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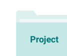

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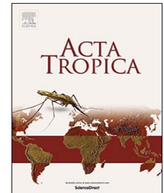

Comparative proteome profiling of hydatid fluid from Algerian patients reveals cyst location-related variation in *Echinococcus granulosus*

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ARTICLE INFO

Keywords:
Echinococcus granulosus
Hydatid fluid proteomics
Anatomical location
Fertility
Antigens

ABSTRACT

Human cystic echinococcosis, an endemic zoonosis in Algeria, is caused by larvae of the cestode *Echinococcus granulosus*. Parasitic modulation of the immune response allows *E. granulosus* to persist in intermediate hosts. Previous *in vitro* and *in vivo* immunological studies have shown differences in host immune responses according to the status and location of the hydatid cysts in the body. In this study, a proteomic analysis of human hydatid fluids was performed to identify the proteins in hydatid cyst fluids. Hydatid fluid was obtained after cystic surgical removal from three patients with these cysts. The study was conducted on fertile hydatid fluids from lungs, vertebra, and infertile paravertebral fluids. Comparisons of the protein compositions of these fluids revealed differences in their protein profiles. These differences are probably related to the cyst location and fertility status of the parasite. Notably, our analysis identified new proteins from the parasite and human host. The identification of host proteins in hydatid fluids indicates that the hydatid walls are permeable allowing a high protein exchange rate between the metacestode and the affected tissue. Interestingly, our study also revealed that parasite antigenic protein expression variations reflect the differences observed in host immunostimulation.

1. Introduction

Human echinococcosis is a parasitic disease caused by *Echinococcus granulosus* metacestodes. It is characterized by long-term growth of the larval stage in the intermediate host (Zhang et al., 2003). It constitutes a major health problem in North Africa, particularly in Algeria. The annual incidence is 1.11/100,000 Algerian population. *Echinococcus granulosus* is a small tapeworm that lives firmly attached to the mucosa of the small intestine in their definitive hosts (e.g. dogs and wild carnivores).It grows into adult stage. The shedding of gravid proglottids resulting into eggs production in the feces occurs within 4–6 weeks after infection of the definitive host. Ingestion of eggs by intermediate host animals such as sheep or human results in the release of an oncosphere into the gastrointestinal tract, which travels via blood or lymph to reach the most frequent organs the liver and lungs, and less frequent in spleen, soft tissues, bones, breast, heart and spinal extra-dural space where cystic development begins (Farmer et al., 1990; Zhang et al., 2003; Siracusano et al., 2012a,b). In most cases, the larvae remain viable within their host for many years despite the host's immune response. The hydatid cyst is unilocular, filled with hydatid fluid which might be fertile or sterile. Fertile cysts contain protoscoleces parasites produced by the germinal layer via asexual reproduction. The cyst is entirely covered by a laminated layer with an acellular carbohydrate-rich surface of variable thickness, which protects the parasite from the immunological and physiological reactions occurring in the host (Diaz et al., 2011). Hydatid fluid is a complex mixture of excretory and secretory products derived from protoscoleces and the germinal layer which confers antigenic properties to the parasite (Siracusano et al., 2012a,b). Parasite molecules play an important role in the chronic establishment of the infection and interfere with the functional activity of the host's immune cells (Hewitson et al., 2009). *E. granulosus* has evolved a range of strategies to impair the host immune

Abbreviations: E. granulosus, *Echinococcus granulosus*; *E. multilocularis*, *Echinococcus multilocularis*; PHF, pulmonary hydatid fluid; VHF, vertebral hydatid fluid; PVHF, paravertebral hydatid fluid; ESP, excretory/secretory products; *S. kowalevskii*, *Saccoglossus kowalevskii*; *D. dendriticum*, *Dicrocoelium dendriticum*

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http://dx.doi.org/10.1016/j.actatropica.2017.03.034
Received 29 December 2016
Available online 13 April 2017
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response, such as antigenic variation, protease production, immunosuppression, and skewing of the Th1/Th2 cytokine profile (Touil-Boukoffa et al., 1997, 1998; Zhang et al., 2008; Mezioug and Touil-Boukoffa, 2009).

In 2013, Zheng et al., sequenced the genome of *E. granulosus* revealing new genes which products are essential for host interaction and immune system perversion opening the field for new therapeutic targets which may help for future antihelmintic drug development (Zheng et al., 2013). The post-genomic studies were conducted and based on proteomic characterization of the different development stages of *E. granulosus*. Chemale et al. (2003). This team was the first to identify protoscoleces and hydatid fluid proteins by coupling two-dimensional gel electrophoresis and mass spectrometry. This work was completed by Monteiro et al. (2010) who identified proteins from the components of *E. granulosus* metacestode (protoscolex, germinal layer and hydatid cyst fluid) from bovine as well as host proteins in association with the hydatid cyst revealing new insights into parasite-host interaction. Most recently, the excretory/secretory products and antigenic proteins of *E. granulosus* adult worms from infected dogs were also characterized using two-dimensional LC–MS (Cui et al., 2013; Wang et al., 2015) providing new insights into the mechanisms involved in the establishment of *E. granulosus* infection and the modulation of the immune response.

