 8]. Comparative ES proteomic studies of helminths, including analysis of the same species infecting different hosts, and different genotypes/species/strains infecting a common host, have increased the knowledge about molecular survival strategies adopted by parasites [6, 9]. Furthermore, the discovery of proteins shared by distinct species can allow the identification of conserved mechanisms involved in host-parasite interaction. On the other hand, a species-specific set of proteins can be used as molecular markers for parasite diagnosis. The aim of this work was identify the ES proteins shared between different *Echinococcus* species, in fertile healthy lung cysts, in an attempt to describe proteins involved in conserved mechanisms of parasite survival.

E. granulosus and *E. ortleppi* cysts were obtained from slaughtered cattle at a commercial abattoir in the metropolitan region of Porto Alegre, RS (Brazil). Lung viscera were dissected and HF was aseptically aspirated from cysts and centrifuged at 10.000 g for 15 min at 4°C to sediment PSCs and debris [5]. PSCs were collected and used for species identification, which was done by high-resolution melting (HRM) [10]. Protein concentration of each *E. granulosus* HF sample was determined using Qubit™ and was qualitatively evaluated by 12% SDS-PAGE [11]. We selected three individual *E. granulosus* and three individual *E. ortleppi* HF samples to proceed with further proteomic analysis. Proteins were digested by trypsin and fractionated by strong cation exchange (SCX) [11]. The salt gradient was as follows: Fraction A (phosphate buffer + 75 mM of

KCl), fraction B (phosphate buffer + 125 mM of KCl), fraction C (phosphate buffer + 200 mM of KCl), fraction D (phosphate buffer + 300 mM of KCl) and fraction E fraction (phosphate buffer + 400 mM of KCl). Each fraction was lyophilized using a vacuum lyophilizer and stored at -80°C until Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Each biological SCX fractioned sample was analyzed, totalizing 30 LC−MS/MS runs (Supplementary figure 1).

All samples were analyzed on an LTQ-Orbitrap Velos (Thermo Fisher Scientific, Bremen, GA) connected to an EasynLCII (Thermo Fisher Scientific). Raw files were processed using the software MSconvert (http://proteowizard.sourceforge.net) to generate .mgf files. For protein identification LC-MS/MS data were searched using a local *E. granulosus* and *E. multilocularis* database containing deduced amino acid sequences (21.225) from genome annotation. Search was also carried out using the non-redundant database from NCBI (National Center for Biotechnology Information) restricted to the Mammalia taxonomy (1,471,442 protein sequences, download date: 5/27/13). For the LTQ Orbitrap Velos mass spectrometer, the search parameters consisted of carbamidomethylation as a fixed modification, oxidation of methionine as a variable modification, two trypsin missed cleavage and a tolerance of 10 ppm for precursor and 0.5 Da for fragment ions. Ion type was set as monoisotopic, and peptide charges 2+, 3+ and 4+ were taken into account. Mascot Daemon v. 2.2.2 (Matrix Science) was used to peptide and protein identifications. The identified proteins from three *E. granulosus* (A1, B1 and C3) and three *E. ortleppi* (A5, B5 and C5) cysts were submitted to further *in silico* analyses. The identified parasite proteins were searched for the presence of signal peptide using SignalP 4.1, and presence of non-classical signal for exportation using SecretomeP

2.0. A protein was considered to contain a signal peptide if the D-score was significant and to be non-classically secreted if the NN score was higher than 0.6, but not at the same time be predicted to contain a signal peptide. To determine protein topology, we used Phobius. Gene ontology (GO) terms were searched to each protein using Blast2GO with the default parameters and following the GO second and third level hierarchy.

In an attempt to shed some light on HF protein components and to verify possible differences in the protein repertoires from *E. granulosus* and *E. ortleppi* fertile cysts with low albumin content, we performed a proteomic survey (Supplementary figures 1A, B). We were able to identify 285, 321 and 344 proteins in A1, B1 and C1 samples, respectively, amounting 549 *E. granulosus* proteins (Supplementary figure 1C). We identified from *E. ortleppi* samples a total of 703 proteins, which 236, 472 and 536 from A5, B5 and C5, respectively (Supplementary figure 1D). A total of 842 distinct *Echinococcus* spp. proteins were identified (Figure 1A; Supplementary Table 1). This represents a larger number of protein identifications compared to previous studies focused on identifying the HF content of *Echinococcus* spp. [5, 6, 11].

In order to highlight the most frequent proteins in HF, we selected those present in at least two samples from the same species, amounting 415 proteins, which 43 and 142 of them present exclusively in *E. granulosus* and *E. ortleppi* samples, respectively (Figure 1B). Furthermore, we selected the 162 proteins present in at least five of the six samples of *Echinococcus* spp. (Supplementary Table 2) and classified them as core proteins (Figure 1B). This protein core consists of 98 proteins present in all six samples, 26 found in three *E. granulosus* and two *E. ortleppi* samples, and 38 identified in three *E. ortleppi* and two *E. granulosus* samples (Figure 1C).

Among the 162 identified proteins, 81 (50%) had a signal peptide (66 non-cytoplasmic and 15 transmembrane). We highlight the presence of two cathepsin D and basement membrane specific heparan sulfate, three protocadherin and one cadherin. This may indicate that the cysts are able to modulate cell adhesion through matrix remodeling (Supplementary figures 2, 3), improving the molecular basis for generating individual cellular diversity [11]. We found four antigen B subunits, responsible for parasite fatty acids uptake, protease inhibitor, and immunomodulation. [12]. As previously described in the literature, AgB8/1 was the most abundant subunit [13].

Among the protein core, 15 (9%) were found to be secreted via the non-classical pathway (14 non-cytoplasmic or transmembrane) (Figure 2). We identified seven expressed conserved proteins and hypothetical proteins with alternative secretion signals. While their functions are not yet known, we hypothesize that they may be relevant to parasite survival and should be potential subjects for future studies. We highlight here galactosyltransferase protein which is required for the synthesis of O-glycans [14], glycoproteins described to play a key role in maintaining mucosal barrier in *Echinococcus* spp. (Supplementary figures 2, 3), function through carbohydrate-dependent interactions with cell surface mucins [15].

The remaining 66 proteins (41%) did not have their specific secretory pathway determined, from which 45 are non-cytoplasmic (Figure 2). Here we emphasize thioredoxin peroxidase that act as cytoprotective antioxidant enzyme, protecting cells against deleterious oxidation of DNA, proteins and lipids, caused by physiological or pathophysiological production of extracellular reactive oxygen species and reactive nitrogen species [16]. Oxidative damage was already described to promote cyst infertility

in *Echinococcus* [17]. Proteins that are not predicted to be secreted by signal peptide or alternative secretion are good targets to be investigated as possible markers for extracellular vesicles (Supplementary figure 3).

In an attempt to find possible differences between *E. granulosus* and *E. ortleppi* we subdivided the 842 proteins in three subsets: shared subset, where we found 140 proteins (34%) secreted by a signal peptide, 59 (14%) to be secreted via non-classical pathway and 211 (52%) were non determined. Secretion pathway of proteins exclusively found in *E. ortleppi* subset have shown that 205 (70%) were not described to be secreted neither by signal peptide nor by alternative secretion. Instead, in the *E. granulosus* subset, 47% of the proteins are predicted to be secreted either by signal peptide or by alternative secretion (Figure 3), which is similar to that found in shared subset (Figure 2). We highlight seven heat shock proteins found exclusively in at least two *E. ortleppi* samples and with non-determined secretion pathway. This family of proteins is critical in the maintenance of cellular proteostasis by folding, refolding, aggregation suppression, translocation, and degradation of proteins [18]. Moreover, it has already been described as a component of exosomes, improving the protein-folding environment in other cells and even at the organismal level [19].

Remarkably we identified in all *E. ortleppi* samples, without any identification in *E. granulosus*, the WD repeat containing protein 52, belonging to one of the most abundant protein classes in eukaryotic organisms, involved in a variety of cellular processes, such as signal transduction, cytoskeleton assembly, or cell cycle control. This protein shares a common function by acting as a scaffold for protein–protein interactions, thereby mediating the formation of protein complexes and, in consequence, coordinating

downstream events [20]. Even using a dataset with *E. granulosus* and *E. multilocularis* genomes, we identified more *E. ortleppi* than *E. granulosus* proteins, besides that fewer *B. taurus* proteins were identified in *E. ortleppi* samples (Supplementary figure 4). This fact is quite interesting, since *E. ortleppi* is specifically adapted to parasite bovine lungs [21].

In our study, we describe the proteomic analysis and comparison of HF samples from three *E. granulosus* and *E. ortleppi* samples from the *B. taurus* lungs. With the use SCX followed by a LC-MS/MS approach, performed in an Orbitrap Velos mass spectrometer, we identified 842 parasite proteins. The comparison of the identified protein repertoire from these species allowed us to identify a core of 162 proteins, and some degree of infection specialization from *E. ortleppi* in lung bovine cysts.

Competing interests

There are no conflicts of interest.

Acknowledgments

This work was supported by CAPES, FAPERGS and CNPq. GBS and MEB are recipients of CAPES-REUNI and PIBIC/CNPq-UFRGS fellowships, respectively.

References

[1] Galindo, M., Gonzalez, J.M., and Galanti, N., *Echinococcus granulosus* protoscolex formation in natural infections. Biol Res 2002, 35(3-4), 365-71.

[2] McManus, D.P., Zhang, W., Li, J., Bartley, P.B., Echinococcosis. Lancet 2003, 362(9392), 1295-304.

[3] Thompson, R.C., Lymbery, A.J., Constantine, C.C., Variation in *Echinococcus*: towards a taxonomic revision of the genus. Adv Parasitol 1995, 35, 145-76.

[4] Siracusano, A., Delunardo, F., Teggi, A., Cystic echinococcosis: aspects of immune response, immunopathogenesis and immune evasion from the human host. Endocr Metab Immune Disord Drug Targets 2002, 12(1), 16-23.

[5] Monteiro, K.M., Carvalho, M.O., Zaha, A., Ferreira, H.B., Proteomic analysis of the *Echinococcus granulosus* metacestode during infection of its intermediate host. Proteomics 2010, 10, 1985-99.

[6] Aziz, A., Zhang, W., Li, J., Loukas, A. *et al*., Proteomic characterization of *Echinococcus granulosus* hydatid cyst fluid from sheep, cattle and humans. J proteomics 2011, 74(9), 1560-72.

[7] Cao, X., Fu, Z., Zhang, M., Han, Y., *et al*., iTRAQ-based comparative proteomic analysis of excretory-secretory proteins of schistosomula and adult worms of *Schistosoma japonicum*. J Proteomics 2016, 138, 30-39.

[8] Dvorak, J., Fajtova, P., Ulrychova, L., Lontovic, A., Excretion/secretion products from *Schistosoma mansoni* adults, eggs and schistosomula have unique peptidase specificity profiles. Biochimie 2016, 122, 99-109.

[9] De la Torre escudeiro, E., Manzano-Román, R., Valero, L., Oleaga, A., *et al*., Comparative proteomic analysis of *Fasciola hepatica* juveniles and *Schistosoma bovis* schistosomula. J proteomics 2011, 74(9), 1534-44.

[10] Santos, G.B., Espínola, S.M., Ferreira, H.B., Margis, R., Zaha, A. Rapid detection of *Echinococcus* species by a high-resolution melting (HRM) approach. Parasit Vectors 2013, 6(1), 327.

[11] Santos, G.B., Monteiro, K.M., Silva, E.D., Battistella, M.E. Excretory/secretory products in *Echinococcus granulosus* metacestode: is the intermediate host complacent with infection caused by the parasite's larval form? Manuscript submeted to BBA general subjects 2016.

[12] Silva-Álvarez, V., Folle, A.M., Ramos, A.L., Zamarreño, F. *Echinococcus granulosus* antigenB: a hydrophobic ligand binding protein at the host–parasite interface. Protaglandins Leukot Essent Fatty Acids 2015, 93, 17-23.

[13] Monteiro, K.M., Cardoso, M.B., Follmer, C., da Silveira, N.P. *Echinococcus granulosus* antigen B structure: subunit composition and oligomeric states. PloS Negl Trop Dis 2012, 6(3), e1551.

[14] Argüeso, P., Guzman-Aranguez, A., Mantelli, F., Cao, Z. Association of cell surface mucins with galectin-3 contributes to the ocular surface epithelial barrier. J Biol Chem 2009, 284(34), 23037-45.

[15] Del Puerto, L., Rovetta, R., Navatta, M., Fontana, C. Negligible elongation of mucin glycans with Gal β1-3 units distinguishes the laminated layer of *Echinococcus*

multilocularis from that of *Echinococcus granulosus*. Int J Parasitol 2016, Epub ahead of print.

[16] Knoops, B., Argyropoulou, V., Becker, S., Ferté, L., Kuznetsova, O. Multiple roles of peroxiredoxins in inflammation. Mol Cells 2016, 39(1), 60-4.

[17] Cabrera, G., Cabrejos, M.E., Morassutti, A.L., Cabezón, C., DNA damage, RAD9 and fertility/infertility of *Echinococcus granulosus* hydatid cysts. J Cell Physiol 2008, 216(2), 498-506.

[18] Bukau, B., Weissman, J., Horwich, A. Molecular chaperones and protein quality control. Cell 2006, 125(3), 443-51.

[19] Takeuchi, T., Suzuki, M., Fujikake, N., Popiel, H.A., Intercellular chaperone transmission via exosomes contributes to maintenance of protein homeostasis at the organismal level. Proc Natl Acad Sci U S A 2015, 112(19), E2497-506.

[20] Neer, E.J., Schmidt, C.J., Nambudripad, R., Smith, T.F. The ancient regulatory-protein family of WD-repeat proteins. Nature 1994, 371(6495), 297-300.

[21] Balbinotti, H., Santos, G.B., Badaraco, J., Arend, A.C., *et al*. *Echinococcus ortleppi* (G5) and *Echinococcus granulosus* sensu stricto (G1) loads in cattle from Southern Brazil. Vet parasitol 2012, 188(3-4), 255-60.

Figures

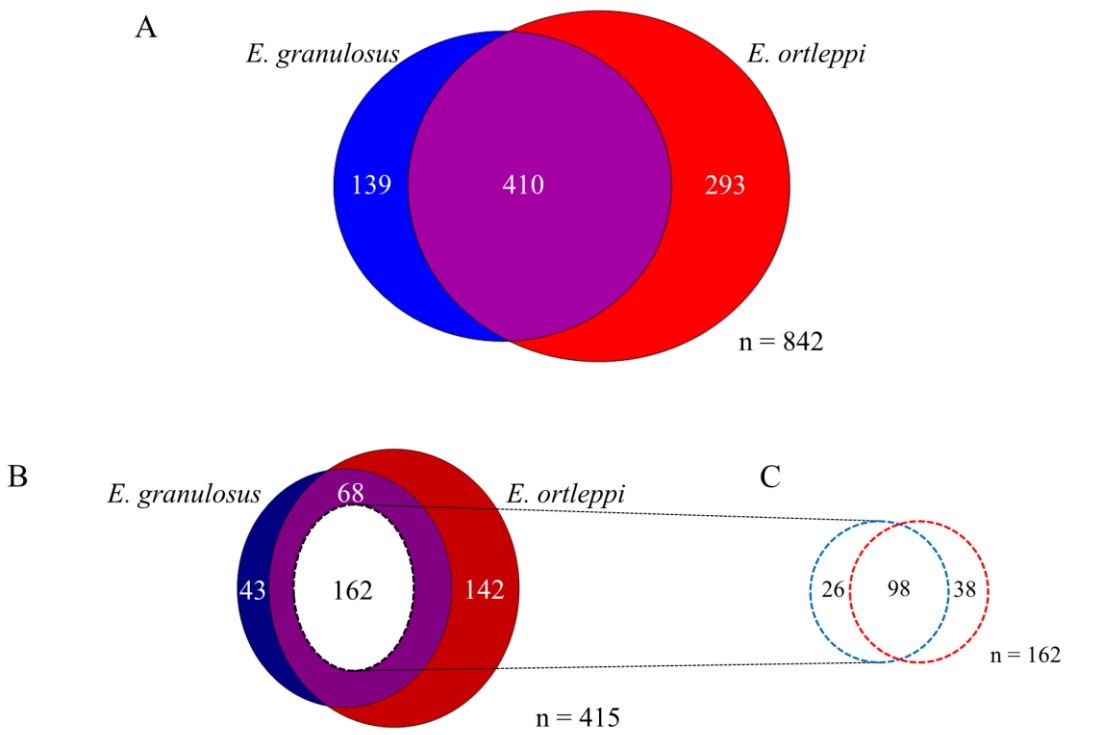


Figure 1. *E. granulosus* and *E. ortleppi* proteins identified in HF. (A) Comparison of protein identifications from three *E. granulosus* and three *E. ortleppi* HF. A total of 842 proteins were identified, 410 in both species while 139 and 293 exclusively in *E. granulosus* and *E. ortleppi* cysts, respectively. (B) Venn diagram of the 415 proteins present in at least two samples from the same species. (C) Venn diagram of the 162 proteins present in at least five of the six samples of *Echinococcus* spp. defined as core proteins.

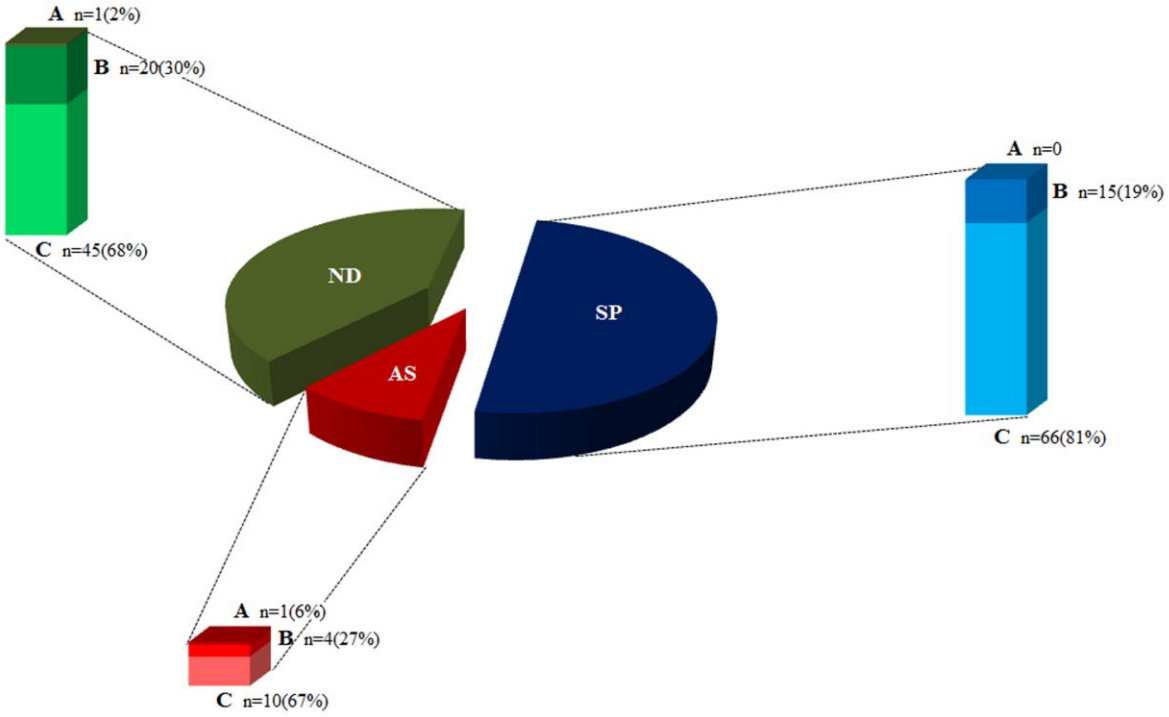


Figure 2. Core proteins associated with their secretion pathway. Core proteins (n=162) with possible secretion through signal peptide (SP) and alternative secretion (AS) assigned using SignalP and SecretomeP, respectively. Proteins without an identified secretion pathway were defined as ND (non-determined proteins).Each group of pathway secretion was uploaded into Phobius tool: Protein subcellular localization prediction was retrieved as cytoplasmic (A), Transmembrane (B) and Non-cytoplasmic (C).

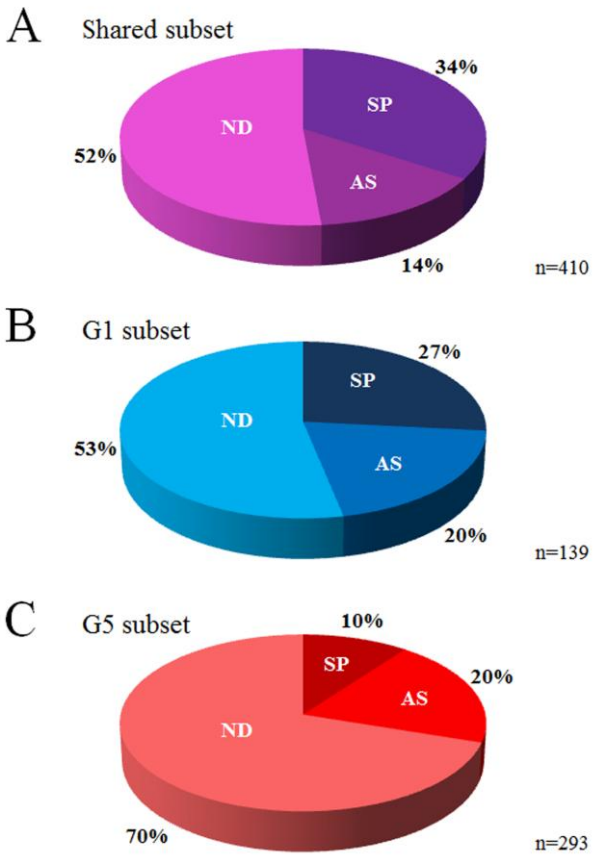


Figure 3. Parasite proteins associated with their secretion pathway. Proteins from shared, G1 and G5 subsets (n=842) with possible secretion through signal peptide (SP) and alternative secretion (AS) assigned using SignalP and SecretomeP, respectively. Proteins without an identified secretion pathway were defined as ND (non-determined).

Supplementary Tables

Supplementary Table 1. Proteins from *E. granuslosus* (A1, B1, C1) and *E. ortleppi* (A5, B5, C5) identified by LC-MS/MS analyses in fertile lung cysts. NI, not identified.

