Comparative Proteomics of Excretory-Secretory Proteins Released by the Liver Fluke *Fasciola hepatica* in Sheep Host Bile and during *in Vitro* Culture ex Host*†*

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Livestock infection by the parasitic fluke *Fasciola hepatica* causes major economic losses worldwide. The excretory-secretory (ES) products produced by *F. hepatica* are key players in understanding the host-parasite interaction and offer targets for chemo- and immunotherapy. For the key players in understanding the host-parasite interaction that causes major economic losses worldwide, the excretory-secretory (ES) products produced by an ovine host bile, with classical *ex host in vitro* ES methods. Only cathepsin L proteases from *F. hepatica* were identified in our ovine host bile preparations. Several host proteins were also identified including albumin and enolase with host trypsin inhibitor complex identified as a potential biomarker for *F. hepatica* infection. Time course *in vitro* analysis confirmed cathepsin L proteases as the major constituents of the *in vitro* ES proteome. In addition, detoxification proteins (glutathione transferase and fatty acid-binding protein), actin, and the glycolytic enzymes enolase and glyceraldehyde-3-phosphate dehydrogenase were all identified *in vitro*. Western blotting of *in vitro* and *in vivo* ES proteins showed only cathepsin L proteases were recognized by serum pooled from *F. hepatica*-infected animals. Other liver fluke proteins released during *in vitro* culture may be released into the host bile environment via natural shedding of the adult fluke tegument. These proteins may not have been detected during our *in vivo* analysis because of an increased bile turnover rate and may not be recognized by pooled liver fluke infection sera as they are only produced in adults. This study highlights the difficulties identifying authentic ES proteins *ex host*, and further confirms the potential of the cathepsin L proteases as therapy candidates. *Molecular & Cellular Proteomics* 6:963–972, 2007.

The parasitic fluke *Fasciola hepatica* infects humans and ruminant livestock worldwide. An estimated 2.4 million people are infected with *Fasciola* species, and a further 180 million are at risk (1). In addition, *F. hepatica* causes an estimated loss of $3 billion worldwide per annum through livestock mortality, especially in sheep, and by decreased productivity via reduction of milk and meat yields in cattle (2). Presently the rate of fluke infection in parts of northwest Europe has been estimated as greater than 30%. In the absence of commercial vaccines, the benzimidazole derivative triclabendazole (TCBZ,® Fasinex®) is the drug most extensively used against *Fasciola*. Unlike other fasciolicides, TCBZ shows activity against both juvenile flukes, which are responsible for the damage to the liver of acute fasciolosis, and the mature flukes, which cause the debilitation of chronic fasciolosis. Recently resistance to TCBZ has been reported in several countries suggesting that chemotherapeutic control of this infection may soon be compromised (3–5).

The most common diagnostic field method used to detect *Fasciola* infection requires counting fluke eggs in fecal samples. However, egg counts are time-consuming and expensive and have limited ability to diagnose early acute stages of infection. New immunological diagnostic methods with commercially protected antigen identification are available in the form of a rapid on-site field test, DriDot™ (Biotechnology Ireland) based on latex agglutination and a companion ELISA for laboratory veterinary applications. Further diagnostics-based research is required to freely validate current tests and to provide alternative molecular approaches for liver fluke populations.

In well characterized cattle models, the immune response to *F. hepatica* infection is proinflammatory lasting for about 4–6 weeks postinfection. This immune response appears to be...
Proteomics of *F. hepatica* ES Products in Vivo and in Vitro

be switched off at around the time adults enter the bile duct. The presence of flukes modify Th1 helper T cell responses, inhibiting the induction of the protective immune system, which makes the host more susceptible to further infections. It is likely that liver fluke secretions modify host macrophage-based signaling events to switch the proinflammatory immune response “off” before the infection has been appropriately controlled.

Juvenile *F. hepatica* develop and mature within the intrahepatic bile duct and gall bladder of infected hosts, continuing to release their excretory-secretory (ES) survival products that can ultimately lead to fibrosis and calcification of host tissues. In vitro biochemical studies have predicted that ES products of *F. hepatica* have roles in feeding behavior, detoxification of bile components, and the evasion of the immune system. In addition, in vitro proteomics (6) support the release of several major protein superfamilies from liver fluke. For example, general phase II detoxification GST with proposed immune evasion roles were found to be secreted by in vitro cultured liver flukes (7). Multiple forms of cathepsin L proteases are also secreted in vitro by adult *F. hepatica* (6, 8). These proteases can cleave host hemoglobin (9) and matrix proteins (10) for nutrition and are also capable, at least in vitro, of cleaving host IgG (11), indicating immune evasion roles.

