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Ortleppascaris sp. and your host *Rhinella marina*: A proteomic view into a nematode–amphibian relationship

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ABSTRACT

The success of the helminth–host relationship depends on a biochemical molecular arsenal. Perhaps the proteome is the largest and most important set of this weaponry, in which the proteins have a crucial role in vital processes to the parasite/host relationship, from basic metabolism and energy production to complex immune responses. Nowadays, the bioproducts expressed by the parasites are under the "spotlight" of immunoassays and biochemical analysis in helminthology, especially in proteomic analysis, which has provided valuable information about the physiology of the infecting agent. Looking into this point of view, why not turn to the infected agent as well? This study characterised the proteomic profile of fluid-filled fibrous cysts of encapsulated *Ortleppascaris* sp. larvae in the hepatic parenchyma of their intermediate host, the amphibian *Rhinella marina*. The proteins were separated by two-dimensional electrophoresis and identified by MS with the aid of *Peptide Mass Fingerprint*. A total of 54 molecules were analysed in this system, revealing a complex protein profile with molecules related to basic metabolic processes of the parasite, energy production, oxi-reduction and oxidative stress processes as well as molecules related to the host response. This study contributes to proteomic studies of protein markers of the development, infectivity, virulence and co-existence of helminths and their hosts.

1. Introduction

The parasite survival depends on the relationship established between them and the host (McKay, 2009) and this relationship starts to increase in the complexity when the larval helminths and their intermediate hosts have elaborate life cycles and transmission strategies to definitive hosts (Chubb et al., 2010).

The secretome, the set of products excreted/secreted by a cell or organism, can tell us a co-evolutionary life history on the dynamics of a parasite, including how it interacts and carries out its functions in the host "milieu" (Hewitson et al., 2008; Nagaraj et al., 2008; Ranganathan and Garg, 2009; Bourke et al., 2011).

Bioactive molecules found in helminth extracts or in excretion/secretion products are responsible for modulating and suppressing the host immune response to favour co-existence of both organisms (Johnston et al., 2009). According to Craig et al. (2006) and Soblik et al. (2011), these substances are derived from the body surface or from specialised excreting/secreting glands in helminths and are often released during specific stages.

This set of products, especially the proteins, has been intensely studied. They can play an important role in the infection and in host–parasite interactions, assisting the survival of parasites within hosts (Ranganathan and Garg, 2009; Liao et al., 2011).

Proteomics has been producing data on a large scale; however due to the scarcity of helminth genomes, there is a need for the production of more data on "helminth-derived" substances. This is especially true when considering the large number of species and the plasticity of these organisms in terms of their biology, morphology, development in different hosts and infection and transmission modes (Rebello et al., 2011; Mutapi, 2012).

Despite this biological variety, the murines are the current animal models of choice in the biological sciences; however, other biological systems *in vivo* can be promising study models and also to contribute to the elucidation of the host–parasite relationship (Bolker, 2012; Sotillo et al., 2012; Robinson et al., 2013).

Herein we explore the parasitism of *Ortleppascaris* sp. larvae in the liver of the amphibian *Rhinella marina*. These larvae are characteristically found within fibrous cysts filled with a viscous fluid stimulating severe pathological alterations in the tissue infected

