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Fasciola hepatica and *Fasciola gigantica*: Cloning and characterisation of 70 kDa heat-shock proteins reveals variation in HSP70 gene expression between parasite species recovered from sheep

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Abstract

Fasciola hepatica and *Fasciola gigantica* are trematode parasites responsible for fasciolosis, a disease of ruminant animals which is also increasingly recognised as a disease in humans. By biochemical and *in silico* methods, we have cloned and characterised the 70 kDa heat-shock proteins (HSP70s) of *F. hepatica* and *F. gigantica*. The nucleotide and protein sequences for HSP70 were found to be 98% and 99% identical between liver fluke species, respectively, and to encode conserved amino acid motifs that are of putative functional importance. Western blot analysis demonstrated that HSP70 proteins were expressed at a higher level in *F. gigantica* recovered from sheep relative to *F. hepatica*, but HSP70 was not detected in the excretory–secretory products of these liver fluke samples. Real-time reverse-transcriptase PCR analysis of HSP70 expression in parasites from sheep, but not cattle, showed HSP70 expression to be higher in *F. gigantica* than *F. hepatica.* These results suggest that hosts refractory to *F. gigantica* are associated with higher HSP70 expression by this parasite and that HSP70 expression may represent a biochemical marker of the stress response of *F. gigantica*.

Index Descriptors and Abbreviations: HSP70; *Fasciola hepatica*; *Fasciola gigantica*; Fasciolosis; Liver fluke; Parasite; Trematode; ESP, excretory–secretory products; HSP, heat-shock protein; ITT, Indonesian Thin Tail; rFhHSP70, recombinant *F. hepatica* 70 kDa heat-shock protein; WWL, whole worm lysate

1. Introduction

Fasciola hepatica and *Fasciola gigantica* or ''liver flukes'' are parasitic flatworms and aetiological agents of liver fluke disease (fasciolosis). Fasciolosis affects mainly ruminants and is responsible for losses to global agriculture of at least $US3.2 billion each year (Spithill et al., 1999). Additionally, 2.4–17 million people are infected with *Fasciola* species world-wide and 180 million are at risk from infection (Hopkins, 1992; WHO, 1995): the prevalence of human fasciolosis in some villages of the Bolivian Altiplano may reach 70% (Mas-Coma et al., 1999).

Fasciola parasites undergo a multi-stage life cycle involving a snail and mammalian host and are most commonly found in sheep, cattle and buffalo. The ability of parasites to survive in a host and establish successful infections is likely to depend on host factors, such as differences in the type, timing and magnitude of immune mechanisms, as well as parasite factors, such as differences between liver fluke species in the expression of immunomodulatory factors, or stress-response molecules that are protective to

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the fluke (reviewed in Spithill et al., 1999; Piedrafita et al., 2004, 2007).

The 70 kDa heat-shock proteins (HSP70s) are intracellular proteins found in all organisms. The HSP70s have key roles in mediating the effects of environmental stresses and cellular homeostasis: they fold and assemble proteins, refold mis-folded and aggregated proteins and translocate proteins (reviewed in Mayer and Bukau, 2005) and are critical for parasite survival (reviewed in Polla, 1991). For a parasitic organism such as *Fasciola*, which resides within a hostile host environment comprising bile salts, immune molecules and gastric secretions, stress-response proteins, including HSP70 proteins, are likely to play an important role in parasite homeostasis.

In this paper, HSP70s from *F. hepatica* and *F. gigantica* have been cloned and characterised by biochemical methods and *in silico*. The expression of HSP70 in *F. hepatica* and *F. gigantica* recovered from a variety of host backgrounds, both refractory and susceptible to *Fasciola* infections, has been compared by real-time reverse-transcriptase PCR to determine whether there is any association between parasite HSP70 expression levels and host background.