The aim of our current study was to characterize the proteome of the fluids from human hydatid cysts and to compare the protein composition of them according to their location and fertility status. In this work, the proteomic analysis was conducted on pulmonary, vertebral and paravertebral hydatid cystic fluids with the aim of clarifying the host-parasite interactions occurring in the different stages and how the parasites adapt to the different host tissue locations. Comparing the protein compositions of the hydatid fluids from different host locations could potentially lead to improvements in the diagnosis, prognosis and treatment of human cystic echinococcosis.

2. Materials and methods

2.1. Sample preparation

Human cysts were obtained from three Algerian patients with primary infection. The patients were admitted at the thoracic surgery department of Mustapha Bacha University Hospital, Algiers, Algeria. Patients were from the high plateau and north-central provinces. They came from rural and grazing areas with a common presence of dogs. The patient with pulmonary cysts suffered cough, fever and chest pain. The patient with paravertebral hydatidosis was admitted with symptoms of paraplegia and showed an osteolysis in hydatid cyst area. The pulmonary, vertebral and paravertebral human hydatid cysts containing fertile and infertile fluids (3–8 cm in diameter) were collected from patients after their surgical removal. None of the patients had received pharmacological treatment. All subjects were informed of the study objectives and signed official consent forms. The study was conducted according to the guidelines of the local Ethics Working Group of the Thematic Research Agency in Health Science. Hydatid fluids were aseptically aspirated from individual cysts and centrifuged at 10,000 × g for 15 min at 4 °C to recover the fluid from the pelleted protoscoleces. Proteins present in the hydatid fluids were precipitated overnight with ice-cold acetone (1 V/3 V), and then recovered by centrifugation at 10,000 × g for 15 min at 4 °C. The protein pellet was washed three times with ice-cold acetone, air-dried, and stored at −80 °C until use.

2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein identification by liquid chromatography-tandem mass spectrometry (LC–MS/MS)

Proteins were resuspended in SDS-PAGE loading buffer (60 mM Tris-HCl, pH 6,8; 8% SDS; 10% glycerol; 0,01% bromophenol blue; 5% β-mercaptoethanol) and boiled for 5 min. Proteins were separated by SDS-PAGE (12%) run at 30 V for 120 min, 90 V o/n and 120 V for 120 min. Proteins were revealed by home-made Coomassie blue-staining (0,1% R250, 50% methanol). Protein bands were manually excised from the gels, reduced with 10 mM dithiothreitol, alkylated with 55 mM iodacetamid, and trypsin digested with sequencing-grade modified trypsin (Promega, Leiden, Holland) as described by Shevchenko et al. (1996). The resulting tryptic peptides were fractionated on a nano-HPLC EASY-nLCII instrument (Thermo Fisher Scientific, Odense, Denmark) coupled to a QTOF Ultima Global instrument (Waters, Zellik, Belgium). Peptides were loaded on a 10 cm long column with a 75 μm inner diameter, packed with 3 μm C_{18} particles. Reverse-chromatography was performed with a binary buffer system consisting of 0.1% formic acid (FA) (Buffer A) and 95% acetonitrile in 0.1% FA (Buffer B) for a one hour gradient run with a flow rate of 300 nl/min. The QTOF instrument was operated in the data-dependent mode and the three most abundant peptides with +2 and +3 charges were selected. The raw data files were processed using the Mascot Daemon platform (Matrix Science Ltd, London, UK). The fragmentation spectra were searched against NCBInr-All (https://www.ncbi.nlm.nih.gov/guide/all/) with the parent ion mass tolerances set to 100 ppm. Database search parameters were the following: trypsin as the digestion enzyme with 1 tryptic miscleavage allowed; carbamidomethylation of cysteine was set as fixed modification and methionine oxidation and pyroglutamic acid as variable modifications. For database searching, Mascot individual search algorithms internal estimates using a 95% confidence cutoff were used to calculate Mascot scores. Hence, proteins with scores greater than 54 were significant. Protein identifications were then manually inspected and protein hits were retained when matched with at least two peptides.

2.3. Bioinformatic analysis

Host proteins, identified by searching the NCBInr database, were organized in groups by the PANTHER® classification system (Protein ANalysis THrough Evolutionary Relationships Version 9.0, release date Jan 20, 2014). The classification System Version 9.0 was designed to facilitate high-throughput analysis. Proteins in this database are classified according to their molecular functions and biological processes. The parasite proteins were identified by NCBInr database searches and then organized in groups by gene ontology (http://www.geneontology.org).Each protein was organized in groups related to biological processes and molecular functions.

