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Characterization of HcMSP, A Novel Gender Specific Gene from Parasitic Nematode *Haemonchus contortus*

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Abstract: Major Sperm Protein (MSP) is one of the important components of nematode sperms and the characters of this protein were widely studied in *Caenorhabdit elegans* and *Ascaris suum*. However, no knowledge of homology proteins in *Haemonchus contortus* was reported. In the present research, the *MSP* gene of this parasite (HcMSP) was first cloned and characterized. A pair of primer targeted the conserved sequence of nematode MSPs was designed and a partial of *HcMSP* gene was obtained by reverse transcription PCR (RT-PCR). Then, the 5' and 3' ends of *HcMSP* gene were cloned by 5' Rapid Amplification of cDNA ends (RACE) and 3' RACE. The full length of cDNA was obtained by overlapping the sequences of 3' and 5' extremities. The Open Reading Frame (ORF) was amplified by RT-PCR and expressed in prokaryotic cell.

Key words: *Haemonchus contortus*, major sperm protein, RACE, differential expressions, gene

INTRODUCTION

Haemonchus contortus is a gastrointestinal parasitic nematode that infects ruminants such as cattle, sheep and goats. This parasite is a threat to the production of small ruminants worldwide and causes great economic losses. Infection with this parasite causes anemia, weight loss and death, especially in lambs (Knox *et al*., 1993). The current methods for the control of gastrointestinal nematodes rely heavily on chemicals but this has led to an increase of anthelmintic resistant parasites (Wolstenholme *et al*., 2004). With the growing concern of residual chemicals in the environment and food chain, the situation has resulted in the need to search for alternative or supplementary means for controlling this parasite. Great progress has been made on the immunology of *H. contortus* (Newton and Munn, 1999). However, researchers still have no commercial vaccines. Research on the biology such as the development and sex determination of *H. contortus* would be helpful for looking for innovative control measures for this parasite.

Major Sperm Protein (MSP) was the most abundant protein in nematode sperms which makes up about 40% of the soluble proteins (Roberts and Stewart, 1995). MSP plays important roles in the reproduction for the helminthes: as cytosolic component, MSP is responsible for the crawling movement of the mature sperm and once MSP was released, it acts as hormone on the female germ cells where it triggers oocyte maturation and stimulates the oviduct wall to contract to bring the oocytes into position for fertilization (Baker *et al*., 2002; Tarr and Scott, 2005; Yang *et al*., 2010).

Most research on nematode MSP was focused on *A. suum* and *C. elegans*. Recently, *MSP* genes from nematodes *Oesophagostomum dentatum* (Cottee *et al*., 2004), *Brugia malay* (Ghedin *et al*., 2007), *Dictyocaulus viviparous* (Strube *et al*., 2009) and *Nippostrongylus brasiliensis* (Arizono *et al*., 2011) were cloned and part of the functions were studied. However, no knowledge of homology proteins in *H. contortus* was reported. In the present research, the *MSP* gene of this parasitie (HcMSP) was first cloned and characterized.

MATERIALS AND METHODS

Parasites and RNA extraction: *H. contortus* strain was originally obtained from Nanjing, Jiangsu Province, China and maintained by serial passage in the laboratory. Adult *H. contortus* worms were collected from the abomasums of the artificial infection goats. Eggs from feces were isolated and purified. Third stage Larval (L3) was yield by culturing the feces at 28°C. The detailed protocols were described as earlier (Muleke *et al*., 2006). Total RNA was extracted from pooled adult worms utilizing the single step protocol (Chomczynski and Sacchi, 1987).

Cloning of Full length *HcMSP* gene: Primers used for cloning of HcMSP gene were listed in Table 1 which were designed based on *C. elegans MSP* gene (GenBank Accession No. 133903986) and the primers provided in the RACE kit (TaKaRa Biotech, Dalian, China). The oligonucleotides were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China).