Protein code/name	G1	G5
EgrG_000040900.1..pep|Tubulin alpha 1 chain	A1	NI
EgrG_000042200.1..pep|Tubulin alpha 1 chain	A1	B5xC5
EgrG_000042700.1..pep|tubulin alpha 1C chain	A1	C5
EgrG_000058100.1..pep|receptor type tyrosine protein phosphatase	A1	NI
EgrG_000060800.1..pep|nipped b protein	A1	Shared_G5
EgrG_000075800.1..pep|epidermal growth factor receptor	A1	C5
EgrG_000077700.1..pep|Kunitz:Bovine pancreatic trypsin inhibitor	A1	NI
EgrG_000085000.1..pep|serine:arginine repetitive matrix 1	A1	B5xC5
EgrG_000101500.1..pep|dual specificity	A1	Shared_G5
EgrG_000111700.1..pep|complement C1q tumor necrosis factor	A1	NI
EgrG_000112900.1..pep|protocadherin alpha 6	A1	C5
EgrG_000115400.1..pep|hypothetical protein	A1	NI
EgrG_000118700.1..pep|polyribonucleotide 5~ hydroxyl kinase Clp1	A1	NI
EgrG_000136400.1..pep|semaphorin 5B	A1	NI
EgrG_000144800.1..pep|n acyl phosphatidylethanolamine hydrolyzing	A1	NI
EgrG_000155900.1..pep|aldo keto reductase family 1 member B4	A1	NI
EgrG_000184100.1..pep|sarcoplasmic calcium binding protein	A1	B5xC5
EgrG_000226000.1..pep|titin	A1	Shared_G5
EgrG_000227100.1..pep|jumonji domain containing 1A	A1	NI
EgrG_000228100.1..pep|protocadherin	A1	NI
EgrG_000253000.1..pep|glutaminyl peptide cyclotransferase	A1	NI
EgrG_000334500.1..pep|hypothetical protein	A1	NI
EgrG_000347500.1..pep|adam	A1	C5
EgrG_000381100.1..pep|Tapeworm specific antigen B	A1	NI
EgrG_000418900.1..pep|glycosyltransferase 14 family member	A1	NI
EgrG_000443100.1..pep|procollagen lysine2 oxoglutarate 5	A1	NI
EgrG_000443200.1..pep|procollagen lysine2 oxoglutarate 5	A1	B5
EgrG_000444100.1..pep|disks large 1	A1	Shared_G5
EgrG_000517900.1..pep|protein elys	A1	B5

EgrG_000522600.1..pep|collagen type iv alpha 3 binding protein	A1	B5xC5
EgrG_000532800.1..pep|Glucosidase II beta subunit	A1	NI
EgrG_000545700.1..pep|beta 13 n galactosyltransferase	A1	NI
EgrG_000551000.1..pep|fatty acids and retinol binding protein	A1	C5
EgrG_000555100.1..pep|hypothetical protein	A1	NI
EgrG_000569600.1..pep|expressed conserved protein	A1	NI
EgrG_000636500.1..pep|frizzled	A1	C5
EgrG_000643400.1..pep|Immunoglobulin	A1	NI
EgrG_000651200.1..pep|n6 adenosine methyltransferase 70 kDa	A1	NI
EgrG_000653900.1..pep|Myophilin	A1	Shared_G5
EgrG_000654600.1..pep|cysteine protease	A1	NI
EgrG_000655700.1..pep|receptor type tyrosine protein phosphatase	A1	B5
EgrG_000678900.1..pep|bifunctional heparan sulfate	A1	NI
EgrG_000693400.1..pep|CC2D1B protein	A1	NI
EgrG_000724500.1..pep|expressed protein	A1	NI
EgrG_000737300.1..pep|zinc transporter foi	A1	NI
EgrG_000759860.1..pep|hypothetical protein	A1	NI
EgrG_000761600.1..pep|WD repeat protein	A1	B5
EgrG_000763300.1..pep|paramyosin	A1	B5xC5
EgrG_000766700.1..pep|protein kinase C binding protein 1	A1	B5
EgrG_000772200.1..pep|60S ribosomal protein L19	A1	A5
EgrG_000773400.1..pep|Pleckstrin y	A1	Shared_G5
EgrG_000822600.1..pep|28S ribosomal protein S22 mitochondrial	A1	NI
EgrG_000868200.1..pep|ceramide kinase	A1	NI
EgrG_000872100.1..pep|t complex protein 1 subunit gamma	A1	NI
EgrG_000878500.1..pep|protocadherin 9	A1	B5
EgrG_000888900.1..pep|anosmin 1	A1	NI
EgrG_000904400.1..pep|carbonic anhydrase	A1	C5
EgrG_000919100.1..pep|3~partial|multidrug and toxin extrusion	A1	NI
EgrG_000932000.1..pep|tyrosine protein kinase Fyn	A1	NI
EgrG_000949780.1..pep|transmembrane protein 132E	A1	B5
EgrG_000961100.1..pep|slit 2 protein	A1	NI
EgrG_000970900.1..pep|histone deacetylase 6	A1	NI
EgrG_000979800.1..pep|UDP glucuronic acid decarboxylase 1	A1	B5xC5
EgrG_001004900.1..pep|calponin	A1	B5xC5
EgrG_001022300.1..pep|protein disulfide isomerase A3	A1	NI
EgrG_001026000.1..pep|protein of unknown function DUF953	A1	C5
EgrG_001082100.1..pep|protein YIPF5 YIP1 family	A1	NI
EgrG_001096300.1..pep|alpha actinin sarcomeric	A1	B5

EgrG_001120500.1..pep|cache domain containing protein	A1	B5
EgrG_001127800.1..pep|Speract scavenger receptor	A1	NI
EgrG_001157000.1..pep|lymphocyte antigen 75	A1	NI
EgrG_001178700.1..pep|PIK kinase FAT and Armadillo domain	A1	NI
EgrG_002002600.1..pep|alpha mannosidase 2	A1	NI
EmuJ_000005200.1..pep|hypothetical protein	A1	NI
EmuJ_000054700.1..pep|death domain containing protein	A1	Shared_G5
EmuJ_000069410.1..pep|apoptosis inducing factor 1 mitochondrial	A1	C5
EmuJ_000275300.1..pep|glycosyltransferase protein LARGE2	A1	NI
EmuJ_000315600.1..pep|expressed protein	A1	NI
EmuJ_000443300.1..pep|procollagen lysine,2 oxoglutarate	A1	NI
EgrG_000056700.1..pep|EGF region	A1xB1	NI
EgrG_000068100.1..pep|laminin	A1xB1	B5xC5
EgrG_000096600.1..pep|tissue type plasminogen activator	A1xB1	B5xC5
EgrG_000116900.1..pep|lysosomal protein NCU G1 B	A1xB1	NI
EgrG_000164500.1..pep|Immunoglobulin	A1xB1	B5xC5
EgrG_000178100.1..pep|tgf beta family	A1xB1	C5
EgrG_000205000.1..pep|protocadherin 1	A1xB1	B5xC5
EgrG_000236200.1..pep|neurexin 1 alpha	A1xB1	B5xC5
EgrG_000239500.1..pep|nephrin	A1xB1	B5xC5
EgrG_000279300.1..pep|enhancer of polycomb	A1xB1	C5
EgrG_000297300.1..pep|BC026374 protein S09 family	A1xB1	B5
EgrG_000357600.1..pep|lysosomal acid lipase:cholesteryl ester	A1xB1	NI
EgrG_000363400.1..pep|3~partial|cell adhesion molecule	A1xB1	B5xC5
EgrG_000389000.1..pep|protocadherin 1	A1xB1	C5
EgrG_000390400.1..pep|protocadherin 1	A1xB1	B5xC5
EgrG_000393900.1..pep|hypothetical protein	A1xB1	B5
EgrG_000412100.1..pep|neuroendocrine convertase 2	A1xB1	B5xC5
EgrG_000443800.1..pep|prohormone 4	A1xB1	C5
EgrG_000461400.1..pep|tyrosine protein kinase transmembrane	A1xB1	NI
EgrG_000468100.1..pep|palmitoyl protein thioesterase 1	A1xB1	B5xC5
EgrG_000476300.1..pep|procollagen galactosyltransferase 2	A1xB1	B5xC5
EgrG_000479300.1..pep|myosin heavy chain non muscle	A1xB1	B5
EgrG_000486600.1..pep|Spectrin alpha actinin	A1xB1	NI
EgrG_000522900.1..pep|neurexin 1 alpha	A1xB1	NI
EgrG_000534700.1..pep|Kunitz protease inhibitor	A1xB1	Shared_G5
EgrG_000543800.1..pep|immunogenic protein ts11	A1xB1	B5xC5
EgrG_000550400.1..pep|expressed protein	A1xB1	Shared_G5
EgrG_000591200.1..pep|Pfam-B_8122 and DUF4381 domain	A1xB1	NI

EgrG_000601800.1..pep|Apple	A1xB1	B5xC5
EgrG_000605100.1..pep|mannosyl oligosaccharide 12 mannosidase	A1xB1	B5xC5
EgrG_000606700.1..pep|GPI anchored surface glycoprotein	A1xB1	B5xC5
EgrG_000624850.1..pep|CDP diacylglycerol glycerol 3 phosphate	A1xB1	B5xC5
EgrG_000638000.1..pep|netrin 1	A1xB1	B5xC5
EgrG_000644850.1..pep|cell adhesion molecule	A1xB1	NI
EgrG_000656900.1..pep|expressed conserved protein	A1xB1	Shared_G5
EgrG_000722600.1..pep|Calcium binding protein	A1xB1	NI
EgrG_000732400.1..pep|acetylcholinesterase	A1xB1	B5xC5
EgrG_000741700.1..pep|beta galactosidase	A1xB1	NI
EgrG_000751800.1..pep|folate receptor beta	A1xB1	B5xC5
EgrG_000759050.1..pep|neuropeptide	A1xB1	C5
EgrG_000846700.1..pep|protocadherin 11	A1xB1	B5xC5
EgrG_000854900.1..pep|protocadherin 2 gamma c6 sCP1	A1xB1	C5
EgrG_000945000.1..pep|protocadherin 1	A1xB1	B5xC5
EgrG_000959800.1..pep|voltage dependent calcium channel subunit	A1xB1	NI
EgrG_000989200.1..pep|cathepsin l cysteine peptidase	A1xB1	NI
EgrG_001001100.1..pep|fasciclin 1	A1xB1	B5xC5
EgrG_001045100.1..pep|protocadherin 9	A1xB1	B5xC5
EgrG_001063900.1..pep|aminopeptidase N	A1xB1	B5xC5
EgrG_001079800.1..pep|multiple inositol polyphosphate phosphatase	A1xB1	B5
EgrG_001096100.1..pep|EF hand domain containing protein	A1xB1	NI
EgrG_001097100.1..pep|expressed conserved protein	A1xB1	Shared_G5
EgrG_001133800.1..pep|receptor type tyrosine protein phosphatase	A1xB1	B5xC5
EgrG_001169400.1..pep|iron:zinc purple acid phosphatase protein	A1xB1	Shared_G5
EmuJ_000097600.1..pep|alpha mannosidase 2	A1xB1	B5xC5
EmuJ_000140000.1..pep|collagen alpha(iv) chain	A1xB1	Shared_G5
EmuJ_000525100.1..pep|secreted antigen Ts8B1	A1xB1	C5
EmuJ_000538400.1..pep|expressed conserved protein	A1xB1	B5xC5
EmuJ_000655200.1..pep|inositol monophosphatase	A1xB1	NI
EmuJ_001136700.1..pep|proteinase inhibitor I2, Kunitz metazoa	A1xB1	B5
EgrG_000174000.1..pep|c-Jun N-terminal kinases	A1xC1	B5
EgrG_000211500.1..pep|von Willebrand factor type C	A1xC1	C5
EgrG_000227300.1..pep|expressed conserved protein	A1xC1	C5
EgrG_000237100.1..pep|tektin 1	A1xC1	B5xC5
EgrG_000305400.1..pep|expressed protein	A1xC1	Shared_G5
EgrG_000374700.1..pep|nucleolar pre ribosomal associated protein	A1xC1	B5xC5
EgrG_000471400.1..pep|expressed conserved protein	A1xC1	Shared_G5
EgrG_000508000.1..pep|ankyrin domain repeat containing protein	A1xC1	B5xC5

EgrG_000569000.1..pep|tubulin subunit beta	A1xC1	NI
EgrG_000597700.1..pep|titin	A1xC1	B5xC5
EgrG_000650120.1..pep|spectrin beta chain, erythrocyte	A1xC1	B5xC5
EgrG_000893500.1..pep|cysteine and glycine rich protein 1	A1xC1	B5xC5
EgrG_000910400.1..pep|nesprin 1	A1xC1	B5xC5
EgrG_001013800.1..pep|myotubularin protein 3	A1xC1	B5xC5
EgrG_001055200.1..pep|expressed conserved protein	A1xC1	B5xC5
EgrG_001081100.1..pep|hypothetical protein	A1xC1	Shared_G5
EgrG_001085050.1..pep|Heat shock 70 kDa protein 4	A1xC1	A5
EgrG_001094600.1..pep|serine:threonine protein kinase TAO1	A1xC1	Shared_G5
EgrG_001188700.1..pep|px19 protein	A1xC1	Shared_G5
EgrG_000056900.1..pep|bone morphogenetic protein antagonist noggin	B1	Shared_G5
EgrG_000087900.1..pep|expressed conserved protein	B1	C5
EgrG_000097500.1..pep|polyu specific endoribonuclease b	B1	B5xC5
EgrG_000119200.1..pep|neuroendocrine protein 7b2	B1	NI
EgrG_000120300.1..pep|expressed conserved protein	B1	A5xC5
EgrG_000170000.1..pep|Ras GTPase activating protein	B1	C5
EgrG_000237600.1..pep|hypothetical protein	B1	NI
EgrG_000296900.1..pep|hypothetical protein	B1	C5
EgrG_000347700.1..pep|neuropeptide	B1	NI
EgrG_000355800.1..pep|TSP1	B1	A5
EgrG_000358700.1..pep|serine incorporator 5	B1	NI
EgrG_000359800.1..pep|Thioredoxin fold	B1	C5
EgrG_000419300.1..pep|TMEM9	B1	NI
EgrG_000439600.1..pep|expressed conserved protein	B1	B5xC5
EgrG_000440600.1..pep|alanyl tRNA synthetase mitochondrial	B1	A5xB5
EgrG_000460400.1..pep|intraflagellar transport protein 81	B1	C5
EgrG_000487400.1..pep|inorganic pyrophosphatase	B1	C5
EgrG_000524400.1..pep|EGF region	B1	NI
EgrG_000583700.1..pep|protocadherin 11	B1	C5
EgrG_000617300.1..pep|melanoma receptor tyrosine protein kinase	B1	NI
EgrG_000645800.1..pep|NADP dependent malic enzyme	B1	B5xC5
EgrG_000647000.1..pep|expressed conserved protein	B1	C5
EgrG_000650200.1..pep|histone H2A	B1	B5xC5
EgrG_000672200.1..pep|Tubulin beta 2C chain	B1	Shared_G5
EgrG_000700220.1..pep|protein fat free	B1	B5
EgrG_000724600.1..pep|expressed conserved protein	B1	NI
EgrG_000769500.1..pep|discoidin domain containing receptor 2	B1	B5xC5
EgrG_000778400.1..pep|glypican	B1	B5xC5

EgrG_000814100.1..pep|expressed conserved protein	B1	B5xC5
EgrG_000814400.1..pep|subfamily M14A unassigned peptidase	B1	B5
EgrG_000838600.1..pep|hypothetical protein	B1	C5
EgrG_000843500.1..pep|UTP glucose 1 phosphate uridylyltransferase	B1	Shared_G5
EgrG_000861900.1..pep|protocadherin 11	B1	NI
EgrG_000877700.1..pep|expressed protein	B1	B5
EgrG_000882500.1..pep|actin modulator protein	B1	B5xC5
EgrG_000886400.1..pep|tubulin alpha chain	B1	Shared_G5
EgrG_000887000.1..pep|cadherin	B1	NI
EgrG_000909300.1..pep|Glycoside hydrolase chitinase	B1	B5xC5
EgrG_000912600.1..pep|EF hand domain containing family member A2	B1	NI
EgrG_000941000.1..pep|dynein light chain	B1	B5xC5
EgrG_000955100.1..pep|Tubulin beta 2C chain	B1	NI
EgrG_000984300.1..pep|cupin 2 barrel domain containing protein	B1	C5
EgrG_000991000.1..pep|dynein light chain	B1	C5
EgrG_001028100.1..pep|dipeptidyl peptidase 3	B1	B5
EgrG_001028800.1..pep|Lysosomal Pro X carboxypeptidase	B1	B5xC5
EgrG_001060100.1..pep|14-3-3 protein zeta:delta	B1	B5xC5
EgrG_001069200.1..pep|ectonucleotide pyrophosphatase	B1	NI
EgrG_001069300.1..pep|disintegrin and metalloproteinase	B1	B5xC5
EgrG_001120400.1..pep|expressed conserved protein	B1	NI
EgrG_001145700.1..pep|NADP dependent malic enzyme	B1	Shared_G5
EgrG_001163400.1..pep|Armet protein	B1	Shared_G5
EgrG_001163700.1..pep|hydrocephalus inducing	B1	B5
EmuJ_000067800.1..pep|dystrophin,s A:C:F:G:H	B1	NI
EmuJ_000155400.1..pep|aldo keto reductase family 1, member B4	B1	Shared_G5
EmuJ_000216800.1..pep|expressed conserved protein	B1	C5
EmuJ_000229800.1..pep|neuropeptide	B1	C5
EmuJ_000374800.1..pep|glutathione peroxidase	B1	Shared_G5
EmuJ_000407300.1..pep|actin	B1	C5
EmuJ_000422400.1..pep|hemicentin 1	B1	NI
EmuJ_000474600.1..pep|expressed conserved protein	B1	A5xB5
EmuJ_000550000.1..pep|fatty acid binding protein FABP2	B1	B5xC5
EmuJ_000739300.1..pep|protein vprbp	B1	C5
EmuJ_000760400.1..pep|protein AHNAK2	B1	Shared_G5
EmuJ_000799500.1..pep|phosphoglycerate mutase	B1	B5xC5
EmuJ_000950100.1..pep|transmembrane protein 132E	B1	B5
EmuJ_000956500.1..pep|expressed conserved protein	B1	NI
EmuJ_001129500.1..pep|expressed protein	B1	NI

EgrG_000008700.1..pep|heat shock protein heat shock protein 90	B1xC1	B5xC5
EgrG_000069900.1..pep|beta tubulin	B1xC1	C5
EgrG_000076900.1..pep|adenosylhomocysteinase	B1xC1	Shared_G5
EgrG_000089300.1..pep|bone morphogenetic protein antagonist noggin	B1xC1	C5
EgrG_000103100.1..pep|transketolase	B1xC1	B5xC5
EgrG_000170200.1..pep|lysosomal protective protein	B1xC1	Shared_G5
EgrG_000182400.1..pep|dynein light chain	B1xC1	B5xC5
EgrG_000202600.1..pep|Tubulin beta 2C chain	B1xC1	B5xC5
EgrG_000206800.1..pep|MAM	B1xC1	Shared_G5
EgrG_000212300.1..pep|tyrosine protein kinase otk	B1xC1	Shared_G5
EgrG_000231300.1..pep|14-3-3 protein epsilon	B1xC1	B5xC5
EgrG_000236500.1..pep|major egg antigen	B1xC1	B5xC5
EgrG_000239700.1..pep|expressed conserved protein	B1xC1	B5xC5
EgrG_000342900.1..pep|Cysticercus cellulosae specific antigenic	B1xC1	B5xC5
EgrG_000364000.1..pep|14-3-3 protein beta:alpha	B1xC1	B5xC5
EgrG_000372400.1..pep|tegumental protein	B1xC1	Shared_G5
EgrG_000393000.1..pep|sj ts4 protein	B1xC1	Shared_G5
EgrG_000406900.1..pep|Actin cytoplasmic A3	B1xC1	Shared_G5
EgrG_000485800.1..pep|H17g protein tegumental antigen	B1xC1	B5xC5
EgrG_000492700.1..pep|immunogenic protein	B1xC1	C5
EgrG_000501500.1..pep|glycogen phosphorylase	B1xC1	B5xC5
EgrG_000501600.1..pep|glycogen phosphorylase	B1xC1	B5xC5
EgrG_000521700.1..pep|heat shock protein 70	B1xC1	B5xC5
EgrG_000527500.1..pep|nuclear pore complex protein Nup133	B1xC1	B5
EgrG_000538900.1..pep|glutathione S transferase	B1xC1	B5xC5
EgrG_000550800.1..pep|endophilin B1	B1xC1	B5xC5
EgrG_000596700.1..pep|dynein heavy chain	B1xC1	A5xB5
EgrG_000610500.1..pep|titin	B1xC1	Shared_G5
EgrG_000617000.1..pep|Tubulin beta 2C chain	B1xC1	NI
EgrG_000638300.1..pep|superoxide dismutase 1 soluble	B1xC1	B5xC5
EgrG_000644200.1..pep|ras protein Rab 26	B1xC1	B5xC5
EgrG_000677200.1..pep|peptidyl prolyl cis trans isomerase E	B1xC1	Shared_G5
EgrG_000688950.1..pep|cop9 complex subunit	B1xC1	Shared_G5
EgrG_000790300.1..pep|cathepsin b	B1xC1	Shared_G5
EgrG_000901500.1..pep|tyrosyl tRNA synthetase	B1xC1	NI
EgrG_000908900.1..pep|n acetylated alpha linked acidic dipeptidase	B1xC1	Shared_G5
EgrG_000935200.1..pep|synaptic vesicle membrane protein VAT 1	B1xC1	Shared_G5
EgrG_000982200.1..pep|elongation factor 1 alpha	B1xC1	B5xC5
EgrG_000990800.1..pep|dynein light chain	B1xC1	B5xC5

EgrG_000990900.1..pep|dynein light chain	B1xC1	Shared_G5
EgrG_000991200.1..pep|Dynein light chain type 1 2	B1xC1	Shared_G5
EgrG_001023000.1..pep|secreted frizzled protein 5	B1xC1	Shared_G5
EgrG_001024500.1..pep|hypothetical protein	B1xC1	C5
EgrG_001028500.1..pep|citrate synthase	B1xC1	Shared_G5
EgrG_001065500.1..pep|Ndr	B1xC1	B5xC5
EgrG_001085400.1..pep|Heat shock 70 kDa protein 4	B1xC1	B5xC5
EgrG_001097500.1..pep|kinesin protein KIF16B	B1xC1	NI
EgrG_001117000.1..pep|nucleoside diphosphate kinase	B1xC1	Shared_G5
EgrG_001138700.1..pep|huntingtin	B1xC1	B5xC5
EgrG_001185000.1..pep|malate dehydrogenase	B1xC1	B5xC5
EgrG_001196600.1..pep|EGF region	B1xC1	Shared_G5
EmuJ_000027400.1..pep|histone H2B	B1xC1	B5xC5
EmuJ_000031300.1..pep|histone H4	B1xC1	Shared_G5
EmuJ_000140100.1..pep|Collagen alpha 1(V) chain	B1xC1	Shared_G5
EmuJ_000141000.1..pep|lysosomal alpha glucosidase	B1xC1	Shared_G5
EmuJ_000156000.1..pep|aldo keto reductase family 1, member B4	B1xC1	B5xC5
EmuJ_000212700.1..pep|Major egg antigen (p40)	B1xC1	B5xC5
EmuJ_000314700.1..pep|heat shock protein 70 related	B1xC1	Shared_G5
EmuJ_000331300.1..pep|E3 ubiquitin protein ligase UBR5	B1xC1	Shared_G5
EmuJ_000422300.1..pep|hemicentin 1	B1xC1	B5xC5
EmuJ_000554100.1..pep|heat shock protein 70	B1xC1	B5xC5
EmuJ_000920900.1..pep|myosin heavy chain	B1xC1	B5xC5
EmuJ_000984800.1..pep|UDP glucose 4 epimerase	B1xC1	B5xC5
EmuJ_000991300.1..pep|Dynein light chain, type 1 2	B1xC1	Shared_G5
EmuJ_001051900.1..pep|expressed conserved protein	B1xC1	Shared_G5
EmuJ_001086400.1..pep|expressed protein	B1xC1	Shared_G5
EmuJ_001201600.1..pep|diagnostic antigen gp50	B1xC1	B5xC5
EgrG_000020150.1..pep|heat shock protein 70	C1	Shared_G5
EgrG_000041100.1..pep|Tubulin beta 2C chain	C1	NI
EgrG_000041200.1..pep|annexin	C1	NI
EgrG_000043000.1..pep|sperm associated antigen 1	C1	NI
EgrG_000058900.1..pep|timeless	C1	B5xC5
EgrG_000060700.1..pep|protein tyrosine kinase	C1	NI
EgrG_000071400.1..pep|dynein light chain	C1	C5
EgrG_000075000.1..pep|GAS2 protein 3	C1	NI
EgrG_000129900.1..pep|Kinesin protein CG14535	C1	NI
EgrG_000149500.1..pep|expressed conserved protein	C1	Shared_G5
EgrG_000160800.1..pep|hypothetical protein	C1	A5xC5