In vitro studies can only attempt to mimic the protein content secreted by *F. hepatica* into host bile. There is concern that the ES proteome of *F. hepatica* will be altered by parasite removal from natural host tissues and maintaining the parasite in a chemical mixture. As proteomics can identify individual proteins from a mixture of host and parasite proteins this offers the potential to validate in vitro ex host studies in vivo and provide the possibility for real time analysis. In vivo proteomics during parasite infection also offers the possibility of identifying new targets and validating old therapy targets, providing protein probes for sensitive and selective diagnosis and increasing our understanding of how the parasite modulates the immune system. Proteomics investigations of sheep bile have yet to be reported, and a protein-based study of the immune system. Proteomics investigations of sheep bile have yet to be reported, and a protein-based study of the immune system. Proteomics investigations of sheep bile have yet to be reported, and a protein-based study of the immune system. Proteomics investigations of sheep bile have yet to be reported, and a protein-based study of the immune system. Proteomics investigations of sheep bile have yet to be reported, and a protein-based study of the immune system. Proteomics investigations of sheep bile have yet to be reported, and a protein-based study of the immune system.
were then resuspended in 50 mM Tris, pH 8.8, 6 M urea, 30% (v/v) glycerol, and 2% (w/v) SDS (18) with the addition of DTT (Melford) at 10 mg ml\(^{-1}\) followed by a second equilibration with iodoacetamide (Sigma) at 25 mg ml\(^{-1}\). The IPG strips were separated in the second dimension on the Protean II system (Bio-Rad) using 12.5% polyacrylamide gels run at 40 mA for \(-1\) h until through a stacking gel followed by 60 mA through the resolving gel until completion. The resulting gels were Coomassie Blue-stained (PhastGel Blue R, Amersham Biosciences) and imaged via a GS-800 calibrated densitometer (Bio-Rad). Imaged 2DE gels were analyzed using Progenesis PG220 version 2006 (previously Phoretix 2D Evolution version 2005). Analysis was performed using the mode of non-spot background subtraction on average gels created from a minimum of three biological replicates. Normalized spot volumes were achieved using total spot volume multiplied by total area and were also used to determine any increase or decrease in protein abundance between comparisons (with significance set at \(\pm 2\)-fold change). Unmatched protein spots were also detected between appropriate gel comparisons. Protein spots of interest were excised and digested with trypsin (modified trypsin, sequencing grade, Roche Applied Science). Protein tryptic fragments were then eluted according to the technique of Shevchenko et al. (19). Samples were then resuspended in 5 \(\mu\)l of 0.1% (v/v) TFA for mass spectrometry work.

**Immunoblotting**—In vitro ES and bile proteins separated via 2DE were transferred to Hybond-C nitrocellulose membrane (Amersham Biosciences) using a Trans-blot Cell (Bio-Rad) at 20 V overnight. To assess the efficiency of transfer, membranes were stained for 1 min using 0.1% Amido Black in 10% acetic acid and 25% isopropanol. Background staining was reduced using 10% acetic acid and 25% isopropanol. Following destaining, the membranes were blocked in TBS (0.1 M Tris, pH 7.5, and 0.9% (w/v) NaCl) with 1% Tween 20 (TTBS) containing 5% (w/v) skimmed milk powder for a minimum of 4 h (also removing the last traces of Amido Black staining). The blocked membranes were washed in TTBS for 10 min and then incubated with a primary antibody, either *F. hepatica*-challenged bovine serum or control/naïve bovine serum both diluted at a ratio of 1:3000 in TTBS containing 5% skimmed milk for a minimum of 4 h (also removing the last traces of Amido Black staining). The resulting gels were stained for 1 min and were transferred to Hybond-C nitrocellulose membrane (Amersham). Protein tryptic fragments were then digested with trypsin (modified trypsin, sequencing grade, Roche Applied Science). Protein tryptic fragments were then eluted according to the technique of Shevchenko et al. (19). Samples were then resuspended in 5 \(\mu\)l of 0.1% (v/v) TFA for mass spectrometry work.

