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Short Communication

Immunoproteomic approach for identification of *Ascaris suum* proteins recognized by pigs with porcine ascariasis

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ABSTRACT

Ascaris suum, the causative agent of porcine ascariasis, is responsible for marked economic losses in pig farms worldwide. Despite recent advances in research, including the characterization of the genome of *A. suum*, knowledge about the parasite/host relationship in porcine ascariasis at the molecular level is scarce and chemotherapy is the only effective option for parasite control. The aim of this study was to identify immunogenic proteins of *A. suum* somatic antigens associated with the pathogenicity/survival mechanisms of the parasite, by using two-dimensional (2-D) electrophoresis, 2-D Western blot and mass spectrometry (MS). A total of 24 parasite proteins recognized by serum samples from pigs naturally infected with *A. suum* were identified. Most of them (23/24) were identified as being involved in parasite survival mechanisms, including functions related to energy generation (12 proteins) and redox processes (5 proteins). These results may aid the search for effective chemo-therapeutic targets in porcine ascariasis. Further studies are needed, however, to illustrate the effect of the host immune response on the survival mechanisms of *A. suum*.

1. Introduction

Ascariasis caused by nematode parasites of the genus *Ascaris* represents one of the major public health problems in the world. *Ascaris suum*, a pig parasite, is responsible for significant economic losses in swine by interfering with the health and development of the parasitized animal (Dold and Holland, 2011).

Pigs contract *A. suum* infection via the fecal–oral route. Following the ingestion of infective ova, the eggs hatch in the small intestine and larvae penetrate the mucosa and migrate to the portal venous system to reach the liver. After hatching, the larvae advance to the lungs, penetrate the alveolar space and move to the pharynx where they are swallowed. They then return to the small intestine at 8–10 days post-infection, where they complete their development and reach sexual maturity. *A. suum* adult worms can live in the intestine for 1 year, evading the host response (Dold and Holland, 2011).

Studies of the parasite/host relationship in porcine ascariasis at the molecular level are still scarce. No vaccines have so far been developed and the use of antihelmintic

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drugs remains the only effective option for controlling this infection (Chen et al., 2012). Proteomic studies represent an alternative strategy to study the protein expression pattern of an organism, using cross-species databases to interpret mass spectrometry data (Cui et al., 2013). Furthermore, the recent characterization of the *A. suum* genome provides new hope for the development of control strategies for this parasite (Jex et al., 2011). From an immunological point of view, it has been reported that *A. suum* induces a strong Th2 response, typical of gastrointestinal parasites, which can be measured both systemically (e.g. blood eosinophilia, IL4) and locally (increase in IL4, IL6, IL10 and IL13) (Roepstorff et al., 2011).

The aim of this study was to identify immunogenic proteins of *A. suum* and to associate them with molecular mechanisms governing porcine ascariasis. Proteomic, immunomic and mass spectrometry techniques were employed.

2. Materials and methods

2.1. Parasites and serum samples

Adult worms of *A. suum* were obtained at slaughter during autopsy of naturally infected pigs from an industrial pig farm located in the Lombardy region of NE Italy. Serum samples taken from 10 pigs diagnosed as having porcine ascariasis (from the same farms above mentioned) and from 10 healthy pigs (Università degli Studi di Parma, Italy) born and raised in secure facilities were used to carry out the experiments. The pigs were large white breed and blood samples were taken at approximately 4–6 months of age. The parasitological status of the infected pigs was tested by coprological assay for *A. suum* eggs and an indirect ELISA for anti-*A. suum* IgG (Roepstorff, 1998).

2.2. Two-dimensional electrophoresis (2-DE) of AsSA extract and immunoblot assay

A. suum somatic antigen (AsSA) extract was prepared from 6 adult worms (3 males and 3 females) with lengths from 118 to 275 mm and diameters from 2 to 4.5 mm. Homogenization was carried out by maceration of the adult worms using a tissue-grinder in the presence of phosphate-buffered saline solution (PBS), pH 7.4, and sonication of the resulting suspension on ice for 5 cycles of 1 min each at 75 kHz.