2. Materials and methods

2.1. Rapid amplification of cDNA Ends-PCR (RACE-PCR)

A nucleotide fragment of 180 bp was obtained from *F. hepatica* during a cDNA Representational Difference Analysis of gene expression between adult *F. hepatica* and *F. gigantica* (data not shown). From this nucleotide fragment, full-length HSP70 cDNAs from *F. hepatica* and *F. gigantica* were obtained by RACE-PCR using *Fasciola* poly(A)$^+$ RNA (isolated with the QuickPrep™ *Micro* mRNA Purification Kit [Amersham Biosciences]) and the 2nd Generation 5′/3′ RACE Kit (Roche Applied Science). After RACE-PCR, PCR primers to the 5′ and 3′ termini of the *F. hepatica* and *F. gigantica* HSP70s were designed and used to amplify *Fasciola* HSP70 cDNAs with *Vent*® DNA polymerase (New England Biolabs). PCR products representing full-length *Fasciola* HSP70s were then A-tailed by *Taq* DNA polymerase in the presence of 0.2 mM dATP, cloned into the pGEM®-T Easy Vector System (Promega) and transformed into electrocompetent DH5α *Escherichia coli*. For each *Fasciola* species, the plasmid DNA of six recombinant bacterial clones was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega) and *Fasciola* HSP70 insert DNAs were sequenced using BigDye (v3.1; ABI) and pGEM®-T Easy vector-specific primers.

2.2. Sequence analysis and phylogram construction

The molecular weight and isoelectric point (p*I*) of *Fasciola* HSP70 were calculated at the exPASY server (Gasteiger et al., 2005). NetNGlyc 1.0 and YinOYang 1.2 programs were used to identify amino acids with the potential to be glycosylated (Gasteiger et al., 2005). Position-Specific Iterated-BLAST analysis of the *F. hepatica* HSP70 with default parameters and the non-redundant protein database identified homologous HSP70s in other species (Altschul et al., 1997). A multiple sequence alignment of the *Fasciola* protein with its homologues from other invertebrate species and the mammalian hosts of *Fasciola* was generated using ClustalW (Thompson et al., 1994). Functionally important amino acids and domains within the *Fasciola* protein were identified by their homology to previously characterised HSP70s of other species. Using the multiple sequence alignment, an unrooted phylogenetic tree of *Fasciola* HSP70 and its homologues were created by the neighbour-joining method. The Newick format of the resultant tree was used to create a phylogram in Phylodendron (Gilbert et al., 1990).

2.3. Western blotting

SDS–PAGE and Western transfer proceeded according to standard protocols. Antigens used were whole worm lysate (WWL) and excretory–secretory products (ESPs) of *F. hepatica* and *F. gigantica*, obtained from Merino sheep and prepared as described (Estuningsih et al., 2004; Raadsma et al., 2007). Recombinant *F. hepatica* HSP70, used as a positive control antigen, was generated by subcloning *F. hepatica* HSP70 cDNA into the *Bgl*II site of the YEpFLAG expression vector (Sigma–Aldrich) and transforming BJ3505 *Saccharomyces cerevisiae* yeast with YEpFLAG/HSP70 clones as described (Elble, 1992). Recombinant *F. hepatica* HSP70 protein was secreted into the growth medium of *S. cerevisiae* and purified as described (Law et al., 2003). Primary serum for detecting *Fasciola* HSP70 was generated in rats vaccinated with rFhHSP70 in Quil A adjuvant. Negative control serum was collected from rats unexposed to rFhHSP70. Secondary sera were anti-rat antibodies conjugated to horse-radish peroxidase enzyme (Sigma–Aldrich). Western blots were autoradiographed and then developed using ECL™ Western Blotting Detection Reagents (Amersham Biosciences).

2.4. Real-time reverse-transcriptase PCR analysis of HSP70 expression

RNA used in real-time reverse-transcriptase PCR was isolated from adult *F. hepatica* liver fluke obtained from Merino sheep and Friesian cattle in Victoria, Australia and from Indonesian Thin Tail (ITT) sheep in Java, Indonesia. Adult *F. gigantica* RNA was isolated from Merino sheep, ITT sheep and Ongole cattle from Java, Indonesia. The samples from ITT sheep were recovered at 12 weeks after infection: parasites from cattle were collected at an abattoir. Collection and preparation of motile *Fasciola* parasites from liver tissue has been described (Law et al., 2003). Parasites were snap-frozen on dry ice and stored