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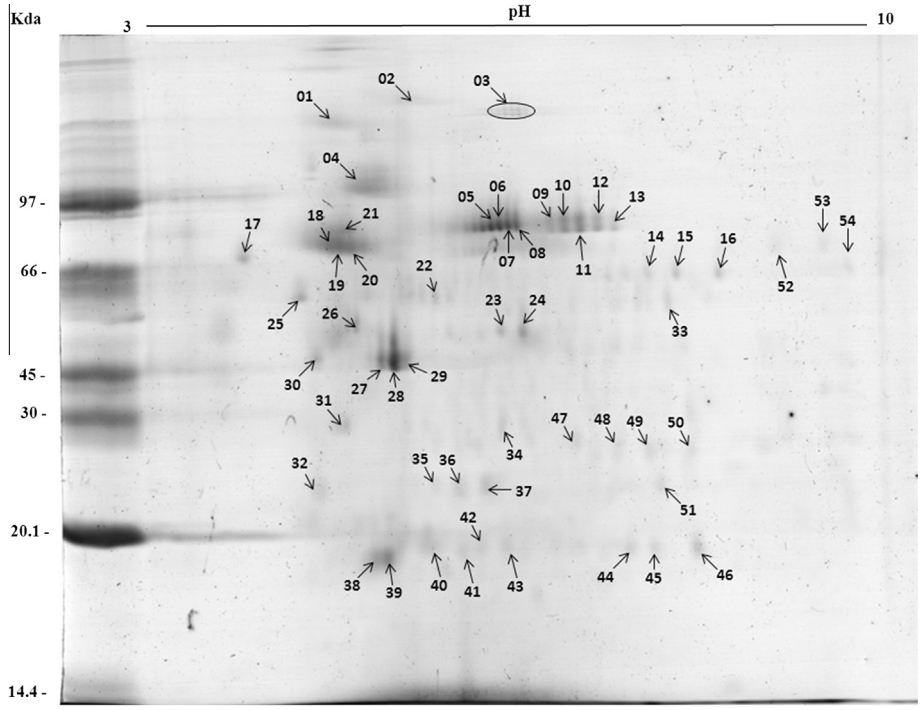

Fig. 1. Two-dimensional gel of proteins originating from cystic fluid of *Ortleppascaris* sp. encystment.

(Silva et al., 2013a,b). The current study used proteomic tools to identify the protein composition of this fluid found within the fibrous cysts and to analyse the biochemical and biological aspects of parasitism by these larval nematodes in the liver of *R. marina*.

2. Materials and methods

The present study was approved by the Animal Research Ethics Committee of the Federal University of Pará (UFPA) through authorisation CEPAEUFPA: BIO010-10.

2.1. Cystic fluid

Cysts of the hepatic parenchyma were removed and the fluid was collected by suction using approximately 200 cysts with the aid of a disposable syringe and stored in extraction buffer (7 M Urea, 2 M Thiourea, 2% CHAPS) at −20 °C.

2.2. Preparation of the cystic fluid proteins

The collected fluids in extraction buffer were centrifuged at 13,000*g* for 15 min at 4 °C to obtain the supernatant, which was used directly for protein analysis.

2.3. Two-dimensional electrophoresis and in-gel tryptic digestion and mass spectrometry

Two-dimensional electrophoresis of the fluid, tryptic digestion procedures and mass spectrometry analysis were performed as described in Silva et al. (2014).

2.4. Database searching and gene ontology analysis

The search for proteins by PMF (peptide mass fingerprint) homology was performed in the NCBI [National Center for Biotechnology Information (NCBInr)] public database with the aid of the online version of Mascot (Matrix Science). The taxonomic parameter was restricted to *Xenopus laevis*. The following search parameters were used: up to two lost cleavage sites, error of 0.1 Da in peptide identification, cysteine carbamidomethylation as a fixed modification and methionine oxidation as a variable modification. To infer the biological process in which the identified molecules are involved, the PMF data analysis was enriched by comparing it with Gene Ontology (GO) terms by means of the UniProt online database. The identified proteins were grouped into functional categories and subcategories according to their ontology.

3. Results

To characterise the fluid's protein profile, the most abundant spots detected with Image Master 2D Platinum software were analysed, shown in Fig. 1 and indicated as spots 1–54.

The spots observed in the two-dimensional gel (Fig. 1) and their respective proteins are summarised in Table 1, according to the database used in this study. Of these spots, 47 were identified, 4 were uncharacterised (03, 13, 34, 36), and 3 spots (35, 37, 49) did not generate good quality spectra for identification because they not presented sufficient similarity to any protein in the database.

The list of marked spots and the respective proteins are summarised in Table 1. These proteins found in the fluid make up a set of molecules that may be grouped into general categories of functions important for cellular functioning (such as redox balance and vesicular traffic), in addition to a group of molecules related to specific processes.

Prolyl endopeptidase-like (spot 01) belongs to the S9A subfamily and has probable serine endopeptidase activity. Subunit beta of the proteasome (spot 15) also has endopeptidase activity and is involved in ATP/ubiquitin-dependent non-lysosomal proteolytic activity.

