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Cloning and Expression of *Fasciola gigantica* Cathepsin-B Recombinant Proteins

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ABSTRACT

Fasciola gigantica cathepsin (cysteine) proteases are potential diagnostic antigens for animal and human fasciolosis. These include cathepsin-L proteases that have been exploited in the diagnosis of animal fasciolosis. However, no scientific data on the diagnostic potential of *F. gigantica* cathepsin B proteases is available. Therefore, three recombinant antigens of *F. gigantica viz*. cathepsin (cat) B-1, cat B-2 and cat B-3 were expressed in prokaryotic expression system. The recombinant antigens were purified under denaturing conditions by Nickel affinity chromatography and an optimal level of the recombinant proteins was obtained. These recombinant proteins will further be evaluated for their potential in the early prepatent diagnosis of *F. gigantica* infection in domestic ruminants.

Key words: Cathepsin B, *Fasciola gigantica*, Recombinant antigen, Serodiagnosis.

INTRODUCTION

Parasitic diseases are major health hazards and cause of huge economic losses to the livestock sector. *Fasciola* (liver fluke) is a major cause of production losses to the livestock and meat industries due to clinical disease, reduced weight gain, drop in milk production, condemnation of infected livers and deaths (Chick *et al*., 1980; Mehra *et al*., 1999; Charlier *et al*., 2007; Mezo *et al*., 2011; Bardhan *et al*., 2014; Nyirenda *et al*., 2019). Fasciolosis, caused by *F. gigantica,* is a disease of prime concern on account of larger animal populations at risk and frequent outbreaks of the disease in domestic animals in our country. Vaccination is a viable strategy for controlling the disease but no vaccine has been commercialized. Therefore, effective control of fasciolosis depends on the timely detection of the infection in the definitive host that helps in early treatment. But lack of sensitive tests for the diagnosis of infection in large herds has hampered adoption of the proper control measures for the disease. Diagnosis of animal fasciolosis is largely based on microscopic demonstration of parasite ova in the host faeces from 10th-14th week post-infection (Kleiman *et al*., 2005; Graham-Brown *et al*., 2019). But due to low sensitivity of the coprological detection, it is not suitable for analyzing the disease condition in large herds. Further, *F. gigantica* has a longer pre-patent period (12-14 weeks) for detection of eggs in the host faeces. Pre-patent and pre-clinical diagnosis of *F. gigantica* infection in ruminants is important for better management of the disease. Serological diagnosis is preferred since anti-*Fasciola* antibodies can be detected at an early stage of infection and can thus facilitate early chemotherapeutic intervention.

Of the several antigens evaluated in serodiagnosis of *Fasciola* infection in domestic ruminants, cysteine proteases have shown better potential both in human and animals. Cysteine proteases of *Fasciola* are important molecules involved in several biological processes of the parasite (Dowd *et al*., 1994; Berasain *et al*., 1997; Dalton *et al*., 2003) and the major cysteine proteases secreted by *Fasciola* are cathepsin-L proteases (Dowd *et al*., 1994). Several workers have successfully expressed functional cathepsin-L proteases that have shown diagnostic potential for fasciolosis in animals and human. In India, several reports on the potential of *F. gigantica* cathepsin-L protease in the pre-patent diagnosis of *F. gigantica* infection in buffaloes, sheep and cattle are available (Dixit *et al.,* 2002, 2003; Raina *et al*., 2006; Sriveny *et al*., 2006; Varghese *et al.,* 2012). However, identification of the diagnostic antigens expressed by the parasite at the initial stage of the host infection would add a new dimension to the diagnosis of early prepatent *F. gigantica* infection. Cathepsin (cat)-B cysteine 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 proteases are expressed in *Fasciola*. Cat-B1 transcript is detected in all stages, whereas cat-B2 and cat-B3 transcripts are expressed in metacercariae (infective stage), newly excysted juvenile (NEJ) and juvenile parasites only (Meemon *et al*., 2004). As the parasite

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matures expression of the cat-Ls is predominant with a decline in the expression of cat-Bs (Chantree *et al*., 2012). Hence, strategy of using both cat-B and cat-L antigens in a diagnostic assay will enhance the sensitivity of the detection assay. Therefore, present study was undertaken to generate three recombinant cat-B antigens including cat-B1, Cat-B2 and cat-B3 of *F. gigantica* for their further evaluation in the serodetection of *F. gigantica* infection in livestock.