**Fluke-infected and -uninfected Bile Subproteome**—The bile proteomes produced from both infected and uninfected fluke individual bile samples produced reproducible 2DE arrays (Fig. 1, A and B). The matching levels using Progenesis software averaged 40% matching between replicate gels, most likely related to increases in protein trails commonly seen with serum proteins. In both infected and uninfected fluke bile samples a prominent protein cluster between the 30 and 97 kDa markers in the second dimension and between pH 5.5 and 7.5 in the first dimension was visualized along with a further protein trail spanning approximately from pH 7 to 10 at the 30-kDa marker. Image analysis of the fluke-free bile samples yielded a total of 53 protein spots present in all 2DE arrays. The fluke-infected bile array showed a total of 60 protein spots consistently visualized on all 2DE arrays. A cluster of 15 protein spots located at the 30-kDa marker and spreading from approximately pH 5 to 6 was visible in all the fluke-infected bile samples; these protein spots were absent in the fluke-free bile samples (15 of 23 spots in total from pH 3 to 10 shown in Fig. 1C).

**In Vitro ES Product Time Course Proteomes**—The ES products released during *F. hepatica in vitro* culture were separated by 2DE after 2, 4, 8, and 16 h postculture (Fig. 2). The in vitro derived 2DE profiles were compared with the *in vivo* bile fluke ES subproteome profile (Fig. 1) to validate the applicability of *in vitro* culture methods currently used by parasitic worm research communities. For up to 8 h ex host, the *in vitro* culture 2DE profile was similar to the *in vivo* profile.
of infected bile samples (Fig. 2), although a limited proteome was produced of which the majority of the protein spots visualized migrated to the 30-kDa marker between pH 5 and 6. Overall complexity of the in vitro ES profiles increased after 8 h ex host with the most noticeable increases present between 45 and 66 kDa. In particular two protein spots, spot 16 estimated at 49 kDa and spot 17 estimated at 46 kDa (Fig. 2), showed significant increases after 16 h in culture (Fig. 3; spot 16 showed a 2.4-fold increase, and spot 17 showed a 3.6-fold increase). In addition, two protein spots (Fig. 2, two spots both identified as spot 13) present between 14.4 and 20 kDa, estimated at 14.8 kDa using Progenesis, also increased over time (Fig. 3; after 8 h of culture a 3.8-fold increase for one and a 2.8-fold increase for the other). In relation to previous ES studies on F. hepatica the cluster of protein spots present at the 30-kDa marker are in agreement with that found by Jef-
However, the remaining proteins seen by Jefferies et al. (6) only begin to appear after time, especially in the 16-h profile. All of the ES profiles analyzed were reproducible (80–94% matching between in vitro ES product replicates) as only viable flukes were taken to produce accurate 2DE in vitro assays. Analysis of the dead fluke cultured ES proteome (Fig. 4) showed an increase in total overall protein spots visualized and yielded a total of 107. Matching between live ES products and the dead ES proteome was relatively poor when compared with the live ES products (13.1% matching versus 2-h ES proteome and 22.4% matching versus 16-h proteome) and was related to the increase in protein spots detected. The large cluster of proteins observed between pH 5 and 6 and migrating to the 30-kDa marker in live ES proteomes was vastly reduced in the dead ES proteome with large increases in protein abundance seen all over the remainder of the array.

Identification of Protein Spots from in Vivo and in Vitro Approaches—A total of 90 protein spots from all assays were cut from gels for identification via PMF. This included spots from fluke-infected (20 spots) and fluke-free bile proteomes (10 spots as marker proteins) and in vitro culture proteomes (25 spots from the 16-h profile and 35 spots from the dead ES proteome). For identification through MASCOT several factors were exploited to assign a significant identification, i.e., expectancy (e) value, percent coverage, M, pl, and the error expressed in ppm. MASCOT also has the added advantage of being able to assign statistical significance of which a minimum of $p < 0.05$ was taken (wherever possible significance was increased to $p < 0.01$ or $p < 0.001$). Positive identifications were made on 67 occasions (Table I) with 15 from infected bile proteomes (Fig. 1A), six from uninfected bile proteomes (Fig. 1B), 17 from in vitro culture proteomes (Fig. 2), and a further 25 from the dead ES proteome (Fig. 4).