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Table 1: Primers used in the research

Primer name	Sequence (5'-3')	Length (bp)	Description
HcM-EST-F	GCCCCGTACGACGACAAGCACA	22	Forward primer for HcMSP EST amplification
HcM-EST-R	CGGCGGACCATACCATCTCCTTG	23	Reverse primer for HcMSP EST amplification
MSP-3-F1	TCAACGCCTCTGGCCGACGTATC	23	Forward primer specific for 3' end of HcMSP in primary PCR
MSP-3-F2	TTAGACCCTAAGGAAGCCACTCT	23	Forward primer specific for 3' end of HcMSP in second PCR
3' outer primer	TACCGTCGTTCCACTAGTGATTT	23	Reverse primer for 3' end of HcMSP in primary PCR (3' RACE kit)
3' inner primer	CGCGGATCCTCCACTAGTGATTT CACTATAGG	32	Reverse primer for 3' end of HcMSP in second PCR (3' RACE kit)
MSP-5-R1	GGAACCATTCTCGTCGGAACTGC	23	Reverse primer specific for 5' end of HcMSP in primary PCR
MSP-5-R2	CGCAGGAGACAGCCATAAGAGTG	23	Reverse primer specific for 5' end of HcMSP in second PCR
5' outer primer	CATGGCTACATGCTGACAGCCTA	23	Forward primer for 5' end of HcMSP in primary PCR (5' RACE kit)
5' inner primer	CGCGGATCCACAGCCTACTGATGA TCAGTCGATG	34	Forward primer for 5' end of HcMSP in second PCR (5' RACE kit)
MSP-ORF-F	CAAGAATTCATGTCTTCAGTTCCTC	25	Forward primer for amplification of HcMSP ORF, contained a EcoR I site (underlined)
MSP-ORF-R	CACAAGCTTTCAAGGATTGTATTCG	25	Reverse primer for amplification of HcMSP ORF, contained a Hind III site (underlined)
Qua-Tub-F	GAGCCGAGCTAGTTGATAACGTAC	24	To amplify 169 bp DNA fragments of the housekeeping gene (β-Tubulin) in quantitative PCR
Qua-Tub-R	GCCATAATTCTATCAGGGTACTCTTC	26	
Qua-MSP-F	TATGGCTGTCTCCTGCGATG	20	To amplify 166 bp DNA fragments of *HcMSP* gene in quantitative PCR
Qua-MSP-R	GTATTCGATGGGAAGGTTCTTTC	23	

A partial fragment of HcMSP of *H. contortus* (named as EST) was obtained by RT-PCR using a pair of gene specific primers (HcM-EST-F and HcM-EST-R, listed in Table 1) which targeted the conserved sequence of nematode MSPs. The EST of HcMSP was cloned into pM18-T vector (TaKaRa Biotech, Dalian, China) and sequenced by Invitrogen Biotech Co., Ltd. (Shanghai, China).

The 3' end of HcMSP cDNA was amplified by 3' full RACE kit (TaKaRa Biotech, Dalian, China) using the forward gene specific primers MSP-3-F1 and MSP-3-F2 (Table 1). The primary PCR system (final volume, 25 μL) contained 2 μL of 3'-RACE-Ready cDNA, 2.5 U LA Taq® polymerase (TaKaRa Biotech, Dalian, China), 40 μM 1×cDNA Dilution Buffer II, 50 μM 10×LA PCR Buffer II (Mg^{2+} Free), 3.0 mM $MgCl_2$ and 400 nM of each primer. The primary PCR was performed using MSP-3-F1 and 3'outer primer followed by treatment at 94°C (3 min), 30 cycles at 94°C (30 sec), 55°C (30 sec) and 72°C (1 min) and a final extension at 72°C for 10 min. The reaction product was used as a template in the second PCR with primers MSP-3-F2 and 3' inner primer. This second PCR system contained the same ingredients as the first except that it lacked the 40 μM 1×cDNA Dilution Buffer II and contained 400 μM dNTP mixtures. The parameters for the second PCR were the same as those in the first run. The HcMSP 3' end fragment was then obtained.

The 5' end of the cDNA was amplified by 5' RACE PCR using the same method as the 3' RACE PCR. The primary PCR was performed using MSP-5-R1 and 5' outer primer with MSP-5-R2 and 5' inner primer in the second PCR.