A protein similar to cyclin-dependent kinase inhibitor, p16, (spot 02) was found, which are involved in the regulation of the eukaryotic cell cycle and is considered a tumor suppressor.

Table 1
Summary of proteins from cystic fluid identified by PMF using NCBI database. Search restricted taxonomically to *Xenopus laevis*. (See Supplementary Material Table 1 for more details).

Spot	Protein
01	Prolyl endopeptidase-like
02	CDKN2A interacting protein N-terminal like
03	Uncharacterized protein LOC779198
04	MGC84728 protein
05, 16	Cytoglobin
06	Cysteine-rich with EGF-like domain protein 2-B precursor
07	Chain A, Crystal Structure Of *X. laevis* T-Cadherin Ec1
08	LOC733224 protein, partial
09, 10, 11, 12, 22	TraB domain containing
13, 34	Uncharacterized protein LOC379167
14	Regulation of nuclear pre-mRNA domain containing 1B
15	Proteasome subunit beta type-7
17, 25	Phosphopantothenoyl cysteine synthetase
18	Peroxisome proliferator-activated receptor alpha
19	Fidgetin-like protein 1
20	LOC446946 protein, partial
21	LOC733282 protein
23	Microtubule-associated protein 1 light chain 3 gamma
24	Cell division cycle 73, Paf1/RNA polymerase II complex component, homolog
26	Cas/HEF-likeprotein1
27	Structure-specific endonuclease subunit slx1
28	Actin, cytoplasmic type 5
29	Unknown (protein for MGC:181785)
30	Gamma-crystallin-3
31	Autophagy protein 5
32	Nitrogen fixation cluster-like
33	Vacuolar protein sorting 33 homolog A
36	Uncharacterized protein LOC734830
38	LOC398774 protein
39	Pro-opiomelanocortin B
40	LOC443709 protein, partial
41	LOC443713 protein, partial
42, 52	Copper chaperone for superoxide dismutase
43, 50	ATPase, H + transporting, lysosomal 50/57 kDa, V1 subunit H
44	RAB3A, member RAS oncogene Family
45	Aspartoacylase-2
46	Tubulin tyrosine ligase-like family, member 7
47	MARK3 protein
48	5-Aminoimidazole-4-carboxamide ribonucleotide formyl transferase/IMP cyclohydrolase
51, 53	LOC414512 protein, partial
54	Methylthioadenosine phosphorylase

Molecules involved in basic biochemical processes were found, such as cytoglobin (spots 05, 16), which is involved in oxygen transport; cadherin (spot 07) participating in intracellular adhesion processes and fidgetin-like 1 (spot 19) protein that is a member of the large AAA ATPase family.

Hydrolases responsible for ATP breakdown and proton transport were found in spots 43 and 50, and aspartoacylase was found in spot 45.

Another ubiquitous molecules expressed in eukaryotes were also found, e.g., cytoplasmic actin type 5 (spot 28), proteins related to vesicular traffic (spots 33, 44) and molecules with protein-binding (spot 38), zinc-binding (spot 40), protein modification (spot 46) and phosphorylation (spot 47) functions.

Proteins involved in the oxi-reduction of fatty acids were also present, such as acyl-CoA dehydrogenase (spot 08) and PPAR-alpha (spot 18) (peroxisome proliferator-activated receptor alpha). Other molecules were detected in addition to these: the copper chaperone for superoxide dismutase protein (CCS) in 2 spots (42, 52) and the nitrogen fixation cluster-like protein (NifU), which operates in the mounting system of "iron-sulphur" compounds that are important for electron transfer.

Proteins with as yet unclear functions are also present in our dataset, such as the protein domain TraB, identified in five spots (9, 10, 11, 12, 22), and nucleic acid-binding molecules, such as LOC733282 protein (spot 21) and cdc73 (spot 24) (cell division cycle 73, Paf1/RNA polymerase II complex component, homolog), believed to be an accessory factor of RNA polymerase II. In addition to endonucleases responsible for DNA repair and recombination, such as the structure-specific endonuclease subunit slx1 (spot 27) and LOC443713 protein (spot 41), other molecules associated with nucleic acids were also found, including molecules involved in purine biosynthesis (spot 48), nucleic acid binding (spots 51, 53) and methylthioadenosine phosphorylase (MTAP) (spot 54).

