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Amplification and cloning of exon regions from 1-4 of cathepsin B-like cysteine protease gene of *Haemonchus contortus* and immune response in lambs against cathepsin B-like cysteine protease fraction

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ABSTRACT

Haemonchus contortus is an economically important, highly pathogenic blood-sucking nematode parasite of ruminants. A study was conducted to characterize the cathepsin B-like cysteine protease gene of *H. contortus* of Indian origin, which has been explored as potential target for vaccine development. Adult *H. contortus* worms were collected from the abomasum of sheep and goats and whole genomic DNA was extracted and amplified using appropriate primers. The expected amplicon of 767 bp was cloned into T-vector using GeNei^{TM} Instant cloning kit. Immunization study was conducted to examine the effect of cysteine protease fraction on the faecal egg counts in lambs. It was found that the egg per gram of faeces was lower in immunized group as compared to control group (at 10, 11, 12, and 15 week). At post mortem, the worms recovered from the abomasa were lower in immunized group (800.40±101.8) as compared to control group (950.93±300.29). The results indicated that the immunization with cysteine protease fraction in lambs reduces the faecal egg count, but did not remove all the worms from lambs.

Key words: Cysteine proteases, *Haemonchus contortus*, Small ruminants, Vaccine

In India, parasitic infections particularly gastrointestinal parasites are considered as major entity responsible for morbidity and mortality in sheep flocks (Gupta *et al*. 1987, Singla 1995, Jithendran and Bhat 1999, Singh *et al*. 2002). *Haemonchus contortus* is one of the major nematode parasite causing substantial economic losses in small ruminant farming. The most widely used method for control of the *H. contortus* is through use of anthelmintics group of drugs. However, the extensive and indiscriminate use of the anthelmintics have resulted in the emergence of anthelmintic resistance in *H. contortus* parasite throughout the world, including India (Sanyal 1998, Singh *et al.* 2002, Das and Singh 2005). There are also few reports of very high degree of resistance in the parasites against multiple drugs (Burke and Miller 2006). Hence, development of alternative strategies for control of the parasite is warranted for management of haemonchosis.

A number of highly effective antigens with protease activity have been identified in *Haemonchus contortus* (Knox *et al.* 2003). Proteases produced by larval and adult gastrointestinal nematodes play an important role in assuring parasite invasiveness and completion of the nematode lifecycle in appropriate host. A protease from *H. contortus* (termed Pep1), which is similar to mammalian pepsinogen is localized in the gut of adult *H. contortus*, where it is believed to be involved in the digestion of blood meal (Longbottom *et al.* 1997). Pep1 is a component of host protective integral membrane protein complex, (H-gal-GP), which reduces the faecal egg count by 93% and worm burden by 75% when used as a vaccine in sheep (Smith *et al*. 2000). The most common class of cysteine proteases is clan CA. The clan CA proteases are further divided into two families: C1, which comprises cathepsin B and L-like proteases, and C2 which comprises calpain-like proteases. Cysteine proteases possess an essential cysteine residue that forms a covalent intermediate complex with substrates (Sajid and McKerrow 2002). Cheng *et al.* (2012) identified a novel gene encoding a cathepsin B-like cysteine protease (AcCBL1) from the cDNA library of *Angiostrongysus cantonensis* fourth-stage larvae (L4) and characterized its biological role in the parasite. Similarly, Lee *et al.* (2014) have cloned and characterized the *Naegleria gruberi* cathepsin B (nfcpb) and cathepsin B-like (nfcpb-L) genes. The full-length sequences of nfcpb and nfcpb-L genes were 1,038 and 939 bp (encoded 345 and 313 amino acids), and the molecular weights were 38.4 and 34 kDa, respectively.

Proteases are now considered as the future drug and vaccine targets for control of the parasites (Dalton *et al.* 2003). Successful vaccination against the nematodes may be the most effective strategy for prevention and control of the parasites. There is dearth of reports on cathepsin B-like cysteine protease gene of *H. contortus* in India. Keeping

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this in view, this preliminary study was undertaken to clone the part of cathepsin B-like cysteine protease gene from *H. contortus* and to study the effect of immunization of cysteine protease fraction on the faecal egg count in lambs adapted in the Bikaner region of Rajasthan.

MATERIALS AND METHODS

Experimental materials: Adult male and female of *H. contortus* were used in this study. Adult *H. contortus* worms were collected from abomasums of sheep and goats slaughtered in abattoir at Bikaner and processed according to the method of Donald *et al.* (1978) and Dobson *et al*. (1990). The abomasum was cut open along its greater curvature and the mucosa was carefully rinsed with running tap water. The content was collected into separate beakers. The solution was stirred carefully and stored at - 20^0C until the samples were examined. Finally, the contents were filtered through sieve and worms were recovered.