3. Results

3.1. Protein profiles of the hydatid fluids

The SDS-PAGE and Coomassie-stained protein profiles of the three hydatid fluids (pulmonary hydatid fluid, PHF; vertebral hydatid fluid, VHF; and paravertebral hydatid fluid, PVHF) are shown in Fig. 1. The SDS-PAGE analysis of the human hydatid fluids showed, in each case, a complex mixture of proteins ranging from 170 kDa to less than 10 kDa with a major band of 40–70 kDa corresponding to albumin (Fig. 1). A protein band with a molecular weight larger than 170 kDa can be seen in the fertile fluid samples (corresponding to ferritin as shown in Fig. 1). Interestingly, the infertile PVHF preparation showed fewer bands compared with fertile PHF and VHF, although the albumin signal is conserved.

3.2. Proteomic analysis

The proteomic analysis of the different hydatid fluids from the larval stage of *E. granulosus* revealed the presence of both host and parasite proteins as already observed by previous studies. In such

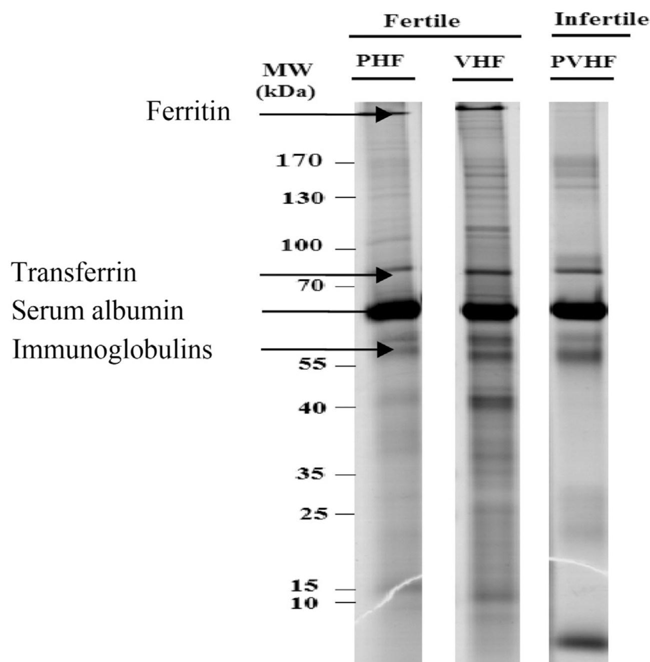

Fig. 1. Protein profile of human hydatid fluids from pulmonary (PHF), vertebral (VHF) and paravertebral (PVHF) cysts. Total proteins, electrophoresed on a 12% SDS-PAGE gel, were stained with Coomassie blue. Molecular weight markers are shown on the left side of the gel. Each lane was loaded with 20 μg of protein.

situation, assigning a protein to the parasite or the host is impossible if the protein is identified with peptides common between the two species. However, the identification of proteins by their unique peptides not only allows the confirmation that this particular isoform is present in the sample but also to assign it to the appropriate species hence alleviating this problem. Hence, protein identification using a unique peptide (unique peptides, Table 1) confirms the presence of the protein in the sample, while protein identification with shared peptides (zero unique peptides, Table 1) indicates that all isoforms sharing the matched peptides are all potentially present. In such condition, we choose to show the first match proposed by Mascot. When the same peptides are shared by both host and parasite, we decided to show both identifications (e.g., human actin, gi|28336 in Table 1 and parasite actin gi|3182894 in Table 2).

3.3. Host protein identification

In this proteomic analysis, new proteins were identified in the human hydatid fluids, some of which are location specific. Thus, serum albumin precursor (gi|6013427), alpha-1-antitrypsin (gi|24438gi|177827), antithrombin III (gi|179161), histone H2A type 1 (gi|4504239), two heat shock 70 kDa proteins (gi|194388088, gi|34419635), two immunoglobulins (gi|323432985, gi|323432992), and alpha-1-B-glycoprotein (gi|69990) and lactoferrin (gi|186833) were specifically present in the PHF, while chain A, apo-human serum transferrin (gi|110590597), C9 complement protein (gi|179726), immunoglobulins (gi|10334541, gi|8918518, gi|62871078, gi|1913916), chitinase (gi|23512215) and some interestingly metabolic enzymes such asfructose-1,6-biphosphate (gi|182311), enolase (gi|31179), and glycogen phosphorylase (gi|183353) were exclusively present in the VHF. In contrast, although very few proteins were identified in the infertile fluid sample, most of them were immunoglobulins (gi|12054074, gi|184759, gi|1827928, gi|468243, gi|170684576, gi|21669343, gi|9968499)and these were present exclusively in this fluid type. Other serum proteins, such as serum albumin (gi|28592, gi|157830361), serotransferrin precursor (gi|4557871) and Chain A, human serum albumin (gi|157830361) were common to all three human hydatid fluids (Fig. 2).