Molecules associated with autophagy were also detected: Microtubule-associated protein 1 light chain 3 gamma, also known as map1lc3b or LC3 (spot 23), a member of the protein family ATg8 ubiquitin-like (autophagy protein) and autophagy protein 5 (spot 31).

Pro-opiomelanocortin B (spot 39), an important neurotransmitter hormone and precursor to other neuropeptides, was identified among this set of proteins from the cystic fluid.

4. Discussion

According Poulin et al. (2003) potential interactions among larval helminths in intermediate hosts have not generated as much interest, possibly because of the apparent 'dormant' nature of many larval helminths. The presence of *Ortleppascaris* in the liver of *R. marina* creates a typical scenario of helminthic tissue infections: an encapsulation generating a fibrous cyst, but ironically,

Table 2
Summary of the identified proteins from 2D electrophoresis of the cystic fluid and the category of biological process in GO terms. See Supplemental Table 1 for additional information.

GO term	Process/biological function	Spot	Protein
Metabolic process and energy production molecules			
GO:0006508/ GO:0004252	Proteolysis/serine-type endopeptidase activity	01	Prolylendopeptidade-like
GO:0015671	Oxygen transport	05, 16	Cytoglobin
GO:0004222/ 0060322	Metalloendopeptidase activity/head development	9,10,11,12, 22	TraB domain containing
GO:0051603/ GO:0004252	Proteolysis involved in cellular protein catabolic process/serine-type endopeptidase activity	15	Proteasome subunit beta type
GO:0046034	ATP metabolic process	19	Fidgetin-like protein 1
GO:0005856	Cytoskeleton	28	Actin, cytoplasmic type 5
GO:0005515	Protein binding	38	LOC398774 protein
GO:0016192	Vesicle-mediated transport	33	Vacuolar protein sorting 33 homolog A
GO:0008270	Zinc ion binding	40	LOC443709 protein, partial
GO:0015991	ATP hydrolysis coupled proton transport	43, 50	ATPase, H + transporting, lysosomal 50/57 kDa, V1 subunit H
GO:0015031	Protein transport	44	RAB3A, member RAS oncogene family
GO:0016787	Hydrolase activity	45	Aspartoacylase-2
GO:0006464	Cellular protein modification process	46	Tubulin tyrosine ligase-like family, member 7
GO:0006468	Protein phosphorylation	47	MARK3 protein
Oxi-reduction and oxidative stress process molecules			
GO:0055114/ GO:0003995	Oxidation–reduction/Acyl-CoA dehydrogenase activity	08	LOC733224 protein, partial
GO:0008289	Lipid binding	18	Peroxisome proliferator-activated receptor alpha
GO:0016226	Iron–sulfur cluster assembly	32	Nitrogenfixation cluster-like
GO:0055114/ GO:0006801	Oxidation–reduction/superoxide metabolic process	42, 52	Copper chaperone for superoxide dismutase
Associated with nucleic acids molecules			
GO:0003676	Nucleic acid binding	21	LOC733282
GO:0006355	Regulation of transcription, DNA-templated	24	Cell division cycle 73, Paf1/RNA polymerase II complex component, homolog
GO:0006281	DNA repair	27	Structure-specific endonuclease subunit slx1
		41	LOC443713
GO:0006164	Purine nucleotide biosynthetic process	48	5-Aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase
GO:0000166	Nucleic acid binding	51, 53	LOC414512 protein, partial
GO:0009116	Nucleoside metabolic process	54	Methylthioadenosine phosphorylase
Host response molecules			
GO:0006914	Autophagy	31	Autophagy protein 5
GO:0007218	Neuropeptide signaling pathway	39	Pro-opiomelanocortin B

the attempt of the host to isolate the parasites within cysts enables the development of the infective agent. The process of encystment allows the permanence of the larvae immersed in a fluid material, this matrix is perhaps the main interface between the nematode and the amphibian and contains a complex protein profile with molecules related to the parasite's basic metabolism, energy production, oxi-reduction and oxidative stress processes, in addition to molecules related to the host response. These molecules are potential molecular markers for development, infectivity, virulence and co-existence of helminths and their hosts.