From both bile proteomes, six proteins were identified as host proteins, potential bile-based biomarkers of fluke infection. Three of the identifications were based on significant hits on Bos taurus due to the lack of or limited sequences for Ovis aries. Two of these identifications based on B. taurus, transferrin and enolase, were subjected to sequence alignment with available O. aries sequence to further support identification. Further verification of enolase as a host protein and not
parasite enolase was required. To confirm enolase as a host protein the short sequence of *O. aries* (GenBank accession number AAF60279), the *B. taurus* (GenBank accession number NP_776474) sequence hit from PMF, the top enolase BLAST (22) hit sequence from *B. taurus* (GenBank accession number AAJ02989), and the top *F. hepatica* (GenBank accession number AAF60279) BLAST hits were subjected to sequence alignment. Matching peptides (most importantly the peptide at m/z 1556.8; sequence VVIGMDVAASEFYR) from PMF analysis corresponded to conserved sequences from both *B. taurus* sequences and that of the *O. aries* sequence but not that of *F. hepatica* confirming identification of enolase as a host derived protein.2 The final identification based on *B. taurus* sequences was that of regucalcin with no sequence available for *O. aries*; identity was assigned by the e value of a protein highly conserved in mammals (23).

Of the 15 identified proteins found in *Fasciola*-infected bile 10 were identified as *Fasciola* cathepsin L proteases. However, only two of these identifications could be significantly assigned to a specific entry in the public database, confirming problems in proteomics identification by PMF (6). The two significant identifications corresponded to a cathepsin L-like protease (GenBank accession number CAA80446) and secreted cathepsin L2 (GenBank accession number AAC47721) (Fig. 1, spots 1 and 2, respectively). These two could be distinctly separated from the remaining cathepsin hits due to unique peptides appearing in the PMFs, for CAA80446 a peptide of *M* *r* 2449.07/2449.05 (monoisotopic) corresponding to the peptide DQGQCGSCWAFSTTGAVEGQFR and for AAC47721 a peptide of *M* *r* 2449.07 (monoisotopic) corresponding to NQGQCGSCWAFSTTGAVEGQFR. Due to the high conserva-

![Molecular & Cellular Proteomics](image)

**Fig. 4.** Representative 2DE protein array of *F. hepatica* proteins nonspecifically released from *in vitro* incubation of dead adults. Proteins were separated across a linear pH range of 3–10 using IEF in the first dimension and 12.5% SDS-PAGE in the second dimension and Coomassie Blue-stained. Numbered and circled spots correspond to putative identifications located in Table I.