3.4. Parasite protein identification

Interestingly, our results showed a difference in protein profile of the parasitic proteins depending on the location and fertility status of the hydatid fluid (Table 2). Fertile hydatid fluid samples contained more proteins than the infertile sample (Fig. 3). A single parasite protein corresponding to citrate synthase (gi|674572145) was identified in the PVHF; it was shared with PHF while various parasite metabolic enzymes, including stress-related, structural and antigenic proteins, were detected in the fertile hydatid fluids (PHF and VHF, Table 2). The proteins identified also showed differential expression patterns depending on the cyst locations. Although most of these proteins are isoforms of the same protein, their presence was validated by sequencing their unique peptides, such as for malate dehydrogenase, which was present as a single isoform in PHF (gi|556520106) and three isoforms in VHF (gi46406288, gi|6016537, gi|62178022). Other proteins belonging to functionally different gene ontology families were differentially expressed depending on the cyst location (Table 2). Hence, the biological processes and their associated proteins that are unique to the parasite and present in PHF cystsare as follows: pentose phosphate shunt (transaldolase), galactose metabolism (UDP glucose 4 epimerase), amino acid metabolism (glutamate dehydrogenase and ornithine aminotransferase), peptide hydrolysis (calpain), DNA translation (threonyl tRNA synthetase C), chaperones (HSP20, HSP70 and protein disulfide isomerase), membrane binding (annexins)and actin binding (gelsolin, myosin, filamin and actinin). Few proteins were unique to the parasites present in VHF compared to PHF, but those that were included those related to peptide binding (cyclophilin), protein biosynthesis (EF1 alpha) and, notably, two antioxidant enzymes (2-cys peroxiredoxin and thioredoxin peroxidase). In this analysis, differences in the antigen composition of the human fluids were also observed. Antigen B was present in the PHF cyst and antigen S was present in the VHF cyst, while antigen 5 and 14-3-3 proteins were common to both cyst locations (Table 2).

4. Discussion

This proteomic identification reveals new host and parasite proteins in hydatid cyst fluids. Most of the proteins identified in our study have already been described elsewhere (Chemale et al., 2003; Monteiro et al., 2010; Aziz et al., 2011; Virginio et al., 2012). However, proteins such asalpha-1-antitrypsin, antithrombin III, chitinase 3-like 1 and heat shock 70 kDa protein 6were identified in human hydatid fluid for the first time. Xu et al. (1997) reported that heat shock proteins (HSPs) play physiological roles in mediating protective responses to nitric oxide stress. More importantly, chitinase 3-like 1, which was identified specifically in VHF, plays major roles in inflammation, repair and remodeling responses (Areshkov et al., 2012; He et al., 2013). Identification of chitinase 3-like1 indicates that a potential protein exchange occurs between the vertebrate microenvironment and the hydatid cyst, thus indicating adaptation of the cyst to its location. The protective role of this protein has been highlighted previously in several studies in the parasitic diseases field (Choi et al., 2001; Nair et al., 2006; Sutherland et al., 2014).

The identification of human histone in this work may indicate alteration of the host tissue. However, this alteration is probably related to the chronic production of nitrogen species such as peroxynitrite, which can induce apoptosis (Virag et al., 2003). We also identified Fc and Fab fragments in the fertile and infertile fluids, which are similar to those produced by papain digestion. Indeed, IgGl, IgG2, IgG3 and IgG4 subclasses were found to be sensitive to enzymatic digestion.In the same context, it has been shown that protoscoleces binds to and cleaves IgG (Baz et al., 1998). It has also been reported that Fc binding and proteolysis occurs on the surface of the protoscolex (Baz et al., 1998).

Table 1
Host proteins identified in the PHF, VHF, and PVHF as determined by LC/MS-MS and organized by PANTHER® according to their biological processes and molecular function.