Worms were collected and differentiated with respect to sex and developmental stages. The species of *Haemonchus* were identified according to the method developed by Jacquit *et al*. (1995).

Isolation of genomic DNA from adult Haemonchus contortus: DNA was extracted from adult male and female *Haemonchus contortus*. The phenol-chloroform extraction followed by ethanol precipitation with slight modification was used (Sambrook and Russel 2001). The quality and quantity of DNA was measured by agarose gel electrophoresis and spectrophotometer, respectively. DNA concentrations of the samples were adjusted to100 ng/µl.

Primer details, PCR amplification and purification: The cathepsin B-like cysteine protease gene of *H. contortus* consists of 12 exons. Among the 12 exons, the exon regions from 1-4 were chosen in the present work. The total length of the cathepsin B-like cystiene protease gene was 3177 bp, whereas the length of the first 1-4 exon region was 767 bp including introns between them (starting from 1bp to 767 bp of the gene; Pratt *et al.* 1990). The primers were synthesized from Clontech, USA and GCC Biotech., India. These were reconstituted in sterile TE buffer to make the stock of 100 mmol. Cathepsin B-like cysteine protease gene of *H. contortus* (Exons 1-4) was amplified using primer pairs as follows: Forward primer 5'-GTT CCG CAC AGG ATG AAC ATA TTT GGT TTA-3' and Reverse primer 5'-AGT GAG GCG ATT GAC AAG AGA GGT ACT CAC-3' (Genbank accession No. AH001064). PCR reaction was carried out in 50µl volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 picomole of each primer, 3U of Taq DNA polymerase and 100 ng *H. contortus* DNA in PCR tube. PCR reaction conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. The PCR product was loaded in 1% low melting agarose gel and the desired band was carefully excised with the help of a sterile scalpel under UV illumination. The PCR products were purified by phenol chloroform extraction method or gel extraction kit.

Cloning of PCR product and transformation: Plasmid DNA from *E. coli* DH5α cells was isolated by the alkali lysis method as described in Sambrook and Russel (2001) with slight modifications. The plasmid DNA was air dried and suspended in 50 µl of filtered quartz water and stored at –20°C till further use. Restriction endonuclease (RE) digestion of either plasmid DNA or PCR product was done with the help of restriction enzyme (*NcoI*) simultaneously as per the manufacturer's recommendations. About 1µg of plasmid DNA (1µg/10µl) and 2µg of PCR product was digested with 5µl of *NcoI* enzyme in a 50µl reaction volume at 37°C for 3 h. The digested product was analyzed in 1.0% agarose gel using DNA molecular weight marker run alongside the samples. The digested DNA was purified in low melting agarose gel on preparatory scale as described earlier and purified by phenol chloroform extraction method. The concentration of purified DNA was assessed by spectrophotometer.

E. coli (DH5α) cells were made competent for transformation by the calcium chloride method as described by Sambrook and Russel (2001). The cloning was done by using the instant cloning kit. The DNA fragment and the T- (carrying two unique *NcoI* sites in the T-vector) were digested with *NcoI* enzyme (C∇CATGG) to generate compatible ends (Sticky) for ligation. The individual DNA was mixed so as to have a vector and then DNA was inserted in the ratio of 1:3. The ligation was done in the reaction volume of 20 µl containing 2µl of 10 X T4 DNA Ligase buffer 400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP (pH 7.8 at 25°C)], and 2U of T4 DNA ligase. The contents were mixed, spun down in a micro centrifuge for 3-5 seconds and incubated at 22^0C for 1 h. The reaction was stopped by heat inactivating the enzyme at 65°C for 10 min. The ligation mixture was either stored at –20°C for future use or used directly for transformation.

The frozen aliquots of competent cells were thawed on ice for 30 min and mixed with appropriate quantity of ligated mixture (10 µl) and incubated on ice for 30 min. The *E. coli* cells were given heat shock at 42°C for 50s and then cooling on ice for 2 min. Immediately 1 ml of prewarmed SOC/LB broth was added to the cells and the suspension was incubated at 37°C for 1 h in a shaking incubator. Subsequently, the cells were pelleted by centrifugation at 8000 × g for 5 min at room temperature and the pellet was resuspended in 100 µl of fresh LB broth. The cells were plated on LB agar plates containing ampicillin (50 µg/ml) and incubated at 37°C for 16 to 18 h. Colonies that appeared were screened for the presence of plasmids and colony lysis method as per procedure described in Promega protocols (using the same conditions of amplification applied for gene or as specified for the vector specific primers).