**TABLE I**

<table>
<thead>
<tr>
<th>Spot N.</th>
<th>MASCOT e Value</th>
<th>Sequence Coverage (%)</th>
<th>Matched Peptide (Total)</th>
<th>Theoretical M/z (p)</th>
<th>Calculated M/z (p)</th>
<th>GenBank Accession N.</th>
<th>Species Description</th>
<th>Western Blotting (WB)</th>
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<tbody>
<tr>
<td>1</td>
<td>0.003**</td>
<td>39</td>
<td>12(35)</td>
<td>24184/1556.8</td>
<td>37534/5.43</td>
<td>CA85546</td>
<td><em>F. hepatica</em> L-like protease</td>
<td>V, B, W</td>
</tr>
<tr>
<td>2</td>
<td>0.015**</td>
<td>43</td>
<td>10(43)</td>
<td>24996/1356.8</td>
<td>37024/5.54</td>
<td>AAC47721</td>
<td>Secreted Cathepsin L 2</td>
<td>V, B, W</td>
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<tr>
<td>3</td>
<td>0.9</td>
<td>25</td>
<td>5(38)</td>
<td>24457/5.16</td>
<td>37524/5.55</td>
<td>AAFF8356</td>
<td>Cathepsin L</td>
<td>V, B, W</td>
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<tr>
<td>4</td>
<td>Av. 0.68</td>
<td>Av. 25</td>
<td>Av. 6(36)</td>
<td>25741/2583</td>
<td>5.55/5.93</td>
<td>AAFF8356</td>
<td>Cathepsin L</td>
<td>V, B, W</td>
</tr>
<tr>
<td>5</td>
<td>Av. 7.6</td>
<td>Av. 17</td>
<td>Av. 5(26)</td>
<td>25586/6.50</td>
<td>24003/5.01</td>
<td>AA441584</td>
<td>Cathepsin L</td>
<td>V, B, W</td>
</tr>
<tr>
<td>6</td>
<td>Av. 0.33</td>
<td>Av. 43</td>
<td>Av. 8(64)</td>
<td>24458/6.48</td>
<td>35067/5.79</td>
<td>AA811657</td>
<td>Cathepsin L</td>
<td>W</td>
</tr>
<tr>
<td>7</td>
<td>0.037**</td>
<td>59</td>
<td>8(45)</td>
<td>11984/6.50</td>
<td>15723/5.16</td>
<td>P06673</td>
<td>O. aries Superoxide Dismutase (Cu-Zn)</td>
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</tr>
<tr>
<td>8</td>
<td>1.45E-05**</td>
<td>94</td>
<td>3(49)</td>
<td>28887/7.01</td>
<td>29797/8.47</td>
<td>P09022</td>
<td>O. aries Carbonic Anhydrase II (Carbonic anhydrase II (CA-II))</td>
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<td>9</td>
<td>0.0041**</td>
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<td>16(45)</td>
<td>54170/5.57</td>
<td>33039/5.54</td>
<td>NP_775082</td>
<td>B. taurus Regucalcin (Bioscience marker protein-00)</td>
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<tr>
<td>10</td>
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<td>13(36)</td>
<td>45094/6.71</td>
<td>42727/6.44</td>
<td>NP_775074</td>
<td>B. taurus Enolase 1</td>
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<tr>
<td>11</td>
<td>1.80E-11</td>
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<td>21(67)</td>
<td>72704/6.10</td>
<td>60188/5.80</td>
<td>P46439</td>
<td>O. aries Serum albumin precursor</td>
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<tr>
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<td>83094/5.86</td>
<td>72703/5.73</td>
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<td>12(34)</td>
<td>17093/5.16</td>
<td>14805/5.95</td>
<td>OTMK051</td>
<td><em>F. hepatica</em> Fatty Acid-Binding Protein Type II</td>
<td>V, B, W</td>
</tr>
<tr>
<td>14</td>
<td>0.039</td>
<td>46</td>
<td>15(65)</td>
<td>23812/7.15</td>
<td>23873/5.88</td>
<td>AA28745</td>
<td>Glutathione S-transferase 26 KD 51 (GSHT51)</td>
<td>V, B, W</td>
</tr>
<tr>
<td>15</td>
<td>0.0044**</td>
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<td>13(40)</td>
<td>34758/6.21</td>
<td>23087/7.04</td>
<td>AA222387</td>
<td><em>F. hepatica</em> Glycolate Dehydrogenase</td>
<td>V, B, W</td>
</tr>
<tr>
<td>16</td>
<td>8.98E-07</td>
<td>50</td>
<td>22(56)</td>
<td>48221/6.65</td>
<td>49177/6.59</td>
<td>AA5685</td>
<td><em>F. hepatica</em> Phosphohydrolase Hydrolase (Elongase)</td>
<td>V, B, W</td>
</tr>
<tr>
<td>17</td>
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<td>10(51)</td>
<td>47269/6.36</td>
<td>41677/6.58</td>
<td>AA822603</td>
<td><em>B. taurus</em> Actin</td>
<td>V, B, W</td>
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<tr>
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<td>0.001**</td>
<td>57</td>
<td>9(45)</td>
<td>19846/6.95</td>
<td>14671/5.02</td>
<td>CA611051</td>
<td><em>F. hepatica</em> Fatty Acid-Binding Protein Type III</td>
<td>V, B, W</td>
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</table>

a BLAST score derived from searches using MSMS data.
b Peptides sequence using MSMS; underlined regions indicate matches to the top scoring sequence.
c TIC was identified as a host protein; although it was recognized by Western blotting, it is not derived from *F. hepatica*.

1 Serum albumin and FABP type III were identified *in vitro* in dead ES products only.

2 Data not shown.

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Western blot profile later identified as trypsin inhibitor complex (Table I, spots A and B). A secondary antibody (anti-bovine IgG) to many of the host proteins within the bile proteome. Areas displaying antigenic properties and were identified as *F. hepatica* cathepsin L proteases. Circled spots outline a major contributor to the Western blot profile later identified as trypsin inhibitor complex (Table I, spots A and B).