Protein function	Accession #	MASCOT score			# of matched peptides	# of unique peptides
		PHF	VHF	PVHF		
Transport						
Serumalbumin	gi|28592	294	165	267	6-5-6	3-2-6
Albumin	gi|119626083	–	–	165	5	1
Chain A, human serum albumin	gi|157830361	818	184	332	17-7-9	9-5-6
Serum albumine precursor	gi|6013427	1187	–	–	21	1
Transferrin	gi|553788	–	113	–	3	2
Serotransferrinprecursor	gi|4557871	294	1004	506	7-20-13	3-1-8
Chain A, Apo-human serum transferrin	gi|110590597	–	995	–	19	4
Lactoferrin	gi|186833	104	–	–	3	3
Hemopexin precursor	gi|386789	96	114	–	3-4	3-4
Gluconeogenesis						
Fructose-1,6-bisphosphatase	gi|182311	–	81	–	2	2
Glycolysis						
2-phosphopyruvate-hydratase alpha-enolase carbonate dehydratase	gi|693933	–	69	–	3	2
Enolase	gi|31179	–	58	–	2	1
Glycogenmetabolicprocess						
Glycogen phosphorylase	gi|183353	–	67	–	2	2
Polysaccharide metabolicprocess						
Chitinase 3-like 1 (cartilage glycoprotein-39)	gi|23512215	–	78	–	3	2
Proteaseinhibitor						
Alpha-1-antitrypsin	gi|177827	108	65	–	2-3	3-3
Alpha-1-antitrypsin	gi|24438	130	–	–	2	2
Antithrombin III	gi|179161	130	–	–	3	2
Defense/immunity/protease						
C9 complement protein	gi|179726	–	95	–	2	2
Haptoglobin precursor	gi|306882	76	94	–	3-2	3-2
Antioxidantactivity						
Peroxiredoxin-4	gi|5453549	–	59	–	3	1
Structural constituent of cytoskeleton						
Mutant beta-actin	gi|28336	114	497	–	4-9	1-0
Predicted similar to actin alpha 1 skeletal muscle protein	gi|169213772	–	61	–	2	2
Chromatinorganization/DNA bindingprotein						
Histone H2A type 1	gi|4504239	86	–	–	1	1
Immune system process						
Heat shock 70 kDa protein 1A/1B	gi|194388088	133	–	–	3	1
Heatshock 70 kDa protein 6	gi|34419635	167	–	–	2	2
Inflammatoryresponse						
Alpha-1-acid glycoprotein 1 (AGP1)	gi|112877	–	81	–	3	1
Unknownontology						
Beta-globin	gi|183817	–	–	146	2	1
Immunoglobulin heavy chain	gi|10334541	–	178	–	5	1
Immunoglobulin gamma heavy chain	gi|8918518	–	142	–	3	1
Immunoglobulin heavy chain constant region gamma 4	gi|12054078	133	120	–	3-3	1-1
Immunoglobulin alpha heavy chain variable region	gi|62871078	–	68	–	2	2
Immunoglobulin heavy chain constant region gamma 2	gi|12054074	–	–	92	4	1
Immunoglobulin gamma-4 heavychain	gi|184759	–	–	75	3	2
Ig gamma-1 chain C region	gi|121039	92	–	90	3-5	1-1
Chain L, Igg Fab (human IgG1, kappa)	gi|1827928	–	–	166	3	1
Immunoglobulin kappa light chain	gi|468243	–	–	144	3	1
Immunoglobulin kappa 4 light chain	gi|170684576	–	–	135	3	1
Immunoglobulin kappa light chain VLJ region	gi|21669343	–	–	121	3	1
Immunoglobulin kappa chain variable region	gi|9968499	–	–	68	3	2
Immunoglobulin variable region	gi|323432985	115	–	–	2	1
Immunoglobulin variable region	gi|323432992	84	–	–	2	1
Anti-HIV-1 gp120 immunoglobulin heavy chain	gi|272982598	315	444	–	8-9	1-1
Alpha-1-B-glycoprotein	gi|69990	62	–	–	2	2
Immunoglobulin Fd chain	gi|1913916	–	136	–	3	1

Interestingly, immunoglobulins were more abundant in the infertile fluid, possibly suggesting a role in cyst infertility. The presence of annexins exclusively in the PHF may suggest a role in the calcification process of cysts (Diaz et al., 2000). We also observed that the PHF was less fertile than the VHF. Cytoskeleton protein such as gelsolin, myosin, filamin and actin were also identified and their antigenic effect was highlighted in protoscoleces.

SDS-PAGE analysis showed that far fewer bands were present in the infertile PVHF as compared with the fertile PHF and the HVF (Fig. 1), this observation is consistent with that of Latif et al. (2013). In the infertile PVHF, citrate synthase, a protein expressed by cyst walls, was the only protein of parasite origin. The abundance of this protein appears to be low in the infertile PVHF (mascot score, 62), compared with the fertile PHF (mascot score, 479), suggesting a possible relationship with cystic fertility. However, its absence from the fertile VHF undermines this hypothesis for cyst location. Similarly, a transcriptomic analysis revealed that the germinal layer possesses a high metabolic activity involving the Krebs pathway (Parkinson et al., 2012). The coexistence of antioxidant proteins of both host and parasite origin highlights the impact of a stressful microenvironment. Parasite survival relies on effective defense against reactive oxygen and nitrogen species produced by the host immune system. Indeed, it has been shown that

Table 2
Parasite proteins identified in PHF, VHF, and PVHF, as organized into biological and molecular functions by Gene Ontology annotation.