A cocktail of protease inhibitors was added to the homogenates (Maizels et al., 1991) and an ultracentrifugation at 100,000 × *g* for 1 h at 4 °C was performed. The supernatants with the soluble proteins were dialyzed against water for 24 h and the protein concentrations were measured by DC protein assay commercial kit (Bio-Rad). The AsSA extract was concentrated and purified with the ReadyPrep 2-D Cleanup Kit (Bio-Rad), following the manufacturer's instructions and re-suspended in rehydration buffer 2-D (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)). The samples were divided into 125 μl aliquots (containing 40 μg of protein) and stored at −20 °C until being used.

The 2-DE of the AsSA extract was performed as described before by González-Miguel et al. (2010) with minor modifications. Briefly, AsSA extract aliquots were supplemented with ampholytes, incubated and centrifuged, and then applied to 7-cm IPG strips (Bio-Rad) with linear pH ranges of 3–10, 5–8 and 7–10, using a Protean IEF Cell (Bio-Rad) for isoelectric focusing (IEF). After IEF, strips were reduced and alkilated, and second dimension was done in 12% acrylamide gels. Gels were then silver stained with the PlusOne Silver Staining Kit, Protein (GE Healthcare).

To determine which proteins of the AsSA extract were immunoreactive an immunoblot was performed (González-Miguel et al., 2010). The 2-D gels were transferred to nitrocellulose membranes, which were blocked, and then incubated overnight at 4 °C with a pool of 10 sera from healthy pigs or a pool of 10 sera from naturally infected pigs at 1:100 dilution. A horseradish peroxidase-labeled anti pig IgG (Fitzgerald) at 1:5000 dilution was also used and immunoreactive proteins were revealed with 4-chloro naphthol. Samples were analyzed in triplicate to assess the overall reproducibility of the protein and immunogen spot patterns.

The 2-D gels and membranes were scanned and analyzed with the software Quantity One Software v.4.6.5 (Bio-Rad). Matching of 2-D gels with the homologous Western blot to identify immunoreactive proteins was analyzed using the PDQuest Software v.8.0.1 (Bio-Rad).

2.3. MS and protein identification

In gel digestion of proteins and MS analysis were done as described before by González-Miguel et al. (2012). The selected spots containing immunogenic proteins were excised manually from the gels and sent to the Unit of Proteomics of the Centro de Investigación Príncipe Felipe (Valencia, Spain) for MS analysis. For peptide mass fingerprinting and the acquisition of LIFT TOF/TOF spectra, an aliquot of the digestion of each spot was deposited onto a 600 μm Anchor Chip MALDI probe (Bruker-Daltonics). Peptide mass fingerprint spectra were measured on a Bruker Ultraflex TOF/TOF MALDI mass spectrometer (Bruker-Daltonics) in positive-ion reflector mode. The measured tryptic peptide masses were transferred through the MS BioTools program (Bruker-Daltonics) as inputs to search the National Center for Biotechnology Information non-redundant database (NCBInr) using Mascot software (Matrix Science). When necessary, MS/MS data from the LIFT TOF/TOF spectra were combined with MS peptide mass fingerprint data for database searches. The molecular function and biological processes of the identified proteins were assigned according to the gene ontology database (http://www.geneontology.org) and the Swiss-Prot/UniProt database (http://beta.uniprot.org).

3. Results

3.1. Two-dimensional gel electrophoresis

The AsSA extract was first electrofocused using 3–10 linear immobilized pH gradient strips. Silver nitrate

