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Analysis of Cathepsin-L and Cathepsin-B Gene Expression in Embryonated Eggs of *Fasciola gigantica* and Effect of Various Protease Inhibitors on Miracidial Hatching

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ABSTRACT

Background: *Fasciola gigantica* is a helminth parasite of veterinary importance and a constraint on the growth and productivity of domestic livestock in the tropical countries of Asia and Africa. Present study aimed at determining the expression of four cysteine protease genes including cathepsin (cat) L-1 D and cat-Bs in the embryonated egg stage of *F. gigantica* by polymerase chain reaction (PCR). In addition to the analysis of expression of the above genes in the embryonated eggs, inhibition of *in vitro* hatching of the miracidia from the eggs was carried out using three protease inhibitors *viz.* serine, cysteine and leucine aminopeptidase inhibitors to understand the possible role of these proteases in the hatching of the miracidia from the eggs.

Methods: Cat- L-1D, cat B-1, cat B-2 and cat B-3 genes were amplified by reverse transcriptase and quantitative-PCR, respectively for determining their expression in the embryonated eggs. Inhibition of *in vitro* hatching of the miracidia from the eggs was carried out by exposing the embryonated eggs to different concentrations of iodoacetamide, phenyl methyl sulphonyl fluoride and bestatin as cysteine, serine and leucine aminopeptidase inhibitors.

Result: Results showed that cat L1-D, cat B-1 and cat B-3 genes were expressed at the embryonated egg stage but cat B-2 gene was not expressed at this stage of the parasite. Iodoacetamide and phenyl methyl sulphonyl fluoride (cysteine and serine protease inhibitors) inhibited the miracidial hatching at different concentrations, indicating probable role of cysteine and serine proteases in its hatching process. However, leucine aminopeptoidase does not seem to influence the miracidial hatching from the eggs as bestatin did not inhibit its hatching.

Key words: *Fasciola gigantica,* Cathepsin L-1D, Cathepsin-B, Egg hatching, PCR, Protease inhibitors.

INTRODUCTION

Fasciola gigantica is the causative agent of fasciolosis in domestic ruminants in tropical countries including India. Fasciolosis cause major economic losses to the livestock industry in terms of weight loss, decline in feed conversion efficiency, reduced milk and wool production and mortality (Mehra *et al*., 1999). A larger animal population is at the risk of picking up of this infection and frequent outbreaks of the disease are reported in India. Control of fasciolosis is based on reducing the snail populations, treatment of the definitive host and timely detection of the infection in the host (Fairweather, 2011). Vaccination is a viable strategy for controlling the disease but no vaccine has been commercialized due to strong immune evasion mechanisms developed by the parasite. Parasite derived cysteine proteases play key roles in haemoglobin hydrolysis, immune-evasion, enzyme activation, virulence, tissue and cellular invasion as well as excystment, hatching and molting (Sajid and McKerrow, 2002). Cysteine proteases modulate macrophage activity and suppress Th1 responses in the host (O'Neill *et al*., 2001; Donnelly *et al*., 2010). Several cathepsin protease classes have evolved to perform diversified roles in the parasite biology with endopeptidase (cat L and F), exo-and endopeptidase (cat B) and dipeptidyl peptidase activities (cat C) (Dalton *et al.,* 2006). Mass spectrometry based proteomics has identified a vast array of peptidases secreted by *F. hepatica* that are developmentally regulated, correlating with the migration and maturation of the parasite within its host (Robinson *et al*., 2009). Cathepsin proteases are thought to be the major targets for drug and vaccine development. Cat-B proteases are expressed by *Fasciola* at the host gut penetration stage and their expression is continued till the flukes are immature (Cancela *et al*., 2008; Sethadavit *et al*., 2009). Several variants of the cat-B are expressed in *Fasciola*. Cat-B genes are differentially expressed in *F. gigantica* with metacercaria and newly excysted juvenile (NEJ) stages containing abundant cat-B transcripts but less transcripts found in older stages of the juveniles and adults (Meemon *et al*., 2004).