MATERIALS AND METHODS

Collection of *F. gigantica* flukes and host serum

Buffaloes slaughtered at a local abattoir at Bareilly, U.P were screened for *F. gigantica* infection and adult flukes were collected from the infected livers. The flukes were washed with physiological saline (0.9% w/v NaCl) and transported to the laboratory in physiological saline at room temperature. Blood samples (5-10 ml each) were collected from *F. gigantica* positive and negative buffaloes for retrieval of the sera. The sera were retrieved from the blood, marked and stored at -20°C for screening of the antibodies in immunoassays. Also, sera retrieved from buffaloes, free from *F. gigantica* infection, maintained in the experimental sheds of the institute (ICAR-Indian Veterinary Research Institute, Izatnagar) were used as negative control.

Isolation of total RNA from flukes and cDNA synthesis

The adult flukes retrieved from the buffalo liver were processed for RNA isolation with Trizol reagent (Invitrogen, USA) following standard protocols. Briefly, 1/3rd portion of an adult fluke was teased in a sterile, nuclease free mortar pestle in 1 ml of Trizol reagent. The parasite tissue was manually homogenized with the sterile pestle, freeze-thawed at -80°C for multiple cycles together with manual homogenization to completely lyze the parasite tissue. Chloroform (0.2 ml) was added to the Trizol treated parasite homogenate, mixed well and suspension centrifuged at 10,000 rpm to separate the aqueous phase from the organic phase. Total RNA in the aqueous phase was precipitated at -80°C for 2 h after adding 0.5 volumes of isopropyl alcohol and centrifuged at 12,000 rpm at 4°C for 15 min. The RNA pellet was washed with 70% ethanol, air dried and reconstituted in nuclease free water. The concentration of RNA was quantified by Nanodrop spectrophotometer and divided into aliquots and stored at -80°C for complementary DNA (cDNA) synthesis. RNA was converted to cDNA using cDNA synthesis kit (Revert Aid H Minus first strand cDNA synthesis kit; Thermo Scientific, USA) as per the manufacturer's protocol.

Cloning of *F. gigantica* cat B-1, cat B-2 and cat B-3 cDNA

The cDNA coding for cat-B1, cat-B2 and cat-B3, respectively was amplified by polymerase chain reaction (PCR). Cat B-2 gene was PCR amplified with forward and reverse primers (cat B-2-For and cat B-2-Rev). A common set of forward and reverse primers (cat B-1 and cat B-3-For and Rev) for cat-B1 and cat-B3 genes was synthesized for PCR amplification of these cDNAs as the nucleotide sequence at N and C-termini of the cDNA coding for these two proteins are conserved (Table 1).These PCR amplicons were then identified as cat-B1 and cat B-3 after their cloning and sequencing of the individual clones. The above primers were designed as per the published gene sequences (accession numbers: AY227673, AY227674, AY227675), respectively and custom synthesized. The PCR amplification was carried out at 60°C annealing temperature for all the three target genes as per the standard protocol. The PCR products were purified using QIAquick® Gel Extraction kit (Qiagen, Germany) following manufacturer's protocol and were cloned into p^{DRIVE} cloning vector (Qiagen, Germany) for sequencing of the genes.

Expression of cat B-1, cat B-2 and cat B-3 recombinant proteins in prokaryotic expression system

The three recombinant proteins cat B-1, cat B-2 and cat B-3 were expressed in prokaryotic expression vector p$^{PROEXHT-b}$ (Thermo Scientific, USA). The cDNA coding for the cat B-2 protein was PCR amplified with forward cat B-2-For-Ex and reverse cat B-2-Rev-Exp primers tailed with *Nco*1 and *Hin*d III restriction enzyme sites and cloned in frame with N-terminal histidine fusion tag of the expression vector (Table 1). The cDNAs coding for cat B-1 and cat B-3 were amplified with forward cat B-1and B-3-For-Exp and reverse cat B-1and cat B-3-Rev-Exp primers, respectively designed with *Nco*I and *Hin*d III restriction enzyme sites (Table 1) and cloned in frame with the N terminal fusion tag of the expression vector. The forward primers for each gene were designed from 46th nucleotide to delete the signal sequence of 15 hydrophobic amino acids. The competent *Escherichia coli* BL21 DE3 pLys S cells were transformed with

Table 1: Primer sequences for PCR amplification of *F. gigantica* cat B-1, cat B-2 and cat B-3 genes and expression of recombinant proteins.

