Polymerase chain reaction (PCR) based amplification of *hmcp3* and *hmcp6* cysteine protease genes of *Haemonchus contortus* from small ruminants

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Received: 20 February 2015 / Accepted: 10 March 2015 / Published online: 21 March 2015
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Abstract *Haemonchus contortus* is a haematophagous nematode in small ruminants population and causes anaemia, weakness and mortality especially in young animals. In the present study, *hmcp3* partial gene of 836 bp and *hmcp6* full length gene of 1041 bp were amplified from the cDNA of Bareilly isolate of adult male *H. contortus* by polymerase chain reaction. Further gene characterization and expression studies are warranted to know the immunoprophylactic potential of *hmcp3* and *hmcp6* proteins of *H. contortus*.

Keywords *Haemonchus contortus* · *hmcp3* · *hmcp6* · Cysteine protease · PCR

Introduction

Haemonchus contortus is highly pathogenic blood sucking nematode of small ruminants in different parts of the world. The infection haemonchosis is manifested by anaemia, hypoproteinemia, weakness, malnutrition with progressive loss of weight, wool in adults and stunted growth in young

animals throughout India (Sood 1981). Estimates of the average blood loss per worm per day range from approximately 0.003 to 0.004 ml (Dargie and Allonby 1975) to 0.05 ml (Soulsby 1982) and 0.07 ml of blood/day (Malviya et al. 1979). Cost of anthelmintic treatment against *H. contortus* alone in India has been estimated to be \$103 million per annum (McLeod 2004). However, widespread selection of resistant *H. contortus* strains against anthelmintics has accelerated the need for studies on the development of alternative strategies (Wolstenholme et al. 2004). Many efforts have been directed towards the development of vaccines against *Haemonchus* and panel of promising vaccine candidates have been identified (Knox et al. 1993, 1995; Longbottom et al. 1997; Smith 1999) without expected degree of success till today. Significant among these antigens are H11 (Smith et al. 1997), cysteine proteases (Knox et al. 1993) and H-gal-GP (Smith et al. 1994).

Cysteine proteases are secreted by larval and adult parasites and its presumed functions are tissue penetration, feeding and defense against effector mechanisms of the host immune response (Karanu et al. 1993; Tort et al. 1999). Cysteine proteases are vaccine candidate molecules in *H. contortus* capable of digesting nutrients, Hb, fibrinogen, collagen and immunoglobulin G (Knox et al. 1993). Three genes *hmcp1*, *hmcp4* and *hmcp6* were controlling production of gut derived cysteine proteases in UK strain of *H. contortus* are important for immunoprophylactic potential (Knox et al. 2005). Rehman and Jasmer (1998) isolated cysteine protease *gcp7* from USA strain of *Haemonchus*. To develop a promising vaccine against *H. contortus* of Indian strain, it would be essential to consider the presence and variations of cysteine protease genes of *H. contortus* and their immunoprophylactic potential.

Diversity among *Haemonchus* cysteine proteases has been observed between USA and Kenyan isolates (Karanu

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et al. 1993). Suchita (2012) identified immunodominant polypeptides of 31, 43, 60 kDa in purified native cysteine protease fractions of adult male and female *H. contortus*. Cysteine protease activity was identified at different range in male and female *H. contortus* (43–66 kDa in male and 31–43 kDa in female). This difference in the cysteine protease activity in *H. contortus* was of great significance for development of anthelmintic drugs as well as developing protease based immunoprophylactic agent against *H. contortus*. Prasad et al. (2013) studied the genetic polymorphism of cysteine proteases of *H. contortus* which confirmed the presence of *hmcp4* gene of UK strain in the Indian strain. Cysteine protease genes of both UK strain and USA strain have been identified recently in different polymorphic Indian isolate of *H. contortus* by Allaie et al. (2014).

Cysteine protease genes polymorphism needs to be investigated further in the Indian strain of *H. contortus* about which we lack information presently other than the reports by Prasad et al. (2013) and Allaie et al. (2014). Therefore, it is in great need to know the presence of number of cysteine protease genes and its characterization in Indian isolates, which may give ideas for control of *H. contortus* in future. As a preliminary work, the study has been tried for PCR amplification of *hmcp3* and *hmcp6* genes in Indian strain of *H. contortus*.

