



Intraspecific and gender variation in *hmcp4* gene of cysteine proteinase in *Haemonchus contortus*

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Sheep and cattle farming worldwide, is subjected to severe economic losses from gastro-intestinal nematode (Steppek *et al.* 2004). In India, haemonchosis due to *Haemonchus contortus* is recognized as a parasite of considerable economic importance in sheep and goats (Sood 1981), and losses due to anthelmintic treatment against *H. contortus* in India are estimated to be \$ 103 million annually (McLeod 2004, Waller and Chandrawathani 2005). Much effort is directed towards the development of a vaccine against *Haemonchus* and a number of promising candidate vaccine antigens were identified (Smith 1999). Cysteine proteinase is found to be a good candidate vaccine antigen (Knox *et al.* 1993). Three genes *hmcp1*, *hmcp4* and *hmcp6* controlling production of gut derived cysteine proteinases in UK strain of *H. contortus* are important for immunoprophylactic potential (Knox *et al.* 2005). Vaccination studies also suggested that the effect of immunisation is more on female parasite than on male parasite (Newton 1995). Major objective of the study was to clone and characterize *hmcp4* gene of cysteine proteinase in male and female *H. contortus* and to record nucleotide heterogeneity in the Indian isolate, if any, for further expression study to exploit immunoprophylaxis potential. Further, the study was diversified in female in which there are 2 common morphotypes having vulva covered with linguiform flap (lingui form type) and the other without flap (button form type).

Parasite collection and RNA isolation: Adult *H. contortus*, collected from abomasa of slaughtered sheep were used for RNA isolation. Male and female worms were identified and separated into linguiform and buttonform individuals based upon morphology of vulva. Eggs were evacuated and empty female worms were kept in RNA later solution at –20°C till further use. Similarly male worms were kept in RNA later

solution at –20°C as female worms. Total RNA was extracted directly from the male, button-form and linguiform *H. contortus*, separately using Trizol reagent as per the manufacturer's instructions. The yield and the purity of RNA were measured by absorbance at 260 and 280 nm.

Reverse transcription- polymerase chain reaction (RT-PCR) : First strand cDNA was synthesized for both the morphotypes and male, separately from 200ng total RNA using Mu-MLV reverse transcriptase. Based on the published nucleotide sequence of *hmcp4* of *H. contortus* (Skuce *et al.* 1999), primers were designed to amplify *hmcp4* from cDNA of the both morphotypes and male (Forward primer: 5'-GAC ATC CCA GAA AGT TTC GAC- 3'; Reverse primer: 5'-TCA GAC GTG TCC GGC TAC AA- 3') by PCR using the Taq DNA polymerase in a thermal cycler as follows: initial denaturation at 95°C for 2 min, followed by 33 cycles of denaturation at 95°C for 45sec, annealing at 60°C for 45sec and extension at 72°C for 1 min with a final extension at 72°C for 10 min. The amplified product was run in 1.5% agarose gel and the confirmation of the product was done based upon its size in the gel.

Cloning of *hmcp4* gene: The purified amplicons were cloned in pDRIVE T/A cloning vector (Sambrook *et al.* 1989). The recombinant plasmids were transformed into competent *Escherichia coli* DH5 α . Positive clones were selected by blue white colony screening. Finally, the recombinant plasmids were extracted from the transformed DH5 α cells following standard protocol (Sambrook *et al.* 1989). For confirmation of the insert in the clones, restriction digestion reactions was carried out using *EcoRI* restriction enzyme as well as with colony PCR. The positive clones were custom sequenced for nucleotides by Sanger's di deoxy method.

Nucleotide sequence and deduced aminoacid sequence analysis of *hmcp4* gene: Comparison of buttonform, linguiform morphotypes and male of Indian isolate *H. contortus* and UK strain *H. contortus* *hmcp4* nucleotide sequences were made using the ClustalV pair distance software package. The per cent homology of *hmcp4* gene of

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buttonform (Genbank Accession No. GQ223788), linguiform female (Genbank Accession No. GQ223787) and male (Genbank Accession No. GU979002) Indian isolate of *H. contortus* with UK strain *H. contortus* (Genbank Accession No: Z69345) and among themselves were determined. The deduced amino acid sequences of the *hmcp4* gene from male, buttonform and linguiform Indian isolate *H. contortus* were compared with that of UK strain and also between themselves.