EgrG_000175800.1..pep|exportin 4	C1	A5xB5
EgrG_000241100.1..pep|hypothetical protein	C1	C5
EgrG_000243100.1..pep|transcriptional adapter 2 beta	C1	NI
EgrG_000243300.1..pep|hypothetical protein	C1	B5xC5
EgrG_000243500.1..pep|spectrin beta chain	C1	C5
EgrG_000244000.1..pep|annexin	C1	C5
EgrG_000246200.1..pep|Thymosin beta 4	C1	C5
EgrG_000249600.1..pep|Heat Shock protein family member hsp 3	C1	B5xC5
EgrG_000249700.1..pep|THO complex subunit 5 protein	C1	A5
EgrG_000305000.1..pep|expressed protein	C1	B5xC5
EgrG_000316600.1..pep|irregular chiasm C roughest protein	C1	A5xC5
EgrG_000332900.1..pep|bromodomain containing 2	C1	B5xC5
EgrG_000342100.1..pep|exocyst complex component 5	C1	NI
EgrG_000355700.1..pep|tetraspanin	C1	B5xC5
EgrG_000366900.1..pep|heat shock protein 70	C1	NI
EgrG_000373050.1..pep|RAB6A member RAS oncogene family	C1	NI
EgrG_000375100.1..pep|hypothetical protein	C1	NI
EgrG_000389900.1..pep|Pfam-B_8674	C1	NI
EgrG_000413800.1..pep|expressed protein	C1	C5
EgrG_000416400.1..pep|triosephosphate isomerase	C1	B5xC5
EgrG_000427400.1..pep|transportin 1	C1	B5
EgrG_000434300.1..pep|hypothetical protein	C1	C5
EgrG_000435000.1..pep|valyl tRNA synthetase	C1	NI
EgrG_000440800.1..pep|hypothetical protein	C1	NI
EgrG_000462900.1..pep|caspase 3 apoptosis cysteine peptidase	C1	B5
EgrG_000491400.1..pep|CalModulin family member cmd 1	C1	NI
EgrG_000520000.1..pep|glutamyl tRNA synthetase cytoplasmic	C1	NI
EgrG_000525400.1..pep|secreted antigen Ts8B1	C1	A5xC5
EgrG_000527300.1..pep|RNA polymerase II associated protein 1	C1	A5xC5
EgrG_000528400.1..pep|integrin beta 2	C1	NI
EgrG_000532100.1..pep|bromodomain adjacent to zinc finger domain	C1	NI
EgrG_000579800.1..pep|expressed protein	C1	A5xB5
EgrG_000584200.1..pep|kelch protein 7	C1	B5
EgrG_000589100.1..pep|glutamate dehydrogenase	C1	A5
EgrG_000590100.1..pep|dynein light chain	C1	NI
EgrG_000600800.1..pep|nuclear factor of activated T cells 5	C1	NI
EgrG_000621000.1..pep|dynein heavy chain	C1	Shared_G5
EgrG_000631600.1..pep|hypothetical protein	C1	NI
EgrG_000639400.1..pep|myosin va	C1	NI

EgrG_000665600.1..pep|lipoxygenase domain containing protein	C1	NI
EgrG_000679700.1..pep|heat shock protein	C1	B5xC5
EgrG_000694380.1..pep|ribosomal protein S23 putative	C1	NI
EgrG_000697700.1..pep|EF Hand type	C1	Shared_G5
EgrG_000707700.1..pep|atlastin 2	C1	A5
EgrG_000710600.1..pep|ankyrin domain repeat containing protein	C1	C5
EgrG_000719700.1..pep|calpain	C1	NI
EgrG_000724900.1..pep|cAMP dependent protein kinase	C1	NI
EgrG_000732300.1..pep|DNA double strand break repair rad50	C1	NI
EgrG_000735900.1..pep|hypothetical protein	C1	NI
EgrG_000770100.1..pep|cpg binding protein	C1	NI
EgrG_000786600.1..pep|protein unc 80	C1	C5
EgrG_000799300.1..pep|insulin growth factor binding	C1	NI
EgrG_000803800.1..pep|1-Phosphatidylinositol	C1	NI
EgrG_000819000.1..pep|protein fam13a	C1	C5
EgrG_000825200.1..pep|myoferlin	C1	NI
EgrG_000826850.1..pep|hypothetical protein	C1	B5xC5
EgrG_000863900.1..pep|hypothetical protein	C1	NI
EgrG_000902300.1..pep|G protein coupled receptor fragment	C1	NI
EgrG_000906200.1..pep|importin 7	C1	B5
EgrG_000911200.1..pep|calpain A	C1	B5xC5
EgrG_000916620.1..pep|Alpha helical coiled-coil rod protein	C1	B5
EgrG_000936600.1..pep|Cytoskeleton associated protein CAP	C1	C5
EgrG_000940900.1..pep|dynein light chain	C1	B5xC5
EgrG_000941100.1..pep|dynein light chain	C1	B5xC5
EgrG_000949820.1..pep|tumor protein d52	C1	Shared_G5
EgrG_000986900.1..pep|expressed conserved protein	C1	NI
EgrG_000998500.1..pep|gtp binding protein 2	C1	A5xB5
EgrG_001001800.1..pep|tegumental antigen	C1	C5
EgrG_001003300.1..pep|ras protein rab 8b	C1	NI
EgrG_001064900.1..pep|proteasome subunit beta t family	C1	A5
EgrG_001077100.1..pep|TSP5	C1	NI
EgrG_001078200.1..pep|protein strawberry notch 1	C1	NI
EgrG_001097550.1..pep|erythrocyte membrane protein	C1	B5
EgrG_001104800.1..pep|Acidic leucine rich nuclear phosphoprotein	C1	NI
EgrG_001122500.1..pep|growth arrest specific protein 8	C1	Shared_G5
EgrG_001126150.1..pep|tubulin beta 4A class IVa	C1	NI
EgrG_001147900.1..pep|phenylalanyl tRNA synthetase alpha chain B	C1	B5
EgrG_001164100.1..pep|chaperone protein DnaJ	C1	A5xB5

EgrG_001176700.1..pep|ras protein Rab 10	C1	NI
EgrG_001178600.1..pep|protein of unknown function DUF2146	C1	C5
EgrG_001185200.1..pep|integrator complex subunit 2	C1	C5
EgrG_001199000.1..pep|succinyl coenzyme A synthetase alpha subunit	C1	NI
EgrG_001201800.1..pep|ankyrin domain repeat containing protein	C1	B5xC5
EgrG_002050100.1..pep|actin	C1	NI
EgrG_002051400.1..pep|histone H2B	C1	NI
EmuJ_000027600.1..pep|histone H2A	C1	Shared_G5
EmuJ_000044200.1..pep|actin	C1	Shared_G5
EmuJ_000064150.1..pep|conserved hypothetical protein	C1	Shared_G5
EmuJ_000085100.1..pep|coiled coil domain containing protein 164	C1	NI
EmuJ_000116800.1..pep|Zinc finger, CCCH type	C1	NI
EmuJ_000142000.1..pep|major vault protein	C1	NI
EmuJ_000151600.1..pep|Spectrin alpha actinin	C1	B5xC5
EmuJ_000178800.1..pep|ccaat enhancer binding protein delta	C1	B5
EmuJ_000189800.1..pep|atpase inhibitor protein	C1	NI
EmuJ_000211300.1..pep|WNT	C1	NI
EmuJ_000240900.1..pep|cop9 signalosome complex subunit 7b	C1	NI
EmuJ_000291900.1..pep|heat shock 70 kda protein 1a	C1	A5xB5
EmuJ_000321550.1..pep|Biotin lipoyl attachment	C1	Shared_G5
EmuJ_000357500.1..pep|heat shock protein 71 kDa protein	C1	NI
EmuJ_000444600.1..pep|short transient receptor potential channel 6	C1	C5
EmuJ_000452900.1..pep|expressed protein	C1	NI
EmuJ_000476900.1..pep|GDP L fucose synthase	C1	Shared_G5
EmuJ_000484000.1..pep|voltage gated potassium channel	C1	NI
EmuJ_000533500.1..pep|enolase	C1	C5
EmuJ_000534100.1..pep|propionyl coenzyme A carboxylase alpha	C1	B5xC5
EmuJ_000537700.1..pep	C1	C5
EmuJ_000569700.1..pep|TBC1 domain family	C1	C5
EmuJ_000603600.1..pep|WD repeat containing protein 78	C1	NI
EmuJ_000675100.2..pep	C1	NI
EmuJ_000704300.1..pep|S1 RNA binding domain containing protein 1	C1	A5xB5
EmuJ_000730000.1..pep|heat shock protein 70	C1	B5xC5
EmuJ_000821100.1..pep|cyclic nucleotide gated cation channel	C1	A5
EmuJ_000866200.1..pep|Replication factor C 38 kDa subunit	C1	A5xB5
EmuJ_000880300.1..pep|membrane bound acyltransferase:hhat	C1	NI
EmuJ_001038700.1..pep|Zinc finger, C2H2 type family protein	C1	C5
EmuJ_001086300.1..pep|expressed protein	C1	NI
EmuJ_001107000.1..pep|ankyrin :unc	C1	C5

EmuJ_001134100.1..pep|aspartate aminotransferase, mitochondrial	C1	A5xC5
EmuJ_001145800.1..pep|myeloid:lymphoid or mixed lineage leukemia	C1	A5xB5
EgrG_000043400.1..pep|Pancreatic hormone	NI	C5
EgrG_000064200.1..pep|hypothetical protein	NI	C5
EgrG_000071500.1..pep|dynein light chain	NI	C5
EgrG_000080600.1..pep|solute carrier family 12	NI	C5
EgrG_000084300.1..pep|Bcl 2 ous antagonist:killer	NI	C5
EgrG_000090500.1..pep|reticulon 4 interacting protein 1	NI	C5
EgrG_000092800.1..pep|transaldolase	NI	A5
EgrG_000095900.1..pep|lysine specific histone demethylase 1A	NI	B5
EgrG_000096900.1..pep|tubulin polymerization promoting protein	NI	C5
EgrG_000103600.1..pep|hypothetical protein	NI	B5
EgrG_000104300.1..pep|alpha adducin	NI	C5
EgrG_000109400.1..pep|phd:f box containing protein	NI	B5
EgrG_000113800.1..pep|eukaryotic translation elongation factor 1	NI	C5
EgrG_000114400.1..pep|carbonyl reductase 1	NI	B5xC5
EgrG_000115200.1..pep|carbonyl reductase 1	NI	C5
EgrG_000120500.1..pep|expressed protein	NI	B5
EgrG_000122100.1..pep|Profilin allergen	NI	C5
EgrG_000127300.1..pep|Na+ dependent nucleoside transporter	NI	B5xC5
EgrG_000127900.1..pep|zinc finger protein 1	NI	B5xC5
EgrG_000128000.1..pep|histone H4	NI	C5
EgrG_000135900.1..pep|protein DJ 1	NI	C5
EgrG_000142500.1..pep|major vault protein	NI	B5xC5
EgrG_000149200.1..pep|microfibrillar associated protein 1	NI	B5
EgrG_000149300.1..pep|DNA polymerase zeta catalytic subunit	NI	B5
EgrG_000156400.1..pep|aldo keto reductase family 1 member B4	NI	B5xC5
EgrG_000161650.1..pep|heat shock protein 70	NI	Shared_G5
EgrG_000170500.1..pep|Translation machinery associated TMA7	NI	C5
EgrG_000174700.1..pep|chromobox protein 2	NI	Shared_G5
EgrG_000179600.1..pep|rhophilin 2	NI	B5
EgrG_000179700.1..pep|enolase	NI	C5
EgrG_000182300.1..pep|rho guanine nucleotide exchange factor 7	NI	B5xC5
EgrG_000184400.1..pep|small subunit processome component 20	NI	C5
EgrG_000186700.1..pep|tyrosine kinase	NI	B5
EgrG_000193700.1..pep|annexin	NI	B5xC5
EgrG_000194000.1..pep|glycogenin 1	NI	C5
EgrG_000196200.1..pep|basic fibroblast growth factor receptor 1 A	NI	Shared_G5
EgrG_000211900.1..pep|kinesin light chain 4	NI	B5

EgrG_000212100.1..pep|coiled coil domain containing protein 151	NI	B5
EgrG_000212600.1..pep|DNA replication licensing factor MCM2	NI	B5
EgrG_000213900.1..pep|dis3 exonuclease 2	NI	B5xC5
EgrG_000223550.1..pep|26s proteasome regulatory particle subunit	NI	B5
EgrG_000225800.1..pep|Papilin	NI	B5
EgrG_000226500.1..pep|Galectin carbohydrate recognition domain	NI	B5
EgrG_000229100.1..pep|hypothetical protein	NI	C5
EgrG_000235050.1..pep|muscle myosin heavy chain	NI	C5
EgrG_000240100.1..pep|AP 1 complex subunit beta 1	NI	C5
EgrG_000245300.1..pep|calumenin	NI	A5
EgrG_000247500.1..pep|hypothetical protein	NI	C5
EgrG_000253700.1..pep|protein of unknown function DUF1683	NI	A5
EgrG_000257700.1..pep|Fragile site associated protein C terminal	NI	A5xB5
EgrG_000257800.1..pep|dna ligase 4	NI	C5
EgrG_000261600.1..pep|fructose 16 bisphosphatase 1	NI	C5
EgrG_000262050.1..pep|granulins	NI	C5
EgrG_000301800.1..pep|expressed protein	NI	C5
EgrG_000303200.1..pep|hypothetical protein	NI	C5
EgrG_000304800.1..pep|diagnostic antigen gp50	NI	B5
EgrG_000314800.1..pep|heat shock protein 70	NI	A5xB5
EgrG_000320300.1..pep|forkhead box protein P4	NI	B5xC5
EgrG_000320800.1..pep|heat shock 10 kDa protein 1	NI	C5
EgrG_000322250.1..pep|large subunit ribosomal protein 6	NI	C5
EgrG_000330300.1..pep|annexin	NI	C5
EgrG_000360300.1..pep|thioredoxin mitochondrial	NI	C5
EgrG_000376700.1..pep|neuroblastoma amplified sequence	NI	A5xC5
EgrG_000376800.1..pep|selenoprotein t	NI	B5
EgrG_000379300.1..pep|methyltransferase C20orf7	NI	B5xC5
EgrG_000392000.1..pep|uridine 5~ monophosphate synthase	NI	C5
EgrG_000409600.1..pep|KIF1 binding protein	NI	B5
EgrG_000422900.1..pep|lisH domain and HEAT repeat protein	NI	A5xB5
EgrG_000457600.1..pep|phosphatidylinositol 3 and 4 kinase	NI	A5xB5
EgrG_000471600.1..pep|transitional endoplasmic reticulum atpase	NI	C5
EgrG_000480500.1..pep|cohesin subunit SA 1	NI	C5
EgrG_000487300.1..pep|RNA directed DNA polymerase transcriptase	NI	C5
EgrG_000492000.1..pep|bestrophin 3	NI	A5xC5
EgrG_000500700.1..pep|hypothetical protein	NI	B5
EgrG_000503900.1..pep|Dynein intermediate chain	NI	C5
EgrG_000507900.1..pep|coiled coil domain containing protein 148	NI	B5

EgrG_000510700.1..pep|DNA replication licensing factor mcm7	NI	C5
EgrG_000511100.1..pep|protein piezo2	NI	B5
EgrG_000511750.1..pep|ATP dependent RNA helicase DDX52	NI	B5xC5
EgrG_000517500.1..pep|structural maintenance of chromosomes	NI	A5
EgrG_000520950.1..pep|heat shock protein 70	NI	Shared_G5
EgrG_000528900.1..pep|actin depolymerizing factor	NI	B5xC5
EgrG_000539800.1..pep|LIM zinc bindingdomain containing protein	NI	C5
EgrG_000540200.1..pep|E3 ubiquitin protein ligase UBR4	NI	B5xC5
EgrG_000549800.1..pep|fatty acid binding protein FABP2	NI	B5
EgrG_000564000.1..pep|5~partial|diagnostic antigen gp50	NI	C5
EgrG_000566700.1..pep|diagnostic antigen gp50	NI	B5xC5
EgrG_000586300.1..pep|coiled coil domain containing protein 40	NI	A5
EgrG_000588700.1..pep|protein furry	NI	B5
EgrG_000589500.1..pep|expressed conserved protein	NI	C5
EgrG_000594300.1..pep|SH3 and multiple ankyrin repeat domains	NI	C5
EgrG_000596000.1..pep|dynein heavy chain	NI	B5
EgrG_000602500.1..pep|hypothetical protein	NI	B5
EgrG_000606800.1..pep|Clusterin associated protein 1	NI	B5
EgrG_000607900.1..pep|manganese superoxide dismutase	NI	A5xC5
EgrG_000615300.1..pep|hypothetical protein	NI	C5
EgrG_000627900.1..pep|E3 ubiquitin protein ligase listerin	NI	C5
EgrG_000633300.1..pep|splicing factor 3b subunit 3	NI	B5
EgrG_000634800.1..pep|L lactate dehydrogenase B chain	NI	B5xC5
EgrG_000637800.1..pep|potassium voltage gated channel subfamily H	NI	A5
EgrG_000641200.1..pep|macroglobulin:complement	NI	C5
EgrG_000644500.1..pep|glycogen debranching enzyme	NI	C5
EgrG_000651400.1..pep|heat shock kda protein	NI	C5
EgrG_000655300.1..pep|dystrobrevin	NI	B5
EgrG_000669300.1..pep|hypothetical protein	NI	B5xC5
EgrG_000684700.1..pep|dynein light chain	NI	B5xC5
EgrG_000689700.1..pep|Spectrin alpha actinin	NI	A5
EgrG_000698000.1..pep|Pleckstrin y	NI	A5
EgrG_000703800.1..pep|ecotropic viral integration site	NI	C5
EgrG_000728000.1..pep|SNW domain containing 1	NI	B5
EgrG_000733800.1..pep|death associated protein kinase 1	NI	B5
EgrG_000736000.1..pep|talin	NI	B5
EgrG_000736050.1..pep|apolipoprotein A I binding protein	NI	C5
EgrG_000736500.1..pep|heat shock protein 70	NI	C5
EgrG_000737500.1..pep|Immunoglobulin	NI	B5xC5

EgrG_000748500.1..pep|roundabout 2	NI	C5
EgrG_000753300.1..pep|EGF region	NI	B5xC5
EgrG_000755700.1..pep|OCIA domain containing protein 1	NI	C5
EgrG_000757300.1..pep|expressed conserved protein	NI	B5
EgrG_000758700.1..pep|Helicase SANT associated DNA binding	NI	C5
EgrG_000759000.1..pep|Ankyrin repeat domain containing protein	NI	B5
EgrG_000761550.1..pep|nucleolar MIF4G domain containing protein 1	NI	A5
EgrG_000767200.1..pep|rab gdp dissociation inhibitor	NI	C5
EgrG_000780100.1..pep|deoxyhypusine synthase	NI	C5
EgrG_000793800.1..pep|cysteinyl tRNA synthetase	NI	B5
EgrG_000798700.1..pep|acyl coenzyme A binding protein	NI	C5
EgrG_000800300.1..pep|tafazzin	NI	B5
EgrG_000806000.1..pep|hypothetical protein	NI	A5
EgrG_000806200.1..pep|expressed conserved protein	NI	C5
EgrG_000815400.1..pep|transcription elongation factor spt6	NI	C5
EgrG_000824100.1..pep|Estrogen regulated protein EP45	NI	B5
EgrG_000824300.1..pep|enhancer of polycomb	NI	B5
EgrG_000825450.1..pep|hsp70 binding protein	NI	A5
EgrG_000828800.1..pep|protocadherin gamma c4	NI	B5
EgrG_000838700.1..pep|secreted frizzled protein	NI	A5
EgrG_000846400.1..pep|6 phosphogluconate dehydrogenase	NI	C5
EgrG_000849000.1..pep|spectrin beta chain	NI	B5
EgrG_000856100.1..pep|alpha actinin sarcomeric	NI	C5
EgrG_000860100.1..pep|CCR4 NOT transcription complex subunit 3	NI	A5
EgrG_000865300.1..pep|elongation factor 2	NI	C5
EgrG_000866000.1..pep|small nuclear ribonucleoprotein G	NI	C5
EgrG_000866600.1..pep|heat shock protein 75 kDa mitochondrial	NI	B5
EgrG_000874400.1..pep|zinc finger MYND domain 1	NI	B5xC5
EgrG_000880900.1..pep|WD repeat containing protein 52	NI	Shared_G5
EgrG_000881700.1..pep|elongator complex protein 1	NI	C5
EgrG_000882300.1..pep|gelsolin	NI	B5xC5
EgrG_000887700.1..pep|ribosomal protein L40	NI	B5
EgrG_000894900.1..pep|Immunoglobulin	NI	A5
EgrG_000901300.1..pep|ATP binding cassette subfamily B	NI	B5
EgrG_000904900.1..pep|pre mRNA splicing factor	NI	B5
EgrG_000905700.1..pep|cytochrome B5	NI	C5
EgrG_000906000.1..pep|Histone H1 delta	NI	B5xC5
EgrG_000907600.1..pep|regulator of microtubule dynamics protein	NI	C5
EgrG_000909900.1..pep|stress 70 protein mitochondrial	NI	B5xC5

EgrG_000918550.1..pep|Heat shock 70 kDa protein 4	NI	B5xC5
EgrG_000920400.1..pep|CDC37 cell division cycle 37	NI	C5
EgrG_000932900.1..pep|Sedlin	NI	A5
EgrG_000943900.1..pep|phosphoglucomutase	NI	B5xC5
EgrG_000946400.1..pep|RNA polymerase I associated factor A49	NI	C5
EgrG_000946900.1..pep|dnl2 protein	NI	C5
EgrG_000958100.1..pep|tropomyosin	NI	C5
EgrG_000968600.1..pep|splicing factor 3b subunit 1	NI	B5xC5
EgrG_000973500.1..pep|lamin dm0	NI	B5
EgrG_000980900.1..pep|ubinuclein:yemanuclein	NI	B5
EgrG_000981300.1..pep|insulin growth factor 1 receptor beta	NI	C5
EgrG_000988200.1..pep|integrator complex subunit 11	NI	B5
EgrG_000993000.1..pep|histone lysine N methyltransferase MLL3	NI	A5
EgrG_000999000.1..pep|transmembrane protein C6orf70	NI	B5xC5
EgrG_000999800.1..pep|ES1 protein mitochondrial	NI	C5
EgrG_001000600.1..pep|membrane associated phosphatidylinositol	NI	B5
EgrG_001001300.1..pep|Calcium binding EF hand domain	NI	C5
EgrG_001002600.1..pep|ATP dependent DNA helicase Q4	NI	A5
EgrG_001009600.1..pep|coiled coil domain containing protein 34	NI	C5
EgrG_001011000.1..pep|cytoplasmic polyadenylation element binding	NI	C5
EgrG_001013600.1..pep|sterol regulatory element binding protein 1	NI	C5
EgrG_001026700.1..pep|par cell polarity protein partition defective	NI	C5
EgrG_001028600.1..pep|dynein heavy chain	NI	B5xC5
EgrG_001036900.1..pep|protein of unknown function DUF602	NI	C5
EgrG_001042700.1..pep|transforming growth factor beta	NI	B5
EgrG_001045700.1..pep|tubulin polyglutamylase TTLL13	NI	C5
EgrG_001046200.1..pep|subfamily S1A unassigned peptidase	NI	B5
EgrG_001047400.1..pep|coiled coil domain containing 19	NI	B5
EgrG_001047900.1..pep|terminal uridylyltransferase 7	NI	B5xC5
EgrG_001052900.1..pep|ATP binding cassette sub family E	NI	C5
EgrG_001060400.1..pep|dynein light chain	NI	B5
EgrG_001075400.1..pep|acetylcholinesterase	NI	C5
EgrG_001075850.1..pep|nuclear pore complex protein Nup205	NI	C5
EgrG_001078100.1..pep|pyruvate kinase	NI	C5
EgrG_001085100.1..pep|heat shock protein 71 kDa protein	NI	C5
EgrG_001094800.1..pep|MACRO domain containing protein 2	NI	B5
EgrG_001096800.1..pep|testis expressed sequence 9 protein	NI	A5
EgrG_001099700.1..pep|tubulin gamma 1 chain	NI	B5xC5
EgrG_001101100.1..pep|glyceraldehyde 3 phosphate dehydrogenase	NI	C5