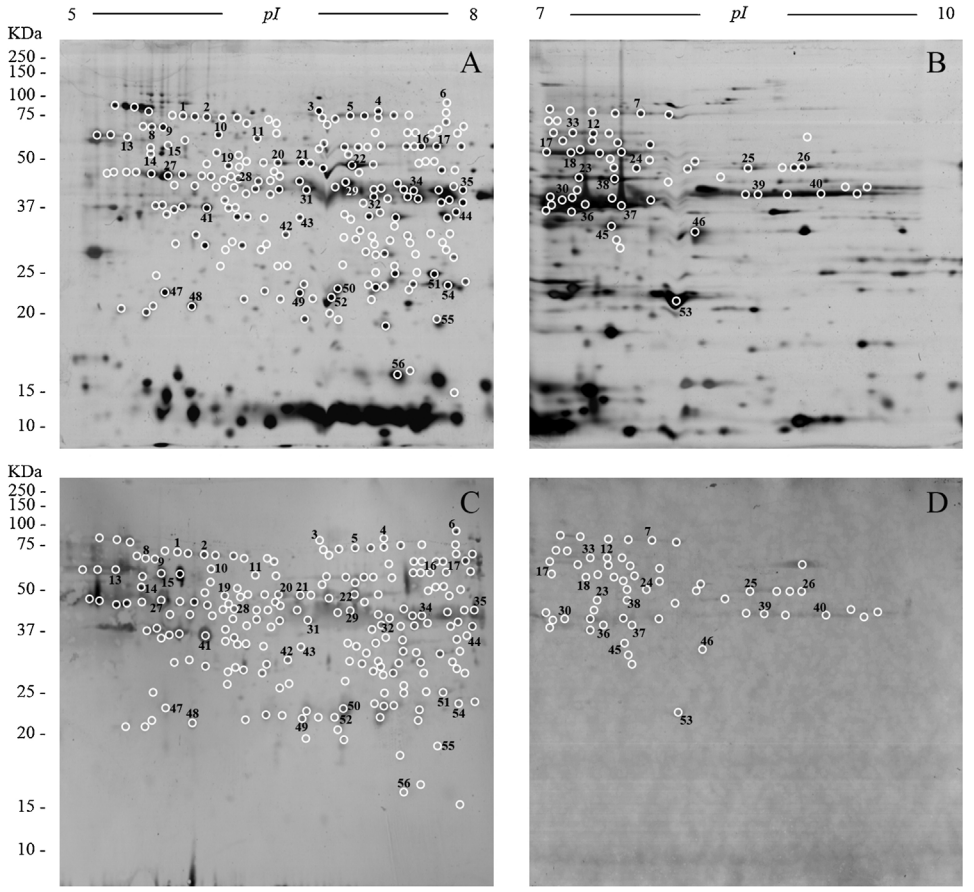

Fig. 1. Representative two-dimensional electrophoresis of 40 μg of the AsSA extract from adult *A. suum* worms (A and B). The gels were in the 5–8 and 7–10 pH ranges, 12% polyacrylamide and silver-stained, and 2-D Western blot showing the antigenic spots of the AsSA extract (C and D) revealed by pools of serum samples from naturally infected pigs. Reference molecular masses are indicated on the left. The antigenic spots analyzed by MS are circled and numbered.

staining of the 2-D gels revealed 605 spots in the proteome of *A. suum*, many sparsely settled, with pIs between 5 and 10, and a broad range of MWs (5–120 kDa). Only 35 spots were observed with pI < 5 (not shown). In order to improve spot resolution and detection, the AsSA was electrofocused in 5–8 (Fig. 1A) and 7–10 (Fig. 1B) IPG strips. With these new conditions, silver staining revealed a total of 682 spots, most of them located between pH 5 and 8. One hundred and ninety-eight spots of *A. suum* had pIs between 8 and 10.

3.2. Antigenic spots revealed by the serum samples from naturally infected pigs with A. suum

Immunoblot analysis of the AsSA carried out with serum samples from naturally infected pigs with *A. suum* revealed around 293 major antigenic spots (Fig. 1C and D). This represented a recognition rate of 42.9% for total spots revealed in the 2D gels. In terms of distribution, most of the antigenic spots of *A. suum* were located in a narrower range of MWs (20–85 kDa) and pIs (5–8). Twenty-three spots of AsSA were found below these ranges. Serum samples from healthy individuals (negative control) did not reveal any spots (not shown).

3.3. Identification of A. suum immunoreactive proteins

The matching of spots revealed by Western blotting with their homologous in the silver stained 2-D gels allowed for a selection of 56 antigenic spots of *A. suum* which were manually excised from 2-D gels and submitted to analysis by MS. Thirty-six of 56 spots were identified (64.3%) and corresponded to 24 different proteins. Between 1 and 5 isoforms of each protein were identified.

Table 1 shows the identity of these proteins, their theoretical MW and pIs, the access number to the homologous protein available in the NCBI database and the Mascot score. Most of the identified spots corresponded to proteins of *A. suum* deposited in databases (30 of 36 spots). The 6 remaining spots corresponded to proteins from other nematodes (*A. lumbricoides*, *Caenorhabditis elegans*, *Brugia malayi* and *Onchocerca volvulus*) or from other groups of parasites (*Fasciola hepatica*). In addition Table 1 shows the molecular function and biological processes in which the 24 proteins identified are involved. All proteins showed catalytic activity except actin 2 (structural activity). Among these, most were related to energy generation and metabolic pathways (12 proteins) and to redox processes (5 proteins).

