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Discovery and validation of serum biomarkers expressed over the first twelve weeks of *Fasciola hepatica* infection in sheep

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Abstract

Serum biomarkers associated with *Fasciola hepatica* infection of Corriedale sheep were analysed during the first 12 weeks of infection using surface-enhanced laser desorption ionisation time of flight mass spectrometry (SELDI-TOF MS). In the discovery phase of analysis, pooled sera collected at week 0 and at each week p.i. to week 12 were fractionated by anion-exchange chromatography and the protein mass fingerprints obtained in individual fractions were in the M/z range 1.5–150 kDa. A total of 2302 protein clusters (peaks) were identified that varied between time-points following infection with peaks increasing or decreasing in intensity, or showing transient variation in intensity, during the 12 weeks of parasite challenge. In the validation phase, candidate biomarkers in sera of individual sheep at weeks 3 and 9 p.i. were analysed, identifying 100 protein peaks, many of which are small peptides <10 kDa in size: 54% of these peaks were up-regulated in intensity at week 3 or 9 p.i. Twenty-six biomarkers were chosen for further study, ranging in size from 1832 to 89,823 Da: six biomarkers were up-regulated at weeks 3 and 9 p.i., 16 biomarkers were up-regulated only at week 9 p.i. and four biomarkers were down-regulated at week 9 p.i. Two biomarkers up-regulated at week 9 were identified as transferrin (77.2 kDa) and Apolipoprotein A-IV (44.3 kDa), respectively. The results show that the interaction between the host and *F. hepatica* is complex, with changes in biomarker patterns beginning within 3 weeks of infection and either persisting to weeks 9–12 or showing transient changes during infection. Identification of biomarkers expressed during ovine fasciolosis may provide insights into mechanisms of pathogenesis and immunity to *Fasciola* and may assist in the rational development and delivery of vaccines.

Keywords: *Fasciola hepatica*; Liver fluke; Fasciolosis; Sheep; SELDI-TOF MS; Biomarkers; Serum; Transferrin; Apolipoprotein A-IV

1. Introduction

Fasciolosis is a disease affecting sheep, cattle and humans caused by *Fasciola hepatica* in temperate climates and *Fasciola gigantica* in the tropics. It is estimated that 250 million sheep, 350 million cattle and 180 million humans worldwide are at risk of infection, with production losses of over USD 3 billion per year (Hillyer and Apt, 1997; Mas-Coma et al., 1999, 2005; Spithill et al., 1999). *Fasciola* spp. parasites have complex interactions with their host. The initial phase of the disease includes parasite excystment in the small intestine and migration to the liver where subsequent movement and feeding in the liver parenchyma causes extensive tissue inflammation and necrosis. This acute phase of disease occurs during the first 5 weeks

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of infection in sheep and, at high worm burdens, can cause death. Sexually mature parasites migrate to the bile ducts of sheep at about 8–10 weeks after infection, where they feed on the blood and duct mucosa of the host. This chronic phase is characterised by liver fibrosis, anaemia and subsequent production losses.

Proteomic studies of *Fasciola* spp. have recently expanded from the analysis of subclasses of proteins such as cathepsins (Dalton et al., 2003) and glutathione *S*-transferases (Hillyer, 2005; Chemale et al., 2006), among others, to the identification of subsets of proteins of certain parasitic products such as ES products released by adult *F. hepatica* in vitro (Jefferies et al., 2001) and a comparison between ES products detected in vitro and in bile of infected sheep (Morphew et al., 2007) by two-dimensional gel electrophoresis.

Recent advances in mass spectrometry, namely the development of electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI), have extended our ability to unravel proteomes. Surface-enhanced laser desorption ionisation time of flight mass spectrometry (SELDI-TOF MS) allows sample binding to chemically active ProteinChip® surfaces, such as ion-exchange or immobilized metal affinity capture (IMAC) surfaces. As with MALDI, different matrices can be used to facilitate the uniform ionisation and desorption of molecules from the ProteinChip array surface (Merchant and Weinberger, 2000). SELDI technology allows the rapid and quantitative profiling of proteins in complex samples under different biological conditions and identification of markers of a biological state, termed 'biomarkers' (Xiao et al., 2005).