EgrG_001110400.1..pep|hypothetical protein	NI	B5
EgrG_001130600.1..pep|Vacuolar protein sorting associated protein	NI	C5
EgrG_001138900.1..pep|HIV Tat specific factor 1	NI	C5
EgrG_001149950.1..pep|Receptor type tyrosine protein phosphatase	NI	C5
EgrG_001151200.1..pep|multiple pdz domain protein	NI	A5
EgrG_001163200.1..pep|aldo keto reductase family 1 member B4	NI	B5xC5
EgrG_001166800.1..pep|26S proteasome regulatory subunit N11	NI	B5
EgrG_001172100.1..pep|WD40 repeat domain containing protein 83	NI	C5
EgrG_001176300.1..pep|hypothetical protein	NI	A5
EgrG_001181300.1..pep|serine:threonine protein kinase PAK 3	NI	B5xC5
EgrG_001195600.1..pep|ubiquitin	NI	A5xC5
EgrG_001198000.1..pep|GMP synthase glutamine hydrolyzing	NI	B5
EgrG_001200800.1..pep|Vam6:Vps39 protein	NI	C5
EgrG_002052600.1..pep|heat shock protein 70	NI	Shared_G5
EgrG_002053700.1..pep|hypothetical protein	NI	C5
EgrG_002066900.1..pep|aldo keto reductase family 1, member B4	NI	C5
EmuJ_000002100.1..pep|expressed protein	NI	C5
EmuJ_000005400.1..pep|glutathione S transferase	NI	B5
EmuJ_000008800.1..pep|expressed conserved protein	NI	A5
EmuJ_000056650.1..pep|EGF region	NI	B5
EmuJ_000061600.1..pep|discoidin domain containing receptor 2	NI	C5
EmuJ_000076600.1..pep|ATPase family AAA domain	NI	B5xC5
EmuJ_000093100.1..pep|FMRFamide activated amiloride sensitive	NI	A5xB5
EmuJ_000112900.2..pep	NI	B5
EmuJ_000113500.1..pep|carbonyl reductase 1	NI	C5
EmuJ_000121600.1..pep|DnaJ (Hsp40) subfamily C member	NI	B5xC5
EmuJ_000134200.1..pep|ankyrin domain repeat containing protein	NI	C5
EmuJ_000137700.1..pep|transforming growth factor beta 1 induced	NI	C5
EmuJ_000137900.1..pep|voltage dependent calcium channel	NI	B5
EmuJ_000145600.1..pep|goliath e3 ubiquitin ligase	NI	C5
EmuJ_000156050.1..pep|aldo keto reductase	NI	C5
EmuJ_000156100.1..pep|aldo keto reductase family 1, member B4	NI	C5
EmuJ_000156200.1..pep|aldo keto reductase	NI	A5xC5
EmuJ_000163000.1..pep|immunoglobulin binding protein 1	NI	B5
EmuJ_000175500.1..pep|ubiquitin specific peptidase (c family)	NI	A5
EmuJ_000191600.1..pep|hypothetical protein	NI	C5
EmuJ_000196000.1..pep|expressed protein	NI	B5
EmuJ_000223900.1..pep|histone lysine N methyltransferase MLL5	NI	B5
EmuJ_000239850.1..pep|glucan (1,4 alpha), branching enzyme 1	NI	B5

EmuJ_000242700.1..pep|serine:threonine protein phosphatase 2A	NI	C5
EmuJ_000261200.1..pep	NI	B5
EmuJ_000276900.1..pep|Gap Pol polyprotein	NI	B5
EmuJ_000279100.1..pep|histone H2B	NI	A5
EmuJ_000280300.1..pep|homeobox protein	NI	B5
EmuJ_000292000.1..pep|heat shock protein	NI	C5
EmuJ_000303000.1..pep|expressed protein	NI	B5
EmuJ_000313500.1..pep	NI	A5
EmuJ_000322600.1..pep|expressed protein	NI	C5
EmuJ_000334550.1..pep|fk506 binding protein	NI	B5xC5
EmuJ_000339200.1..pep|conserved hypothetical protein	NI	C5
EmuJ_000346700.1..pep|microtubule associated protein 1S	NI	C5
EmuJ_000364900.1..pep|conserved hypothetical protein	NI	B5
EmuJ_000373400.1..pep	NI	B5
EmuJ_000375800.1..pep|Threonyl tRNA synthetase, C	NI	C5
EmuJ_000381500.1..pep|Tapeworm specific antigen B	NI	C5
EmuJ_000421800.1..pep|26S proteasome non ATPase	NI	B5
EmuJ_000476100.1..pep|down syndrome cell adhesion	NI	A5xC5
EmuJ_000523700.1..pep|ATP binding cassette sub family F	NI	B5
EmuJ_000537900.1..pep|expressed protein	NI	C5
EmuJ_000540300.1..pep|t complex associated testis	NI	B5
EmuJ_000573400.1..pep|ATPase, F1 V1 A1 complex	NI	B5
EmuJ_000584700.1..pep|tyrosine protein kinase Blk	NI	B5
EmuJ_000631100.1..pep|small g protein signaling modulator 3	NI	A5
EmuJ_000638400.1..pep|Ankyrin	NI	B5
EmuJ_000647100.1..pep|expressed protein	NI	C5
EmuJ_000649900.1..pep	NI	B5xC5
EmuJ_000674600.1..pep|guanine nucleotide binding protein	NI	C5
EmuJ_000675100.1..pep|aminoacylase 1	NI	B5
EmuJ_000683300.1..pep|expressed protein	NI	B5xC5
EmuJ_000685900.1..pep|glutathione S transferase	NI	C5
EmuJ_000698600.1..pep|inhibitor of apoptosis protein	NI	B5
EmuJ_000700900.1..pep|conserved hypothetical protein	NI	C5
EmuJ_000710100.1..pep|heat shock protein 71 kDa protein	NI	B5xC5
EmuJ_000733200.1..pep|heat shock protein 70b	NI	C5
EmuJ_000733500.1..pep|heat shock protein 70	NI	C5
EmuJ_000734700.1..pep|troponin i	NI	B5xC5
EmuJ_000753600.1..pep|Vesicle transport protein SEC20	NI	C5
EmuJ_000779500.1..pep|serine:threonine protein phosphatase PP1	NI	C5

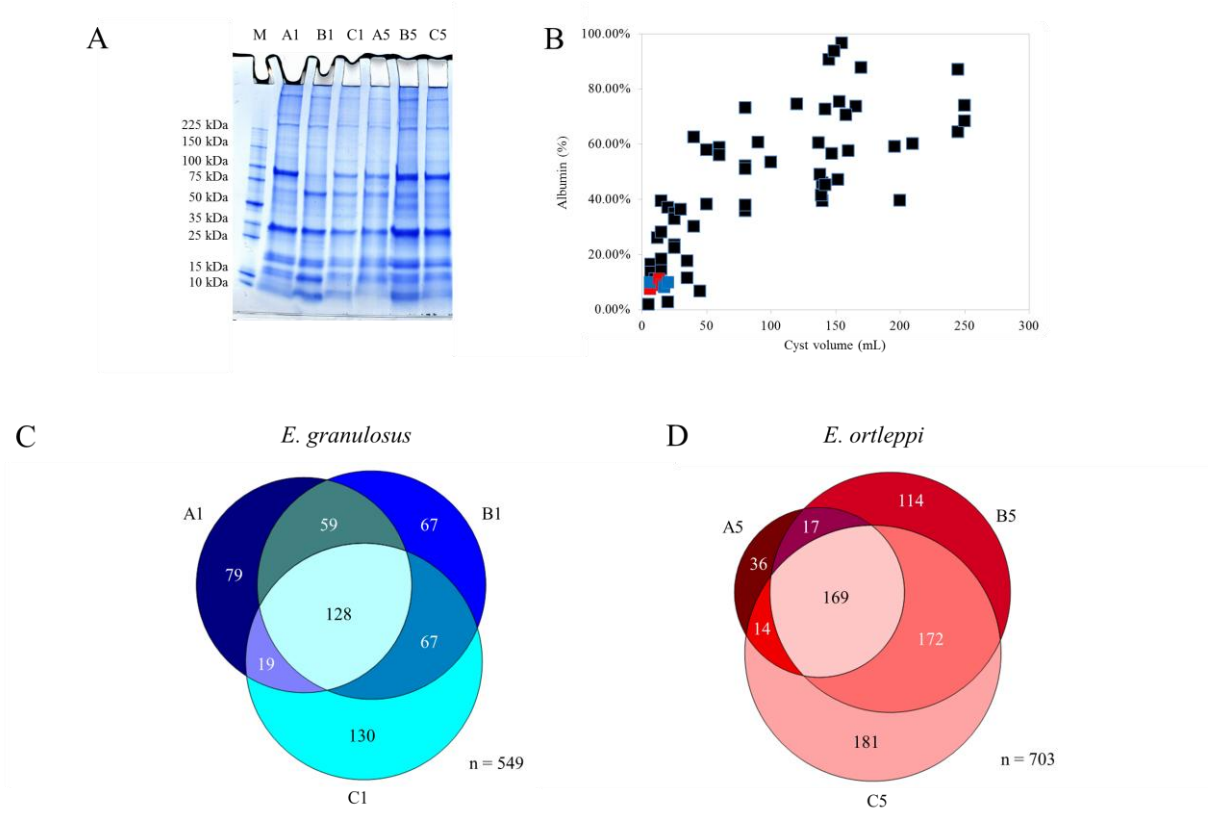
EmuJ_000810400.1..pep|expressed protein	NI	C5
EmuJ_000827600.1..pep|leucine rich repeat and fibronectin type III	NI	B5
EmuJ_000849100.1..pep|PHD and RING finger domain	NI	B5
EmuJ_000859700.1..pep|filamin	NI	C5
EmuJ_000881300.1..pep|coiled coil domain containing protein 41	NI	B5xC5
EmuJ_000890800.1..pep|FERM domain containing protein 3	NI	C5
EmuJ_000892900.1..pep|ATP dependent RNA helicase DHX34	NI	B5
EmuJ_000897500.1..pep|transmembrane emp24 domain	NI	A5
EmuJ_000899100.1..pep|histone lysine N methyltransferase MLL3	NI	C5
EmuJ_000917000.1..pep|Heat shock 70 kDa protein 4	NI	C5
EmuJ_000946700.1..pep|dynein light chain	NI	B5xC5
EmuJ_000954800.1..pep|ribosome biogenesis protein BMS1	NI	B5
EmuJ_000960400.1..pep|26S proteasome non ATPase regulatory	NI	B5xC5
EmuJ_001001400.1..pep|tegumental antigen	NI	C5
EmuJ_001002700.1..pep|protein disulfide isomerase A6	NI	B5
EmuJ_001028700.1..pep|BRCT	NI	C5
EmuJ_001033600.1..pep|rna polymerase II transcriptional coactivator	NI	B5
EmuJ_001115100.1..pep|ankyrin repeat containing	NI	B5xC5
EmuJ_001137100.1..pep|expressed protein	NI	C5
EmuJ_001139400.1..pep|hypothetical protein	NI	A5
EmuJ_001149700.1..pep|Receptor type tyrosine protein phosphatase	NI	C5
EmuJ_001153600.1..pep|Heat shock 70 kDa protein	NI	B5xC5
EgrG_000055100.1..pep|expressed protein	Shared_G1	C5
EgrG_000061200.1..pep|actin	Shared_G1	Shared_G5
EgrG_000061400.1..pep|agrin	Shared_G1	Shared_G5
EgrG_000067000.1..pep|disks large 1	Shared_G1	Shared_G5
EgrG_000079700.1..pep|expressed protein	Shared_G1	Shared_G5
EgrG_000085400.1..pep|Mastin	Shared_G1	B5xC5
EgrG_000085600.1..pep|hypothetical protein	Shared_G1	Shared_G5
EgrG_000086300.1..pep|fras1 related extracellular matrix protein	Shared_G1	B5xC5
EgrG_000088000.1..pep|expressed conserved protein	Shared_G1	B5xC5
EgrG_000088500.1..pep|Diacylglycerol kinase zeta	Shared_G1	B5xC5
EgrG_000097200.1..pep|expressed conserved protein	Shared_G1	B5xC5
EgrG_000117200.1..pep|cadherin	Shared_G1	B5xC5
EgrG_000122500.1..pep|protocadherin gamma a8	Shared_G1	B5xC5
EgrG_000143500.1..pep|lysosomal alpha glucosidase	Shared_G1	Shared_G5
EgrG_000144300.1..pep|Collagen alpha 1V chain	Shared_G1	Shared_G5
EgrG_000144350.1..pep|collagen alpha 1(IV) chain	Shared_G1	Shared_G5
EgrG_000144400.1..pep|abnormal EMBroygenesis family member	Shared_G1	Shared_G5

EgrG_000155600.1..pep|aldo keto reductase family 1 member B4	Shared_G1	Shared_G5
EgrG_000176400.1..pep|fras1 related extracellular matrix protein	Shared_G1	Shared_G5
EgrG_000184900.1..pep|glycoprotein Antigen 5	Shared_G1	Shared_G5
EgrG_000203400.1..pep|Collagen alpha 1V chain	Shared_G1	Shared_G5
EgrG_000217900.1..pep|lysyl oxidase	Shared_G1	Shared_G5
EgrG_000223300.1..pep|fibulin	Shared_G1	B5xC5
EgrG_000236300.1..pep|hypothetical protein	Shared_G1	B5xC5
EgrG_000241300.1..pep|glypican	Shared_G1	Shared_G5
EgrG_000254600.1..pep|glyceraldehyde 3 phosphate dehydrogenase	Shared_G1	Shared_G5
EgrG_000255800.1..pep|egf domain protein	Shared_G1	Shared_G5
EgrG_000264700.1..pep|hypothetical protein	Shared_G1	Shared_G5
EgrG_000292700.1..pep|phosphoenolpyruvate carboxykinase	Shared_G1	Shared_G5
EgrG_000302100.1..pep|expressed protein	Shared_G1	Shared_G5
EgrG_000302900.1..pep|expressed protein	Shared_G1	Shared_G5
EgrG_000316400.1..pep|expressed protein	Shared_G1	Shared_G5
EgrG_000317300.1..pep|vesicular amine transporter	Shared_G1	Shared_G5
EgrG_000318600.1..pep|Netrin receptor DCC	Shared_G1	B5xC5
EgrG_000340400.1..pep|hypothetical protein	Shared_G1	B5xC5
EgrG_000340500.1..pep|Alpha N acetylgalactosaminidase	Shared_G1	B5xC5
EgrG_000343000.1..pep|neurogenic locus notch protein	Shared_G1	Shared_G5
EgrG_000359000.1..pep|expressed conserved protein	Shared_G1	C5
EgrG_000373600.1..pep|notch	Shared_G1	Shared_G5
EgrG_000381200.1..pep|Tapeworm specific antigen B	Shared_G1	Shared_G5
EgrG_000381400.1..pep|Tapeworm specific antigen B	Shared_G1	Shared_G5
EgrG_000381600.1..pep|Tapeworm specific antigen B	Shared_G1	Shared_G5
EgrG_000381800.1..pep|Tapeworm specific antigen B	Shared_G1	B5xC5
EgrG_000412400.1..pep|expressed conserved protein	Shared_G1	Shared_G5
EgrG_000412500.1..pep|expressed conserved protein	Shared_G1	Shared_G5
EgrG_000413200.1..pep|tubulin alpha 1C chain	Shared_G1	B5xC5
EgrG_000415200.1..pep|expressed protein	Shared_G1	A5xC5
EgrG_000417100.1..pep|cytosolic malate dehydrogenase	Shared_G1	Shared_G5
EgrG_000417600.1..pep|collagen alpha 1IV chain	Shared_G1	Shared_G5
EgrG_000422350.1..pep|hemicentin 1	Shared_G1	Shared_G5
EgrG_000456150.1..pep|Lysosomal Pro X carboxypeptidase	Shared_G1	Shared_G5
EgrG_000458400.1..pep|Laminin subunit gamma	Shared_G1	Shared_G5
EgrG_000485000.1..pep|SLIT and NTRK protein 5	Shared_G1	Shared_G5
EgrG_000514200.1..pep|enolase	Shared_G1	Shared_G5
EgrG_000516500.1..pep|polyubiquitin	Shared_G1	Shared_G5
EgrG_000523100.1..pep|expressed conserved protein	Shared_G1	Shared_G5

EgrG_000524200.1..pep|collagen type XI alpha 2	Shared_G1	Shared_G5
EgrG_000530400.1..pep|amiloride sensitive amine oxidase	Shared_G1	Shared_G5
EgrG_000548500.1..pep|beta 13 n galactosyltransferase	Shared_G1	B5xC5
EgrG_000549500.1..pep|discoidin domain containing receptor 2	Shared_G1	B5xC5
EgrG_000549850.1..pep|fatty acid binding protein FABP2	Shared_G1	Shared_G5
EgrG_000570400.1..pep|protocadherin 1	Shared_G1	B5xC5
EgrG_000575900.1..pep|basement membrane specific heparan sulfate	Shared_G1	Shared_G5
EgrG_000596300.1..pep|expressed conserved protein	Shared_G1	Shared_G5
EgrG_000602100.1..pep|structural maintenance of chromosomes	Shared_G1	Shared_G5
EgrG_000618900.1..pep|fucosidase alpha L 1 tissue	Shared_G1	B5xC5
EgrG_000626300.1..pep|glucose 6 phosphate isomerase	Shared_G1	Shared_G5
EgrG_000640700.1..pep|Tolloid protein 1	Shared_G1	B5xC5
EgrG_000641100.1..pep|hypothetical protein	Shared_G1	Shared_G5
EgrG_000660800.1..pep|lactate dehydrogenase a	Shared_G1	Shared_G5
EgrG_000682900.1..pep|Niemann Pick C2 protein	Shared_G1	Shared_G5
EgrG_000684200.1..pep|Lipid transport protein N terminal	Shared_G1	Shared_G5
EgrG_000701800.1..pep|basement membrane specific heparan sulfate	Shared_G1	Shared_G5
EgrG_000704400.1..pep|lysosomal alpha mannosidase	Shared_G1	Shared_G5
EgrG_000712600.1..pep|gynecophoral canal protein	Shared_G1	Shared_G5
EgrG_000716600.1..pep|neutral alpha glucosidase AB	Shared_G1	Shared_G5
EgrG_000729300.1..pep|collagen alpha 1(XV) chain	Shared_G1	Shared_G5
EgrG_000733100.1..pep|prosaposin a preproprotein	Shared_G1	Shared_G5
EgrG_000733600.1..pep|peroxidasin	Shared_G1	B5xC5
EgrG_000766600.1..pep|peptidase inhibitor 16	Shared_G1	Shared_G5
EgrG_000789900.1..pep|beta mannosidase	Shared_G1	Shared_G5
EgrG_000790200.1..pep|cathepsin b	Shared_G1	Shared_G5
EgrG_000791700.1..pep|thioredoxin peroxidase	Shared_G1	Shared_G5
EgrG_000796300.1..pep|Coagulation factor 5 8 type C terminal	Shared_G1	Shared_G5
EgrG_000806300.1..pep|gamma glutamyltranspeptidase 1	Shared_G1	Shared_G5
EgrG_000823800.1..pep|collagen alpha 2I chain	Shared_G1	Shared_G5
EgrG_000824000.1..pep|Estrogen regulated protein EP45	Shared_G1	Shared_G5
EgrG_000824400.1..pep|gynecophoral canal protein	Shared_G1	Shared_G5
EgrG_000828400.1..pep|metal transporter cnnm2	Shared_G1	Shared_G5
EgrG_000842900.1..pep|fgfr protein	Shared_G1	Shared_G5
EgrG_000849600.1..pep|proteinase inhibitor I25 cystatin	Shared_G1	Shared_G5
EgrG_000875100.1..pep|calnexin	Shared_G1	Shared_G5
EgrG_000879900.1..pep|beta D xylosidase 2	Shared_G1	Shared_G5
EgrG_000880000.1..pep|laminin subunit beta	Shared_G1	Shared_G5
EgrG_000901900.1..pep|beta hexosaminidase subunit alpha	Shared_G1	Shared_G5

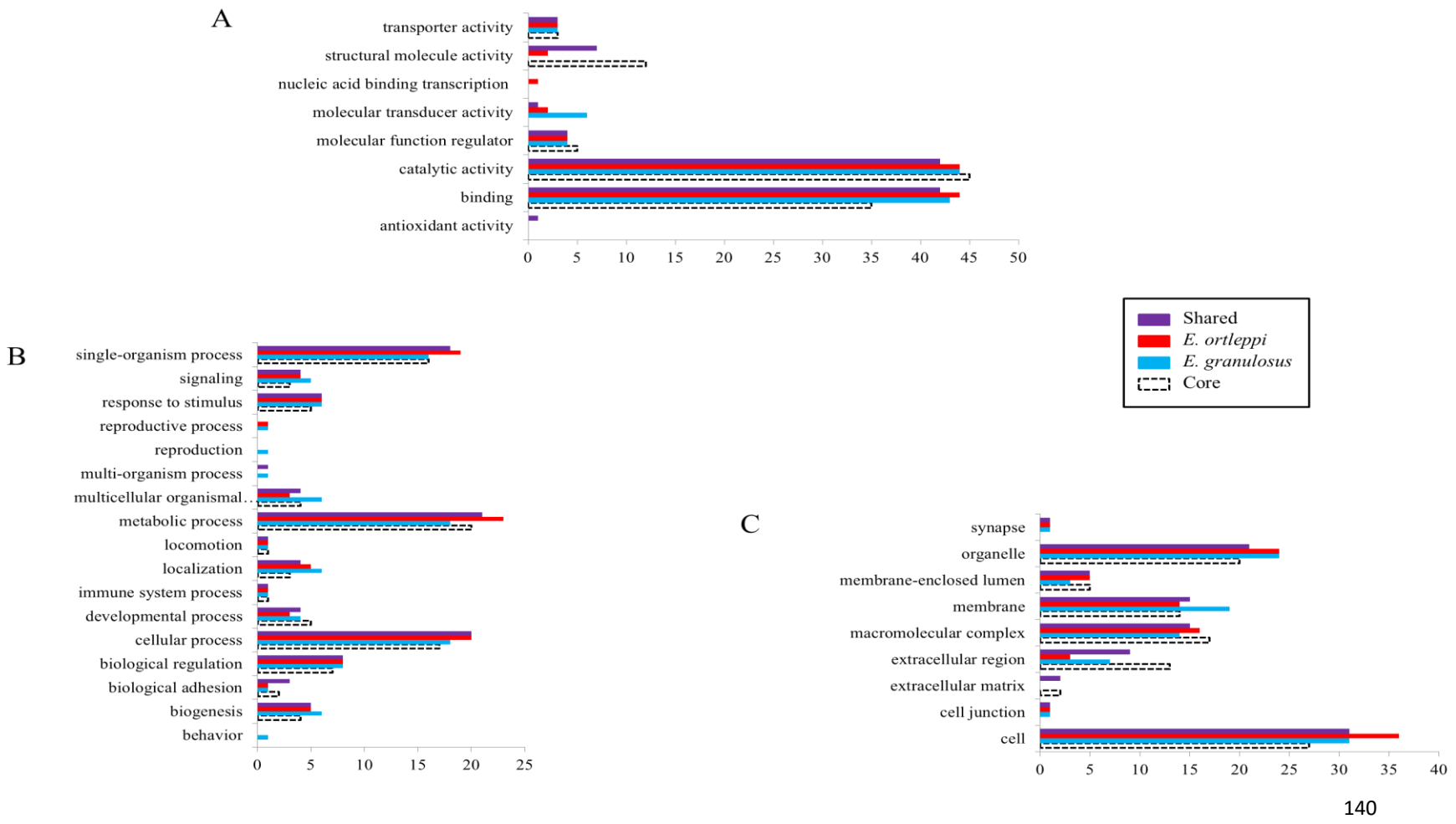
EgrG_000903100.1..pep|calsyntenin 1	Shared_G1	Shared_G5
EgrG_000905600.1..pep|fructose 16 bisphosphate aldolase	Shared_G1	Shared_G5
EgrG_000920600.1..pep|expressed protein	Shared_G1	Shared_G5
EgrG_000929500.1..pep|SPONdin extracellular matrix glycoprotein	Shared_G1	Shared_G5
EgrG_000937300.1..pep|Immunoglobulin	Shared_G1	B5xC5
EgrG_000968100.1..pep|syndecan	Shared_G1	Shared_G5
EgrG_000969100.1..pep|adamTS protein 3	Shared_G1	Shared_G5
EgrG_000970500.1..pep|cathepsin d lysosomal aspartyl protease	Shared_G1	Shared_G5
EgrG_000981200.1..pep|hypothetical protein	Shared_G1	Shared_G5
EgrG_000992700.1..pep|roundabout 2	Shared_G1	Shared_G5
EgrG_001032200.1..pep|ornithine aminotransferase	Shared_G1	Shared_G5
EgrG_001043100.1..pep|phosphoglycerate kinase 1	Shared_G1	Shared_G5
EgrG_001054000.1..pep|fibrillin 2	Shared_G1	B5xC5
EgrG_001058700.1..pep|hypothetical protein	Shared_G1	B5xC5
EgrG_001060600.1..pep|Type II collagen B	Shared_G1	Shared_G5
EgrG_001060700.1..pep|fibrillar collagen chain FAp1 alpha	Shared_G1	Shared_G5
EgrG_001061900.1..pep|expressed conserved protein	Shared_G1	Shared_G5
EgrG_001071800.1..pep|Zinc finger RanBP2 type	Shared_G1	B5xC5
EgrG_001132400.1..pep|laminin	Shared_G1	Shared_G5
EgrG_001132700.1..pep|polyu specific endoribonuclease	Shared_G1	Shared_G5
EgrG_001133400.1..pep|protein l isoaspartate o methyltransferase	Shared_G1	Shared_G5
EgrG_001180700.1..pep|protocadherin fat 1	Shared_G1	B5xC5
EgrG_001181950.1..pep|Papilin	Shared_G1	Shared_G5
EgrG_001190600.1..pep|collagen type i ii iii v xi alpha	Shared_G1	Shared_G5
EgrG_001192500.1..pep|14-3-3 protein	Shared_G1	Shared_G5
EgrG_001200300.1..pep|Desert hedgehog protein	Shared_G1	Shared_G5
EmuJ_000036300.1..pep|actin, cytoplasmic type 5	Shared_G1	Shared_G5
EmuJ_000139900.1..pep|abnormal EMBroygenesis family member	Shared_G1	A5xC5
EmuJ_000239200.1..pep|Type III secretion protein EspF	Shared_G1	Shared_G5
EmuJ_000407200.1..pep|Actin, cytoplasmic A3	Shared_G1	Shared_G5
EmuJ_000534800.1..pep|expressed protein	Shared_G1	Shared_G5
EmuJ_000538300.1..pep|glutathione S-transferase	Shared_G1	B5xC5
EmuJ_000653100.1..pep|calreticulin	Shared_G1	Shared_G5