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Cat B-1 transcript is detected in all stages, whereas cat B-2 and cat B-3 transcripts are expressed in metacercariae, NEJ and juvenile parasites only (Meemon *et al*., 2004). Presence of these transcripts in the early developmental stages has been reported in *F. hepatica* (Law *et al.,* 2003). The abundance of cat-B transcripts in metacercariae and early juvenile suggests that these proteases are important in the excystment of metacercariae and in the parasite penetration of host gut and liver. Expression of several members of cat-B gene family (cat B1-B7) has been reported in *F. gigantica* as the protein may be performing diversified functions in the parasite. With the maturation of the parasite, expression of the cat-Ls is predominant with a decline in the expression of cat-Bs (Chantree *et al*., 2012).

The role of cysteine proteases (cat Ls and cat Bs) in the hatching of the miracidium from the parasite egg has not been elucidated. Identifying the target molecules relevant to hatching of the *Fasciola* eggs and development of intervention approaches to inhibit the hatching process of the eggs can reduce infection of the snail intermediate host. But proteins responsible for hatching of the *Fasciola* egg have not been identified. Leucine aminopeptidase (LAP) plays a central role in the hatching of the miracidium from the egg in *S. mansoni* (Xu and Dresden, 1986; Rinaldi *et al*., 2009). However, expression of LAPs in the eggs of *F. gigantica* has not been determined and role of these proteases in the miracidial hatching is not known in *Fasciola*. In fact, proteins responsible for egg hatching process in *Fasciola* are not known. The aim of the present study was to determine the expression of four cathepsin proteases *viz.* cat-LI-D and cat-Bs in the embryonated eggs in *F. gigantica.* Three protease inhibitors were tested for inhibition of the miracidial hatching from the eggs to determine possible role of the targeted proteases in the egg hatching process.

MATERIALS AND METHODS

In vitro embryonation of *F. gigantica* eggs

Live, adult *F. gigantica* flukes were collected from the livers of buffaloes slaughtered at a local abattoir in Bareilly, U.P, India and transported to the laboratory at room temperature in normal saline (0.9% sodium chloride). The flukes were washed extensively in the normal saline for removal of bile and tissue debris and were cultured in RPMI-1640, supplemented with gentamycin @ 50 µg/ml of medium, at 37°C for 4 h for release of the eggs. The eggs were retrieved and given several washes in tap water. The cleaned egg masses were incubated in 100 ml glass beakers at 28°C in dechlorinated tap water in BOD incubator (Macro Scientific Works, Delhi, India) for a period of 12-15 days for the development of miracidia. The water was changed periodically and eggs examined under microscope for the development of miracidia.

Isolation of total RNA from eggs and cDNA synthesis

The *in vitro* egg culture batches with 70-80% of the eggs showing miracidial development were processed for the isolation of total RNA. Total RNA was isolated from these eggs using RNAqueous Micro-RNA isolation kit (Thermo Scientific, USA), following protocols prescribed by the manufacturer. Briefly, RNA was isolated from ~5000 eggs. The eggs were centrifuged and egg pellet lysed by addition of 100 µl of lysis buffer in RNase free microcentrifuge tubes. The parasite lysate was added with 50 µl of 100% ethanol and vortexed. The lysate/ethanol mixture was loaded on the micro-filter cartridge assembly and centrifuged at 10,000 rpm for 30 sec. The cartridge was given washings as per the recommendation of the manufacturer. Total RNA was eluted and subsequently digested with DNase. Total RNA free of DNA contamination was stored at -80°C for further use. The RNA was quantified by Nanodrop spectrophotometer and used for cDNA synthesis with oligo-dT primer and M-MLV reverse transcriptase enzyme kit (Thermo Scientific, USA) following standard protocols of reverse transcription reaction.

Polymerase chain reaction amplification of cDNA in the embryonated eggs

Cat L-1D, cat B-1, cat B-2 and cat B-3 genes were studied for their expression in the embryonated eggs. The cDNA synthesized from the total RNA was subjected to PCR amplification of full length cat L-1D (accession no: AF239266), cat B-1 (accession no: (AY227673), cat B-2 (accession no: (AY227674) and cat B-3 (accession no: AY227675) genes using primers specific to these genes (Table1). Primers used in the PCR amplification of the respective genes were designed as described in Anandanarayanan *et al.* (2017). A single set of primers was designed on conserved N- and C- termini of cat B-1 and cat B-3 genes for amplification of these two genes. The cDNA was synthesized from the adult stage of the parasite as well and the above primers were tested for amplification of these genes.The PCR was standardized with denaturation at 94°C for 1 min, annealing at 50-52°C for 45 sec for respective primers and primer extension at 72°C for 1 min for 35 cycles; with initial denaturation of the cDNA at 94°C for 3 min. The PCR products were analyzed on 1% agarose gel by

Table 1: Primers used for PCR amplification of *F. gigantica* cat B-1, cat B-2, cat B-3 and cat L-1D.