Table 1
Antigenic protein spots from AsSA extract recognized by naturally infected pigs and identified by MALDI-TOF MS. Mascot score is the score given as $S = -10 \times \log(P)$, where P is the probability that the observed match would be a random event. Mascot score values above 80 are considered significant ($P < 0.05$). Molecular function and biological process in which the antigenic proteins of AsSA extract are involved was assigned according to the Gen Ontology and Swiss-Prot/Uniprot databases. Theor, theoretical; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PE, phosphatidyl-ethanolamine.

Spot number	Accesion code	Protein identification	Species	MW (KDa) theor	pI theor	Mascot Score	Molecular function	Biological process
							Structural activity	
13	XP_001898433	Actin 2	*B. malayi*	42.1	5.3	321	–	Cell motility
							Catalytic activity	
1	gi|17971973	Similar to HSP70	*A. suum*	20.9	5	115	ATP binding	Stress response
3, 4	gi|23263210	Similar to Immunosupresive ovarian message protein	*A. suum*	12.7	9.3	120, 125	Chitin binding	Chitin metabolic process
5	Q05893	Phosphoenolpyruvate carboxykinase	*A. suum*	73.0	6.3	451	Lyase	Gluconeogenesis
7	Q19842	Propionyl-CoA carboxylase alpha chain	*C. elegans*	80.2	7.6	96	Ligase	CoA metabolism
9, 10	gi|17989720	Similar to chaperonine HSP60	*A. suum*	22.7	9.2	80, 107	ATP binding	Protein refolding
12	P27443	NAD-dependent malic enzyme	*A. suum*	73.2	8.4	105	Oxidoreductase	Malate metabolism
17, 18	AAD30450	Dihydrolipoyl dehydrogenase	*A. suum*	53.4	6.9	192, 109	Oxidoreductase	Redox homeostasis
19	BAC66617	Inorganic pyrophosphatase	*A. suum*	40.6	6.7	126	Diphosphatase	Phosphate metabolism
22, 23	AAP81756	Enolase	*O. volvulus*	47.5	6.0	97	Lyase	Glycolysis
25, 26	AAP51177	Fumarase	*A. suum*	50.8	8.1	80, 99	Lyase	Tricarboxylic acid cycle
27	P26269	Pyruvate dehydrogenase	*A. suum*	39.7	5.8	496	Oxidoreductase	Glycolysis
29, 30	gi|24467934	Similar to Fructose-bisphosphate aldolase	*A. suum*	22.6	6.2	117, 184	Lyase	Glycolysis
31, 32	gi|17990578	Similar to Aldehyde reductase C07D8.6	*A. suum*	24.2	6.1	171, 96	Oxidoreductase	Locomotion
33–37	BAB68543	GAPDH	*A. suum*	36.3	6.8	79, 264, 307, 321, 392	Oxidoreductase	Glycolysis
38	AAZ17561	Phosphoglycerate kinase	*F. hepatica*	47.4	9.0	94	Transferase	Glycolysis
41	gi|171288831	Similar to DHS-15	*A. suum*	25.0	7.2	248	Oxidoreductase	Redox process
47	gi|17993818	Similar to Thioredoxin peroxidase	*A. suum*	22.6	6.0	140	Oxidoreductase	Redox process
49	gi|23260162	Similar to AV25 protein	*A. lumbricoides*	23.7	6.5	166	–	Stress response
50	gi|171283860	Similar to Triosephosphate isomerase	*A. suum*	25.1	6.6	82	Isomerase	Glycolysis
52, 53	P46436	Glutathione S-transferase 1	*A. suum*	23.6	6.9	423, 105	Transferase	Redox process
54	gi|17971162	Similar to Glutathione S-transferase 2	*A. suum*	28.6	6.8	148	Transferase	Redox process
55	gi|24468119	Similar to PE-binding protein	*A. suum*	20.7	7.0	160	Lipid binding	–
56	gi|17989859	Similar to Myoglobin	*A. suum*	19.0	6.9	160	Oxygen binding	Oxygen transport