Primer name	Primer sequence
Cat B-1 and Cat B-3 (For)	5´- atgagttggttgctcatatttg-3
Cat B-1 and B-3 (Rev)	5´- ttaaggtaatccggcattaatcc-3'
Cat B-2 (For)	5´- atgaactggttaactgtgtttgc-3´
Cat B-2 (Rev)	5´- tcaaagacgtggcattccggc-3´
Cat B-1 and B-3 For-Exp	5'- agg*ccatgg*cacc aaaccacaaa ccgcaat-3´
Cat B-1 and B-3 Rev-Exp	5´- cgc*aagctt*aaggtaatccggcattaatcc-3'
Cat B-2 For-Exp	5´- ata*ccatgg*ccaaaccaa accataaacc gcaat-3´
Cat B-2 Rev-Exp	5'- gcg*aagctt* caaagacgtggcattccggc-3´

*Italicized sequences: restriction enzyme sites.

recombinant clones as per the standard protocols (Sambrook and Russell, 2001) and were grown on LB agar plates supplemented with 100 µg /ml of ampicillin overnight at 37°C. The master plates supplemented with 100 µg /ml ampicillin were prepared for each target gene by streaking the randomly selected colonies from the LB agar plates on these plates. The master plates were incubated at 37°C overnight and screened for the positive clones expressing the recombinant proteins.

The recombinant cat B-1, cat B-2 and cat B-3 clones were induced with Isopropyl thio-β-D-galactoside (IPTG) for protein expression. Six recombinant clones for each target gene were randomly screened for protein expression. The bacterial culture (100 ml) was induced with IPTG at the final concentration of 1mM and incubated for 12 h post-induction at 37°C. Aliquots were prepared from both IPTG induced and uninduced cultures at 2 h intervals, centrifuged at 12,000 rpm for 5 min and the corresponding pellets were checked for protein expression by SDS-PAGE (Laemmli, 1970). The gels were stained with Coomassie brilliant blue R-250 and subsequently destained for checking the expression of the recombinant proteins.

Purification of recombinant proteins

The recombinant proteins were purified by Ni-NTA affinity chromatography. The recombinant clones were individually cultured in 500 ml of LB broth supplemented with 100 µg / ml ampicillin and were induced for protein expression with 1 mM IPTG. Each induced bacterial culture was divided into aliquots of 250 ml, centrifuged and pellet stored at -20°C. All the buffers for protein purification were prepared following the QIAexpressionist™ manual (Qiagen, Germany) with some minor modifications. The *E.coli* cells were lyzed in 5 ml of lysis buffer (pH 8.0) for 3h with constant shaking and intermittent vortexing at room temperature. The cell debris was centrifuged at 10,000 rpm for 10 min at 4°C and the clear supernatant was incubated with the Ni-NTA resin (1 ml) and recombinant protein allowed to bind with affinity resin on a rotary shaker at room temperature for 2 h. Following this step, the resin was washed with wash buffer (pH 7.0) and affinity resin bound recombinant protein was eluted in 1 ml fractions of elution buffer (pH 4.2). The compositions of the lysis, wash and elution buffers used in the purification steps of each recombinant protein were 10 mM tris and 100 mM potassium dihydrogen phosphate containing 6 M guanidine hydrochloride or 8 M urea as protein denaturant.