Primer name	Primer sequence
Fgcat L1-D-F1	5′-ATG CGG TGC TTC GTA TTA GCC-3′
Fgcat L-1 D-R2	5′-TCA CGG AAA TCG TGC CAC CAT C-3′
Fgcat L1-D-F2	5′-TGA CGA TTT GTG GCA TGA ATG-3′
Fgcat B1+B3-F	5′-ATG AGT TGG TTG CTC ATA TTT GC- 3′
Fgcat B1+B3-R	5′-TCA AGG CAA TCC CGC ATT AAT CC-3′
Fgcat B1+B3-F2	5′-TCC AGT TGT AGT TCG TGT TGG-3′
Fgcat B1+B3-R3	5′-TTG GGG AAC GGG TAG GGT AAA C-3′
Fgcat B2-F	5′-ACT GGT TAA TCG TGT TTG CCA TT- 3′
Fgcat B2-R	5′-TCA AAG ACG TGG CAT TCC GGC GAC-3′

electrophoresis and purified from the agarose gel using Gel Extraction Kit (Qiagen,Germany).The PCR products were cloned in p^{DRIVE} cloning vector (Qiagen,Germany) following standard cloning protocols. The recombinant clones were custom sequenced at the Department of Biochemistry, Delhi University, South Campus, New Delhi. The nucleotide sequences were analysed for their identity using NCBI-BLAST (www.ncbi.nlm.nih.gov/BLAST).

Quantitative Real-time PCR

Real-time PCR was carried out in Applied Bio Systems 7500 v 2.3 Stepone plus and Applied Bio Systems 7500 v 2.0.6 fast Real-time PCR System. Reactions were performed with initial incubation of the reaction mixture at 50°C for 2 min, followed by 10 min exposure at 94°C and cycling conditions of 94°C for 15 sec; 60°C for 30 sec and 72°C for 30 sec (40 cycles) using Maxima SYBR Green / ROX qPCR Master Mix Kit (Thermo Scientific, USA). Fluorescence was detected during the extension step and melting curve analysed for the presence of a single peak.

Inhibition of egg hatching by protease inhibitors

In vitro egg hatching assay was set up for studying the effect of phenyl methyl sulphonyl fluoride (PMSF), iodoacetamide and bestatin on miracidial hatching. These protease inhibitors were procured from Sigma Aldrich, USA and used as serine, cysteine and aminopeptidase inhibitors, respectively. Eggs with fully developed miracidia (~70-80%) were treated with these protease inhibitors at 10-12 days of incubation at 28°C. Eggs (~1000-2000) were treated with different concentrations of PMSF and iodoactamide for 1-1.5 h and with bestatin overnight in sterile distilled water in 2 ml microcentrifuge tubes at 28°C. Following treatment with inhibitors, eggs were washed with several changes of dechlorinated tap water and incubated further at 28°C overnight in tap water for allowing the miracidia to hatch. Untreated control groups of the embryonated eggs were maintained along side treated groups. Miracidial hatching was stimulated by exposure of the microcentrifuge tubes to the electric bulb light (100 watts) for 30 min at room temperature. Miracidia were scored as hatched by counting the number of empty egg shells in inhibitor treated versus untreated groups. Percentage hatching of the miracidia was calculated as-

$$\frac{\text{No of empty egg shells} \times 100}{\text{Total number of embryonated eggs counted}}$$