Supplementary Figures



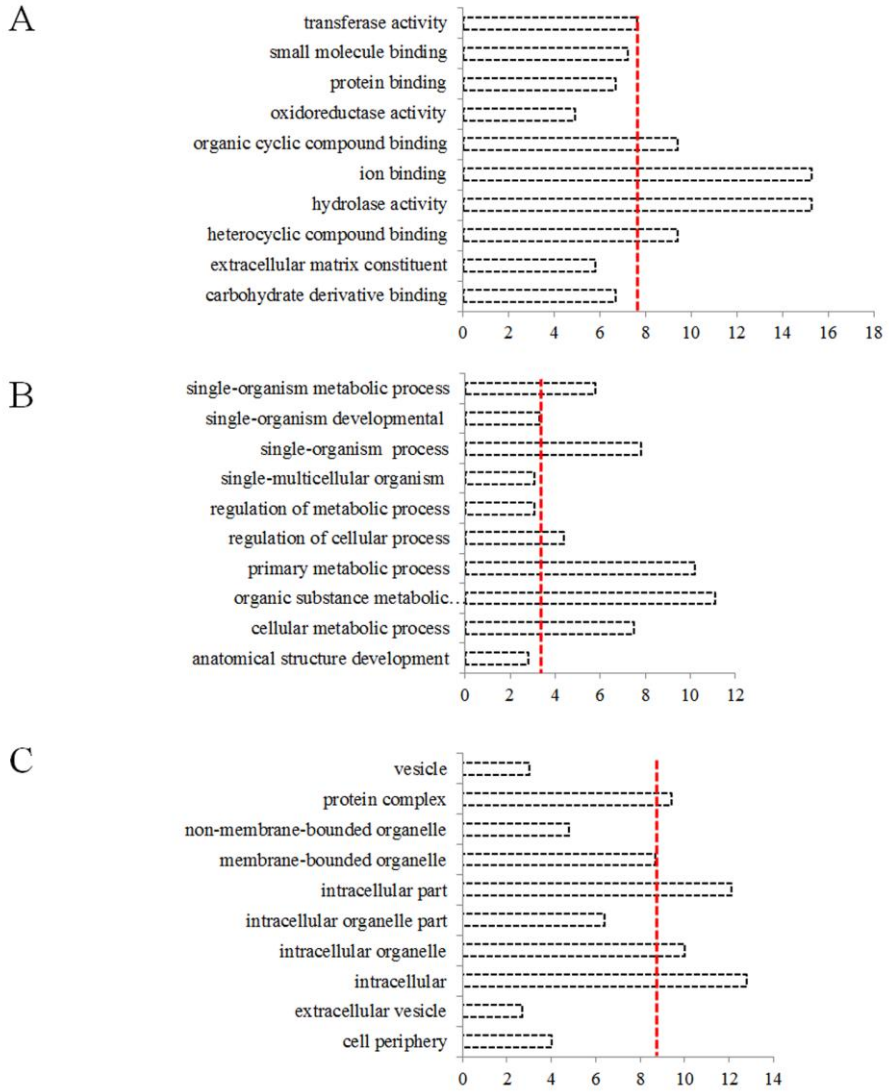
Supplementary figure 1. *E. granulosus* and *E. ortleppi* hydatid fluid protein comparison. (A) 50 µg of protein sample evaluated by 12% SDS-PAGE gel of hydatid fluid from tree *E. granulosus* (A1, B1 and C1) and *E. ortleppi* (A5, B5 and C5) samples. For each sample was possible to identify stained proteins from 10 kDa to 250 kDa. Marker is indicated on the left. (B) Correlation between cysts volume and intensity of bovine albumin band, using IMAGEJ to quantify band intensity and Spearman's rank correlation test to estimate correlation. After this, six HF samples were selected and subjected to the

LC-MS/MS runs in LTQ-Orbitrap Velos. The trap column (50 mm x 100 μm I.D) was packed with Jupiter C18 resin (10 μm, Phenomenex, Torrance, CA), and the analytical column (100 mm x 75 μm I.D.) was packed with ACQUA C18 resin (5 μm, Phenomenex). The sample was delivered to the trap column at 2 μL /min in 100% solvent A (0.1% formic acid [Sigma]). Solvent B consisted of 0.1% formic acid in acetonitrile. Gradient elution was performed as follows: 5-35%% solvent B over 60 min, 35-85% solvent B over 5 min, 85% solvent B for 2 min, followed by a return to the initial condition at a flowrate of 200 nL/min. The source was operated in positive-ionization mode, with the voltage and temperature adjusted to 1.8 kV and 200 °C, respectively. The mass spectrometer was programmed in data dependent acquisition mode, with a scanning mass of 300−1800 m/z using the LTQ-Orbitrap analyzer with a resolution of 30 000, followed by collision-induced dissociation (CIDusing the ion-trap analyzer) of the 10 most intense ions, with a dynamic exclusion time of 90 s. After, the Mascot Daemon v. 2.2.2 (Matrix Science) was used to peptide and protein identifications. (C) Venn diagram of the 549 proteins from *E. granulosus* identified in three samples. (D) Venn diagram of the 703 proteins from *E. ortleppi* identified in three samples.


A
transporter activity
structural molecule activity
nucleic acid binding transcription
molecular transducer activity
molecular function regulator
catalytic activity
binding
antioxidant activity
0 5 10 15 20 25 30 35 40 45 50
B
single-organism process
signaling
response to stimulus
reproductive process
reproduction
multi-organism process
multicellular organismal..
metabolic process
locomotion
localization
immune system process
developmental process
cellular process
biological regulation
biological adhesion
biogenesis
behavior
0 5 10 15 20 25
Shared
E. ortleppi
E. granulosus
Core
C
synapse
organelle
membrane-enclosed lumen
membrane
macromolecular complex
extracellular region
extracellular matrix
cell junction
cell
0 5 10 15 20 25 30 35 40

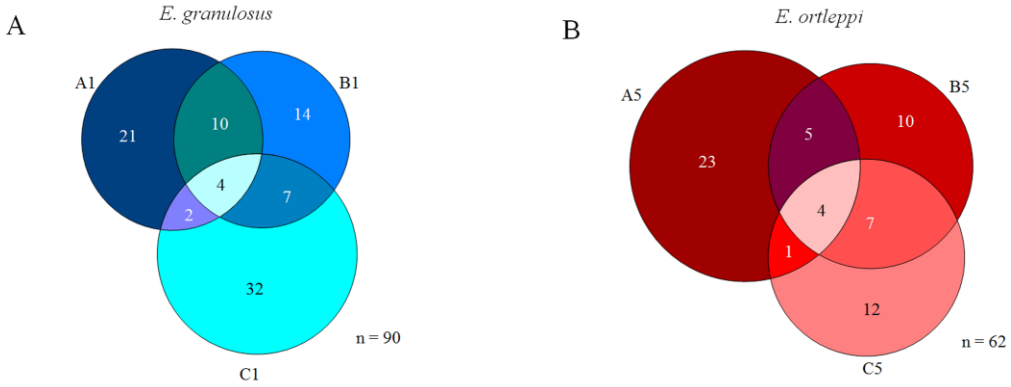
Supplementary figure 2. Gene ontology of shared, G1, G5 and core subsets.

Blast2GO was used to clarify the most relevant functional categories found in *E. granulosus* and *E. ortleppi* samples. To better illustrate similarities and differences we used the fallowing subsets: shared, exclusively found in *E.granulosus* (G1), exclusively found in *E. ortleppi* (G5) and core proteins. A total of 402 (48%) *E. granulosus* and 505 (60%) *E. ortleppi* proteins sequences were annotated with GO terms in three independent categories: biological processes, molecular functions, and cellular components. We normalized each category by the total number of GO terms. (A)In the molecular function category (level 2), binding (GO:0005488) and catalytic activity (GO:0003824) that were the two major categories considering each of the analyzed subsets. Considering the core subset at level 3, ion binding (GO:0043167), hydrolase activity (GO:0016787), were the major categories. (B) In biological processes (level 2), metabolic process (GO:0008152), cellular process (GO:0009987) and single-organism process (GO:0044699) were the three major categories.(C) In the Cellular Component analysis (level 2), cell (GO:0005623), membrane (GO:0016020) and Organelle (GO:0043226) were the three major categories. This data are consistent with previous GO analyses from HF studies (Aziz *et al.*, 2011; Santos *et al.*, 2016).



Supplementary figure 3. Gene ontology of the ten most abundant categories of core proteins at level 3. Proteins from core subset were annotated with GO terms, at level 3, in three independent categories: biological processes, molecular functions, and cellular components. We normalized each category by the total number of GO terms. (A) In the molecular functions five categories comprise 50% of the ontologies, which ion binding

(GO:0043167), hydrolase activity (GO:0016787) the major categories. (B) In the biological process, seven categories comprise 50% of the ontologies, which organic substance metabolic (GO:0071704) and primary metabolic process (GO:0044238) the major categories. (C) In the Cellular Component five categories comprise 50% of the ontologies, which intracellular (GO:0005622) and intracellular part (GO:0044424) the major categories. Interestingly extracellular vesicle (GO:1903561) appears among the ten most abundant functions looking at core subset at level 3. Red dash line indicates the cutoff to the 50% most abundant GO terms in each category.



Supplementary figure 4. *B. taurus* proteins identified in *E. granulosus* and *E. ortleppi* HF. Venn diagram of the 90 and 62 proteins from *B. Taurus* identified identified in *E. granulosus* (A) and *E. ortleppi* (B), respectively.

3. Discussão geral, conclusões e perspectivas

Nos últimos anos melhorias substanciais nos métodos de genotipagem mudaram as abordagens utilizadas para identificação das mais diferentes espécies. Este passo envolveu grandes avanços em bioinformática, e mesmo na automação dos métodos de genotipagem. Outro fator preponderante para tal melhoria são as bases de dados genômicos, as quais atualmente encontram-se em constante atualização e permitem avaliações rápidas e fáceis entre dados obtidos por distintos laboratórios, facilitando comparações fidedignas entre distintos grupos de pesquisa. Todos estes fatores juntos permitem que técnicas alternativas ao sequenciamento de DNA sejam desenvolvidas e empregadas com maior eficiência e precisão auxiliando na identificação e diferenciação de espécies.

O DNA mitocondroal é largamente utilizado para discriminação de organismos intimamente relacionados, devido a sua taxa de evolução relativamente rápida, se comparado à maioria dos genes nucleares em um dado organismo, e sua herança exclusivamente materna. O sequenciamento do gene cox1, utilizado desde 1992 nas análises moleculares com o intuito de identificar as variantes genotipicas existentes entre as espécies do gênero *Echinococcus*, tem sido empregado para a determinação de um grande número de espécies. Como resultado do sequenciamento em massa do fragmento de cox1 o GenBank, banco de dados de acesso público, possui atualmente mais de 214.000 sequências do gene, sendo a espécie *E. granulosus* a décima oitava com maior número de sequencias depositadas, totalizando 798 (05/04/16). Embora o gênero *Echinococcus* seja atualmente compreendido por dez espécies, o número de sequencias depositadas para as nove espécies restantes restringe-se a 319. Se levarmos em conta a existência de variações haplotípicas descritas para diferentes populações das espécies de *Echinococcus*,

visualizaremos um panorama dispendioso financeiramente, para um laboratório interessado em identificar corretamente o material com que trabalha rotineiramente. Além disso, outro agravante do sequenciamento de DNA é o tempo dispendido tanto para a preparação das amostras como para a identificação e diferenciação dos indivíduos de uma população.

Com o intuito de facilitar o processo de varredura de novos genótipos e a identificação dos já descritos, diferentes técnicas de genotipagem vem sendo empregadas, as quais diminuem os custos e o tempo associados à análise, devido à redução substancial (geralmente 70-90%) do número de amostras a serem sequenciadas (Jabbar & Gasser, 2013). A rotina de trabalho com amostras provenientes do cisto hidático inclui a identificação do genótipo e/ou espécie associado ao material coletado. Existem na fase larval deste parasito, duas frações biológicas das quais são possíveis à extração de DNA em quantidades suficientes para a identificação do parasito, os protoescólices e a camada germinativa (McManus, 2006; Balbinotti *et al.*, 2012). Este fator é um limitante para muitos estudos funcionais, os quais necessitam usar o material parasitário coletado para a obtenção de RNAs e proteínas. Uma alterntiva para tal problema deu-se com a experimentação e posterior emprego de uma rotina na qual a extração de DNA passou a ser realizada a partir de uma quantidade mínima de material biológico, 10-20 protoescólices (Haag *et al.*, 2004; Balbinotti *et al.*, 2012). O emprego desta metodologia levou a uma diminuição considerável do material utilizado para genotipagem, possibilitando a utilização do mesmo para extração de outras biomoléculas.

Atualmente o custo aproximado de uma reação de sequenciamento é de 20 reais, sendo que para a obtenção de uma sequência nucleotídica de boa qualidade e consequentemente confiável o suficiente para a identificação da espécie do parasito, são

necessários ao menos duas reações distintas de sequenciamento, direta e reversa, o que eleva o custo por amostra biológica a cerca de 40 reais. Mais que o valor agregado ao sequenciamento, o tempo empregado na marcação, purificação, injeção da amostra, além da análise computacional podem totalizar mais 48 horas de procedimento. Cada abordagem metodológica possui vantagens e desvantagens, sendo que uma ou mais abordagens para genotipagem podem ser utilizadas, dependendo do ambiente do laboratório, apoio financeiro disponível, e da disposição de material biológico. Com o intuito de diminuir os custos e principalmente o tempo empreendido em tal procedimento, a técnica de HRM surgiu como alternativa ao processo de amplificação seguido de sequenciamento.

Em estudo onde a alteração de uma única base foi sistematicamente investigada em fragmentos que variavam de 50 pb até 1000 pb com conteúdo GC entre 40-60% concluiu-se que para produtos de PCR de até 400 pb a sensibilidade e especificidade para detecção de polimorfismos de base única por HRM é de 100%. Já para produtos de PCR de 400 até 1000 pb a sensibilidade e especificidade ficaram respectivamente em 96 e 99% (Reed & Wittwer, 2004). Também é importante levar em consideração o uso do fluoróforo mais adequado, que forneça maior flexibilidade de uso, custo reduzido, e que permita a análise precisa das curvas de dissociação geradas a partir da separação dos produtos de PCR (Ririe *et al.*, 1997; Buh *et al.*, 2010). Apesar da grande variedade de fluoróforos comercialmente disponíveis, a imensa maioria das reações de PCR quantitativo em tempo real é realizada utilizando-se o corante SYBR Green. Isso se deve em grande parte ao fato do SYBR Green exibir um sinal fluorescente muito forte e, portanto facilmente detectável mesmo em baixas concentrações. Contudo já se mostrou que o mesmo é capaz de inibir a reação de PCR

além de diminuir a reprodutibilidade da mesma quando comparado a outros fluoróforos (Gudnason *et al.,* 2007).

A análise da forma da curva gerada pela desnaturação da molécula de DNA usando o corante SYBR Green é complicada, pois o mesmo é descrito por alterar a temperatura de desnaturação do DNA e por ser capaz de se redistribuir ao longo da molécula de dupla fita intacta à medida que as regiões de fita simples de DNA o despreendem (Giglio *et al.,* 2003; Varga & James, 2006). Tal característica não saturante da molécula em questão, aliado aos fatores supracitados, proporcionaram o surgimento de corantes alternativos, sendo muitos deles para uso específico na técnica de HRM. Dentre as várias moléculas atualmente disponíveis para o uso em HRM-PCR, destacam-se os corantes da família Syto (Gudnason *et al.,* 2007). Tais corantes são mais hidrofóbicos do que a grande maioria dos demais fluoróforos existentes no mercado, além de ligarem-se preferencialmente na região do sulco menor da molécula de dupla fita de DNA. Dentro desta família de corantes existem variações em relação, ao custo e excitação de cada molécula, na emissão dos espectros, e na seletividade e afinidade de ligação ao DNA. Os corantes Syto 13 e 16 são considerados os melhores corantes no que concerne à ausência de inibição da reação de PCR e efeitos na desnaturação da dupla fita de DNA (Eischeid, 2011). Como mencionado acima o método de HRM é útil, reproduzível, e vantajoso finaceiramente, acrescentando o valor de 1 (um) real por reação de PCR (Li *et al.,* 2010). Desde que respeitado suas limitações, a técnica de HRM pode ser rotineiramente empregada em laboratórios que trabalhem com genotipgem, especialmente em áreas onde duas ou mais espécies do coexistam.

No presente trabalho a análise do fragmento de 444 pb do cox1 permitiu a distinção entre seis espécies do gênero *Echinococcus*, *E. granulosus*, *E. ortleppi*, *E. multilocularis*,

E. canadensis, *E. vogeli* e *E. oligarthra* além da espécie *T. hydatigena* (Santos *et al.*, 2013). A utilização de uma espécie do gênero *Taenia* levanta a possibilidade da utilização do marcador mitocondrial da técnica de HRM para os demais membros da família Taenidae. Além disso, os resultados levantados abrem possibilidade para o uso da técnica como ferramenta em estudos de identificação de polimorfismos e como metodologia alternativa para genotipagem de indivíduos em estudos de genética de população em larga escala, substituindo assim metodologias como RFLP e SSCP.

Atualmente o artigo apresentado neste trabalho, Santos *et al*. (2013), possui seis citações. Safa *et al.* (2015) usaram uma região do gene cox1 contendo um único polimorfismo e com sucesso empregaram a técnica de HRM para distinguir as espécies *E. granulosus* e *E. canadensis* (G6 e G7). Os autores Sady *et al.* (2015), que diferenciaram as espécies *Schistosoma mansoni* e *Schistosoma haematobium*, e Wang *et al.* (2015), os quais utilizaram a técnica de HRM para detectar mutações em pacientes com osteogênese imperfeita, utilizaram a metodologia de HRM como ferramenta diagnóstica. Mais recentemente Dehgahni *et al.* (2016) utilizaram o marcador ITS1 e por HRM diferenciaram quatro espécies distintas de parasitos Taeniideos, *T. hydatigena*, *T. multiceps*, *T. ovis* e *E. granulosus*. Em suma a utilização da metodologia de HRM, vinculada ao marcador mitocondrial cox1, vem permitindo o uso mais racional do material biológico, o que possibilitou a seleção de amostras com pouco material para a realização, por exemplo, de experimentos de proteômica em larga escala (Santos *et al.*, 2016a,b).

Neste trabalho utilizamos dois conjuntos distintos de amostras de líquido hidático do parasito *Echinococcus* spp., ambos provenientes de cistos pequenos e com baixa quantidade de material biológico disponível, a fim de analisar possíveis mecanismos

moleculares envolvidos na infertilidade dos cistos e na adaptação de diferentes espécies ao hospedeiro bovino. Para tanto utilizamos uma abordagem proteômica, para identificação de proteínas do parasito e do hospedeiro, constituída do fracionamento das amostras por SCX, seguido de análise por LC-MS/MS. Todas as amostras utilizadas no presente trabalho pertenciam a cistos com pouca quantidade de albumina bovina, o que permitiu que as os experimentos fossem realizados sem a necessidade do uso de técnicas para depleção ou concentração de proteínas, permitindo a análise semi-quantitativa das amostras.

Dentre os resultados obtidos no presente trabalho destacamos: aumento no repertório conhecido de proteínas, do parasito *Echinococcus* spp. e do hospedeiro *B. taurus*, presentes no líquido hidático de cistos das espécies *E. granulosus* e *E. ortleppi*; identificação de um novo mecanismo de secreção de proteínas na fase larval de *E. granulosus*, envolvendo vesículas extracelulares; indícios da existência de uma 'corrida armamentista" entre parasito e hospedeiro coordenada pelas proteínas secretadas no líquido hidático de *Echinococcus* spp.; existência de uma grande número de proteínas com função não identificada ou com prováveis funções alternativas, as quais devem ser alvo de futuros estudos funcionais; identificação de um conjunto de proteínas presente em cistos férteis oriundos dos pulmões de bovinos; e a identificação de proteínas encontradas exclusivamente nas espécies *E. granulosus* e *E. ortleppi*.

A disponibilidade de genomas, transcritomas e proteomas vem fornecendo informações sem precedentes em relação aos parasitos do gênero *Echinococcus*. Em estudo recente Wang *et al.* (2015) identificaram, através da análise *in silico* do genoma de *E. multilocularis*, que 673 proteínas (6,4% do proteoma predito) são possivelmente secretadas pelo parasito. Entretanto, estudos proteômicos anteriores identificaram não mais que 44

proteínas do parasito *E. granulosus* no líquido hidático, mesmo utilizando ferramentas para depleção de proteínas abundantes do hospedeiro, como albumina (Monteiro *et al.*, 2010; Aziz *et al.*, 2011). No presente trabalho foi possível identificar um total de 204 proteínas parasitárias analisando oito frações (resultantes do fracionamento por troca catiônica forte) de cistos férteis e inférteis, e 842 proteínas analisando 30 frações relativas às espécies *E. granulosus* e *E. ortleppi*.

Com o intuito de diminuir ao máximo as interferências externas, aquelas as quais as amostras de cistos hidáticos coletadas de bovinos já possuem intrinsicamente, como origem, alimentação e idade do hospedeiro, decidiu-se coletar somente amostras de cistos oriundos de pulmão de bovino com volume semelhante e baixa quantidade de albumina. Após a coleta de mais de 600 amostras de cisto hidático, definiram-se aquelas a serem utilizadas no presente estudo, diferenciando-se apenas quanto à fertilidade e a espécie do parasito. Além disso, os experimentos não contaram com nenhum tipo de manipulação proteica posterior a coleta, como depleção de albumina, o que acabaria prejudicando a análise qualitativa e quantitativa das proteínas presentes no líquido hidático, devido à inespecificidade intrínseca apresentada por tal metodologia. Aziz *et al.* (2011) também analisaram amostras oriundas de líquido hidático de cistos férteis e inférteis e de distintos hospedeiros, todas pertencentes a espécie *E. granulosus*. Suas amostras eram constituídas de três amostras de cistos férteis de fígado de ovelha, duas amostras de cistos inférteis de fígado de bovino e três amostras de cistos férteis de fígado de humanos. A comparação entre as amostras de cistos férteis e inférteis ficou comprometida pois os cistos escolhidos eram oriundos de diferentes hospedeiros, que pode levar a interpretações errôneas a

respeito da infertilidade. Apenas a comparação entre cistos de diferentes hospedeiros, ovelha e humanos, pôde ser realizada com confiabilidade.