Confirmation of the desired gene by RE digestion: The true recombinants were confirmed by the release of insert of expected molecular size upon RE digestion (*NcoI)* of the plasmid DNAs extracted from positive clones. True recombinant plasmid DNAs were extracted by minipreps

alkali lysis method as described earlier. These were subjected to *NcoI* restriction enzyme digestion under identical conditions as described above. The release of the expected size of fragment confirmed the true recombinants.

Immunization of lambs with cysteine enriched fraction: Male sheep lambs (10), 6- months old, were reared in parasite free conditions. They were fed with dry hay and 200 g lamb ration. Animals were divided in two groups consisting of five lambs in each group. Their faecal samples were examined consecutively for 3 days to ascertain their freedom from parasite. The first group was immunized for 5 consecutive weeks by intra muscular injection of 50, 75, 100, and 300 µg of the cysteine enriched fraction with Freunds complete adjuvant. The second group was given phosphate buffered saline (PBS) instead of antigen. Both the groups were challenged with 8000 *Haemonchus contortus* infective larvae on week seven. Faecal egg count (epg) was performed weekly by McMaster technique from day 15 after challenge until the end of experiment (15 weeks). Mean of faecal egg count of immunized and control groups were compared using t- test for significance. At necropsy, the abomasa were removed and total numbers of worms were counted (MAFF 1986).

RESULTS AND DISCUSSION

Cysteine protease from *H. contortus* has been considered as the potential 'vaccine candidate' for the development of vaccine against *H. contortus* (Dalton *et al.* 2003). Therefore, an attempt was made to characterize the cathepsin B-like cysteine protease gene of *H. contortus.*

In the present study, 767 bp PCR product of cathepsin B-like cysteine protease gene (Exons 1-4) of *H. contortus* of sheep and goats of Indian origin was amplified using forward and reverse primers (Fig 1.). Pathak *et al*. (2012) have analyzed the region of cathepsin B-like cysteine protease gene of *H. contortus* in goats earlier. The partial sequence of the cathepsin B-like cysteine protease m-RNA sequence was found to be 997 bp long which was in concordance with earlier reports (Pathak *et al*. 2012). Cheng *et al.* (2012) identified a novel gene encoding a cathepsin B-like cysteine protease (AcCBL1) from *Angiostrongylus cantonensis* fourth-stage larvae (L4). Its sequence and phylogenetic analysis showed that AcCBL1 is related to other cathepsin B family members. In addition, the sequence also contains a specific "hemoglobinase motif" and might have a hemoglobinase (Hb)-degrading function

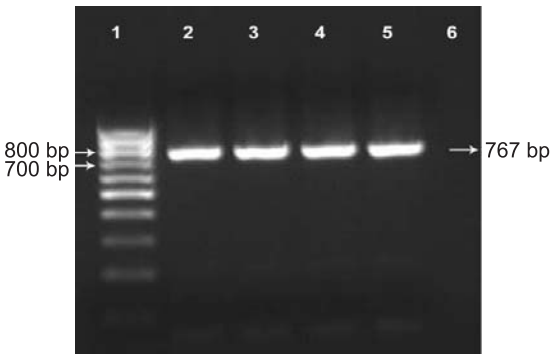

Fig. 1. Separation of the PCR products of Cathepsin B-like cysteine protease gene (Exons 1-4) from adult *H. contortus* parasites. Lane 1: 100 bp ladder, Lane 2-5: expected size of the PCR products (767 bp) from *H. contortus* parasites; Lane 6: negative control.

The PCR product was cloned into T-vector and ligated product was transformed in *E. coli* (DH5α) competent cells. Transformed *E. coli* cells were plated on X-Gal-IPTG-ampicillin agar plate. The blue colonies represent the presence of vector alone that provides intact α-complementation, while white colonies represent true recombinant clones carrying the insert. The white colonies were further confirmed for the presence of insert in vector by alkali lysis method. Plasmid DNAs were extracted from positive white colonies grown in LB-ampicillin broth. It was digested with *NcoI* restriction enzyme and analyzed on 1.2% agarose gel (Fig. 2). Two DNA bands were seen in case of plasmids isolated from positive colonies upon *NcoI* digestion (Lanes 1-3), the less intense lower band may correspond to the insert size. The size of the lower band was deduced from the standard curve drawn from log molecular sizes of the markers against their mobility and it was found to be 767 bp. However, sequence analysis of the PCR product could not be undertaken for the confirmation of specificity of the target region of the gene.