The coding sequence of *H. contortus hmcp4* was retrieved from National Center for Biotechnology Information (NCBI) GenBank (Accession No: Z69345). The partial gene of *hmcp4* was amplified using PCR primers in male and both morphotypes of female. The specificity and size of the amplified product was checked by 1.5% agarose gel electrophoresis and expected size of 762 bp was resolved in male and both morphotypes of females (Fig.1). The amplicon of male and both morphotypes were confirmed with restriction enzyme (RE) analysis with *Clal* enzyme, which yielded the expected size of 439 bp and 323 bp. The *hmcp4* gene was cloned in T/A cloning vector pDRIVE, having MCS (multiple cloning sites) incorporated into a LacZ, a peptide coding region, chosen for easy selection of recombinant were clones. The selection of the *E. coli* DH5 α positive colonies was done by blue-white colony screening method in male and both morphotypes of female.

During the present study, *hmcp4* gene was amplified in male, 2 morphotypes button form and linguiform of female *H. contortus*, separately. Homology between buttonform female *H. contortus* of Indian isolate and published UK strain sequence of *hmcp4* gene was 100% whereas between linguiform female and published UK strain sequence it was 98.8%. Between male *H. contortus* and published UK strain sequence homology was 98.3%. Among Indian strain, 98.8% homology was present between buttonform and linguiform morphotypes. The sequence homology between buttonform female and male was 98.3% whereas between linguiform female and male it was 97.1%. Intra-specific and gender specific variation with respect to *hmcp4* gene of cysteine protease in *H. contortus* is reported for the first time. The deduced amino acid sequence homology between buttonform female *H. contortus* of Indian isolate and published U.K. strain of *hmcp4* gene was 100% whereas between linguiform female and published UK strain sequence it was 98.8%. Between male *H. contortus* and published U.K. strain amino acid sequence homology was 96.5%. Among Indian isolate, 98.8% homology was present between buttonform and linguiform morphotypes. The deduced amino acid sequence homology between buttonform female and male was 96.5% whereas between linguiform female and male it was 95.7% (Fig. 2). Eight amino acid changes were observed in deduced sequence of male compared with female *H. contortus* at 94,95,96, 108,243,246, 250 and 325th positions, the changes were in 2 positions (110 and 200th) when compared button

form and linguiform. Homology between the nucleotide sequence of Indian isolate and U.K. strain was not 100% due to geographical, strain, morphotypes and gender variations.

The principal finding of this study was the characterization of the *hmcp4* gene in male as well as both the morphotypes of female in Indian isolate of *H. contortus*. During the present study, genotypic differences between the male *H. contortus*, buttonform and linguiform female *H. contortus* were studied with respect to *hmcp4* gene. The gene is responsible for expression of the respective enzyme, which is essentially required for survival of parasite and pathogenicity caused by it (Muleke *et al.* 2006). Because database sequence information was also available for the gene, it was possible to investigate genotypic differences of Indian isolate of *H. contortus* with reference to published sequence of U.K. strain as well as genotypic differences between male and two morphotypes of the Indian isolate in simultaneously.

Significance of cysteine proteases is well established with respect to nutrition of *H. contortus* particularly blood feeding, which is due to its capability of digesting haemoglobin, fibrinogen, collagen and IgG (Knox *et al.* 1993). A panel of 6 protease homologues (*hmcp1*–6) were amplified and characterized from U.K. strain of *H. contortus*. Cysteine proteinases expressed by *hmcp1*, 4 and 6 are localized in the gut of the parasite and associated with protective function, which has been established by immunoscreening studies with antisera from S3 TSBP vaccinates (Skuce *et al.* 1999). The expression of the *hmcp1*, 4 and 6 genes, as revealed by RT-PCR, coincides with the onset of blood feeding during the