No presente trabalho, a partir da análise das proteínas identificadas e por microscopia eletrônica de transmissão sugerimos a existência de um novo modo de secreção utilizando vesículas extracelulares (microvesículas e exossomos) tanto pelo parasito *E. granulosus* quanto possivelmente pelo hospedeiro *B. taurus*. A produção e liberação de vesículas extracelulares é atualmente conhecida como uma característica universal de organismos tanto procariotos quanto eucariotos. Vesículas secretadas variam de acordo com sua biogênese, composição e tamanho, sendo divididas em diferentes grupos de acordo com essas características. Os dois principais grupos de vesículas extracelulares são os exossomos (<100 nm), que são formados a partir das vesículas intraluminais dos corpos multivesiculares, e as microvesículas (100 nm – 1 μm), que se originam na membrana plasmática (Silverman & Reiner, 2011; Deolindo *et al*., 2013). Vesículas extracelulares apresentam múltiplas funções biológicas e diversos estudos indicam que seu conteúdo é destinado à comunicação com outras células ou áreas vizinhas, sendo liberadas tanto de maneira fisiológica como em resposta a fatores externos (Deolindo *et al*., 2013).

Estudos recentes têm demonstrado que vesículas extracelulares possuem papel fundamental na relação parasito-hospedeiro, atuando na transferência de moléculas (proteínas, ácidos nucleicos, metabólitos) do parasito para as células do hospedeiro, e vice-versa (Valadi *et al.*, 2007; Novacki *et al.*, 2015). A liberação de vesículas também é uma via importante para secreção de proteínas, pois fornece a vantagem da proteção contra degradação por proteases do hospedeiro e a possibilidade de direcionamento para células

específicas. Em *Leishmania* spp., por exemplo, foi descrito que a liberação de exossomos contendo proteínas imunossupressoras, nas etapas iniciais da infecção, facilita o processo de invasão de macrófagos (Silverman & Reiner, 2012).

Em helmintos parasitas já foram identificadas vesículas extracelulares em três espécies da classe Trematoda, *Fasciola hepatica*, *Echinostoma caproni* e *Dicrocoelium dendriticum*. Marcilla *et al*. (2012) identificaram vesículas do tipo exossomo no tegumento e nos produtos de secreção de *F. hepatica* e *E. caproni*. Os mesmos autores também demonstraram que as vesículas de *E. caproni* são internalizadas por enterócitos em cultura, tipo celular com a qual o verme tem contato durante a infecção. Em exossomos de *D. dendriticum* foram identificados miRNAs com potencial utilização como biomarcadores para diagnóstico da infecção (Bernal *et al*., 2014).

A presença de diferentes moléculas de RNA em vesículas extracelulares parasitárias indica que essa classe de biomoléculas também tem papel importante na interação parasito-hospedeiro. Em *Trypanosoma cruzi* foi demonstrado que pequenos RNAs derivados de tRNA são transferidos para células de mamíferos suscetíveis (Garcia-Silva *et al.*, 2014a,b). Os pequenos RNAs e mRNAs transferidos, via vesículas, para as células do hospedeiro atuam regulando a produção de proteínas e a expressão gênica do hospedeiro em favor do parasito (Valadi *et al.*, 2007). Surpreendentemente, em *Leishmania* spp. e *F. hepatica* observou-se que as proteínas contidas nas vesículas secretadas correspondiam a mais da metade do total de proteínas secretadas, incluindo proteínas que não possuem sequência sinal conhecida de secreção, explicando assim a localização extracelular de tais proteínas (Marcilla *et al*., 2012; Silverman *et al*., 2010).

A contrapartida também é valida, pois recentemente muitos trabalhos têm observado como hospedeiros reagem à infecção ou contato com outros organismos incluindo parasitos por meio da liberação de vesículas extracelulares e exossomos (Ramachandra *et al.,* 2015; Schorey *et al.,* 2015). Baseados nos dados aqui descritos nós hipotetizamos que as vesículas extracelulares encontradas no líquido hidático de cistos inférteis provêm do hospedeiro e que as mesmas poderiam ser aqui explicadas, por terem duas origens distintas, contudo não exludentes.

Internamente o cisto hidático é recoberto pela camada germinativa a qual produz os protoescólices, e que possui como barreira protetora as células e/ou matriz extracelular do hospedeiro a camada laminar, uma capa acelular rica em carboidratos secretada pela prórpia camada germinativa (Díaz *et al.,* 2011a). A camada laminar possui uma espessura de aproximadamente 3 mm na espécie *E. granulosus* (Bortoletti & Ferreti, 1978), sendo considerada uma matriz extracelular especializada encontrada unicamente em parasitos do gênero *Echinococcus,* e evolutivamente designada a manter a integridade física do metacestódeo e proteger a camada germinativa da resposta imune gerada pelo hospedeiro.

Rupturas no cisto hidático durante seu estabelecimento e manutenção nos órgãos do hospedeiro ocorrem com frequência e é justamente a camada laminar que dificulta e muitas vezes impede a entrada de células e moléculas do hospedeiro através de microfissuras causadas pela pressão externa (Brunetti *et al.,* 2010). Embora a camada laminar seja elástica, o crescimento do cisto hidático em até seis ordens de grandeza não pode ser suportado apenas pela característica elasticidade; e, por conseguinte acredita-se que o afrouxamento mecânico e/ou químico da estrutura laminar da camada laminar deva ocorrer (Díaz *et al.,* 2011a). Tal afrouxamento acrescido ao fato da camada laminar permitir a

passagem de nutrientes e algumas proteínas, a caracterizam como uma malha elástica, hidrofílica, a qual permite a difusão de macromoléculas de até 150 kDa (Coltorii & Varela-Díaz, 1974; Díaz *et al.*, 2011b). Sendo assim, a primeira possível origem para a existência de vesículas extracelulares de *B. taurus* dentro dos cistos é a de que tais partículas e mesmo proteínas isoladas ou em complexos proteicos teriam a capacidade de transpassar a camada laminar, durante o crescimento do cisto, ou através de difusão através da malha laminar.

Conhecidamente, neutrófilos estão na linha de frente da defesa inata contra bactérias, atuam principalmente por fagocitose e digestão de bactérias invasoras. Entre as respostas recentemente descritas para neutrófilos estão as NETs, nas quais há liberação de DNA do neutrófilo associado a proteínas antimicrobianas (Brinkmann *et al.*, 2004). Os neutrófilos encontrados no sangue humano também são capazes de controlar o crescimento bacteriano através da liberação de vesículas extracelulares (Timár *et al.*, 2013). Os mesmos autores mostraram que vesículas extracelulares produzidas por neutrófilos encontrados no sangue humano têm tamanho e composição proteica própria quando os mesmos são estimulados pela presença de bactérias. Como resultado, tais autores verificaram que vesículas extracelulares possuem efeito bacteriostático, atuando na redução do crescimento e locomoção bacterianos. Além do efeito bacteriostático, existem indícios de que a secreção de vesículas extracelulares por neutrófilos tenha efeito antibacteriano, aumentando ainda mais a complexidade e diversidade de resposta à presença de bactérias promovida por neutrófilos. Neutrófilos são conhecidos por interagirem com parasitos invasores e por desempenharem papel fundamental na defesa do hospedeiro durante o estabelecimento de tais organismos (Falcone *et al.*, 2001). Interessantemente, neutrófilos já

foram encontrados no líquido hidático de cistos hidáticos coletados de humanos (Zhang *et al.*, 2003), além disso já foi demonstrado *in vitro* que neutrófilos em associação com anticorpos levam a morte da oncosfera e protoescólices de *E. granulosus* (Riley *et al.*, 1986; Rogan *et al.*, 1992). Uma segunda origem para a existência de vesículas extracelulares do *B. taurus* dentro do cisto hidático seria através da entrada de neutrófilos no cisto, os quais secretariam tais vesículas.

O líquido hidático de *E. granulosus* contém uma mistura complexa de produtos de excreção/secreção oriundos da camada germinativa, dos protoescólices, além de proteínas do hospedeiro, indicando uma intensa comunicação parasito-hospedeiro, na qual vesículas extracelulares poderiam ter um papel central (Santos *et al.*, 2016a). Trabalhos anteriores identificaram no líquido hidático e no sobrenadante de cultivo *in vitro* de protoescólices diversas proteínas que não apresentam sinal para exportação (Lorenzatto *et al*., 2012; Monteiro *et al*., 2010; Virginio *et al*., 2012), mas que já foram descritas em outros parasitos sendo secretadas por meio de vesículas, como por exemplo as proteínas aldolase, enolase e histonas. A produção de vesículas extracelulares e o seu conteúdo pode estar relacionado com a capacidade de estabelecimento e sobrevivência do cisto hidático através da atuação em processos básicos de evasão da resposta imune e captação de nutrientes. A caracterização das vesículas extracelulares poderá ajudar a identificar mecanismos moleculares que possam diferenciar espécies muito similares morfologicamente, mas com diferenças nas taxas de desenvolvimento, patogenicidade e especificidade pelo hospedeiro.

Uma proporção grande das proteínas identificadas tanto do parasito quanto do hospedeiro foi caracterizada como peptidases e inibidores de peptidase, o que já foi relatado para outros helmintos, inclusive para a espécie *T. solium* (Gomez *et al.*, 2015). As

proteases encontradas no líquido hidático tanto do parasito (*E. granulosus* e *E. ortleppi*) quanto do hospedeiro, em sua maioria, podem estar atuando na degradação de tecidos e proteínas do hospedeiro (Mckerrow *et al.,* 2006; Jex *et al.,* 2011; Basika *et al.,* 2012). Muitas das peptidases encontradas no líquido hidático também estão relacionadas ao remodelamento da matriz extracelular da camada germinativa e dos protoescólices, possivelmente devido à ação direta do hospedeiro (Margaryan *et al.,* 2010). Por sua vez, os inibidores de peptidase de *E.granulosus* encontrados no líquido hidático atuariam regulando negativamente a ação das peptidases do hospedeiro como já demostrado para helmintos e nematóides (Hartmann & Lucius, 2003; Knox, 2007), sugerindo sua atuação chave na interação com o hospedeiro. É possível que o inverso também seja verdadeiro, e que os inibidores de protease de *B. taurus* encontrados no líquido de cistos inférteis atuem inibindo as peptidases do parasito, como verificado, por exemplo, na relação carrapato-boi, onde inibidores de peptidase bovina atuam impedindo a ação de proteases do ectoparasito em questão (Tirlone *et al.,* 2014).

Neste trabalho também identificamos um grande grupo de proteínas do parasito das quais pouco se sabe, mas que possivelmente tenham relevante função na manutenção do cisto hidático já que estão presentes em grande abundância. As proteínas “secreted conserved proteins” e “ hypothetical proteins” perfazem cerca de 12 % de todo o proteoma predito tanto de *E. granulosus* quanto de *E. multilocularis* (Tsai *et al.,* 2013; Zheng *et al.,* 2013) e necessitam de atenção especial, pois vêm sendo frequentemente identificadas em estudos de expressão gênica (Pan *et al.,* 2014; Debarba *et al.,* 2015). Cada gene em um determinado organismo surge no genoma em determinado tempo durante o processo evolutivo. Neste contexto novos genes são definidos como aqueles que surgem em uma

espécie ou gênero em determinado tempo evolutivo em um locus que até então não existia, e que muitas vezes não possui ortólogos (Chen *et al.,* 2013).

A maioria das espécies do gênero *Echinococcus* possui pouca ou nenhuma estruturação populacional, à exceção da espécie *E. vogeli* (Santos *et al.,* 2012), o que deve-se provavelmente à origem recente do gênero associado a sua rápida expansão que ocorreu logo após o início do processo de domesticação e troca de animais, por volta de 15 mil anos atrás (Nakao *et al.,* 2010; Casulli *et al.,* 2012). Tais processos influenciam diretamente a composição gênica das espécies e podem levar à origem e evolução de novos genes ou à origem de novas funções moleculares e celulares a genes ancestrais. Tais fatores são também determinantes na diversificação entre as espécies no que diz respeito ao nível de expressão de genes (Brobey *et al.,* 2006; Davis *et al.,* 2009), como o que encontramos para a espécie *E. ortleppi* (Santos *et al.,* 2016b). Entender tais mecanismos através do estudo de genes não caracterizados ou investigando novas funções gênicas, é importante para a compreensão da produção da diversidade fenotípica paraistária e consequentemente para o melhor entendimento da relação parasito-hospedeiro (Zhou & Wang, 2008).

Uma imensa parte dos organismos vivos, durante distintos momentos do processo evolutivo, foram evolutivamente selecionados a parasitar outros organismos, aqueles que não seguem tal caminho estiveram ou permanecem sob intenso ataque de parasitos. Tais invasões ocorrem mesmo entre organismos de distintos reinos, tanto de forma intra como extracelular. Assim, o parasitismo tem favorecido e influenciado a especiação dos organismos hospedeiros, além de favorecer a origem de novas funções biológicas através de uma corrida armamentista que teve início a centenas de milhares de anos, que se

perpetua e certamente se manterá ativa enquanto houver organismos vivos (Dawkins & Krebs, 1979).

A partir da identificação de um amplo conjunto proteico tanto do parasito quanto do hospedeiro, da confirmação da existência de vesículas extracelulares, da existência de um grande número de proteínas com função desconhecida, da existência de proteínas espécie-especifica, de um conjunto proteico presente no líquido hidático de cistos férteis de *E. granulosus* e *E. ortleppi*, e principalmente baseando-se no fato de que as amostras utilizadas no presente trabalho são todas oriundas de líquido hidático de cistos de pulmão bovino com baixa quantidade de proteínas séricas, fica evidente a alta complexidade proteica que as espécies do gênero *Echinococcus* e seus respectivos hospedeiro dispõem para seu enfrentamento. Baseado na discussão e conclusão aqui apresentadas abrem-se algumas possibilidades para a continuidade dos estudos da relação *Echinococcus*-hospedeiro. Seguem possíveis perspectivas sobre tal assunto: Seria o perfil proteico do líquido hidático, tanto do parasito quanto do hospedeiro, distinto dependendo do órgão do hospedeiro em que se encontram? Seriam as proteínas secretadas pelo parasito *E. granulosus* distintas de acordo com o hospedeiro, ovelha e bovino? Qual a composição proteica parasitária de cistos férteis, porém com grande quantidade de proteínas séricas? As vesículas extracelulares encontradas no líquido hidático são do parasito, do hospedeiro, de ambos? Qual seria a proporção (parasito versus hospedeiro) de tais estruturas nas mais variadas situações em que o liquido hidático se apresenta? Se as vesículas extracelulares encontradas, principalmente no líquido de cistos inférteis, forem oriundas do hospedeiro, qual seria (m) suas origens? As proteínas identificadas e anotadas como “expressed proteins” e “hypothetical proteins” possuem função relevante no contexto do líquido

hidático? As proteínas espécie-especifica identificadas neste trabalho poderiam ser utilizadas como marcadores moleculares na diferenciação entre espécies? Estas e outras perguntas permanecem sem resposta e merecem a devida atenção, pois podem contribuir para o aumento do conhecimento sobre a relação das espécies do gênero *Echinococcus* com seu hospedeiro e mesmo contribuir para entendimento das relações estabelecidas entre parasitos e hospedeiros de uma forma geral.

4. Referências

ALVAREZ-ROJAS, C. A.; ROMIG, T.; LIGHTOWLERS, M. W. *Echinococcus granulosus* sensu lato genotypes infecting humans-review of current knowledge. *Int J Parasitol*, 44(1): 9-18, 2014.

AZIZ, A.; ZHANG, W.; LI, J.; LOUKAS, A.; MCMANUS, D. P.; MULVENNA, J. Proteomic characterization of *Echinococcus granulosus* hydatid cyst fluid from sheep, cattle and humans. *J Proteomics*, 74(9): 1560-1572, 2011.

BADARACO, J. L.; AYALA, F. J.; BART, J. M.; GOTTSTEIN, B.; HAAG, K. L. Using mitochondrial and nuclear markers to evaluate the degree cohesion among *Echinococcus* populations. *Exp Parasitol*, 119(4): 453-459, 2008.

BALBINOTTI, H.; SANTOS, G. B.; BADARACO, J.; AREND, A. C.; GRAICHEN, D. Â.; HAAG, K. L. ZAHA, A. *Echinococcus ortleppi* (G5) and *Echinococcus granulosus* sensu stricto (G1) loads in cattle from Southern Brazil. *Vet Parasitol*, 188(3-4): 255-260, 2012.

BASIKA, T.; MUNOZ, N.; CASARAVILLA, C.; IRIGOÍN, F.; BATTHYÁNY, C.; BONILLA, M.; SALINAS, G.; PACHECO, J. P.; ROTH, J.; DURÁN, R.; DÍAZ, A. Phagocyte-specific S100 proteins in the local response to the *Echinococcus granulosus* larva. *Parasitology*, 139(2): 271-283, 2012.

BATTELI, G. Echinococcosis: costs, losses and social consequences of a neglected zoonosis. *Vet Res Commun*, 33: 47-52, 2009.

BERNAL, D.; TRELIS, M.; MONTANERS, S.; CANTALAPIEDRA, F.; GALINDO, A.; HACKENBERG, M.; MARCILLA, A.; Surface analysis of *Dicrocoelium dendriticum*. The molecular characterization of exosomes reveals the presence of miRNAs. *J Proteomics*, 105: 232-241, 2014.

BIAVA, M. F.; DAO, A.; FORTIER, B. Laboratory diagnosis of cystic hydatid disease. *World J Surg*, 25(1): 10-14, 2001.

BORTOLETTI, G. & FERRETI, G. Ultrastructural aspects of fertile and sterile cysts of *Echinococcus granulosus* developed in hosts of different species. *Int J Parasitol*, 8(6): 421-431, 1978.

BOWLES, J.; BLAIR, D.; MCMANUS, D. P. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Mol Biochem Parasitol*, 54(2): 165-173, 1992.

BOWLES, J.; MCMANUS, D. P. NADH dehydrogenase 1 gene sequences compared for species and strains of the genus *Echinococcus*. *Int J Parasitol*, 23(7): 969-972, 1993.

BOWLES, J.; BLAIR, D.; MCMANUS, D. P. Molecular genetic characterization of the cervid strain (northern form) of *Echinococcus granulosus*. *Parasitology*, 109(Pt 2): 215-221, 1994.

BRINKMANN, V.; REICHARD, U.; GOOSMANN, C.; FAULER, B.; UHLEMANN, Y.; WEISS, D. S.; WEINRAUCH, Y.; ZYCHLINSKY, A. Neutrophil extracellular traps kill bacteria. *Science*, 303(5663: 1532-1535, 2004.

BROBEY, R. K.; MEI, F. C.; CHENG, X.; SOONG, L. Comparative two-dimensional gel electrophoresis maps for promastigotes of *Leishmania amazonensis* and *Leishmania major*. *Braz J Infect Dis*, 10(1): 1-6, 2006.

BRUNETTI, E.; KERN, P.; VUITTON, D. A. Expert consensus for the diagnosis and treatment of cyst and alveolar echinococcosis in humans. *Acta Trop*, 114(1): 1-16, 2010.

BUDKE, C. M.; DEPLAZES, P.; TORGERSON, P. R. Global socioeconomic impact of cystic echinococcosis. *Emerg Infect Dis*, 12(2): 296-303, 2006.

BUH GASPARIC, M.; TENGS, T.; LA PAZ, J. L.; HOLST-JENSEN, A.; PLA, M.; ESTEVE, T.; ZEL, J.; GRUDEN, K. Comparision of nine diferente real-time PCR chemistries for qualitative nd quantitative applications in GMO detection. *Anal Bioanal Chem*, 396(6): 2023-2029, 2010.

CANCELA, M.; SANTOS, G. B.; CARMONA, C.; FERREIRA, H. B.; TORT, J. F.; ZAHA, A. *Fasciola hepatica* mucin-encoding gene: expression., variability and its potencial relevance in host-parasite relationshiop. *Parasitology*, 142(14): 1673-1681, 2015.

CASULLI, A.; INTERISANO, M.; SRETER, T.; CHITIMIA, L.; KIRKOVA, Z.; LA ROSA, G.; POZIO, E. Genetic variability of *Echinococcus granulosus* sensu stricto in Europe inferred by mitochondrial DNA sequence. *Infect Genet Evol*, 12(2): 377-383, 2012.

CHEMALE, G.; HAAG, K. L.; ZAHA, A. *Echinococcus granulosus* antigen B is encoded by a gene family. *Mol Biochem Parasitol*, 116(2): 233-237, 2001.

CHEMALE, G.; VAN ROSSUM, A. J.; JEFFERIES, J. R.; BARRET, J.; BROPHY, P. M.; FERREIRA, H. B.; ZAHA, A. Proteomics analysis of the larval stage of the parasite *Echinococcus granulosus*: causative agent of cystic hydatid disease. *Proteomics*, 3(8): 1633-1636, 2010.

CHEN, S.; KRINSKY, B. H.; LONG, M. New genes drivers of phenotypic evolution. *Nat Rev Genet*, 14(9): 645-660, 2013.

CHUA, K. H.; LIM, S. C.; NG, C. C.; LEE, P. C.; LIM, Y. A.; LAU, T. P.; CHAI, H. C. Development of high-resolution melting analysis for the diagnosis of human Malaria. *Sci Rep*, 28(5): 15671, 2015.

COHEN, H.; PAOLILLO, E.; BONIFACINO, R.; BOTTA, B.; PARADA, L.; CABRERA, P.; SNOWDEN, K.; GASSER, R.; TESSIER, R.; DIBARBOURE, L.; WEN, H.; ALLAN, J. C.; SOTO DE ALFARO, H.; ROGAN, M. T.; CRAIG, P. S. Human cystic echinococcosis in a Uruguayan community: a sonographic, serologic, and epidemiologic study. *Am J Trop Med Hyg*, 59(4): 620-627, 1998.

COLTORII, E. A.; VALERA-DÍAZ, V. M. *Echinococcus granulosus*: penetration of macromolecules and their localization on the parasite membranes of cysts. *Exp Parasitol*, 35(2): 225-231, 1974.

COSTA, J. M.; CABARET, O.; MOUKOURY, S.; BRETAGNE, S. Genotyping of the protozoan pathogen *Toxoplasma gondii* using high-resolution melting analysis of the repeated B1 gene. *J Microbiol Methods*, 86(3): 357-363, 2011.

CUI, S. J.; XU, L. L.; ZHANG, T.; XU, M.; YAO, J.; FANG, C. Y.; FENG, Z.; YANG, P. Y.; HU, W.; LIU, F. Proteomic characterization of larval and adult developmental stages in *Echinococcus granulosus* reveals novel insight into host parasite interactions. *J Proteomics*, 84: 158-175, 2013.

DAWKINS, R & KREBS, J. R. Arms race between and within species. *Proc R Soc Lond B Biol Sci*, 205(1161): 489-511, 1979.

DAVIS, P. H.; CHEN, M.; ZHANG, X.; CLARK, C. G.; TOWNSEND, R. R.; STANLEY, S. L. Proteomic comparision of *Entamoeba histolytica* and *Entamoeba dispar* and role of *E. histolytica* alcohol dehydrogenase 3 in virulence. *PLoS Negl Trop Dis*, 3(4): e 415, 2009.

DEBARBA, J. A.; MONTEIRO, K. M.; MOURA, H.; BARR, J. R.; FERREIRA, H. B.; ZAHA, A. Identification of newly synthesized proteins by *Echinococcus granulosus* protoscoleces upon induction of strobilation. *PLoS Negl Trop Dis*, 9(9): e0004085, 2015.

DEHGHANI, M.; MOHAMMADI, M. A.; ROSTAMI, S.; SHAMSADDINS, S.; MIRBADIE, S. R.; HARANDI, M. F. High-resolution melting (HRM) for differentiation of four major Taeniidae species in dogs *Taenia hydatigena*, *Taenia multiceps*, *Taenia ovis*, and *Echinococcus granulosus* sensu stricto. *Parasitol Res*, Epub ahead of print, 2016.

DE LA RUE, M. L.; DINKEL, A.; MACKENSTEDT, U.; ROMIG, T. New data on *Echinococcus spp*. in Southern Brazil. *Rev Inst Med Trop São Paulo*, 48(2): 103-104, 2006.

DE LA RUE, M. L.; TAKANO, K.; BROCHADO, J. F.; COSTA, C. V.; SOARES, A. G.; YAMANO, K.; YAGI, K.; KATOH, Y.; TAKAHASHI, K. Infection of humans and animals with *Echinococcus granulosus* (G1 and G3 strains) and *E. ortleppi* in Southern Brazil. *Vet Parasitol*, 177(1-2): 97-103, 2011.