SELDI analyses were initially applied to the discovery of early diagnostic or prognostic biomarkers of cancer, such as prostate cancer (Petricoin et al., 2002b; Lehrer et al., 2003; Semmes et al., 2005), ovarian cancer (Petricoin et al., 2002a; Kozak et al., 2003; Zhang et al., 2004), pancreatic cancer (Rosty et al., 2002; Koopmann et al., 2004) and renal cancer (Won et al., 2003; Tolson et al., 2004; reviewed in Xiao et al., 2005). Recently, this technique has been applied to the study of serum biomarkers of infectious diseases such as Severe Acute Respiratory Syndrome (Poon et al., 2004; Yip et al., 2005), African Trypanosomiasis (Papadopoulos et al., 2004) and several blood borne protozoa (Ndao et al., unpublished data). Such studies have focused on identifying a distinctive configuration of circulating serum proteins that are indicative of a specific pathophysiological state, a so-called ''proteomic fingerprint''.

Here, we believe we report the first proteomic study of biomarkers in serum of sheep infected with *F. hepatica*. The aim of the study was to establish the proteomic fingerprints in sheep serum at intervals during the first 12 weeks of infection with the goals of identifying diagnostic biomarkers for early parasite invasion and gaining insights into host–parasite interactions during establishment of infection and the transition from acute to chronic infection. As acquired resistance to *Fasciola* spp. is expressed during the first few weeks of infection, we were particularly interested to define biomarkers expressed within 5 weeks of infection since these could theoretically be involved in establishment or suppression of host immunity (Spithill et al., 1997; Piedrafita et al., 2004, 2007). Our results highlight the complexity of the changes that occur in the sheep serum protein fingerprint during fasciolosis and reveal multiple biomarkers that are expressed during the acute and chronic phases of disease.

2. Materials and methods

2.1. Sheep serum samples

Serum samples were obtained from eight male Corriedale sheep (2 years old) housed in a paddock with an artificial water supply. The animals were purchased from a fluke-free area and shown to be free of infection by fecal analysis and ELISA using *F. hepatica* cathepsin L1 as a specific antigen (Piacenza et al., 1999). The animals were from the control group of a vaccination trial carried out in Uruguay where eight sheep were immunized with PBS in FCA, followed 4 weeks later by Freund's incomplete adjuvant. After 2 weeks, each sheep was orally challenged with a gelatin capsule containing 300 metacercariae of *F. hepatica* and humanely slaughtered at 12 weeks p.i. The experimental protocol was performed in compliance with national regulations and approved and supervised by the Animal Experimentation Honorary Committee, University of the Republic, Uruguay. Flukes in the main bile ducts and gall bladder were removed. The worm burdens (sheep number) were: 18 (#12), 19 (#24), 20 (#16), 24 (#13), 49 (#22), 50 (#25), 61 (#20) and 70 (#30). Blood was collected from all sheep prior to infection and then weekly until the time of slaughter. The serum was obtained and then stored at −80 °C.

2.2. Serum fractionation

Sera were fractionated using a Ciphergen Q HyperD F strong anion-exchange resin filtration plate. The filtration plate was re-equilibrated by adding 200 µl of rehydration buffer (50 mM Tris–HCl, pH 9.0) and placed on a MicroMix 5 orbital vortex (Beckman Coulter) (form 20 and amplitude 7) for 60 min at room temperature (RT). The rehydration buffer was removed by vacuum and the resin was washed four times with 200 µl rehydration buffer and four times with 200 µl U1 solution (1 M urea, 0.2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM Tris–HCl, pH 9.0). Serum samples were thawed on ice and centrifuged at high speed (17,300*g*) for 5 min at RT to remove particulates. Twenty microlitres of sample were added to a v-bottom 96-well microplate (Costar Corning) with 30 µl of U9 buffer (9 M urea, 2% CHAPS, 50 mM Tris–HCl, pH 9). The microplate was sealed and placed on a MicroMix 5 orbital vortex (form