Protein function	Accession #	Species	MASCOT score			# of matched peptides	# of matched peptides
			PHF	VHF	PVHF		
Ferrric iron binding							
Ferritin	gi|110558962	*E. granulosus*	–	168	–	3	
Ferritin heavy chain	gi|576692417	*E. granulosus*	62	323	–	2-6	
Pentose-phosphate shunt							
Transaldolase	gi|556522327	*E. granulosus*	100	–	–	2	2
Galactose metabolic process							
UDP glucose 4 epimerase	gi|556514599	*E. granulosus*	68	–	–	2	2
Glycolytic process							
Putative glucose phosphate isomerase	gi|62178020	*E. granulosus*	178	–	–	5	5
Glyceraldehyde-3-phosphate dehydrogenase	gi|149364041	*Taenia solium*	89	137	–	3	1
Glyceraldehyde-3-phosphate dehydrogenase	gi|556517778	*E. granulosus*	89	–	–	2	1
Glyceraldehyde-3-phosphate dehydrogenase	gi|6016079	*E. multilocularis*	–	234	–	6	3
Enolase	gi|262192839	*E. granulosus*	199	827	–	4-14	2-8
Enolase	gi|261266611	*Taenia asiatica*	156	564	–	3-6	1-1
Fructose-1,6- bisphosphate aldolase	gi|556514117	*E. granulosus*	641	–	–	12	11
Fructose-bisphosphate aldolase	gi|29336561	*E. multilocularis*	–	576	–	11	11
Lactate dehydrogenase A	gi|375112322	*E. granulosus*	134	–	–	4	4
Tricarboxylic acid cycle							
Malate dehydrogenase	gi|556520106	*E. granulosus*	182	–	–	3	3
Malate dehydrogenase	gi|46406288	*E. granulosus*	–	95	–	2	2
Malat edehydrogenase, cytoplasmic	gi|6016537	*E. granulosus*	–	435	–	8	3
Putative malate dehydrogenase	gi|62178022	*E. granulosus*	–	215	–	6	1
Citrate synthase	gi|674572145	*E. granulosus*	479	–	62	10-2	3-2
Gluconeogenesis							
Phosphoenol pyruvate carboxykinase	gi|338827788	*E. granulosus*	335	–	–	8	5
Phosphoenol pyruvate carboxykinase	gi|338827786	*E. multilocularis*	122	–	–	2	1
Phosphoenol pyruvate carboxykinase	gi|283466470	*E. granulosus*	–	511	–	8	8
Phosphoenol pyruvate carboxykinase	gi|283466482	*Taenia solium*	–	272	–	6	1
Glycogenolysis							
Glycogen phosphorylase	gi|556517139	*E. granulosus*	160	–	–	3	3
Cellular aminoacid metabolic process							
Glutamate dehydrogenase 2	gi|576694207	*E. granulosus*	630	–	–	10	10
Peptide binding							
Cyclophilin (Peptidyl-prolyl *cis*-transisomerase	gi|31077167	*E. granulosus*	–	68	–	2	2
Protein biosynthesis							
Elongation factor 1 alpha	gi|148717321	*E. multilocularis*	–	72	–	2	2
Aminotransferase							
Ornithine aminotransferase	gi|576698640	*E. granulosus*	395	–	–	7	7
ATP binding							
Threonyl tRNAsynthetase C	gi|556521465	*E. granulosus*	381	–	–	8	7
Cytoskeleton component							
Actin-1	gi|543766	*E. granulosus*	–	265	–	8	1
Actin	gi|467215	*D. dendriticum*	221	–	–	5	1
Spectrin alpha actinin	gi|556521777	*E. granulosus*	62	–	–	2	2
Actin filament capping/actin binding							
Actin-filament fragmenting protein	gi|12641925	*E. granulosus*	–	220	–	5	5
Gelsolin	gi|556513882	*E. granulosus*	69	–	–	3	3
Myosin heavy chain	gi|556522013	*E. granulosus*	137	–	–	5	3
Filamin	gi|556513666	*E. granulosus*	95	–	–	2	2
Alpha actinin sarcomeric	gi|576699978	*E. granulosus*	86	–	–	2	2
Chaperon/Stress response							
Heat shock 90 kDa protein	gi|124783236	*Taenia asiatica*	–	292	–	4	1
Predicted heat shock 70 kDa protein 8-like	gi|291230940	*S. kowalevskii*	273	–	–	7	1
Heat shock protein family member hsp 3 (HSP70-like)	gi|556517734	*E. granulosus*	168	–	–	4	1
Heat shock cognate protein(HSP70-like)	gi|576692679	*E. granulosus*	633	–	–	12	2
Putative HSP20 related protein	gi|17065922	*E. multilocularis*	93	–	–	2	2
(Endo)peptidase activity							
Calpain A	gi|556514168	*E. granulosus*	312	–	–	7	7
Calcium ion binding							
Annexin A8	gi|576701205	*E. granulosuss*	134	–	–	4	4
Annexin A7	gi|576695517	*E. granulosuss*	102	–	–	2	2
Annexin A6	gi|576697441	*E. granulosuss*	95	–	–	3	3
Annexin	gi|556519264	*E. granulosuss*	272	–	–	6	6
Annexin	gi|556517680	*E. granulosus*	264	–	–	8	8
Transferase activity							
Glutathione *S*-transferase	gi|2316076	*E. granulosus*	85	108	–	3-3	3-3
UTP glucose 1 phosphate uridylyl transferase	gi|576700328	*E. granulosus*	94	–	–	2	2
Peroxiredoxin activity							
2-Cys peroxiredoxin	gi|158519654	*Taenia solium*	–	150	–	3	1
Cellular oxidant detoxification/antioxidant activity							
Thioredoxin peroxidase	gi|22775336	*E. multilocularis*	–	249	–	5	3
Thioredoxin peroxidase	gi|4104346	*E. granulosus*	–	171	–	3	2