4. Discussion

In the present study, an immunoproteomic analysis was performed in order to identify immunogenic proteins of *A. suum* in pigs naturally infected with the parasite. To date, proteomic studies about *A. suum* and ascariasis are scarce. Several authors have reported a proteomic approach for the identification of candidate chemotherapeutic targets (Kasuga-Aoki et al., 2000; Abebe et al., 2002; Islam et al., 2004, 2006). More recently, the excretory–secretory proteins of the migratory stages of *A. suum* using LC-MS/MS were identified (Wang et al., 2013). Here we have examined the soluble proteome of the *A. suum* adult worm by 2-D Western blot using pools of sera from pigs diagnosed as having porcine ascariasis. Despite the limitations of the techniques applied, a total of 682 spots were observed in the proteome of *A. suum*. This represents a larger number of proteins than that obtained by other authors in similar proteomic studies conducted in *A. suum* or other parasites (Kasuga-Aoki et al., 2000; Abebe et al., 2002; Islam et al., 2004, 2006; Pérez-Sánchez et al., 2006; Martínez-Ibeas et al., 2013). Regarding the immunoproteomic analysis we have identified a total of 24 parasite immunogenic proteins by MS. The exposure of these proteins to the host immune system is probably related to re-infections during which, destruction of larvae can occur. Moreover, like in other nematodes, larval and adult stages share some antigens. For example, five proteins identified in our study are also detected by Wang et al. (2013) as proteins of the excretory/secretory antigens of the L4 of *A. suum* (phosphoenolpyruvate carboxykinase, enolase, fructose-bisphosphate aldolase 1, glutathione S-transferase 1, PE-binding protein). In addition, two of them (enolase and glutathione S-transferase) have been previously postulated as chemotherapeutic targets in *A. suum* (Liebau et al., 1997; Chen et al., 2012).

After comparisons with different databases, each protein was identified as participating in at least one biological process. The most abundant proteins were those associated with different metabolic processes (12 of 24), which could be used by the parasite for energy generation. Among them proteins involved in glycolysis were the most represented (6 proteins) (enolase, pyruvate dehydrogenase, fructose-bisphosphate aldolase, GAPDH, phosphoglycerate kinase and triose phosphate isomerase). The maintenance of this pathway is a key mechanism for parasite survival, since like other anaerobic parasites *A. suum* uses exogenous glucose to generate energy through the glycolytic pathway (Chen et al., 2012).

Five proteins involved in the maintenance of redox homeostasis of *A. suum* were identified (dihydrolipoylde hydrogenase, DHS-15, thioredoxin peroxidase, glutathione S-transferase 1 and glutathione S-transferase 2). Parasitic redox processes are vital for the interactions and adaptations between parasite and host. More studies in this area are needed in order to identify new therapeutic targets, since the host immune response includes an induced oxidative attack that parasites should neutralize and control to survive (Salinas, 2013). The 7 remaining proteins identified were related to other important functions for parasite survival (cell motility, stress response, protein folding, locomotion or oxygen transport).

Aside from the molecular functions described, many of identified proteins have been linked to other important processes in the parasite/host relationships. For example, the involvement of actin, HSP 60, enolase, FBAL and GAPDH in the activation of the fibrinolytic system in other parasitic nematodes has been shown (González-Miguel et al., 2012; Figuera et al., 2013). Activation of this system has also been associated with the degradation of extra-cellular matrix (Vassalli et al., 1991) and, therefore, with the intra-organic migration in different parasites (Jolodar et al., 2003; Bernal et al., 2004). Blocking these proteins may also reduce damage, considering the characteristic migration of *A. suum* larvae in pigs, a fact that has been shown to cause severe liver damage in the host (Roepstorff et al., 2011).

In conclusion, we have identified 24 immunogenic proteins of *A. suum* recognized by the immune system of naturally infected pigs. These proteins are mainly related to metabolic functions and redox processes, associated with parasite survival mechanisms. Energy generation is a key process for the development, fertility and survival of organisms. Moreover, the repair and antioxidant capacity play a key role in stress situations such as long-term infections in competent hosts, contributing to parasite evasion of immune response and repair of damage, so the partial or total blockage of these proteins with antibodies could contribute to control the intra-parasitic population or to decrease the egg production.

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