Table 2 lists the identified proteins with their respective identified spots, grouped into functional categories and subcategories according to their GO terms (see also Supplemental Table 1).

4.1. Metabolic process and energy production molecules

One of the molecules detected was the enzyme Prolyl-peptidase, a dipeptyl peptidase (DPP), which, according to Lone et al., 2010, is important in (patho)physiological processes and for cleavage in proline-rich peptide sites. High levels of proline are found in collagen, which occurs in the cystic capsule and in the *Ortleppascaris*' cuticle. Another prolyl endopeptidase, FAP (fibroblast activation protein), that share a high sequence identity with DPP and may have arisen by gene duplication (Lai et al., 2007), can be involved in this process, FAP have also a collagenolytic activity and can promotes tumor growth and proliferation.

Detection of these proteins, along with the neuroendocrine hormone Pro-opiomelanocortin B, may be related to mechanisms specific to parasitism because, according to Rosenblum and Kozarich (2003), these proteins play an important role in neuroendocrine regulation and signalling under stress in some anurans.

CDKN2A (cyclin-dependent kinase inhibitor 2A), which is also known as p16, whose function has been pointed by Drexler (1998) and Lee et al. (2013) as a tumor suppressor protein, can be related to cell proliferation or tumour development with reshaping of the hepatic parenchyma with cyst expansion.

Another cell proliferation marker found in this proteome was the molecule fidgetin-like 1. The cell proliferation function was pointed by Yang et al. (2005) and in the *Ortleppascaris–R. marina* relationship, this function may be associated with growth of the larval stages, similar to what has been described by Luke-Glaser et al. (2007) for *Caenorhabditis elegans*. These authors report that fidgetin-like 1 may be released into the extracorporeal milieu. Therefore, it is believed that *Ortleppascaris* may be secreting/excreting these molecules into the cystic fluid.

Another important protein of the eukaryotic proteolytic pathway found in this study was proteasome subunit beta. According to Groll and Huber (2003), this proteolytic pathway degrades abnormal proteins and therefore produces the largest source of peptide antigens presented to the immune system through MHC class I. It also regulates degradation in important processes, such as metabolic adaptation. This process may be occurring within

the cyst, where the host needs adequate machinery for antigen presentation and helminth recognition in addition to having to adapt its metabolism in face of the infection.

The compression of blood vessels and the existence of larval changes within the cysts may explain the presence of cytoglobin. This molecule is often associated with low oxygen concentrations, and its function in amphibian metabolism has not yet been well defined (Fuchs et al., 2006). Pesce et al. (2002) and Schmidt et al. (2004) consider that its exacerbated expression is related to tissue hypoxia/ischemia and/or collagen synthesis, factors that are certainly present in the scenario of the infection studied here.

Furthermore, the tissue organisation have been impaired by the parasite (Silva et al., 2013b), thus justifying the expression of cadherins. According to Leckband and Prakasam (2006), cadherins are responsible for maintenance of the tissue structure, among other functions.

Some proteins with unclear functions were pointed, e.g., protein domain TraB, also known as Metalloprotease TIKI1 (Fu et al., 2012), identified in anurans is involved in cell differentiation in the *Xenopus* embryo and frog metamorphosis.

4.2. Oxi-reduction and oxidative stress process molecules

The molecule fidgetin-like 1 takes part in vesicle-mediated transport to the peroxisomes, among other cell activities (Yang et al., 2005). Other molecules obtained from the *Ortleppascaris–R. marina* interaction influence the proliferation of peroxisomes. These molecules include the protein PPAR-alpha, indicating a high metabolism and beta-oxidation reactions related to the fatty acids found in this fluid.

In addition to this set of proteins, other key molecules of the metabolism of lipids, regulators of the fatty acid beta-oxidation pathways, were found in the cystic fluid. They include acyl-CoA dehydrogenase, NifU which is involved in the formation of [Fe–S] complexes and in the transport of electrons (Johnson et al., 2005), and copper chaperone for superoxide dismutase (CCS). Rosenzweig and O'Halloran (2000) believe that CCS is a marker for strong antioxidant reactions.