The most common family of proteases in nematode parasites (particularly in the blood sucking *Strongyles*) is

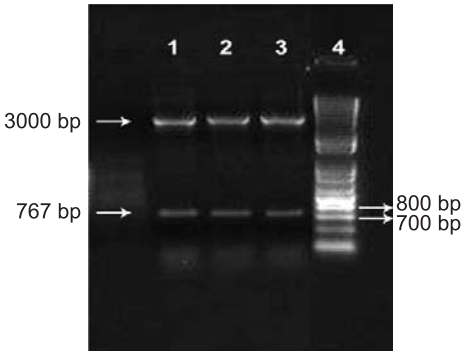

Fig. 2. Digestion of the PCR products of the Cathepsin B-like cysteine protease gene of adult *Haemonchus contortus* parasite. Lane 1-3: Expected size 767 bp released band after *NcoI* digestion. Lane 4: 1 Kb plus DNA ladder.

the papain like cysteine proteases, which belongs to the family C1 that contains the cathepsin B and cathepsin L-like enzymes (Sajid and Mckerrow 2002). Cathepsin B-like cysteine proteases (Cat BS) generally constitute large multigene families in both parasitic and non-parasitic helminthes including *H. contortus*. In parasitic helminthes, Cat BS is expressed in the alimentary canal of the worms, where they play proven roles in the digestion of protein for nutrient uptake. It has been reported that Cat B encoding genes account 16-17% of the intestinal transcripts from *H. contortus* (Jasmer *et al.* 2001, 2004) representing the most abundant and diverse protein family in the gut of *H. contortus*. Thus, cat B encoding transcripts may have similar

type of protective roles and may inhibit the host immune responses. It is speculated that the cathepsin B-like cysteine protease gene of *H. contortus* from sheep and goats may have same gene sequence or it may differ from the cat B sequence of the *H. contortus* existing in other livestock species. It may be due to the different rumen ecosystem of small ruminants which differ from the bovine rumen ecosystem. Jasmer *et al.* (2004) compared the cathepsin B-like cysteine protease (cbl) with other parasitic nematodes, the extreme abundance and diversity of intestinal cathepsin B-like cysteine protease transcripts was recorded in relation to *H. contortus*. Therefore, adaptations related to the nutrient acquisition may vary markedly among parasitic nematodes. Thus, the different ecosystems may upregulate or down regulate the expression of the cathepsin B-like cysteine protease genes in the gut of the parasites. More stress conditions (frequent use of drugs, more temperature, humidity and other disease conditions) may also change the expression of the genes as well may create the mutations in the proteases genes /promotor of the parasite. Further research is required to analyze and compare the cat B sequences of the *H. contortus* of small ruminants and other domestic animals. The whole sequence of the cat B gene needs to be sequenced, which may provide the basis for developing an ideal vaccine or drug target for control of the haemonchosis in India.

Immunization study was conducted to examine the effect of cysteine protease fraction on the faecal egg counts in lambs following immunization. Faecal egg count was performed weekly from day 15 after challenge until the end of experiment (15week) (Table 1). It was found that the eggs per gram of faeces were lower in immunized group of lambs as compared to control group (at 10, 11, 12, and 15 weeks after immunization) but the differences were statistically non significant.. At post mortem, the worms recovered from the abomasa at necropsy were lower in immunized group (800.40±101.8) as compared to control group (950.93±300.29). The results indicated that the immunization with protease fraction in lambs reduced the faecal egg count but did not result in flushing out all worms. The present results were similar to the findings of deVries *et al.* (2009), who tested the cystatin binding fraction (cysteine protease) as an immunogen in lambs, which were vaccinated three times (week 0, 2.5 and 5) and challenged with 8000 L_3 *H. contortus* (week 6). The group vaccinated with cystatin-binding proteins showed 36% and 32% mean worm burden and eggs per gram of faeces (EPG) reductions, respectively, compared to the controls. Significant levels of protection against *H. contortus* was achieved in sheep by vaccination with a cysteine proteinase-enriched fraction (TSBP) isolated from the gut of adult parasites (Redmond and Knox 2004). Protection was associated with three cathepsin B-like cysteine proteinases (hmcp 1, 4 & 6). Lambs vaccinated with these proteinases had significantly reduced (38%) the worm burdens compared to challenge control although, intriguingly, egg output was unaffected. It is concluded from the study that immunization strategies have some moderate effect on the faecal egg counts in immunized lambs.

Table 1. Eggs per gram (epg) of faeces in lambs (mean ±SE)

Week	Immunised group	Infected control	P Value
8	00	00	-
9	00	00	-
10	150±13.5	200±61.5	0.450
11	120.33± 200.9	220.8±100.4	0.667
12	600.89±179.7	750.35±247.9	0.638
15	550.79±100.6	720.22±174.5	0.424
16	800.40±101.8	950.93±300.29	0.648

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