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Proteomic analysis of *Ascaridia galli*. Identification of immunoreactive proteins in naturally and experimentally infected hens

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ABSTRACT

Ascaridia galli, intestinal parasite of domestic fowl, is responsible of economic losses in avian exploitations. However, molecular mechanisms that govern avian ascaridiasis remain largely unknown. The aim of the present work was to identify proteins of *A. galli* recognized by the immune system of naturally and experimentally infected hens, using two-dimensional electrophoresis (2-DE) and mass spectrometry (MS). Sixteen immunoreactive proteins of *A. galli* were identified. These proteins are mainly related to different metabolic processes, cell motility and binding activities. The timing evolution of this recognition pattern was studied using serum samples from experimentally infected hens, allowing us to observe an early recognition of many of these antigens. Many of them were isoforms from lipid and plasminogen-binding proteins. Moreover, plasminogen-binding activity has been related in other parasites with the facilitation of intra-organic migration, which represents an important fact in avian ascaridiasis. This work represents the first proteomic study of *A. galli* and could contribute to explain some aspects of parasite/host relationships of avian ascaridiasis.

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1. Introduction

Ascaridia galli (Schrank, 1788) is an intestinal nematode of domesticated chickens, causing avian ascaridiasis, parasitosis with worldwide distribution in poultry (Wilson et al., 1994; Permin et al., 1999; Magwisha et al., 2002; Martín-Pacho et al., 2005; Luna-Olivares et al., 2006; Marín-Gómez and Benavides-Montano, 2007). The birds become infected after ingesting embryonated eggs which are deposited on the ground with the feces of other infected hosts. The eggs hatch in the small intestine releasing the second stage larvae that live in the intestinal lumen and migrate to the intestinal mucosa to the tenth day post-infection (pi). After two successive molts the larvae back into the intestinal lumen at 17 days pi, reaching sexual maturity and beginning the egg-laying at 50 days pi (Tarazona-Vilas, 1999). The presence of worms in the digestive tract of birds causes diarrhea and bleeding (Simón Vicente, 1956), which produce weight loss and reduction in both growth rate and feed conversion efficiency affecting the poultry production (Ackert and Herrick, 1928; Ramadan and Abou Znada, 1991; Skallerup et al., 2005).

The information available about the molecular aspects of the parasite/host relationships in avian ascaridiasis

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is scarce. The immune response developed by the host against ascarids has been widely studied in mammals (Cooper et al., 2000; Miquel et al., 2005). However, there are no similar studies in avian ascaridiasis infections. The study of the IgG antibody response in experimentally infected hens versus soluble extracts from both embryonated eggs and adult worms of *A. galli*, has revealed a strong response against parasite antigens of 30–34, 44–54 and 58–90 kDa. Moreover, histological analysis revealed an intense inflammatory reaction with leukocyte infiltration in the intestinal wall of parasitized birds (Marcos-Atxutegi et al., 2009). Recently it has been shown that infection by *A. galli* affected the electrogenic transport of alanine and glucose in the gut of the host, where local immune response is also observed caused by infiltration of different populations of T cells in the lamina propria and accumulation of CD4 + lymphocytes in the epithelium (Schwarz et al., 2011). Regarding the identification of parasite/host relationships relevant proteins of *A. galli*, there is a very limited information. It has focused on the purification and characterization of lipid-binding proteins (Timanova et al., 1999; Jordanova et al., 2005a, b). In recent years, the development of different techniques of protein separation and identification has allowed the publication of numerous papers, which identify a large number of proteins of other nematodes analyzing the comprehensively proteome of these species (Robinson et al., 2007; Hewitson et al., 2008; Oleaga et al., 2009; González-Miguel et al., 2010a,b; Moreno et al., 2011; Sotillo et al., 2012).

The objective of this work is the identification of immunoreactive proteins from *A. galli*, the study of the time evolution of their recognition and the possible relationship of these proteins with the pathogenic and survival mechanisms that occur in avian ascaridiasis. For this purpose, proteomic, immunomic and MS techniques were employed.

2. Materials and methods

2.1. Sera

Sera included samples from 10 hens naturally infected with *A. galli* and 10 uninfected hens. Parasitological status of infected hens was tested by ELISA to detect antibodies against *A. galli* (Martín-Pacho et al., 2005), and by coprological assay. Sera from 10 experimentally infected hens collected at 0, 21, 42 and 96 days pi were also used. Experimental infections were performed as described by Marcos-Atxutegi et al. (2009) with minor modifications. Briefly, ten 18 weeks old, Lohmann Brown laying hens (procured from Ibertec, Parque Tecnológico de Boecillo, Valladolid, Spain), born and raised in helminth free conditions, were employed. The hens were orally infected using a plastic Pasteur pipette with individual doses of 250 eggs of *A. galli*. Six hens were maintained uninfected as negative control. All animals received water and food *ad libitum*. Finally, they were followed on a daily basis and examined clinically for signs of the infection. At the end of experiment the disease was confirmed by necropsy.

2.2. Parasite extracts and preparation of samples for 2-D gel electrophoresis

A. galli adult worms were collected from naturally infected hens. Worms were homogenized in phosphate buffered saline (PBS), pH 7.2, and sonicated in ice with 5 cycles of 75 kHz, 1 min each. A mixture of protease inhibitors was added to the resulting suspension (1 mM EDTA, 1 mM NEM, 1 μM Pepstatin A, 1 mM PMSF and 0.1 mM TPMSF) (Maizels et al., 1991) and the homogenate was centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant was dialyzed against distilled water for 24 h. The obtained extract, called AgSB, was assessed for protein concentration with the DC protein assay kit (Bio-Rad). The extract was then concentrated and purified with the ReadyPrep 2-D Cleanup kit (Bio-Rad), and then solubilized in the 2-D rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS). The samples, containing 40 μg of protein, were finally divided into 125 μl aliquots and stored at −20 °C until use.

2.3. Two-dimensional electrophoresis and Western blot

The 2-DE of AgSB was performed as described by González-Miguel et al. (2010b). Briefly, AgSB aliquots were supplemented with ampholites and DTT, incubated and centrifuged to remove all particulate material, 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 alkylated, and second dimension was done in 12% acrylamide gels. Gels were then silver stained with the PlusOne Silver Staining Kit, Protein (GE Healthcare) or transferred to nitrocellulose membranes for Western blot with the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The 2-D images were scanned with the GS-800 Densitometer (Bio-Rad) and analyzed with the Quantity One Software v.4.6.5 (Bio-Rad).

Immunoblot was performed as described before (González-Miguel et al., 2010b). Briefly, membranes were blocked in 2% BSA–0.05% Tween 20 in PBS and then incubated either with a pool of 10 sera from uninfected hens, a pool of 10 sera from naturally infected hens and 4 pools of 8 sera each from experimentally infected hens collected at different time points pi (above-mentioned), at 1:800 dilution. After three washes, blots were incubated with a horseradish peroxidase-labeled anti chicken IgG (Sigma) at 1:4000 dilution. Immunoblots were developed with 4-chloro naphthol. The 2-D images of immunoblots and their homologous silver-stained gels were aligned to isoelectric points (*pI*) and molecular weights (MW) and then matched with the PDQuest Software v.8.0.1 (Bio-Rad) to identify the antigenic spots in the gels.

Samples were analyzed in triplicate to assess the overall reproducibility of the protein and immunogen spot patterns.

2.4. MS and protein identification

In gel digestion of proteins and MS analysis were done as described by González-Miguel et al. (2010b). The spots containing immunogenic proteins were excised manually

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