Both of the products of the second PCRs were cloned into the pMD18-T vector (TaKaRa Biotech, Dalian, China) and sequenced by Invitrogen Biotech Co., Ltd. (Shanghai, China). The complete sequence of the HcMSP cDNA was deduced from the overlapping sequences of both amplification products using BioEdit Version 7.0.1 (T.A. Hall, North Carolina State University, USA).

Sequence analysis: Sequence similarity was studied using the BLASTP and BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi). HcMSP protein sequences were aligned using CLUSTALW1.82. The signal peptide and protein motifs were predicted using approaches accessible on the Internet: SignalP (http://www.cbs.dtu.dk/services/SignalP/) and Motifscan (http://myhits.isb-sib.ch/cgi-bin/motif-scan), respectively. Phylogenetic analyses among MSPs were inferred by the Neighbor Joining (NJ) Method.

Expression and purification of recombinant HcMSP: The ORF of HcMSP was obtained by RT-PCR using the sequence specific primers MSP-ORF-F and MSP-ORF-R (Table 1). The PCR reactions (final volume 25 μL) contained 2 μL cDNA, 2.5 U Taq DNA polymerase (Takara Biotech, Dalian, P.R. China), 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 1×PCR Buffer (Mg^{2+} free) and 100 pmol of each primer. Cycling conditions were: 94°C for 3 min followed by 30 cycles of 94°C (30 sec), 55°C (30 sec), 72°C (45 sec) then a final extension at 72°C for 10 min.

The amplicon was cloned into the pMD18-T vector. Then, the recombinants were digested with EcoR I and Hind and the target gene was subcloned into the bacterial expression vector pET28a (+) (Novagen, USA) using the Standard Method. The recombinant plasmid pET28a (+)/HcMSP was transferred into competent *E. coli* (BL21 strain) and the recombinant protein was include

by addition of 0.8 mM Isopropyl Thiogalactoside (IPTG; Sigma-aldrich, USA) to the cell culture after the OD600 of the culture reached 0.5 at 37°C. The cells were incubated at 37°C for 5 h after the addition of IPTG. The cell lysates were prepared by sonication and analyzed by SDS-PAGE using 12% gels to confirm the distribution of the expressed recombinant protein.

To purify the recombinant protein induced *E. coli* cells were harvested by centrifugation and sonicated for 15 min on ice. After centrifugation at 12 000×g, the supernatant was added to a Ni^{2+}-Nitrilotriacetic Acid (Ni-NTA) column (GE Healthcare, USA) and purified according to the manufacturer's instructions. An elution buffer (300 mM NaCl, 40 mM NaH_2PO_4, pH 8.0) containing 400 mM of imidazole was used to wash the His-tagged proteins from the Ni-NTA column. Purity of the protein was detected by 12% SDS-PAGE and the concentration of purified protein was determined according to the Bradford procedure (Bradford, 1976) using Bovine Serum Albumin (BSA) as a standard. The protein was stored at -20°C for later use.

Generation of polyclonal antibodies: To generation polyclonal antibodies, about 0.3 mg of the purified HcMSP protein was mixed with Freund's complete adjuvant of a 1:1 mixture and injected into SD rats (Qualitied Certificate: SCXK2008-0004; Experimental Animal Center of Jiangsu, China) subcutaneously in multiple places as described (Yanming *et al*., 2007). After the first injection, rats were then boosted four times at 2 weeks intervals with the same dose. The serum, containing specific anti-HcMSP polyclonal antibodies was harvested 10 days following the last injection and then stored at -20°C.

Western blot analysis: Crude somatic extracts of pooled adult worms were separated by SDS-PAGE. Then, the protein was transferred to nitrocellulose membrane (Millipore, USA). After being blocked with 5% (w/v) skimmed milk powder in TBS-Tween-20 for 1 h, the membranes were incubated with the primary antibodies (rat anti-HcMSP serum) for 1 h at 37°C (dilutions 1:1000). After which Horseradish peroxidase (HRP)-conjugated goat anti-rat IgG was added. Finally, the immunoreaction was visualized using freshly prepared diaminobenzidine (DAB, Sigma) as a chromogenic substrate after 2-5 min.