The recombinant proteins (5 ml each) were dialyzed against the decreasing gradient of urea from 8.0 M to 0.0 M concentration in tris-phosphate dialysis buffer (10 mM tris, 100 mM sodium dihydrogen phosphate, pH 7.2) in 10 kDa molecular weight cut-off cellulose membrane (SnakeSkin dialysis tubing, Thermo Scientific, USA). The dialysis membrane was submerged in100 ml of dialysis buffer for 3-4 h at 4°C for each gradient of urea concentration (8.0 M to 0.0 M). Dialysis of the recombinant protein ensured complete removal of urea and refolding of the protein.

Western blotting of recombinant proteins

Western blot was carried out (Towbin *et al*., 1979) using Ni-NTA-HRP conjugate (Sigma Chemicals, USA) for checking the histidine tagged fusion protein expression. Each purified recombinant protein (50 µg) was resolved on SDS-PAGE and electrophoretically transferred to nitrocellulose membrane in chilled transfer buffer at 100 mA constant current for 90 min using Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad, USA). The transfer of the protein to the membrane was confirmed by staining of the recombinant proteins with Ponceau-S stain for 1-2 min. The membrane was washed twice with phosphate buffered saline (PBS) pH 7.2 and blocked with 5% skimmed milk in PBS overnight at 4°C. Following its washing for 10 min (x3) with PBS-Tween-20 (0.05%), the membrane was incubated at room temperature in 1:700 dilution of Ni-NTA-HRP conjugate in PBS at 37°C for 1 h. The membrane was washed 3 times for 10 min each in PBS-T and stained with diamino benzedene substrate solution. The reaction was stopped by rinsing the membrane in distilled water and membrane photographed for reactivity of the recombinant protein.

Similarly, immunoreactivity of the expressed recombinant proteins was analyzed by Western blot using *F. gigantica* positive and negative buffalo sera. The membranes were incubated with buffalo sera at 1:100 dilution and subsequently incubated with rabbit anti-bovine IgG-HRP conjugate (Sigma Chemicals, USA) at 1:1000 dilution following the above described protocol. The immunoreactivity was determined as described above.

RESULTS AND DISCUSSION

Expression of the recombinant cat B1, cat B-2 and cat B-3 proteins

The cDNA coding for cat B-1, cat B-2 and cat B-3 proteins on PCR amplification with gene specific primers generated amplicons of 1048 bp of cat B-2 and 1008 bp of cat B-1 and cat B-3 cDNA, respectively (Fig 1a and 1b). The optimum

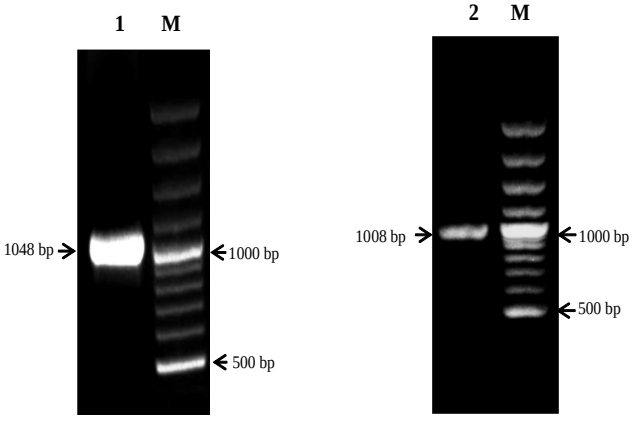


Fig 1: PCR amplification of *F. gigantica* cDNAs coding for cat B-2 (a) and cat B-1 and cat B-3 proteins (b).
Lane M: Molecular weight marker (1kb DNA ladder).
Lane 1: Cat B-2 PCR amplicon (1048 bp).
Lane 2: Cat B-1 and cat B-3 PCR amplicons (1008 bp).

level of expression of the recombinant cat B-1, cat B-2 and cat B-3 proteins was obtained at 12 h post-IPTG induction at 37°C in *E. coli* BL21 (DE3 pLys S) cells (Fig 2a and 2b). The recombinant cat B-2 protein was purified from the *E. coli* cells using lysis buffer supplemented with 8 M urea as mild denaturant. Purification of the recombinant protein with achieved with 11 mM imidazole and 10 mM β-mercaptoethanol supplementation of the lysis buffer. The protein was washed with wash buffer (pH 7.0) supplemented with 7 mM imidazole and recombinant protein eluted with elution buffer at pH 4.2.