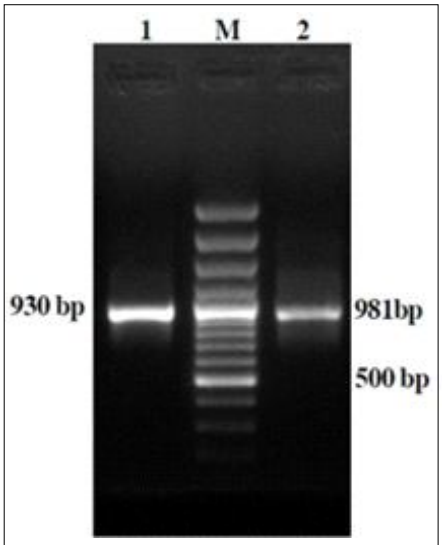
RESULTS AND DISCUSSION

Expression of cat L-1D, cat B-1, cat B-2 and cat B-3 genes in the embryonated eggs

Cat L-1D gene was PCR amplified using two sets of primers (Table 1). The full length cDNA (981bp) was amplified using the primer pair forward F1 and reverse R2 and a shorter fragment of the cDNA (930 bp) was also amplified with primer combination of forward F2 and reverse R2 (Fig 1). But, cat B-1, cat B-2 and cat B-3 genes could not be amplified by reverse transcriptase-PCR with egg cDNA as template. However, quantitative Real-time PCR was highly sensitive and both cat B-1 and cat B-3 genes were amplified with a single set of primers designed on the conserved sequence of the two genes (Fig 2a). Expression of cat B-1 and cat B-3 genes was also confirmed by qPCR amplification of cDNA fragment of 247 bp with primer set forward F2 and reverse R3 (Fig 2b). But cat B-2 gene could not be amplified either by reverse transcriptase-PCR or by qPCR.

Inhibition of egg hatching by protease inhibitors

The *in vitro* treatment of the embryonated eggs with PMSF, iodoacetamide and bestatin showed that bestatin did not cause inhibition of the miracidial hatching up to the



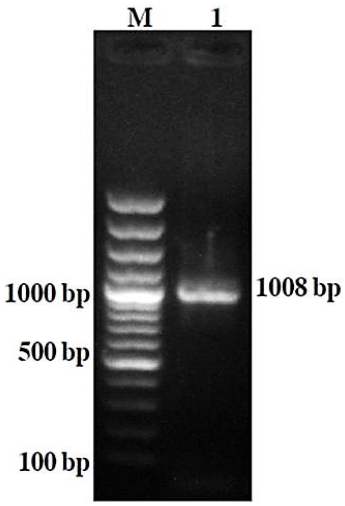
Lane 1: 930 bp fragment amplified with primer pair F2 and R2; Lane 2: 981 bp full length open reading frame amplified with primer pair F1 and R2.

Fig 1: PCR amplification of cat-1D cDNA of embryonated eggs Lane M: 100 bp plus DNA ladder.

Table 2: *In vitro* inhibition of miracidial hatching by PMSF, iodoacetamide and bestatin treatment of embryonated eggs.

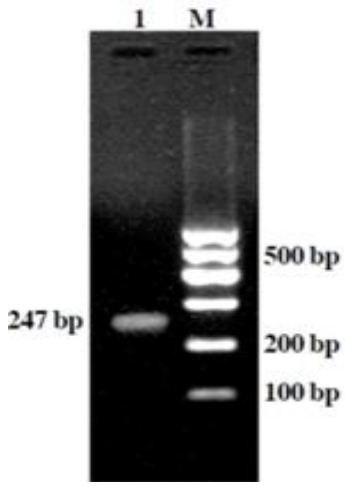
Inhibitor	3 mM	2 mM	1 mM	0.5 mM	0.25 mM	0.125 m	0.065 mM
PMSF	-	-	+	++	++	ND	ND
Iodoacetamide	ND	ND	-	-	-	+	++
Bestatin	++	++	++	++	ND	ND	ND
No treatment control	++	++	++	++	++	++	++

(-) No eggs hatched; (+) ~50% eggs hatched; (++) hatching % age identical in inhibitor treated and un-treated control groups ($p>0.05$); (ND) no inhibitor treatment done.



Lane 1: 1008 bp band representing cat B-1 and cat B-3 genes amplified with a single primer set designed on the conserved sequence of both genes.
Lane M: 100 bp plus DNA ladder.