DEOLINDO, P.; EVANS-OSSES, I.; RAMIREZ, M. I. Microvesicles and exosomes as vehicles betwwen protozoan and host cell communication. *Biochem Soc Trans*, 41(1): 252-257, 2013.

DÍAZ, A.; CASARAVILLA, C.; IRIGOÍN, F.; LIN, G.; PREVIATO, J. O.; FERREIRA, F. Understanding the laminated layer of larval *Echinococcus* I: structure. *Trends Parasitol*, 27(5): 204-213, 2011a.

DÍAZ, A.; CASARAVILLA, C.; ALLEN, J. E.; SIM, R. B.; FERREIRA, A. M. Understanding the laminated layer of larval *Echinococcus* II: immunology. *Trends Parasitol*, 27(6): 264-273, 2011b.

DICKER, A. J.; INGLIS, N. F.; MANSON, E. D.; SUBHADRAS, S.; ILLANGOPATHY, M.; MUTHUSAMY, R.; KNOX, D. P. Proteomic analysis of *Mecistocirrus digitatus* and *Haemonchus contortus* intestinal protein extracts and subsequent efficacy testing in a vaccine trial. *PLoS Negl Trop Dis*, 8(6): e2909, 2014.

ECKERT, J.; THOMPSON, R. C.; BUCKLAR, H.; BILGER, B.; DEPLAZES, P. Efficacy evaluation of epsiprantel (Cestex) against *Echinococcus multilocularis* in dogs and cats. *Berl Munch Tierarztl Wochenschr*, 114(3-4): 121-126, 2001.

ECKERT, J. & DEPLEZES, P. Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. *Clin Microbiol Rev*, 17(1): 107-135, 2004.

EISCHEID, A. C. Syto dyes and EvaGreen outperform SYBRGreen in real-time PCR. *BMC Res Notes*, 4:263, 2011.

FALCONE, F. H.; ROSSI, A. G.; SHARKEY, R.; BROWN, A. P; PRITCHARD, D. I.; MAIZELS, R. M. *Ascaris suum*-derived products induce human neutrophil activation via a G protein-coupled receptor that intercts with the interleukin-8 receptor pathway. *Infect Immun*, 69(6): 4007-4018, 2001.

GARCIA-SILVA, M. R.; CABRERA-CABRERA, F.; DAS NEVES, R. F.; SOUTO-PADRÓN, T.; DE SOUZA, W.; CAYOTA, A. Gene expression changes induced by *Trypanosoma cruzi* microvesicles in mammalian host cells: relevance of tRNA-derived halves. *Biomed Res Int*, 2014: 305239, 2014a.

GARCIA-SILVA, M. R.; SANGUINETTI, J.; CABRERA-CABRERA, F.; FRANZÉN, O.; CAYOTA, A. A particular set of small non-coding RNAs is bound to the distinctive Argonaute protein of *Trypanosoma cruzi*: insights from RNA interference deficiente organisms. *Gene*, 538(2): 379-384, 2014b.

GASSER, R. B.; CHILTON, N. B. Characterisation of taeniid cestode species by PCR-RFLP of ITS2 ribosomal DNA. *Acta Trop*, 59(1): 31-40, 1995.

GASSER, R. B.; HU, M.; CHILTON, N. B.; CAMPBELL, B. E.; JEX, A. J.; OTRANTO, D.; CAFARCHIA, C.; BEVERIDGE, I.; ZHU, X. Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation. *Nat Protoc*, 1(6): 3121-3128, 2006.

GIGLIO, S.; MONIS, P. T.; SAINT, C. P. Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR. *Nucleic Acids Res*, 31(22): e136, 2003.

GOMEZ, S.; ADALID-PERALTA, L.; PALAFOX,-FONSECA, H.; CANTU-ROBLES, V. A.; SOBERÓN, X.; SCIUTTO, E.; FRAGOSO, G.; BOBES, R. J.; LACLETTE, J. P.; YAUNER, L. P.; OCHOA-LEYVA, A. Genome analysis of excretory/secretory proteins in *Taenia solium* reveals their abundance of antigenic regions (AAR). *Sci Rep*, 5: 9683, 2015.

GOTTSTEIN, B.; DEPLAZES, P.; ECKERT, J.; MULLER, B.; SCHOTT, E.; HELLE, O.; BOUJON, P.; WOLFF, K.; WANDELER A.; SCHWIETE, U. Serological (Em2-ELISA) and parasitological examinations of fox populations for *Echinococcus multilocularis* infections. *Zentralbl Veterinarmed B*, 38(3): 161-168, 1991.

GROSSO, G.; GRUTTADAURIA, S.; BIONDI, A.; MISTRETTA, A. Worldwide epidemiology of liver hydatidosis including the Mediterranean area. *World J Gastroenterol*, 18(13): 1425-1437, 2012.

GUARNERA E. A.; ZANZOTTERA E. M.; PEREYRA H.; FRANCO, A. J. Ultrasonographic diagnosis of ovine cystic echinococcosis. *Vet Radiol Ultrasound*, 42(4): 352-354, 2001.

GUARNERA E. A.; PARRA, A.; KAMENETZKY, L.; GARCÍA, G.; GUTIÉRREZ, A. Cystic echinococcosis in Argentina: evolution of metacestode and clinical expression. In various *Echinococcus granulosus* strains. *Acta Trop*, 92(2): 153-159, 2004.

GUDNASON, H.; DUFVA, M.; BANG, D. D.; WOLFF, A. Comparision of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. *Nucleic Acids Res*, 35(19): e127, 2007.

HAAG, K. L.; ZAHA, A.; ARAÚJO, A. M.; GOTTSTEIN, B. Reduced genetic variability within coding and non-coding regions of the *Echinococcus multilocularis* genome. *Parasitology*, 115(Pt 5): 521-529, 1997.

HAAG, K. L.; ALVES-JUNIOR, L; ZAHA, A.; AYALA< F. J. Contigent, non-neutral evolution in a multicellular parasite: natural selection and gene conversion in the *Echinococcus granulosus* antigen B gene family. *Gene*, 333: 157-167, 2004.

HARTMANN, S. & LUCIUS, R. Modulation of host immune responses by nematode cystatins. *Int J Parasitol*, 33(11): 1291-1302, 2003.

HUTTNER, M.; NAKAO, M.; WASSERMANN, T.; SIEFERT, L.; BOOMKER, J. D.; DINKEL, A.; SAKO, Y.; MACKENSTEDT, U.; ROMIG, T.; ITO, A. Genetic

characterization and phylogenetic position of *Echinococcus felids* (Cestoda: Taeniidae) from the African lion. *Int J Parasitol*, 38(7): 861-868, 2008.

JABBAR, A.; GASSER, R. B. Mutation scanning analysis of genetic variation within and among *Echinococcus* species implications and future prospects. *Electrophoresis*, 34(13): 1852-1862, 2013.

JEX, A. R.; LIU, S.; LI, B.; YOUNG, N. D.; HALL, R. S.; LI, Y.; YANG, L.; ZENG, N.; XU, X.; XIONG, Z.; CHEN, F.; WU, X.; ZHANG, G.; FANG, X.; KANG, Y.; ANDERSON, G. A.; HARRIS, T. W.; CAMPBELL, B. E.; VLAMINCK, J.; WANG, T.; CANTACESSI, C.; SCHWARZ, E. M.; RANGANATHAN, S.; GELDHOF, P.; NEJSUM, P.; STERNBERG, P. W.; YANG, J.; WANG, J.; GASSER, R. B. *Ascaris suum* draft genome. *Nature*, 479(7374): 529-533, 2011.

KERN, P.; WEN, H.; SATO, N.; VUITTON, D. A.; GRUENER, B.; SHAO, Y.; DELABROUSSE, E.; KRATZER, W.; BRESSON-HADNI, S. WHO classification of alveolar echinococcosis principles and application. *Parasitol Int*, 55: 283-287, 2006.

KNAPP, J.; NAKAO, M.; YANAGIDA, T.; OKAMOTO, M.; SAARMA, U.; LAVIKAINEN, A.; ITO, A. Phylogenetic relationships within *Echinococcus* and *Taenia* tapeworms (Cestoda: Taeniidae): inference from nuclear protein-coding genes. *Mol Phylogenet Evol*, 61 (3): 628-638, 2011.

KNOX, D. P. Proteinase inhibitors and helminth parasite infection. *Parasite Immunol*, 29(2): 57-71, 2007.

LAVIKAINEN, A.; LEHTINEN, M. J.; MERI, T.; HIRVELÃ-KOSKI, V.; MERI, S. Molecular genetic characterization of the Fennoscandian cervid strain, a new genotypic group (G10) of *Echinococcus granulosus*. *Parasitology*, 127(Pt 3): 207-215, 2003.

LI, Y.; XU, H.; CHEN, J.; GAN, W.; WU, W.; WU, W.; HU, X. Gene cloning, expression, and localization of antigen 5 in the life cycle of *Echinococcus granulosus*. *Parasitol Res*, 110(6): 2315-2323, 2012.

LYMBERY, A. J.; JENKINS, E. I.; SCHURER, J. M.; THOMPSON, R. C. *Echinococcus canadensis*, *E. borealis*, and *E. intermedius*. What's in a name? *Trends Parasitol*, 31(1): 23-29, 2015.

LOOS, J. A.; CUMINO, A. C. In vitro anti-Echinococcal and metabolic effects of metformin envolve activation of AMP-activated protein kinase in larval stages of *Echinococcus granulosus*. *PLos One*, 10(5): e0126009, 2015.

LORENZATTO, K. R.; MONTEIRO, K. M.; PAREDES, R.; PALUDO, G. P.; FONSÊCA, M. M.; GALANTI, N.; ZAHA, A.; FERREIRA, H. B. Fructose-bisphophate aldolase and enolase from *Echinococcus granulosus*: genes, expession, patterns and protein interactions of two potencial moonlighting proteins. *Gene*, 506(1): 76-84, 2012.

LORENZATTO, K. R.; KIM, K.; NTAI, I.; PALUDO, G. P.; CAMARGO DE LIMA, J.; THOMAS, P. M.; KELLER, N. L.; FERREIRA, H. B. Top down proteomics reveals mature proteoforms expressed in subcellular fractions of the *Echinococcus granulosus* preadult stage. *J Proteome Res*, 14(11): 4805-4814, 2015.

MANDAL, S.; MANDAL, M. D. Human cystic echinococcosis: epidemiologic, zoonotic, clinical, diagnostic and therapeutic aspects. *Asian Pac J Trop Med*, 5(4): 253-260, 2012.

MARCILLA, A.; TRELIS, M.; CORTÉS, A.; SOTILLO, J.; CANTALAPIEDRA, F.; MINGUEZ, M. T.; VALERO, M. L.; SÁNCHEZ DEL PINO, M. M.; MUÑOZ-ANTOLI, C.; TOLEDO, R.; BERNAL, D. Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. *PLoS One*, 7(9): e45974, 2012.

MARGARYAN, N. V.; KIRSCHMANN, D. A.; LIPAVSKY, A.; BAILEY, C. M.; HENDRIX, M. J.; KHALKHALI_ELLIS, Z. New insights into cathepsin D in mammary tissue development and remodeling. *Cancer Biol Ther*, 10(5): 457-466, 2010.

MAURELLI, M. P.; RINALDI, L.; CAPUANO, A. G.; PERUGINI, A. G.; CRINGOLI, G. Development of a real-time PCR for the differentiation of the G1 and G2/G3 genotypes of *Echinococcus granulosus*. *Parasitol Res*, 105(1): 255-259, 2009.

MCKERROW, J. H.; CAFFREY, C.; KELLY, B.; LOKE, P.; SAJID, M. Proteases in parasitic diseases. *Annu Rev Pathol*, 1: 497-536, 2006.

MCMANUS, D. P.; RISHI, A. K. Genetic heterogeneity within *Echinococcus granulosus*: isolates from different hosts and geographical areas characterized with DNA probes. *Parasitology*, 99(Pt 1): 17-29, 1989.

MCMANUS, D. P.; ZHANG, W.; LI. J. & BARTLEY, P. B. Echinococcosis. *Lancet*, 362 (9392): 1295-1304, 2003.

MCMANUS, D. P. Molecular discrimination of taeniid cestodes. *Parasitol Int*, 55: 31-37, 2006.

MONTEIRO, K. M.; CARVALHO, M. O.; ZAHA, A.; FERREIRA, H. B. Proteomic analysis of the *Echinococcus granulosus* metacestode during infection of its intermediate host. *Proteomics*, 10(10): 1985-1999, 2010.

MORO, P.; SCHANTZ, P. M. Echinococcosis: a review. *Int J Infect Dis*, 13(2): 125-133, 2009.

NAKAO, M.; MCMANUS, D. P.; SCHANTZ, P. M.; CRAIG, P. S.; ITO, A. A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. *Parasitology*, 134(Pt 5): 713-722, 2007.

NAKAO, M.; LI T,.; HAN, X.; MA, X.; XIAO, N.; QIU, J.; WANG, H.; YANAGIDA, T.; MAMUTI, W.; WEN, H.; MORO, P. L.; GIRAUDOUX, P. CRAIG, P. S.; ITO, A. Genetic polymorphisms of *Echinococcus* tapeworms in China as determined by mitochondrial and nuclear DNA sequences. *Int J Parasitol*, 40(3): 379-385, 2010.

NAKAO, M.; LAVIKAINEN, A.; YANAGIDA, T.; ITO, A. Phylogenetic systematics of the genus *Echinococcus* (Cestoda: Taeniidae). *Int J Parasitol*, 43(12-13): 1017-1029, 2013.

NAKAO, M.; LAVIKAINEN, A.; HOBERG, E. Is *Echinococcus intermedius* a valid species? *Trends Parasitol*, 31(8): 342-343, 2015.

NOWACKI, F. C.; SWAIN, M. T.; KLYCHNIKOV, O. I.; NIAZI, U.; IVENS, A.; QUINTANA, J. F.; HENSBERGEN, P. J.; HOKKE, C. H.; BUCK, A. H.; HOFFMANN,

K. F. Protein and small non-coding RNA-enriched extracellular vesicles are released by the pathogenic blood fluke *Schistosoma mansoni*. *J Extracell Vesicles*, 4: 28665, 2015.

ORITA, M.; IWAHANA, H.; KANAZAWA, H.; HAYASHI, K.; SEKIYA, T. Detection of plymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci USA*, 86(8): 2766-2770, 1989.

PAN, W.; SHEN, Y.; HAN, X.; WANG, Y.; LIU, H.; JIANG, Y.; ZHANG, Y.; WANG, Y.; XU, Y.; CAO, J. Transcriptome profiles of the protoscoleces of *Echinococcus granulosus* reveal that excretory-secretory products are essential to metabolic adaptation. *PLoS Negl Trop Dis*, 8(12): e3392.

PANGASA, A.; JEX, A. R.; CAMPBELL, B. E.; BOTT N.J.; WHIPP, M.; HOGG, G.; STEVENS, M. A.; GASSER, R. B. High-resolution melting curve (HRM) analysis for the diagnosis of cryptosporidiosis in humans. *Mol Cell Probes*, 23(1): 10-15, 2009.

RAMACHANDRARAO, S. P.; MATTHIAS, M. A.; KOKOY-MONDRAGON, C.; AGHANIA, E.; PARK, C.; KONG, C.; ISHAYA, M.; MADRIGAL, A. HORNG, J.; KHOSHABA, R.; BOUNKHOUN, A.; BASILICO, F.; DE PALMA, A.; AGRSTA, A. M.; AWDISHU, L.; NAVIAUX, R. K.; VINETZ, J. M.; MAURI, P. Proteomic analysis of urine exosomes reveals renal tubule response to leptospiral colonization in experimentally infected rats. *PLoS Negl Trop Dis*, 9(3): e0003640, 2015.

REED, G. H.; WITTWER, C. T. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clin Chem*, 50(10): 1748:1754, 2004.

REGEV-RUDZKI, N.; WILSON, D. W.; CARVALHO, T. G.; SISQUELLA, X.; COLEMAN, B. M.; RUG, M.; BURSAC, D.; ANGRISANO, F.; GEE, M.; HILL, A. F.; BAUM, J.; COWMAN, A. F. Cell-cell communication between malaria infected red blood cells via exosome-like vesicles. *Cell*, 153(5): 1120-1133, 2013.

RILEY, E. M.; DIXON, J. B.; JENKINS, P.; ROSS, G. *Echinococcus granulosus* infection in mice: host defense during primary and secondary infection. *Parasitology*, 92(Pt 2): 391-403, 1986.

RIRIE, K. M.; RASMUSSEN, R. P.; WITTWER, C. T. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem*, 245(2): 154-160, 1997.

ROGAN, M. T.; CRAIG, P. S.; ZEHYLE, E.; MASINDE, G.; WEN, H.; ZHOU, P. In vitro killing of taeniid oncospheres, mediated by human sera from hydatid endemic areas. *Acta Trop*, 51(3-4): 291-296, 1992.

ROGAN, M. T.; HAI, W. Y.; RICHARDSON, R.; ZEYHLE, E.; CRAIG, P. S. Hydatid cyst: does every picture tell a story? *Trends Parasitol*, 22(9): 431-438, 2006.

ROMIG, T.; DINKEL, A.; MACKENSTEDT, U. The present situation of echinococcosis in Europe. *Parasitol Int*, 55: 187-191, 2006.

SAARMA, U.; JÕGISALU, I.; MOKS, E.; VARCASIA, A.; LAVIKAINEN, A.; OKSANEN, A.; SIMSEK, S.; ANDRESIUK, V.; DENEGRI, G.; GONZÁLEZ, L. M.; FERRER, E.; GÁRATE, T.; RINALDI, L.; MARAVILLA, P. A novel phylogeny for the genus *Echinococcus*, based on nuclear data, challenges relationships based on mitochondrial evidence. *Parasitology*, 136(3): 317-328, 2009.

SADY, H.; AI-MEKHLAFI, H. M.; NGUI, R.; ATROOSH, W. M.; AI-DELAIMY, A. K.; NASR, N. A.; DAWAKI, S.; ABDULSALAM, A. M.; ITHOI, I.; LIM, Y. A.; CHUA, K. H.; SURIN, J. Detection of *Schistosoma mansoni* and *Schistosoma haematobium* by Real-Time PCR with high-resolution melting analysis. *Int Mol Sci*, 16(7): 16085-16103, 2015.

SAFA, A. H.; HARANDI, M. F.; TAJADDINI, M.; ROSTAMI-NEJAD, M.; MOHTASHAMI-POUR, M.; PESTEHCHIAN, N. Rapid identification of *Echinococcus granulosus* and *E. canadensis* using high-resolution melting (HRM) analysis by focusing on single nucleotide polymorphism. *Jpn J Infect Dis*, epub ahead of print, 2015.

SAKAI, H.; FURUSAWA, R.; OKU, Y.; KAMIYA, M. *Echinococcus multilocularis* coproantigen detection in golden hamster, an alternative definitive host. *Exp Anim*, 45(3): 275-278, 1996.

SANTOS, G. B.; SOARES, M. C.; BRITO, E. M.; RODRIGUES, A. L.; SIQUEIRA, N. G.; GOMES-GOUVÊA, M. S.; ALVES, M. M.; CARNEIRO, L. A.; MALHEIROS, A. P.; PÓVOA, M. M.; ZAHA, A.; HAAG, K. L. Mitochondrial and nuclear sequence polymorphisms reveal geographic structuring in Amazonian populations of *Echinococcus vogeli* (Cestoda: Taeniidae). *Int J Parasitol*, 42(13-14): 1115-1118, 2012.

SANTOS, G. B.; ESPÍNOLA, S. M.; FERREIRA, H. B.; MARGIS, R.; ZAHA, A. Rapid detection of *Echinococcus* species by a high-resolution melting (HRM) approach. *Parasit Vectors*, 6(1): 327, 2013.

SANTOS. G. B.; MONTEIRO, K. M.; SILVA, E. D.; BATTISTELLA, M. E.; FERREIRA, H. B.; ZAHA, A. Excretory/secretory products in *Echinococcus granulosus* metacestode: is the intermediate host complacent with infection caused by the parasite's larval form? *A ser submetido*, 2016a.

SANTOS, G. B.; KITANO, E.; BATTISTELLA, M. E.; MONTEIRO, K. M.; FERREIRA, H. B.; SERRANO, S. M. T.; ZAHA, A. The secreted protein signature of hydatis fluid from pulmonar hydatidosis. *A ser submetido*, 2016b.

SCHOREY, J. S.; CHENG, Y.; SINGH, P. P.; SMITH, V. L. Exosomes and other extracellular vesicles in host-pathogen interactions. *EMBO Rep*, 16(1): 24-43, 2015.

SCOTT, J. C.; STEFANIAK, J.; PAWLOWSKI, Z. S.; MCMANUS, D. P. Molecular genetic analysis of human cystic hydatid cases from Poland: identification of a new genotypic group (G9) of *Echinococcus granulosus*. *Parasitology*, 114(Pt 1): 37-43, 1997.

SHARBATKORI, M.; MIRHENDI, H.; JEX, A. R.; PANGASA, A.; CAMPBELL, B. E.; KIA, E. B.; ESHRAGHIAN,M. R.; HARANDI, M. F.; GASSER, R. B. Genetic categorization of *Echinococcus granulosus* from humans and herbivorous hosts in Iran using an integrated mutation scanning phylogenetic approach. *Electrophoresis*, 30(15): 2648-2655, 2009.

SILES-LUCAS, M.; CUESTA-BANDERA, C.; CÉSAR-BENITO, M. Random amplified plymorphic DNA technique for speciation studies of *Echinococcus granulosus*. *Parasitol Res*, 79(4): 343-345, 1993.

SILES-LUCAS, M.; MERLI, M.; MACKENSTEDT, U.; GOTTSTEIN, B. The *Echinococcus multilocularis* 14-3-3 protein pretects mice against primary but not secondary alveolar echinococcosis. *Vaccine*, 21(5-6): 431-439, 2003.

SILVERMAN, J. M.; CLOS, J.; HORAKOVA, E.; WANG, A. Y. WIESGIGL, M.; KELLY, I. LYNN, M. A.; MCMASTER W. R.; FOSTER, L. I.; LEVINGS, M. K.; REINER, N. E. *Leishmania* exosomes modulate innate and adaptive immune responses through effects on monocytes and dendritic cells. *J Immunol*, 185(9): 5011-5022, 2010.

SILVERMAN J. M. & REINER, N. E. Exosomes and other microvesicles in infection biology: organelles with unanticipated phenotypes. *Cell Microbiol*, 13(1): 1-9, 2011.

SILVERMAN, J. M. & REINER, N. E. *Leishmania* exosomes deliver preemptive strikes to create an environment permissive for early infection. *Front Cell Infect Microbiol*, 1: 26, 2012.

SIQUEIRA, N. G.; ALMEIDA, F. B.; SUZUKI, Y. A.; LIMA, R. N.; MACHADO-SILVA, J. R.; RODRIGUES-SILVA, R. Atypical polycystic echinococcosis without liver involvement in Brazilian patients. *Trans R Soc Trop Med Hyg*, 104(3): 230-233, 2010.

SIQUEIRA, N. G.; SIQUEIRA, C. M.; RODRIGUES-SILVA, R.; SOARES, M. C.; PÓVOA, M. M. Polycystic echinococcosis in the state of Acre, Brazil: contribution to

patient diagnosis, treatment and prognosis. *Mem Inst Oswaldo Cruz*, 108(5): 533-540, 2013.

SIRACUSANO, A.; RIGANÒ, R.; ORTONA, E.; PROFUMO, E.; MARGUTTI, P.; BUTTARI, B.; DELUNARDO, F.; TEGGI, A. Immunomodulatory mechanisms during *Echinococccus granulosus* infection. *Exp Parasitol*, 119(4): 483-489, 2008.

SIRACUSANO, A.; DELUNARDO, F.; TEGGI, A.; ORTONA, E. Host-parasite relationship in cystic echinococcosis: na evolving story. *Clin Dev Immunol*, 2012: 639362, 2012a.

SIRACUSANO, A.; DELUNARDO, F.; TEGGI, A.; ORTONA, E. Cystic echinococcosis: aspects of imune response, immunopathogenesis and imune evasion from the human host. *Endocr Metab Immune Disord Drug Targets*. 12(1): 16-23, 2012b.