Recombinant cat B-1 and cat B-3 expressed in $p^{PROEXHT-b}$ vector could not be purified from the *E. coli* lysate as the proteins aggregated in insoluble product that could not be solubilized with 8 M urea as a mild denaturant. The recombinant proteins were subsequently purified with 6 M guanidine hydrochloride as a strong protein denaturant. *E. coli* cells were lysed in 6 M guanidine hydrochloride in lysis buffer (pH 8.0) supplemented with 8 mM imidazole and 10 mM β-mercaptoethanol. The wash buffer (pH 7.0) was supplemented with 7 mM imidazole and recombinant proteins eluted with elution buffer at pH 4.2. The purified recombinant proteins cat B-1, cat B-2 and cat B-3 resolved at ~37 kDa and ~38 kDa, respectively in the SDS-PAGE (Fig3a and 3b).

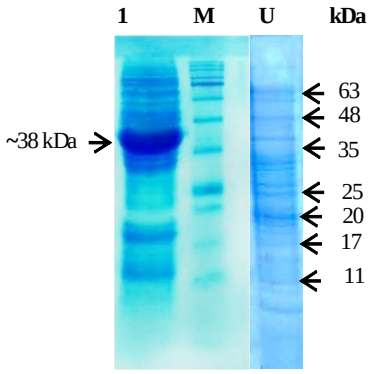


Fig 2a: SDS-PAGE (12%) showing expression of *F. gigantica* recombinant cat B-2 antigen in *E.coli*.

Lane M: Pre-stained protein molecular weight marker.

Lane 1: IPTG induced cell lysate expressing cat B-2 fusion protein at ~38 kDa.

Lane U: Un-induced control.

Western blot analysis of recombinant proteins

The three recombinant proteins when probed with Ni-NTA-HRP conjugate in the Western blot resulted in specific reactive bands at ~37 kDa and ~38 kDa, respectively. These results confirmed that the target proteins were expressed as recombinant histidine tagged fusion proteins (Fig 4a and 4b). Thereafter, the recombinant proteins when probed by *F. gigantica* infected buffalo sera reacted with the protein at ~37 kDa and 38 kDa, respectively. No immunoreaction of the negative sera was observed in the blot.

Meemon *et al*. 2004 reported that cat-B genes are differentially expressed in *F. gigantica* with metacercaria and NEJ stages containing abundant cat-B transcripts and less transcripts found in 6-week old juvenile and adults. The presence of these transcripts in early developmental stages was also reported for cat-B proteins in *F. hepatica* (Law *et al.*, 2003). The abundance of cat-B transcripts in metacercariae and early juvenile suggests that the proteases are important in the excystment of metacercariae and in the parasite penetration of host gut and liver. Meemon *et al.* 2004 demonstrated the stage-specific expression of cat-B1, cat-B2 and cat-B3 with cat-B1 mRNA detected in all analyzed stages, whereas cat-B2 and cat-B3 detected only in NEJ and metacercariae. This may indicate the switching off of the cat-B2 and cat-B3 genes as the parasites mature. Expression of several members of cat-B gene family (cat B1- B7) has been reported for last decade in *F. gigantica* there by indicating that cat-B proteins may be performing