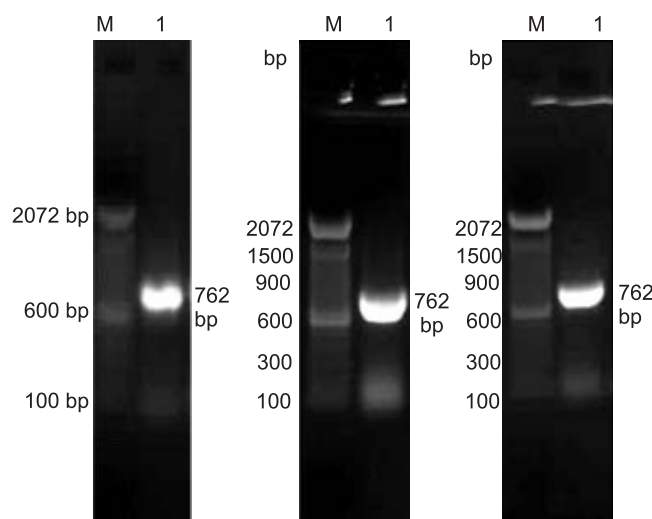


Fig. 1. PCR amplification of partial length *hmcp4* gene of *H. contortus* (Male); Lane M: Marker; Lane 1 : Amplified PCR product (Male). PCR amplification of partial length *hmcp4* gene of *H. contortus* (Linguiform). Lane M: Marker; Lane 1 : Amplified PCR product. PCR amplification of partial length *hmcp4* gene of *H. contortus* (Buttonform). Lane M: Marker; Lane 1 : Amplified PCR product.

A I Q R E I M K N G P V V G A F T V Y E																				Majority	
										170										180	
161	A	I	Q	R	E	I	M	K	N	G	P	V	V	G	A	F	T	V	Y	E	hmcp4 <i>H. contortus</i> Buttonform
161	A	I	Q	R	E	I	M	K	N	G	P	V	V	G	A	F	T	V	Y	E	hmcp4 <i>H. contortus</i> Linguiform
161	A	T	Q	R	E	I	M	K	N	G	P	V	V	G	A	F	T	V	Y	E	hmcp4 <i>H. contortus</i> Male
161	A	I	Q	R	E	I	M	K	N	G	P	V	V	G	A	F	T	V	Y	E	hmcp4 <i>H. contortus</i> U.L strain
D F S Y Y K K G I Y K H T A G K A R G G																				Majority	
										190										200	
181	D	F	S	Y	Y	K	K	G	I	Y	K	H	T	A	G	K	A	R	G	G	hmcp4 <i>H. contortus</i> Buttonform
181	D	F	S	Y	Y	K	K	G	I	Y	K	H	T	A	G	K	A	R	G	G	hmcp4 <i>H. contortus</i> Linguiform
181	D	F	S	Y	Y	K	K	G	I	Y	K	H	T	A	G	K	A	R	G	G	hmcp4 <i>H. contortus</i> Male
181	D	F	S	Y	Y	K	K	G	I	Y	K	H	T	A	G	K	A	R	G	G	hmcp4 <i>H. contortus</i> U.L strain
H A I K I I G W G K E G G V P Y W L I A																				Majority	
										210										220	
201	H	A	I	K	I	I	G	W	G	K	E	G	G	V	P	Y	W	L	I	A	hmcp4 <i>H. contortus</i> Buttonform
201	H	A	I	K	I	I	G	W	G	K	E	N	G	V	P	Y	W	L	I	A	hmcp4 <i>H. contortus</i> Linguiform
201	H	A	I	K	I	I	G	W	G	K	E	G	G	V	P	Y	W	L	I	A	hmcp4 <i>H. contortus</i> Male
201	H	A	I	K	I	I	G	W	G	K	E	G	G	V	P	Y	W	L	I	A	hmcp4 <i>H. contortus</i> U.L strain
N S W H N D W G E N G Y F R I L R G S N																				Majority	
										230										240	
221	N	S	W	H	N	D	W	G	E	N	G	Y	F	R	I	L	R	G	S	N	hmcp4 <i>H. contortus</i> Buttonform
221	N	S	W	H	N	D	W	G	E	N	G	Y	F	R	I	L	R	G	S	N	hmcp4 <i>H. contortus</i> Linguiform
221	N	S	W	H	N	D	W	G	E	N	G	Y	F	R	I	L	C	G	S	N	hmcp4 <i>H. contortus</i> Male
221	N	S	W	H	N	D	W	G	E	N	G	Y	F	R	I	L	R	G	S	N	hmcp4 <i>H. contortus</i> U.L strain
H C G I E E N V V A G H V -																				Majority	
										250											
241	H	C	G	I	E	E	N	V	V	A	G	H	V	-	-	-	-	-	-	-	hmcp4 <i>H. contortus</i> Buttonform
241	H	C	G	I	E	E	N	V	V	A	G	H	V	-	-	-	-	-	-	-	hmcp4 <i>H. contortus</i> Linguiform
241	H	C	G	I	E	E	N	V	V	A	G	H	V	-	-	-	-	-	-	-	hmcp4 <i>H. contortus</i> Male
241	H	C	G	I	E	E	N	V	V	A	G	H	V	-	-	-	-	-	-	-	hmcp4 <i>H. contortus</i> U.L strain