(continued on next page)

Table 2 *(continued)*

Protein function	Accession #	Species	MASCOT score			# of matched peptides	# of matched peptides
			PHF	VHF	PVHF		
Cell redox homeostasis							
Putative proteindisulfide- isomerase ER-60	gi|576698733	*E. granulosus*	189	–	–	3	3
Protein disulfide isomerase	gi|556522176	*E. granulosus*	71	–	–	3	3
Motor activity							
Paramyosin	gi|556512729	*E. granulosus*	1030	–	–	20	5
Paramyosin	gi|547974	*E. granulosus*	–	1222	–	25	25
Myosin heavy chain	gi|556522013	*E. granulosus*	137	–	–	5	3
Antigens							
Antigen S epitope	gi|158840	*E. granulosus*	–	91	–	2	2
Ag5	gi|385682862	*E. granulosus*	363	–	–	5	5
Ag5 precursor	gi|27651943	*E. granulosus*	–	442	–	7	7
22 kDa antigen 5	gi|296012060	*E. granulosus*	–	97	–	2	1
Antigen B subunit 4	gi|34550886	*E. granulosus*	87	–	–	2	2
Putative 14-3-3 protein	gi|62178030	*E. granulosus*	283	181		5-5	5-5
14-3-3 protein epsilon	gi|556517565	*E. granulosus*	126	–	–	2	2

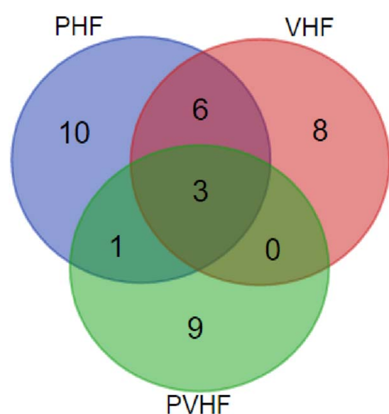

Fig. 2. Venn diagram showing the repartition of the identified proteins from the host in the different hydatid fluids (PHF, VHF and PVHF). Numbers of identified proteins indicate the level of protein overlap between the hydatid fluids. Notable regions include protein groups' specific to only one hydatid fluid while mixed color region show shared proteins.

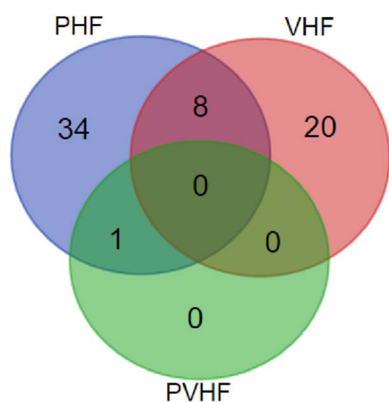

Fig. 3. Venn diagram showing the repartition of the identified proteins from the parasite in the different hydatid fluids (PHF, VHF and PVHF). Numbers of identified proteins indicate the level of protein overlap between the hydatid fluids. Notable regions include protein groups' specific to only one hydatid fluid while mixed color region show shared proteins.

the parasite expresses antioxidants, including glutathione *S*-transferase and thioredoxin peroxidases (TPx) (Li et al., 2004). It is therefore surprising that 2-cys peroxiredoxin and thioredoxin peroxidase were identified exclusively in the VHF of the cyst.