Recombinant HcMSP was electrophoretically transferred from the gel to another nitrocellulose filter (Millipore, USA). After being blocked for 1 h, the membrane was incubated with the primary antibodies (goat sera, collected from goats infected with *H. contortus* naturally) for 1 h at 37°C (dilutions 1:100). Then, it was incubated with the second antibody (HRP-conjugated rabbit anti-goat IgG, Sigma Immuno-Chemicals) and visualized by adding DAB (Sigma) as a chromogenic substrate.

Differential mRNA transcriptions in two genders and three life stages: Quantitative reverse transcript PCR was used to quantify HcMSP expression levels in different genders and life stages of *H. contortus* (male, female, eggs and L3). Total RNA was extracted from male worms, female worms, eggs and L3. The $2^{-\Delta\Delta C_T}$ Method (Livak and Schmittgen, 2001) was used to quantify the expressions of HcMSP. β-tubulin was used as the housekeeping gene (Campbell *et al*., 2010). Three replications for each sample were employed in the study. The primer sets for the real time PCR were listed in Table 1. The assay was performed using SYBR Premix Ex Taq (Takara, Dalian, China) with the sense primer Qua-MSP-F and the antisense primer Qua-MSP-R which are specific for HcMSP. The endogenous reference (β-*tubulin* gene) was amplified using sense primer Qua-tub-F and anti-sense primer Qua-tub-R.

Individual reaction were performed at a final volume of 20 μL which was comprised of 10 μL of the SYBR Premix Ex Taq, 0.4 μL of sense and anti-sense primer, 0.4 μL of ROX Reference Dye, 6.8 μL of the ddH_2O and 2 μL of cDNA. The reactions were carried out at 7500 Real time system (ABI, USA). The reaction steps included an initial denaturation step at 95°C for 30 sec. Each cycle included incubation of samples at 95°C for 10 sec followed by annealing at 60°C for 10 sec and an extension at 72°C for 15 sec. The total number of cycles was 40. Melting curves were performed at the temperature range of 60-95°C.

To measure primer efficiencies, the total cDNA from male worms was 8 fold serially diluted. The average C_T was calculated for each and the ΔC_T determined. Plots of the log plasmid dilution versus ΔC_T were made. Only primer sets with similar efficiencies were accepted, determined by an $R^2 \geq 0.99$ and absolute value of the slope$\leq$0.1. Data was normalized to the reference gene β-tubulin using the $\Delta\Delta C_T$ Method. Fold changes in gene expression were calculated using the comparative C_T Method using the formula $2^{-\Delta\Delta C_T}$. Results were shown as means±SD.

RESULTS AND DISCUSSION

Cloning and sequence analysis of HcMSP: The *EST* gene of HcMSP was obtained by RT-PCR which was about 290 bp long (Fig. 1a). The product of 5'-RACE-PCR

generated a fragment of 274 bp (Fig. 1b). A 17 bp Un-Transcript Region (UTR) was found at 5'-end of this fragment. The 3' RACE-PCR product was 242 bp (Fig. 1c) and contained polyadenylation tail at position 30 bp downstream of stop codon TGA. The full length cDNA of HcMSP (439 bp) was obtained by splicing both 3'- and 5'-RACE fragments (Fig. 2). The 381 bp long ORF encode a protein of 126 amino acids with a molecular mass of 14300 Da (Fig. 2). The deduced theoretical pI of this protein was 7.86. No signal peptide was found in the deduced protein. According to the Motifscan database, a putative Motile-Sperm superfamily site located at position (8-113) was present in this protein (Fig. 3a).