Fig. 2. Decoration 'Decoration #1': Shade (with gray) residues that differ from the Consensus.

parasitic phase of the life cycle, that is, exclusively from fourth stage larvae (L4) and onwards. Knox *et al.* (2005) provided through their findings strong evidence that protection could be attributed to the cysteine proteinase component of TSBP in contrast to water soluble and membrane associated cysteine proteinases, which were without effect as immunogens. Regarding *hmcp4* gene, it needs to be expressed in suitable host for recombinant *hmcp4* production in order to utilize it for immunoprotective potential.

To develop a vaccine against *H. contortus* of Indian isolate it would be essential to consider *hmcp1*, *hmcp4* and *hmcp6* of U.K. strain as well as *gcp7* of USA strain (Rehman and

Jasmer 1998) which is also a gut associated cysteine proteinase and having immunoprophylactic role. Genes other than these if any needs to be investigated in the Indian isolate about which we do not have any information presently. Expression studies correlated to native cysteine proteases will certainly reveal a more reliable profile of Indian strain of *H. contortus* and will strongly help in developing a vaccine. Since, most of the countries having *H. contortus* in their livestock are studying in greater detail polypeptides of immunoprophylactic values and exploring the possible genes expressing these polypeptides. Studies in India also should be taken up to give impetus to vaccine research against haemonchosis. Expression of relevant genes may be helpful

in generating a cocktail vaccine in future against haemonchosis utilising recombinant proteins.

SUMMARY

Different types of cysteine proteinases, viz. hmcp1, hmcp4 and hmcp6 reported in U.K. strain of *Haemonchus contortus* as well as in USA gcp-7, are incriminated in the expression of gut enzymes. In the Indian isolate of *H. contortus*, information on the presence of these genes is lacking. During the present study intraspecific and gender variations of hmcp4 gene was conducted. The gene was cloned and characterized in the 2 commonly occurring morphotypes of females having buttonform and linguiform vulva as well as male worms. RNA was isolated from 2 morphotypes of adult female and male *H. contortus*, separately. The cDNA was synthesized by reverse transcription and the hmcp4 gene was PCR amplified using Taq DNA polymerase. The amplified PCR product was purified and cloned. Sequence homologies between buttonform female and linguiform female *H. contortus* with respect to hmcp4 gene in U.K. strain were 100% and 98.8%, respectively whereas with male *H. contortus* the homology was 98.3%. Intraspecific as well as gender specific variation were also recorded with respect to hmcp4 gene of cysteine proteinase in *H. contortus* for the first time. Amino acid sequence homology between male and female *H. contortus* of Indian isolate and published U.K. strain were also recorded.

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