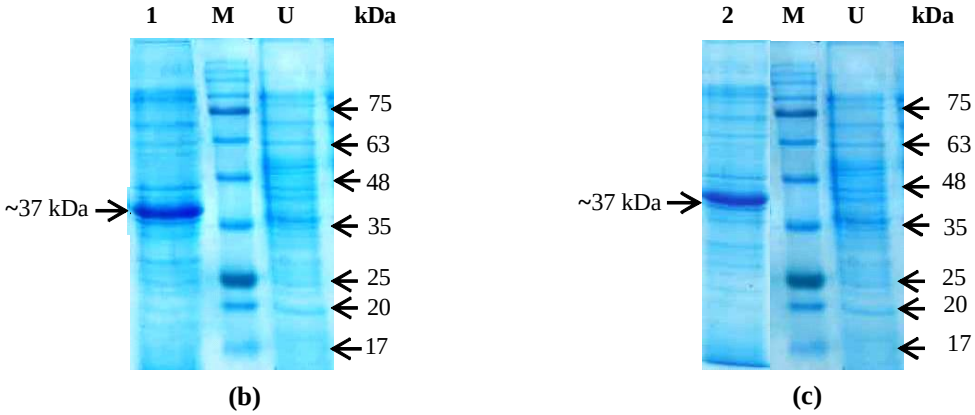
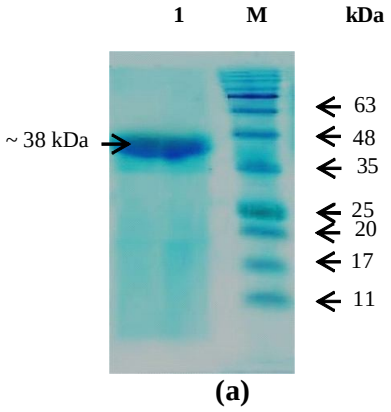


Fig 2b: SDS-PAGE (12%) showing expression of *F. gigantica* recombinant antigens cat B-1(b) and cat B-3 (c) in *E.coli*.

Lane M: Pre-stained protein molecular weight marker.

Lanes 1 and 2: IPTG induced cell lysate expressing cat B-1(b) and cat B-3(c) fusion proteins at ~37 kDa.

Lane U: Un-induced control.



(a)

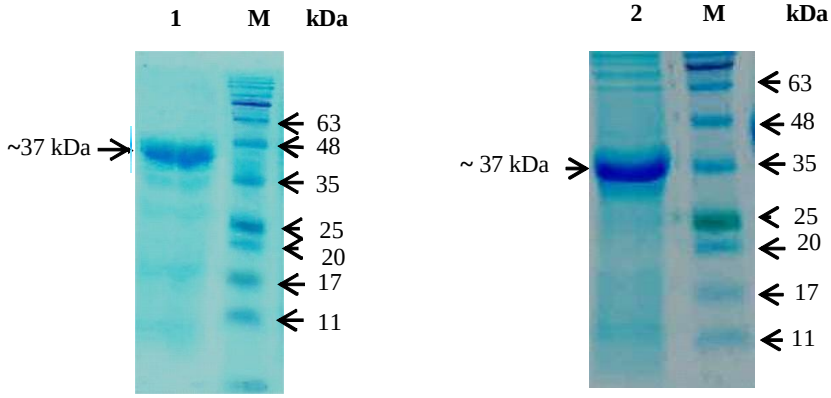
Fig 3a: SDS-PAGE (12%) showing Ni-NTA affinity purified recombinant antigen cat B-2.

Lane M: Pre-stained protein molecular weight marker.

Lane 1: Purified recombinant cat B-2 protein resolved at ~ 38 kDa.

diversified functions in the parasite. These proteins are also secreted like cat-L proteins and are immunogenic. Therefore, these proteins can well be exploited in the diagnostic assays in *Fasciola*. In fact, cat-L proteins that seem to be secreted by all the developmental stages of the parasite can be exploited in the diagnosis of infection at all the stages of the parasite while assays based on cat-B antigens can be used for early prepatent detection of the infection.

In India several workers have reported the potential of cat-L cysteine protease in the diagnosis of *F. gigantica* in cattle, buffaloes and sheep. Dixit *et al.* 2002 isolated cysteine proteinase (28 kDa) from *F. gigantica* excretory-secretory product (ESP) and evaluated the protein in ELISA, Dipstick-ELISA and Western blot and detected *F. gigantica* antibodies in sheep under experimental conditions with high degree of sensitivity. Yadav *et al*. 2005 demonstrated that native protein detected *F. gigantica* experimental infection in sheep and buffaloes as early as 4 weeks post-infection (WPI) by ELISA,

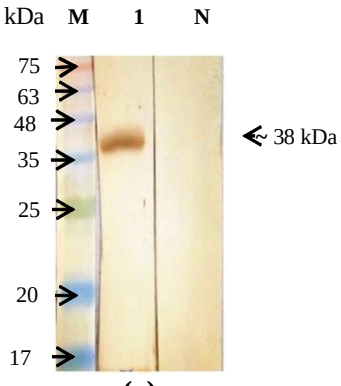


(b) (c)

Fig 3b: SDS-PAGE (12%) showing Ni-NTA affinity purified recombinant antigens cat B-1 (b) and cat B-3(c).