Fig 2a: Real-time PCR amplification of cat B-1 and cat B-3 cDNA of embryonated eggs.



Lane M: 100 bp DNA ladder.
Lane 1: 247 bp band representing cat B-1 and cat B-3 gene fragment amplified with a single primer set forward F2 and R3.

Fig 2b: Real-time PCR amplification of cat B-1 and cat B-3 cDNA of embryonated eggs.

concentration of 3 mM used in the study. Bestatin treatment was given for overnight period to ensure longer exposure to the inhibitor. Strikingly, the miracidia even after hatching remained active for 3-4 h in the bestatin solution. However, treatment of the embryonated eggs with PMSF and iodoacetamide caused 100% inhibition of the miracidial hatching at 2 mM and 250 µM concentrations of the inhibitors, respectively (Table 2). The iodoacetamide treatment indicated that either cysteine proteases are inhibited or proteins with sulfhydral groups, essential for hatching of the eggs, are getting modified. The preliminary data obtained with PMSF and iodoacetamide treatments of the embryonated eggs suggest that both serine and cysteine proteases may have a role in the hatching of miracidia from the eggs.

Present study aimed at determining the expression of cat L-1D, cat B-1, cat B-2 and cat B-3 protease genes in the embryonated eggs of *F. gigantica* and to determine the probable role of various proteases in the hatching process of the parasite eggs. The expression of these genes in the miracidial stage of the parasite was determined at the transcriptional level by reverse transcription-PCR and by qPCR. The qPCR being a highly sensitive quantitative method for the amplification of the low copy number genes in a tissue, amplified cat B-1 and cat B-3 genes that could not be otherwise amplified by conventional PCR of the cDNA in the embryonated eggs. However, cat B-2 gene was not amplified by reverse transcriptase-PCR and qPCR of the cDNA indicating that cat B-2 gene is not expressed in the embryonic stage of the parasite. However, further study on the cat B-2 mRNA expression by northern blotting and protein expression by western blot is required for final confirmation of the expression of cat B-2 protein at this developmental stage of the parasite. The expression of cat L-1D isotype in the embryonated egg was confirmed by both reverse transcriptase-PCR and qPCR.

Several authors have earlier determined expression of various cysteine proteases in different developmental stages of the parasite but literature on the profiling of these proteases in the egg and miracidium has been scanty. Zhang *et al.* (2019) carried out an extensive study on the transcriptional profile of cathepsin proteases (cat-L and cat-B) along with other proteins involved in various metabolic pathways in the life cycle stages of *F. gigantica* including eggs and miracidia and determined expression of several cathepsins in the miracidia stage of the parasite.

Proteomic approaches have identified LAP in a complex repertoire of proteins and glycoproteins within the schistosome eggs (Liu *et al.,* 2006; Jang-Lee *et al.,* 2007). Bestatin, when used as an aminopeptidase inhibitor markedly decreased the escape of miracidia from the eggs suggesting that LAP activity was critical to the hatching process (Xu and Dresden, 1986). This was further established by RNAi in *S. mansoni* where knockdown of the LAP-1 and LAP-2 inhibited the hatching of schistosome eggs proving that both the enzymes are essential to the escape of miracidia from the eggs (Rinaldi *et al*., 2009). However, the role of LAP or other proteases including cysteine and serine proteases in the hatching process of miracidia in *Fasciola* has not been known. In this preliminary study, embryonated eggs were exposed to different concentrations of PMSF, iodoacetamide and bestatin as serine, cysteine and LAP protease inhibitors, respectively. The iodoacetamide treatment indicated that either cysteine proteases are inhibited or proteins with sulfhydral groups, essential for hatching of the eggs, are getting modified. Bestatin did not show any inhibitory effect on the egg hatching at the concentrations

used in this study. These results suggest that cysteine and serine proteases may have a probable role in the hatching of miracidia from the eggs in *F. gigantica* that can be established by further studies.

CONCLUSION

The results of the present study indicated that cat L-1D, cat B-1 and cat B-3 genes are expressed in the embryonated eggs (miracidium) of *F. gigantica*. Serine and cysteine proteases seem to play roles in the release of miracidia from the eggs.

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Conflict of interest

All authors declare that they have no conflict of interest.

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