### Fig. 5. Western blot analysis of ES products from in vivo analysis.

Transferred proteins were probed with Fasciola-challenged and Fasciola-naïve bovine sera to assess the antigenicity of the protein present in bile to identify true secreted proteins. A, Fasciola-infected bile probed with Fasciola-challenged serum. B, Fasciola-infected bile probed with Fasciola-naïve serum, demonstrating the reactivity of the secondary antibody (anti-bovine IgG) to many of the host proteins within the bile proteome. Areas enclosed by a box indicate proteins displaying antigenic properties and were identified as *F. hepatica* cathepsin L proteases. Circled spots outline a major contributor to the Western blot profile later identified as trypsin inhibitor complex (Table I, spots A and B).

As in vivo, cathepsin L proteases could be identified in vitro but in this instance in 15 locations. Only two of these 15 cathepsin L proteases could be significantly identified to a specific entry in the public databases, appearing only once within the ES profile both in vivo and in vitro (GenBank accession numbers CAA80446 and AAC47721; Fig. 2, spots 1 and 2, respectively). In both in vivo and in vitro preparations the cathepsin L, GenBank accession number AAF76330 (Figs. 1 and 2, spot 3), was assigned to the same location but was not significantly confirmed. Two further cathepsin L hits assigned using in vitro methods were again from *F. gigantica* (GenBank accession number AAF44678; Fig. 2, spots labeled 5). The remaining proteins identified from in vitro cultures, with the exception of one, were assigned to proteins from *F. hepatica* sequences. The one protein assigned to an entry not of *F. hepatica* was that of the structural protein actin from the closely related tropical liver fluke, *F. gigantica* (GenBank accession number AAF76330).

As in vivo, partial proteomics of the dead ES subproteome revealed only 15 cathepsin L proteases as immunogenic. The relative contribution of the cathepsin L proteases to the dead ES subproteome was vastly reduced. Interestingly serum albumin from the host was identified in dead ES but not in live cultures.

**Immunoblotting of in Vitro and in Vivo ES Proteomes of F. hepatica**—Western blotting with Fasciola-challenged and naïve bovine sera as the probes was used to compare the antigenicity pattern of the in vivo (Fig. 5) and in vitro ES proteomes of *F. hepatica*. Despite some cross-reactivity with the secondary antibody, anti-bovine IgG, antigenic proteins from in vivo analysis (most likely from *F. hepatica* secretions) could be distinguished. In this manner 27 protein spots were identified as antigenic. Of these 27, 11 were associated with trains of protein that also cross-reacted in the control, leaving a total of 16 that were truly antigenic. Identification of 15 of these antigens corresponded to the *F. hepatica* cathepsin L proteases secreted into the bile.

Two protein spots contributed significantly to the Western blots from *F. hepatica*-infected and, to a lesser extent, uninfected bile proteomes. After failure to identify them using PMF analysis, both spots were subjected to peptide sequencing using MSMS. Following BLAST analysis both were identified as bovine trypsin inhibitor complex (TIC; GenBank accession number 1EB2_A) shown in Table I and Fig. 1, spots A and B. Notably TIC protein spot A was increased in *F. hepatica*-infected bile proteomes by a factor of 3.98, and spot B was absent in control bile. Western blotting of in vitro cultured ES products allowed this profile to be further confirmed for in vivo secretion and exposure to host immune system. Of all of the antigens identified in dead ES, the relative contribution of the cathepsin L proteases to the dead ES subproteome was vastly reduced. Interestingly serum albumin from the host was identified in dead ES but not in live cultures.

**Data not shown. Western blotting of in vitro 16-h ES products revealed only 15 cathepsin L proteases as immunogenic.**
the protein spots in the 16-h profile of ES products, 15 were shown to produce antigenic responses. Again following identification, all 15 were shown to be cathepsin L proteases produced by fluke in vitro. No other proteins displayed antigenic properties. Immunoblotting of the dead ES proteome again revealed the cathepsin L proteases as immunogenic when probed with Fasciola-challenged serum. Actin and enolase were also recognized but by naïve control serum.