SOARES, M. C.; MOREIRA-SILVA, C. A.; ALVES, M. M.; NUNES, H. M.; AMARAL, I. A.; MÓIA, L. J.; CONDE, S. R.; ALMEIDA, F. B.; RODRIGUES,-SILVA, R.; CRESCENTE, J. A. Polycystic echinococcosis in the Eastern brazilian amazona: an update. *Rev Soc Bras Med Trop*, 37: 75-83, 2004.

TAN, L.; YU, X.; ABDULLAHI, A. Y.; WU, S.; ZHENG, G.; HU, W.; SONG, M.; WANG, Z.; JIANG, B.; LI, G. Development of a rapid HRM genotyping method for detevtion of dog derived *Giardia lamblia*. *Parasitol Res*, 114(11): 4081-4086, 2015.

TEICHMANN, A.; VARGAS, D. M.; MONTEIRO, K. M.; MENEGHETTI, B. V.; DUTRA, C. S.; PAREDES, R.; GALANTI, N.; ZAHA, A.; FERREIRA, H. B.

Characterization of 14-3-3 isoforms expressed in the *Echinococcus granulosus* pathogenic larval stage. *J Proteome Res*, 14(4): 1700-1715, 2015.

THOMPSON, R. C. A. Biology and systematics of *Echinococcus*. In: Thompson, R. C. A. & Lymbery, A. J. *Echinococcus* and hydatid disease. Oxon, CAB International, p. 1-50, 1995.

THOMPSON, R. C.& LYMBERY, A. J. The nature, extent and significance of variation within the genus *Echinococcus*. *Adv Parasitol*, 27:209-258, 1988.

THOMPSON, R. C.; LYMBERY, A. J.; CONSTANTINE, C. C Variation in *Echinococcus*: towards a taxonomic revision of the genus. *Adv Parasitol*, 35: 145-176, 1995.

THOMPSON, R. C.; MCMANUS, D. P. Towards a taxonomic revision of the genus *Echinococcus* . *Trends Parasitol*, 18(10): 452-457, 2002.

THOMPSON, R. C. The taxonomic, phylogeny and transmission of *Echinococcus*. *Exp Parasitol*, 119(4): 439-446, 2008.

TIMÁR, C. I.; LORINCZ, A. M.; CSÉPÁNYI-KOMI, R.; VÁLYI-NAGY, G.; BUZÁS, E. I.; IVÁNYI, Z.; KITTEL, A.; POWELL, D. W.; MCLEISH, K. R.; LIGETI, E. Antibacterial effect of microvesicles released from human neutrophilic granulocytes. *Blood*, 121(3): 510-518, 2013.

TIRLONE, L.; RECK, J.; TERRA, R. M.; MARTINS, J. R.; MULENGA, A.; SHERMAN, N. E.; FOX, J. W.; YATES, J. R.; TERMIGNONI, C.; PINTO, A. F.; VAZ IDA, S.

Proteomic analysis of the cattle tick *Rhipicephalus (Boophilus) microplus* saliva: a comparision between partially and fully engorged females. *PLoSOne*, 9(4): e94831, 2014.

TSAI, I. J.; ZAROWIECKI, M.; HOLROVD, N.; GARCIARRUBIO, A.; SANCHEZ-FLORES, A.; BROOKS, K. L; TRACEY, A.; BOBES, R. J.; FRAGOSO, G.; SCIUTTO, E.; ASLETT, M.; BEASLEV, H.; BENNETT, H. M.; CAI, J.; CAMICIA, F.; CLARK, R.; CUCHER, M.; DE SILVA, N.; DAV, T. A.; *et al*. The genomes of four tapeworm species reveal adaptations to parasitism. *Nature*, 496(7443): 57-63, 2013.

VALADI, H.; EKSTROM, K.; BOSSIOS, A.; SJOSTRAND, M.; LEE, J. J.; LOTVALL, J. O. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic Exchange between cells. *Nat Cell Biol*, 9(6): 654-659, 2007.

VAN HERWERDEN L.; GASSER, R. B.; BLAIR, D. ITS-1 ribossomal DNA sequence variants are maintained in different species and strains of *Echinococcus*. *Int J Parasitol*, 30(2): 157-169, 2000.

VARGA, A. & JAMES, D. Real-time RT-PCR and SYBR Green I melting curve analysis for the identification of plum pox virus strains C, EA, and W: effect of amplicon size, melt rate, and dye translocation. *J Virol Methods*, 132(1-2): 146-153, 2006.

VIRGINIO, V. G.; TAROCO, L.; RAMOS, A. L.; FERREIRA, A. M.; ZAHA, A.; FERREIRA, H. B.; HERNÁNDEZ, A. Effects of protoscoleces and AgB from *Echinococcus granulosus* on human neutrophils: possible implications on the parasite´s immune evasion mechanisms. *Parasitol Res*, 100(5): 935-942, 2007.

VIRGINIO, V. G.; MONTEIRO, K. M.; DRUMOND, F.; CARVALHO, M. O.; VARGAS, D. M.; ZAHA, A.; FERREIRA, H. B. Excretory/secretory products from in vitro cultured *Echinococcus granulosus* protoscoleces. *Mol Biochem Parasitol*, 183(1): 15-22, 2012.

VUITTON, D. A. Benzimidazoles for the treatment of cystc and alveolar echinococcosis: what is the consensus? *Expert Rev Anti Infect Ther*, 7(2): 145-149, 2009.

WANG, Y.; LI, Z.; LI, Z.; BO, Y. ZHAO, W. Recombinant ferritin protects mice against challenge with *Echinococcus granulosus*? *Acta Parasitol*, 54: 335-340, 2009.

WANG, J.; REN, X.; BAI, X.; ZHANG, T.; WANG, Y.; LI, K.; LI, G. Identification of gene mutation in patients with osteogenesis imperfect using high-resolution melting analysis. *Sci Rep*, 5: 13468, 2015.

WANG, S.; WEI, W.; CAI, X. Genome-wide analysis of excretory/secretory proteins in *Echinococcus multilocularis*: insights into functional characteristics of the tapeworm secretome. *Parasit Vectors*, 8(1): 666, 2015.

WITTWER, C. T. High-resolution DNA melting analysis: advancements and limitations. *Hum Mutat*, 30(6): 857-859, 2009.

XIAO, N.; QIU, J.; NAKAO, M.; LI, T.; YANG, W.; CHEN, X.; SCHANTZ, P. M.; CRAIG, P. S.; ITO, A. *Echinococcus shiquicus*, a new species from the Qinghai-Tibet plateau region of China: discovery and epidemiological implications. *Parasitol Int*, 55: s233-236, 2006a.

XIAO, N.; NAKAO, M.; QIU, J.; BUDKE, C. M.; GIRAUDOUX, P.; CRAIG, P. S.; ITO, A. Dual infection of animal hosts with different *Echinococcus* species in the eastern Qinghai-Tibet plateau region of China. *Am Trop Med Hyg*, 75(2): 292-294, 2006b.

LI, Y-D.; CHU, Z-Z.; LIU, X-G.; JING, H-C.; LIU, Y-G.; HAO, D-Y. A cost-effective High-resolution melting approach using the evagreen dye for DNA pplymorphism detection and genotyping in plants. *J Integr Plant Biol*, 52(12): 1036-1042, 2010.

YURCHENKO, V.; CONSTANT, S.; EISUNMESSER, E.; BUKRINSKY, M. Cyclophilin-CD147 interactions: a new target for anti-inflamatory therapeutics. *Clin Exp Immunol*, 160(3): 305-317, 2010.

ZAMPIERI, R. A.; LARANJEIRA-SILVA, M. F.; MUXEL, S. M.; STOCCO DE LIMA, A. C.; SHAW, J. J.; FLOETER-WINTER, L. M. High-resolution melting analysis targeting hsp70 as a fast and eficiente method for the discrimination of *Leishmania* species. *PLoS Negl Trop Dis*, 10(2): e0004485.

ZHANG, W.; LI, J.; MCMANUS, D. P. Concepts in immunology and diagnosis of hydatid disease. *Clin Microbiol Rev*, 16(1): 18-36, 2003.

ZHENG, M.; HU, K.; LIU, W.; HU, X.; HU, F.; HUANG, L.; WANG, P.; HU, Y.; LI, W.; LIANG, C.; YIN, X.; HE, Q.; YU, X. Proteomic analysis of excretory secretory products from *Clonorchis sinensis* adult worms: molecular characterization and serological reactivity of a excretory-secretory antigen-fructose-1,6-bisphosphatase. *Parasitol Res*, 109(3): 737-744, 2011.

ZHENG, H.; ZHANG, W.; ZHANG, L.; ZHANG, Z.; LI, J.; LU, G.; ZHU, Y.; WANG, Y.; HUANG, Y.; LIU, J.; KANG, H.; CHEN, J.; WANG, L.; CHEN, A.; YU, S.; GAO, Z.; JIN, L.; GU, W.; WANG, Z.; ZHAO, L.; SHI, B.; WEN, H.; LIN, R.; JONES, M. K.; BREJOVA, B.; VINAR, T.; ZHAO, G.; MCMANUS, D. P.; CHEN, Z.; ZHOU, Y.; WANG, S. The genome of the hydatid tapeworm *Echinococcus granulosus*. *Nat Genet,* 45(10): 1168-1175, 2013.

ZHOU, Q.; WANG, W. On the origin and evolution of new genes – a genomic and experimental perspective. *J Genet Genomics*, 35(11): 639-648, 2008.

5. Anexo

CURRICULUM VITAE

Guilherme Brzoskowski dos Santos
Curriculum Vitae

Dados pessoais

Nome Guilherme Brzoskowski dos Santos
Nascimento 09/09/1985 - Camaquã/RS - Brasil
Endereço profissional Universidade Federal do Rio Grande do Sul, Instituto de Biociências
Av. Bento Gonçalves. Laboratório 204 - Biologia Molecular de Cestodeos
Agronomia - Porto Alegre
91501-970, RS - Brasil
Telefone: 051 3308-6070
Endereço eletrônico: E-mail para contato : guilhermebrzos@gmail.com

Formação acadêmica/titulação

2012 - 2016 Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Análise das proteínas constituintes do secretoma e dos potenciais mecanismos envolvidos na interação da forma larval patogênica de Cestódeos do gênero *Echinococcus* com seu hospedeiro Orientador:
Arnaldo Zaha
Bolsista: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-REUNE)

2010 - 2012 Mestrado em Genética e Biologia Molecular – PPGBM-UFRGS.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Estrutura e dinâmica genética das populações de *Echinococcus vogeli* e *Echinococcus oligarthrus* (Cestoda: Taeniidae), Ano de obtenção: 2012
Orientador: Arnaldo Zaha
Bolsista: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

2005 - 2009 Graduação em Biologia.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título do TCC: Análise comparativa dos genes codificadores de AgB em duas espécies do gênero *Echinococcus* (Rudolphi, 1801) e elucidação da distribuição das mesmas no estado do RS.
Orientador: Karen Haag

Formação complementar

2013 - 2013 Extensão universitária em Curso de Extensão em Expressão Gênica - DOCENTE. (Carga horária: 80h).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2013 - 2013 Extensão universitária em Método lógico para redação científica internacional. (Carga horária: 16h).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2012 - 2012 Curso de curta duração em Análise de dados proteômicos. (Carga horária: 40h).
Fundação Oswaldo Cruz (PR) - Instituto Carlos Chagas, FOC, Brasil

2009 - 2009 Extensão universitária em Evolucao, Cinema e Literatura. (Carga horária: 15h).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2009 - 2009 Monitor da disciplina Genética I. . (Carga horária: 240h).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2007 - 2007 Extensão universitária em XVII Curso de Técnicas Histológicas - Monitor. (Carga horária: 166h).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2006 - 2006 Extensão universitária em XVII Curso de técnicas Histológicas. (Carga horária: 60h).
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

---

Atuação profissional

1. Universidade Federal do Rio Grande do Sul - UFRGS

Vínculo institucional

2015 - 2015 Vínculo: Colaborador , Enquadramento funcional: DOCENTE - bolsista REUNI , Carga horária: 30, Regime: Parcial

2015 - 2015 Vínculo: Colaborador , Enquadramento funcional: DOCENTE - bolsista REUNI , Carga horária: 30, Regime: Parcial

2014 - 2014 Vínculo: Colaborador , Enquadramento funcional: DOCENTE - bolsista REUNI , Carga horária: 30, Regime: Parcial

2014 - 2014 Vínculo: Colaborador , Enquadramento funcional: DOCENTE - bolsista REUNI , Carga horária: 30, Regime: Parcial

2013 - 2013 Vínculo: Colaborador , Enquadramento funcional: DOCENTE - bolsista REUNI , Carga horária: 30, Regime: Parcial

2013 - 2013 Vínculo: Colaborador , Enquadramento funcional: DOCENTE - bolsista REUNI , Carga horária: 30, Regime: Parcial

2012 - 2012 Vínculo: Colaborador , Enquadramento funcional: DOCENTE - bolsista REUNI , Carga horária: 30, Regime: Parcial
2012 - 2012 Vínculo: Bolsista , Enquadramento funcional: Bolsista DOUTORADO , Carga horária: 40, Regime: Dedicação exclusiva
2012 - 2012 Vínculo: Colaborador , Enquadramento funcional: DOCENTE - bolsista REUNI , Carga horária: 45, Regime: Parcial
2010 - 2012 Vínculo: Bolsista, Enquadramento funcional: Bolsista MESTRADO , Carga horária: 40
2009 - 2010 Vínculo: Iniciação Científica , Enquadramento funcional: Bolsista PIBIC/CNPq , Carga horária: 20
2007 - 2009 Vínculo: Iniciação Científica , Enquadramento funcional: Estágio Voluntário , Carga horária: 20, Regime: Parcial
2006 - 2007 Vínculo: bolsista , Enquadramento funcional: Bolsista PIBIC/CNPq , Carga horária: 20, Regime: Dedicação exclusiva

Revisor de periódico

Acta Tropical
Vínculo 2014 - Atual Regime: Parcial

Prêmios e títulos

2009 Mención Al Premio "Raul Mendy" De La Asociación internacional de Hidatidología, Asociación Internacional de Hidatidología

---

Produção bibliográfica
Artigos completos publicados em periódicos

1. **SANTOS, G. B.**, ESPÍNOLA, SERGIO MARTÍN, FERREIRA, HENRIQUE BUNSELMEYER, MARGIS, ROGERIO, ZAHA, ARNALDO
Rapid detection of Echinococcus species by a high-resolution melting (HRM) approach. Parasites & Vectors. , v.6, p.327 - , 2013.

2. **SANTOS, G. B.**, SOARES, MANOEL DO C.P., DE F. BRITO, ELISABETE M., RODRIGUES, ANDRÉ L., SIQUEIRA, NILTON G., GOMES-GOUVÊA, MICHELE S., ALVES, MAX M., CARNEIRO, LILIANE A., MALHEIROS, ANDREZA P., PÓVOA, MARINETE M., ZAHA, ARNALDO, HAAG, KAREN L.
Mitochondrial and nuclear sequence polymorphisms reveal geographic structuring in Amazonian populations of Echinococcus vogeli (Cestoda: Taeniidae). International Journal for Parasitology. , v.42, p.1115 - 1118, 2012.

3. BALBINOTTI, HELIER, AREND, ANA C., HAAG, KAREN L., ZAHA, ARNALDO, BADARACO, JEFERSON, GRAICHEN, DANIEL ÂNGELO S., **SANTOS, G. B.**

Echinococcus ortleppi (G5) and Echinococcus granulosus sensu stricto (G1) loads in cattle from Southern Brazil. Veterinary Parasitology (Print). , v.188, p.255 – 260, 2012.

4. CANCELA, MARTÍN, **SANTOS, G. B.**, CARMONA, CARLOS, FERREIRA, HENRIQUE B., TORT, JOSÉ FRANCISCO, ZAHA, ARNALDO
Fasciola hepatica mucin-encoding gene: expression, variability and its potential relevance in host-parasite relationship. Parasitology (London. Print). , v.142, p.1 - 9, 2015.

Trabalhos publicados em anais de eventos (resumo)

1. **SANTOS, G. B.**, MONTEIRO, K. M., MATIELLO, J., FERREIRA, H. B., ZAHA, A.
Análise proteômica de produtos de excreção-secreção das formas larvais patogênicas de cestódeos do gênero Echinococcus In: XXIII Congresso brasileiro de parasitologia, 2013, Florianópolis-SC.**XXIII congresso brasilerio de parasitologia**. , 2013.

2. **SANTOS, G. B.**, ESPÍNOLA, SERGIO MARTÍN, FERREIRA, H. B., MARGIS, ROGERIO, ZAHA, A.
Rapid detection of species belonging to Echinococcus genus by a high-resolution melting (HRM) approach In: XXIII Congresso brasileiro de parasitologia, 2013, Florianópolis-SC. **XXIII congresso brasilerio de parasitologia**. , 2013.

3. CANCELA, M., **SANTOS, G. B.**, CARMONA, C., FERREIRA, H. B.
Variability in mucin-like gene from F. hepatica In: XXIII Congresso brasileiro de parasitologia, 2013, Florianópolis-SC. **XXIII congresso brasilerio de parasitologia**. , 2013.

4. BARRETO, M. Q., **SANTOS, G. B.**, FERREIRA, H. B., ZAHA, A.
Identification of a putative GAPDH retrogene in the Echinococcus granulosus genome as a target for protein function studies. **4 Congresso Brasileiro de Biotecnologia**, 2012.

5. Meyer, J., **SANTOS, G. B.**, Chies, JAB, Schiengold, M.
Considerações sobre a efetividade dos glossários dos livros didáticos em relação a termos do vocabulário do genima humano. **57° Congresso Brasileiro de genética**, 2011, Águas de Lindóia.

6. **SANTOS, G. B.**, FIGUEIREDO, E. M., SOARES, M., ZAHA, A., HAAG, KAREN L.
Genética populacional de Echinococcus vogeli do norte e sul do Brasil In: XXII Congresso brasileiro de parasitologia, 2011, São Paulo.
Genética populacional de Echinococcus vogeli do norte e sul do Brasil. , 2011.

7. Oliveira C. V., **SANTOS, G. B.**, Graichen, D. A. S., RUE, M. L., HAAG, K. L.
Echinococcus granulosus em suínos. Representa um problema no Rio Grande do Sul. In: 2º Fórum de Extensão Conta, 2009, Santa Maria.
2º Fórum de Extensão Conta, UFSM. , 2009.

8. **SANTOS, G. B.**, Graichen, D. A. S., RUE, M. L., HAAG, K. L.
Invasão de Echinococcus ortleppi, causador da equinococose cística, no rebanho bovino do estado do Rio Grande do Sul In: XXI Congresso brasileiro de parasitologia e II encontro de parasitologia do Mercosul, 2009, Foz do Iguaçu.
Invasão de Echinococcus ortleppi, causador da equinococose cística, no rebanho bovino do estado do Rio Grande do Sul. , 2009.

9. **SANTOS, G. B.**, Graichen, D. A. S., RUE, M. L., HAAG, K. L.
Low level of Echinococcus granulosus Agb genetic varibility in adult worm In: XXII International Congress of Hydatidology, 2009, Colonia del Sacramento.
XXII Congreso Mundial de Hidatidología. , 2009.

10. Sordi, J. O., **SANTOS, G. B.**, Kortmann G. L., Abreu, S. P., SCHIENGOLD, M.
O léxico da Biologia Molecular: uma pesquisa terminográfica. In: XXI Salão de Iniciação Científica da UFRGS, 2009, Porto Alegre.
XXI Salão de Iniciação Científica da UFRGS. , 2009.

11. **SANTOS, G. B.**, Graichen, D. A. S., HAAG, K. L.
Análise comparativa dos genes codificadores de EgAgB4 de Echinococcus granulosus nos haplótipos G1 e G5 In: XX Salão de Iniciação Científica da UFRGS, 2008, Porto Alegre.
XX Salão de Iniciação Científica da UFRGS. , 2008.

12. **SANTOS, G. B.**, BADARACO, J. L, Graichen, D. A. S., HAAG, K. L.
Análise comparativa dos genes codificadores da subunidade 4 do AgB de Echinococcus granulosus. In: XIX Salão de Iniciação Científica, 2007, Porto Alegre.
Livro de Resumos do XVIII Salão de Iniciação Científica da UFRGS, 2007.. , 2007.

13. Nogueira, de Sa Flavia, Ceolin Guilherme, **SANTOS, G. B.**, Sampaio, Jamilla Alves, Grunwald Marcelo Sartori, Costa, Pedro Beschoren, Teixeira, Robson Scheffer, Mattei, Greice
Origin of patchy vegetation in the cost of the South of Brazil In: Linking Tropical Biology with Human Dimensions ATBC 2007, 2007, Morelia.
Origin of patchy vegetation in the cost of the South of Brazil. , 2007.

14. **SANTOS, G. B.,** Estudo do desenvolvimento do órgão copulador em Temnocephala trapeziformis, epibionte de Trichodactylus fluviatilis In: XVIII Salão de Iniciação Científica, 2006, Porto Alegre.
Livro de Resumos do XVIII Salão de Iniciação Científica da UFRGS, 2006. , 2006.

Educação e Popularização de C&T
Curso de curta duração ministrado

1. **SANTOS, G. B.**, CANCELA, M.
Ferramentas em biologia molecular: da genotipagem à expressão gênica, 2015. (Aperfeiçoamento, Curso de curta duração ministrado)

Eventos
Participação em eventos

1. **IV Proteomics Workshop**, 2013. (Seminário)
2. **XXIII Congresso brasileiro de parasitologia**, 2013. (Congresso)
3. **3d Biological Evolution Workshop**, 2011. (Outra)
4. **XXII Congresso brasileiro de Parasitologia**, 2011. (Congresso)
5. **Simpósio Sobre Terapias Inovadoras**, 2010. (Simpósio)
6. **Evolucao, Cinema e Literatura**, 2009. (Outra)
7. **VI JABUFRGS**, 2009. (Encontro)
8. Apresentação de Poster / Painel no(a) **XXI Congresso Brasileiro de Parasitologia e II Encontro de Parasitologia**, 2009. (Congresso)
Invasão de Echinococcus ortleppi, causador da equinococose cística, no rebanho bovino do estado do Rio Grande do Sul.
9. **I Congresso Sul-Americano de Mastozoologia**, 2006. (Congresso)
10. **II Jornada Acadêmica da Biomedicina da FFFCNPA**, 2006. (Encontro)
11. **XIX Congresso Brasileiro de Parasitologia**, 2005. (Congresso)

Organização de evento

1. **SANTOS, G. B.**
VI JABUFRGS, 2009. (Outro, Organização de evento)

Bancas
Participação em banca de trabalhos de conclusão

Graduação

1. **SANTOS, G. B.**, POSSUELO, L. G.
Participação em banca de Elisa Inês Klinger. **Avaliação dos genes il6, crp e fto e sua relação com sobrepeso/obesidade e alterações no perfil lipídico e glicêmico em escolares de Santa Cruz do Sul, RS**, 2015
(Farmácia) Universidade de Santa Cruz do Sul

2. **SANTOS, G. B.**, VALIM, A. R. M.
Participação em banca de Ana Julia Reis. **Genotipagem de cepas de Mycobacterium tuberculosis isoladas de pacientes de um presídio regional do estado do Rio Grande**

do Sul, 2015
(Farmácia) Universidade de Santa Cruz do Sul

3. **SANTOS, G. B.**, Conceição F. R., Borsuk S.
Participação em banca de Filipe Santos Pereira Dutra. **Comparação de metodologias para a genotipagem das espécies Echinococcus granulosus sensu stricto (G1) e Echinococcus ortleppi (G5)**, 2014
(Biotecnologia) Universidade Federal de Pelotas

4. **SANTOS, G. B.**
Participação em banca de Michele Fraga Eisenhardt. **CaracterizaçãoEpidemiológica e Genotipagem de UGT1A1 em Pacientes Portadores de Câncer Coloretal**, 2011
(Farmácia) Universidade de Santa Cruz do Sul

Produção

Produção bibliográfica

Artigos completos publicados em periódico.....4
Indice H.....3
Número de citações.....23

Eventos

Trabalhos publicados em anais de eventos.....14
Apresentações de trabalhos(Simpósio).....4
Curso de curta duração ministrado(aperfeiçoamento).....1
Participações em eventos (congresso).....5
Participações em eventos (seminário).....1
Participações em eventos (simpósio).....1
Participações em eventos (encontro).....2
Participações em eventos (outra).....2
Organização de evento (outro).....1
Participação em banca de trabalhos de conclusão (graduação).....4