In a previous study, we reported an increase in nitric oxide production upon parasite infection (Ait Aissa et al., 2006; Zeghir-Bouteldja et al., 2013), which was related to immune-stimulation by parasite antigens that can leave cysts (Monteiro et al., 2010). In the same way, we observed a difference in nitric oxide production, and this difference was attributed to the different proteins in the hydatid cyst fluids (Zeghir-Bouteldja et al., 2013; Wang et al., 2015). Many antigenic proteins have been identified in fertile cysts. *E. granulosus* antigen B (AgB), which has been identified exclusively in PHF, is associated with a number of immunomodulatory functions in the host, and influences the Th1/Th2 balance. However, it has also been reported that AgB subunit 4 is expressed at a lower level than the other subunits, and is more highly expressed in cyst walls (Parkinson et al., 2012). Antigen 5 (Ag5) and AgB each have major immunodiagnostic value for detecting *E. granulosus* (Lightowlers et al., 1989; Siracusano et al., 2009). The 14-3-3 protein, which was identified herein in both fertile cysts, has been identified in *E. multilocularis* metacestodes (Andrade et al., 2004) and *E. granulosus* adults (Siles-Lucas et al., 2008) and is involved in the reduction of nitric oxide production.

In our study, different HSPs were identified in fertile hydatid fluids. These proteins may be produced in response to host temperature variations. More interestingly, HSPs were detected in protoscoleces ES products after *in vitro* heat treatment. It has been reported that these proteins may act as parasite immune evasion antigens (Del Gudice, 1994; Martínez et al., 1999; Dobbin et al., 2002). *E. granulosus* HSP70 has been reported to stimulate humoral and cell-mediated immune responses. In another study conducted in the same field, it was reported that proteomic analysis of protoscoleces and hydatid fluid extracted from hydatid cysts showed the presence of HSP20 and HSP70 (Chemale et al., 2003). In the present work, we identified elongation factor-1 alpha (EF-1 alpha) and cyclophilin (EA21) exclusively in a VHF cyst.EF-1 alpha was identified previously in *E. granulosus* protoscolex ES products (Virginio et al., 2012), and EA21 has been described as an allergen capable of inducing IgE secretion in *Echinococcus* (Ortona et al., 2001, 2002; Mezioug and Touil-Boukoffa, 2009, 2012). It has also been reported that the latter antigen is probably released into the hydatid fluid only after the protoscoleces degenerates following aging and calcification (Khabiri et al., 2006). These antigens may be found among the main *E. granulosus* antigens used for diagnostic purposes, such as AgB and Ag5.

In the present study, we also identified enzymes involved in gluconeogenesis (fructose-1,6-bisphosphatase and phosphoenol pyruvate carboxykinase PEPCK), glycogenolysis and glycogenesis, which were previously identified by Aziz et al. (2011). Fructose-bisphosphate aldolase and enolase were previously identified in the protoscolex tegument, *in vitro* ES products, and germinal layer cells (Lorenzatto et al., 2012). These enzymes were reported as antigens in human infections with *E. granulosus* (Parkinson et al., 2012). Here, metabolic activity was observed in fertile cysts. Moreover, we observed that in one of the major infection cases, the hydatid cysts located in vascularized

organs such as the liver, lung, and spleen were unilocular and fertile, but those located in organs with less vascularity (i.e., smooth tissue andbone) were infertile cysts with daughter cysts. Thus, this proteomics study has provided new information about hydatid cyst fertility, viability, and adaptation to host organ location. Importantly, the role of some identified proteins in immunoprotection was observed. The existence of parasite endopeptidase inside the cysts and exchange of proteins between host and parasite could contribute to survival and growth of the metacestode. The interruption of this exchange could contitute a therapeutic target.

Collectively, we have shown that the complexity of immune responses in human cystic echinococcosis is influenced by many factors, but the anatomical location of the larval stage of the parasite is particularly relevant. Comparisons of the protein profile of cyst hydatid fluid, according to the cyst location, showed differences in protein expression and point adaptation of the metacestode to its host environment. Proteomic identification of hydatid fluids should help improve our understanding of the biochemical and immunological aspects of human cystic echinococcosis. The characterization of new hydatid proteins involved both in the physiology of the parasite and in host tissue damage may contribute to the development of new therapeutic strategies and new diagnosis tools in hydatidosis. Our results have a potential value in new strategies to improve the therapy, the prevention and diagnosis

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

The authors thank the technical and surgical staff of Mustapha Bacha Hospital (Algiers) for providing the cyst samples. Special thanks go to Professor H. Chaouche. We also thank all the voluntary participants of this study.

This work was supported by Thematic Research Agency in Health Sciences (PNR43/ANDRS/2011).

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