Materials and methods

RNA isolation from *H. contortus*

Adult *H. contortus* collected from abomasa of slaughtered sheep and goats from Bareilly, Uttar Pradesh, India. Male and female worms were identified as per morphology described by Soulsby (1982) and separated followed by washing in phosphate buffered saline. Adult male *H. contortus* worms were kept in RNA later solution at -20°C till further use. Total RNA was extracted directly from the male *H. contortus* 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.

Synthesis of complementary DNA

Complementary DNA (cDNA) was synthesized using 150 ng of total RNA from male *H. contortus* as a template using oligo dT primer and Mu-MLV reverse transcriptase enzyme as per standard protocols of reverse transcription PCR (RT-PCR). The synthesized cDNA was quantified using spectrophotometer and stored in -20°C till further use.

Amplification of *hmcp3* and *hmcp6* genes of *H. contortus* by PCR

The forward and reverse primers were designed based on published *hmcp3* and *hmcp6* nucleotide sequences of *H. contortus* from NCBI using DNA STAR and Gene Tool software.

hmcp3-F: 5'-GGC CAT GGC GAT GGA TCG AAG ATT CAT CAA G-3'

hmcp3-R: 5'-GGA AGC TT TCA TGG TTT GAA TTT CCC GG-3'

hmcp6-F: 5'-GGC CAT GGC GAT GAG GTA CAA CGT AGT TG-3'

hmcp6-R: 5'-GGA AGC TTT CAC AGT AGG ACA TGT CC-3'

The cDNA from male *H. contortus* was used as a template for PCR to amplify the cysteine protease genes. PCR was carried out in a reaction mixture containing $5\times$ GoTaq[®] Flexi DNA Polymerase buffer, 1.5 mM MgCl₂, 200 μM of dNTP, 1 U GoTaq[®] Flexi DNA Polymerase enzyme, 20 pmol of forward and reverse primers and cDNA. The reactions were performed using an automatic Biometra thermal cycler machine with the following cycling conditions; For amplification of *hmcp3* gene, initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 56.5°C for 30 s, elongation at 72°C for 1 min was followed. Final elongation was done at 72°C for 10 min. Similarly, for *hmcp6* gene amplification from cDNA, the cycling conditions were same except the annealing temperature of 55°C was used.

The amplified products were run in 1.5 % agarose gel and the confirmation of the products was done based upon their size in the gel under UV gel-documentation system. The purification of PCR products was done using PCR-Clean up kit method (Sigma Aldrich) and quantification of the PCR product was done using Nanodrop[®] Spectrophotometer. The *hmcp* DNA samples were stored at -20°C until further use.

Results

The adult male and female *H. contortus* worms were identified by their morphology. Male worms were having prominent copulatory bursa with two dark brown spicules having barbed tips (Fig. 1). The female worms were larger than male worms with pointed tail end, white ovaries were spirally located around the red intestine giving the appearance of barber's pole. The vulva was present at posterior part of the body covered by knobbed prominence or tongue like vulval flap (Fig. 2).

Fig. 1 Male *H. contortus* posterior end showing copulatory bursa and barbed spicules ($\times 10$ objective)

Fig. 2 Female *H. contortus* showing eggs in uterus and vulva with knobbed prominence ($\times 10$ objective)

The total RNA isolated from the adult male *H. contortus* was $0.5 \mu\text{g}/\mu\text{l}$. The purity of RNA was checked by measuring optical density between 260 and 280 nm, which yielded a value of 1.7 confirming free from any contaminating protein or DNA. The concentration of cDNA was $19 \text{ ng}/\mu\text{l}$ as quantified using the UV spectrum absorbance at 260 nm. The PCR amplified the *hmcp3* gene with amplicon size of 836 bp (in partial length) and *hmcp6* gene with 1041 bp size (in full length) in 1.5 % agarose gel electrophoresis (Fig. 3). The concentration of the purified PCR products was $227 \text{ ng}/\mu\text{l}$ for *hmcp3* DNA and $166 \text{ ng}/\mu\text{l}$ for *hmcp6* DNA.