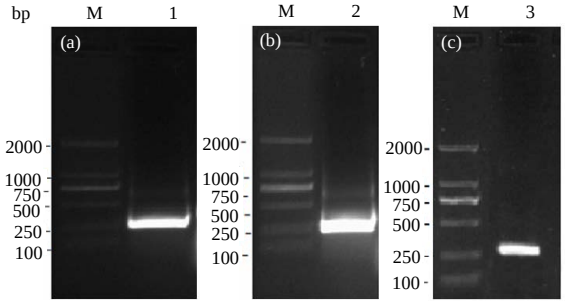

Fig. 1: a) Agarose gel electrophoresis of PCR products for HcMSP EST amplification; b) 5' RACE and c) 3' RACE: Lane M: DNA Marker; Lane 1: RT-PCR products of HcMSP EST (290 bp); Lane 2: PCR product in 5' RACE (274 bp); Lane 3: PCR product in 3' RACE (242 bp)

Alignment of the predicted amino acid sequence of *H. contortus* MSP with these from other nematodes showed that this protein had a significant similarity (60-94% identical at the amino acid level) with MSPs in nematodes. The similarity of this protein to MSP of *O. dentatum* was 94 and 60% to that of *Globodera rostochiensis*. Phylogenetic analysis of various MSP was performed by the Neighbor Joining (NJ) Method (Fig. 3b). It was found that HcMSP was phylogenetically close to the MSP from *O. dentatum* and *D. viviparous*.

Expression and purification of recombinant HcMSP: SDS-PAGE showed that the recombinant protein was

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                                                                gaaaaattcaagaagaa
1    ATGTCTTCAGTTCCTCCCGGCGATATCAACACTCAGCCCAATTCGAAGATCGTCTTCAAC
1     M  S  S  V  P  P  G  D  I  N  T  Q  P  N  S  K  I  V  F  N
61   GCCCCGTACGACGACAAGCACACCTACCACATCAAGATTATCAACGCCTCTGGCCGACGT
21    A  P  Y  D  D  K  H  T  Y  H  I  K  I  I  N  A  S  G  R  R
121  ATCGGATGGCCCATCAAGACCACCAACATGAGGAGGCTTGGTGTCGACCCTGCCTGTGGA
41    I  G  W  A  I  K  T  T  N  M  R  R  L  G  V  D  P  A  C  G
181  GTGTTAGACCCTAAGGAAGCCACTCTTATGGCTGTCTCCTGCGATGTGTTCGACTATGGA
61    V  L  D  P  K  E  A  T  L  M  A  V  S  C  D  V  F  D  Y  G
241  CGTGAGGACACCAACAACGATCGAATCACCGTCGAATGGTGCAACACTCCTGAAGGAGCC
81    R  E  D  T  N  N  D  R  I  T  V  E  W  C  N  T  P  E  G  A
301  GCCAAGCAGTTCCGACGAGAATGGTTCCAAGGAGATGGTATGGTCCGCCGAAAGAACCTT
101   A  K  Q  F  R  R  E  W  F  Q  G  D  G  M  V  R  R  K  N  L
361  CCCATCGAATACAATCCTTGAagacctgtgtgttcaataaattagttatgtaaaaaaaaaa
121   P  I  E  Y  N  P  *
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Fig. 2: Nucleotide sequence of the HcMSP cDNA and its deduced amino acid. The coding region (ORF) was shown in capital letter, starts at nt 1 and ends at nt 381. The ATG start codon and the TAA stop codon were bold. The 5' and 3' UTR were showed in small letters. The polyadenylation at the 3' end was underlined