4.3. Associated with nucleic acids molecules

The protein cdc 73 acts in the context of the RNA PAF1, regulating the transcription event, playing roles in initiation, elongation and post-transcriptional events (Masi et al., 2014). Some important molecules for maintenance of genome integrity, with function for DNA repair and recombination were identified. LOC443713 protein and specially slx1, a highly conserved protein in eukariotes (Fekairi et al., 2009), can play an important role in this biological function but the mechanisms that control structure-specific endonucleases remain poorly understood.

Additionally, two other proteins related to oncogenesis were found. The spot 48, that according to Boccalatte et al. (2009) have considerable attention because of their role in human cancer, and MTAP, an important molecule, whose expression levels of this protein have a significant correlation to collagen expression in diseased human liver tissue, chronic liver disease, malignant transformation, inflammation and fibrosis (Czech et al., 2013), indicating that this parasitism may contribute to a progression of a oncogenic process and/or severe hepatic disease.

4.4. Host response molecules

According to Tanida et al. (2004), LC3 is used as a molecular marker of autophagy in mammals. It is also expressed under a number of stress conditions (Shpilka et al., 2011), for example, in microbial infections, tumour development and food deprivation. Therefore, the presence of LC3, as well as other markers of tumour processes and autophagy (autophagy protein 5 and p16), in the cystic fluid analysed indicates that the hepatic physiology of *R. marina* is under an intense disequilibrium due to the parasitism by *Ortleppascaris*.

Anti-oxidative stress molecules, such as CSS and PPAR-alpha, are also abundant, suggesting a large quantity of free radicals in the cystic fluid. These free radicals may certainly contribute, along with cellular injury and local hypoxia caused by the growth of larvae and their cysts, to the development of autophagy and metaplasia processes in the hepatic parenchyma. These molecules are related to diseases such as cancer, as stated by Kongara and Karantza (2012).

Another molecule involved in cellular stress response processes or even in the amphibian's immune reaction due to changes in the parenchyma is the protein Pro-opiomelanocortin *B*. This molecule has also been found in the anurans *Rana ridibunda* and *Xenopus laevis* by Jenks et al. (2011), who report that this molecule is a precursor of important neuropeptides, such as α-MSH.

Kolk et al. (2002) and Jenks et al. (2003) associate Pro-opiomelanocortin *B* with conditions of intense stress in amphibians: malnutrition, parasitism or the neuroendocrine reflex of skin colour change. Poulin (1994, 2000) consider that many helminths are capable of manipulating the colouration of their intermediate hosts in ways that render them more susceptible to predation by the parasite's definitive host. In fact Silva et al. (2013b) have observed an increased number of melanomacrophages in infected liver, this cells are filled by pigments and among other functions are responsible for colour distribution.

Other important fact to be considered is that the growth of *Ortleppascaris* sp. larvae and cysts in the liver of *R. marina* can diminishes the amphibian's capacity for food metabolism, recruiting nutrients through the parasitism maintenance processes, thus leading to a condition of malnutrition and intense physiological stress.

In conclusion, this study is relevant to broadening the view of parasitism phenomena using an unusual model: amphibian–helminth. The evidences shown here are also an opportunity to observe the co-evolution mechanisms of these organisms in light of the variety and plasticity of the molecules expressed to survival of both organisms.

The immunological strategies employed by the larvae shows an evolutionary adaptation for the survival of these parasites and to achieve the major objective: reach the adult stage and reproduce in the definitive host.

Moreover, the molecular approach to this helminth infection in *R. marina* brings forth a large amount of information on the set of protein antigens found in a synanthropic animal, considered to be an environmental indicator and a potential vector of helminth zoonoses.

Protein mapping of the helminth–amphibian interface may show how the amphibian reacts to the disequilibrium caused by parasitism. Therefore, proteomic analysis and protein identification contribute to advancing the understanding of the parasite–host relationship and its possible molecular markers, thus expanding the field of biomolecule analysis to new study models and providing new insights into helminth infections of human and veterinary interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijppaw.2014.05.004.

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