Discussion

In this study, we observed that the concentration of RNA isolated from the male *H. contortus* was in correlation with Allaie (2009) who isolated total RNA from adult female *H. contortus* $0.6 \mu\text{g}/\mu\text{l}$. The concentration of cDNA synthesized was less as compared to Allaie (2009) who

Fig. 3 PCR amplification of *hmcp3* and *hmcp6* genes of Bareilly isolate of male *H. contortus*. Lanes 1 and 4 1 kb DNA ladder, Lane 2 PCR amplicon of 1041 bp for *hmcp6* gene (full length), Lane 6 PCR amplicon of 836 bp for *hmcp3* gene (partial length), Lanes 3, 5, 7, 8 negative control

prepared cDNA of $27 \text{ ng}/\mu\text{l}$ which may be due to quantity of RNA used and changes in temperature protocol during the cDNA synthesis in laboratory.

The present study amplified the *hmcp3* gene of male *H. contortus* in partial length with amplicon size of 836 bp having $227 \text{ ng}/\mu\text{l}$ DNA concentration while Skuce et al. (1999) amplified 1036 bp in full length *hmcp3* gene of *H. contortus*.

The *hmcp6* gene of male *H. contortus* in full length with amplicon size of 1041 bp was amplified in this study with concentration of DNA in eluted fraction was $166 \text{ ng}/\mu\text{l}$ which is in correlation with the work by Skuce et al. (1999) and Allaie et al. (2014).

Skuce et al. (1999) characterized a panel of six protease homologues (designated *hmcp1* to 6). Three cysteine proteases (*hmcp1*, 4 and 6) were specifically localized to the gut of the parasite and associated with protective function identified through immunoscreening with antisera from S3 TSBP (thiol-Sepharose binding protein) vaccinates.

The *hmcp6* and *hmcp1* genes in linguiform and button-form morphotypes of adult female *H. contortus* of Indian isolate were amplified by Allaie (2009) with PCR products of size 993 and 1038 bp, respectively. Prasad et al. (2013) have studied the genetic polymorphism of cysteine proteases of Indian strain of *H. contortus* which has confirmed presence of *hmcp4* gene of UK strain in the different polymorphic Indian strain. The partial gene of *hmcp4* was amplified in adult male, buttonform and linguiform morphotypes of female *H. contortus* with the amplicon size of 762 bp. Sequence homology of 98.3 % was reported between male *H. contortus* of Indian isolate and UK strain.

Also 98.8 % sequence homology was identified by Prasad et al. (2013) within the button form and linguiform morphotypes of adult female *H. contortus* of Indian strain.

Recently Allaie et al. (2014) reported that cysteine protease genes *hmcp1*, *hmcp4*, *hmcp6* of UK strain and *gcp7* of USA strain have been identified in different polymorphic Indian isolate of *H. contortus*. The 1041 bp nucleotide sequences of *hmcp6* of knobbed and linguiform female revealed only 84.9 and 85 % homology, respectively with the UK strain, however 99.9 % homology was found between the two female morphotypes.

The present study identified the two cysteine protease *hmcp* genes (*hmcp3* and *hmcp6*) in male *H. contortus*. Cysteine protease genes other than these, if any, need to be investigated in the Indian isolates or strain about which we do not have any information presently. Also further characterization and expression studies of different cysteine protease genes in adult and developing larval stages of *H. contortus* are warranted which may give ideas for control of *H. contortus* in future.

Acknowledgments Sincere thanks are due to ICAR, New Delhi and the Director, IVRI, Bareilly, India for providing necessary facilities to the authors. The authors also thank Shri. S. C. Gupta, Principal Scientist, Dr. O. K. Raina, Senior Scientist, Dr. B. C. Saravanan, Senior Scientist, Division of Parasitology and Dr. S. Dandapat, Senior Scientist, Immunology Section and Dr. S. K. Bhure, Senior Scientist, Div. of Animal Biochemistry, IVRI, Bareilly for their support and help during the study.

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