Analysis of all the parasite sequences derived from PMF identifications for signal peptide cleavage sites revealed only a small fraction of the in vivo and in vitro profiles to be secretory. This corresponded to three GenBank protein entries (accession numbers CAA80446, AAC47721, and AAF76330) all of which are Fasciola cathepsin L proteases. No other protein sequences analyzed showed potential cleavage sites for signal peptides.

**DISCUSSION**

A rapid sample preparation strategy for undertaking reproducible 2DE protein arrays from sheep bile from Fasciola-infected individuals has been optimized. In addition, without completed and verified genome support, proteins were identified by PMF from the sheep host, O. aries, and for the first time in vivo its parasite, the liver fluke *F. hepatica*. This is the first experimental in vivo identification of ES products from an adult parasitic worm living in a mammalian host. Our in vivo host bile analysis suggests that the major *F. hepatica* proteins present outside the parasite in host tissues were a variety of proteases, mainly the cathepsin L proteases. This supports previous in vitro studies, suggesting that these proteins play a major role in *F. hepatica* survival in the host gall bladder and bile duct. In vivo proteomics supports the suggested role of cathepsins in providing nutrition with the secreted cathepsin L2 (Figs. 1 and 2, spot 2) cleaving fibrinogen to form blood clots and prevent excessive bleeding at feeding sites (24) and the cathepsin L-led disruption of the immune system via cleavage of host immunoglobulin (25) commonly circulating within the bile (17). These noted differences in enzymatic properties seen in cathepsin L proteases may well be related to changes of amino acid composition in and around the active site where Irving et al. (26) have detailed many sites that are subjected to positive selection pressures.

Cathepsin L proteases were the only *F. hepatica* proteins identified in both bile and in vitro culture. In addition, our in vitro studies confirm that cathepsin L proteases are the major adult ES proteins to at least 16 h postculture. GST (Type 51), FABP (type II), and enolase were also continually released from *F. hepatica* for at least 16 h during in vitro ES culture. These proteins have all been detected previously in vitro ES products from *F. hepatica* (6, 27, 28) but were not observed in our in vivo assays. In addition, *F. hepatica* GST (Type 51), FABP (type II), and enolase have no obvious signal sequence and are not recognized by pooled infection sera. Although GSTs were not identified in vivo, the levels of glutathione in the bile (1–4 mM in mammals with 90% in the reduced form (29–31)) would allow for GST enzymatic activity (*K*ₘ of GSTs, ~0.4 mM) suggesting that the stress-responsive detoxification protein GST could, in theory, function on the surface of the fluke. The known vaccine potential of *F. hepatica* GST may relate to an anti-inflammatory GST being secreted by newly excysted juveniles that are first exposed to the immune system. Enolase has been found on the surface of many other pathogenic species, i.e. bacteria, fungi, and protozoa (32) with immune modulating properties (33), and has been predicted on the surface of the nematode worm Onchocerca volvulus (34) and found in in vitro culture studies in other nematodes, Ascaris suum (35) and Haemonchus contortus (36).

Several proteins showed significant increases in the in vitro system at 16 h postculture, including FABP, actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We believe these proteins are in vitro artifacts, especially given the lack of in vivo validation and absence of immune recognition. We suggest that FABP probably functions in intraparasite lipid transport in adult *F. hepatica* despite being found in ES preparations in previous in vitro studies on *F. hepatica* (6) and Schistosoma mansoni (37). Bile contains high levels of fatty acids that allow the parasite to avoid synthesis and simply take up fatty acids from the host (38). FABP has been located to lipid droplets below the subtegumental region of adult male and female Schistosoma japonicum flatworms but was not present at the surface, suggesting a role in fatty acid transport not uptake per se (38). GAPDH is a major surface antigen in both larval stages and the adult of the blood fluke S. mansoni (39, 40). Therefore, GAPDH may also be present at the surface of the related flatworm *F. hepatica* at least in the adult life cycle stage.