Lane M: Pre-stained protein molecular weight marker.

Lane 1 and 2: Purified recombinant cat B-1(b) and cat B-3 proteins (c) resolved at ~ 37 kDa.



(a)

Fig 4a: Western blot of *F. gigantica* recombinant antigen cat B-2 with Ni-NTA-HRP conjugate for confirmation of histidine tagged fusion protein expression.

Lane M: Pre-stained protein molecular weight marker.

Lane 2: Recombinant cat B-2 protein showing reactivity at ~ 38 kDa.

Lane N: Recombinant cat B-2 with no substrate added and showing no reactivity.

Western blot and Dipstick-ELISA. *Fasciola gigantica* native cat-L cysteine proteinase and recombinant cat L-1 D were assessed for their potential in the immunodiagnosis of *F. gigantica* infection in buffaloes with sensitivity of 97.1% and specificity of 100% in ELISA in naturally infected buffaloes (Raina *et al*., 2006). Native cat-L cysteine proteinase was also evaluated for its potential in the early prepatent detection of experimental infection in bovine calves that detected infection at 4 WPI in ELISA, dipstick-ELISA and Western blotting with 100% sensitivity (Sriveny *et al*., 2006). The protein also detected field infection in buffaloes with a sensitivity of ~90% in Dot-ELISA (Varghese et *al.,* 2012). However, there are no reports on the potential of cat-B proteins as diagnostic antigens against *Fasciola* infection in domestic ruminants. In the present study three members of cat-B *viz.*, cat B-1, cat B-2 and cat B-3 were expressed in *E.coli* as recombinant antigens. All three recombinant proteins were purified under denaturing conditions with optimum yield of each protein. These recombinant proteins will be further analyzed for their potential in the diagnosis of

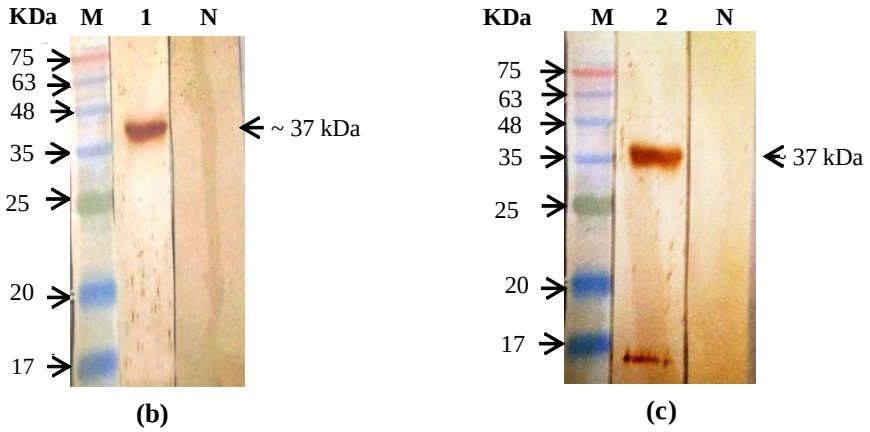


Fig 4b: Western blot of *F. gigantica* recombinant antigen cat B-1(b) and cat B-3 (c) with Ni-NTA-HRP conjugate for confirmation of histidine tagged fusion protein expression.

Lane M: Pre-stained protein molecular weight marker.

Lanes 1 and 2: Recombinant cat B-1(b) and cat B-3(c) proteins showing reactivity at ~ 37 kDa.

Lane N: Recombinant cat B-1 and cat B-3 with no substrate added and showing no reaction.

F. gigantica infection in domestic ruminants including cattle, buffaloes and sheep. Diagnostic assays based on cat-B antigens will enhance the sensitivity of the assay as the antigens are expressed in the very early life of the parasite and when used with cat-L antigens diagnostic sensitivity of the assay will be enhanced.

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