Several host proteins were also identified despite an incomplete genome, taking advantage of cross-species identification (41). Some of these host proteins have been found previously in other bile-based studies (13) and have well characterized functions, such as carbonic anhydrase II (balance and regulation of acid-base levels; Ref. 42), transferrin (iron transport protein) delivered to the intestine via the bile duct system (43), and albumin (a secretory product of the liver used to transport large organic anions; Ref. 44). The glycolytic enzyme enolase was confirmed as a host protein in vivo as others have suggested it is a *Fasciola* ES protein (28). Sheep TIC identified in host bile has been shown to be highly immunogenic (45). TIC inactivates excessive trypsin, and its increase in liver fluke-infected bile may be part of a defense to directly counteract parasite survival roles of *F. hepatica* proteases. An immunogenic TIC may function as a host biomarker for liver fluke infection via antibody-based assays in bulk milk, blood, or feces. This would support current blood-based tests, DriDot, and its companion ELISA that rely on an *F. hepatica* protein to enter the blood circulation via feeding on damaged host tissues. Others are exploring SELDI tech-
ory for parasite (and host) blood-borne biomarkers.

Thus, why are GST, FABP, enolase, actin, and GAPDH released by *F. hepatica* in vitro and are not detected in vivo? Adult *F. hepatica* are thought to continually shed their teguments to evade the host immune system (46). As GST, FABP, enolase, actin, and GAPDH are located at the surface or just below the surface of the fluke, they may be released into the surrounding culture medium as the tegument is sloughed. As *in vitro* time progresses in *ex host* stress response-inducing environments, adult flukes may shed their teguments at a greater rate. Tegumental turnover at an elevated rate facilitates the release of GST, FABP, enolase, actin, and GAPDH.

In a schistosome flatworm proteomics study GST, FABP, enolase, GAPDH, and actin were also identified in soluble extracts of isolated *S. mansoni* teguments (47). The ES proteome from dead cultured *F. hepatica* fluke further confirms that the release of these proteins in *in vitro* is not a specific biological secretion but a physical degradation process. Although found in previous ES studies (6, 48, 49) the predicted helminth antioxidant, immunosuppressant, and vaccine candidate thioredoxin peroxidase (Tpx) was not identified in this study using *in vivo* or *in vitro* techniques. In the flatworm *S. mansoni*, Tpx was located in soluble tegumental preparations and located beneath the surface (47). This indicates that cultured adult liver flukes may need to shed their tegument continuously before Tpx will be seen in ES products or that it results from degradation of cultured flukes. Tpx may be on the surface or secreted by juvenile stages of *F. hepatica* that are more exposed to the immune response, hence its protection properties during vaccine trials (50). As our study suggests, both FABP and GST are not proactively secreted in *in vivo* by adult *F. hepatica* but released via shedding, questioning their inclusion in vaccine formulations or as anti-inflammatory immune modulators as they only appear to be exposed intermittently during shedding.

The ability of proteomics to discriminate between parasite and host in biological fluids, such as in the bile via percutaneous cholecystocentesis (16, 51), will increase understanding of how helminth worms regulate the immune system to establish chronic infections. Other host fluids where *F. hepatica* reside may also be suitable to this type of proteomics analysis. Newly excysted juveniles emerge from their metacercarial cysts in the duodenum where early ES products are released, and migrating juveniles pass through the peritoneal cavity on route to the liver and will again release ES products into the surrounding host fluid. However, in both of these instances the relative abundance of newly excysted juveniles and juvenile ES products in relation to host protein may severely hamper proteomics analysis of these host fluids. Nonetheless the analysis of *in vivo* ES products from intestinal helminth worms may be possible by analyzing intestinal fluid or washes.

In *vivo* proteomics technology has identified for the first time proteins released from the liver fluke *F. hepatica*, a prerequisite to understanding the host-adult parasite interface of this important global parasite. This approach also supports the academic portfolio of the cathepsin L proteases as liver fluke therapeutic candidates and suggests the need to be careful extrapolating findings from *ex host in vitro* studies. In *vivo* proteomics presents an opportunity to validate *in vitro* findings, such as cathepsin L cleavage of host IgG molecules, and although this particular assay was not completed in the present study the potential applications of our technique is demonstrated. Our present study only provides a snapshot of the interaction between host and parasite; a live bile extraction, via a cannula (16, 51), offers opportunities for time course analysis of an experimental infection. The identification of *F. hepatica* proteins in the host from relatively low infection levels (60 flukes) may be translated to biomarker analysis to support current *F. hepatica* infection diagnosis.

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The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

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**REFERENCES**


4 T. W. Spithill, personnel communication.