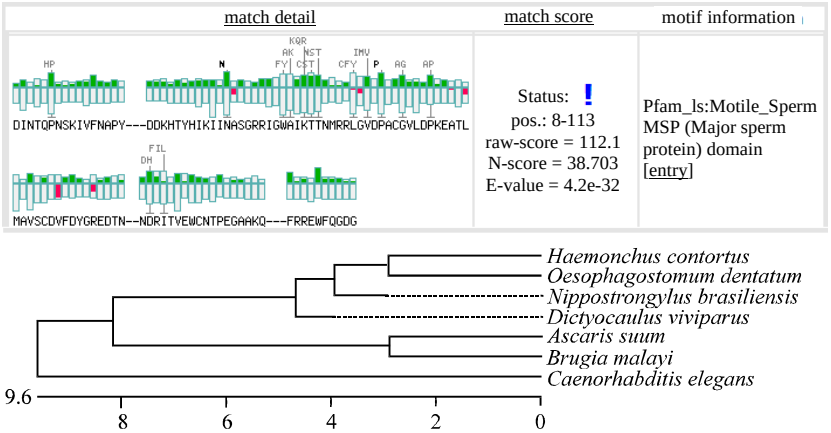

Fig. 3: a) Motifscan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) results showed that HcMSP belonged to the Motile-Sperm superfamily; b) Phylogenic trees inferred from homologies of MSP amino acid sequences utilizing the NJ Method. The scale bar represents one amino acid substitution/100 amino acid residues. The MSPs are represented by the name of their species. The database accession numbers are listed as *Ascaris suum* (P27439.3), *Brugia malayi* (XP_001894184.1), *Caenorhabditis elegans* (AAA28115.1), *Dictyocaulus viviparous* (ABJ97284.1), *Nippostrongyus brasiliensis* (BAI81973.1) and *Oesophagostomum dentatum* (CAF29502.1). NJ analysis was carried out using the program CLUSTAL W Version 1.8

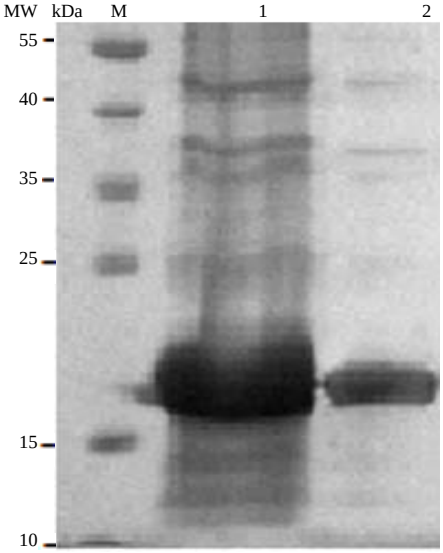

Fig. 4: Expression and purification of recombinant HcMSP. Proteins were resolved by SDS-PAGE on 12% of polyacrylamide gel and stained with Coomassie brilliant blue R250. Lane M: Molecular standards; Lanes 1: Lysate of *E. coli* (Transformed by pET28a (+)/HcMSP) after induced by IPTG for 5 h; Lane 2: Purified recombinant HcMSP fraction (showed with an arrowhead) after Ni-NTA affinity chromatography

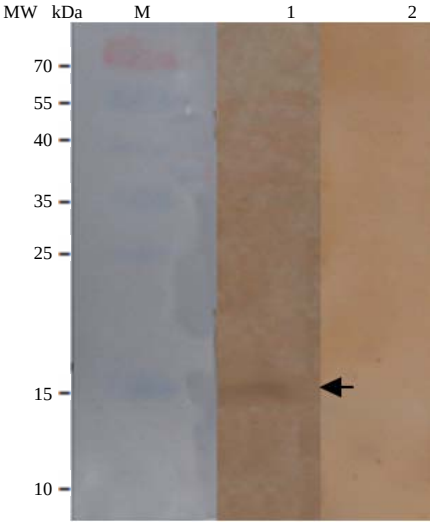

Fig. 5: Western blot analysis. The native HcMSP from adult worms could be recognized by rat anti-rHcMSP serum (Lane 1 showed with an arrowhead). While the recombinant HcMSP could not react with the sera from goat infected with *H. contortus* naturally (Lane 2). Lane M: Standard protein molecular weight marker

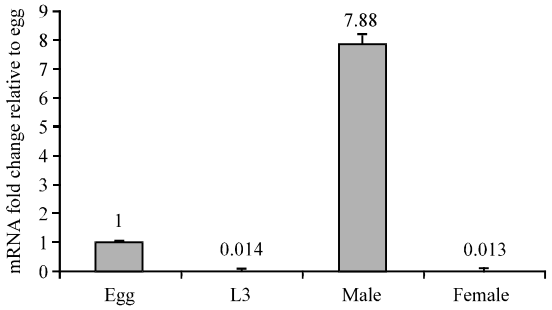

Fig. 6: Stage and gender differential expressions of HcMSP mRNA in *H. contortus*. Fold changes in gene expression relative to egg (= 1) were calculated using the comparative C_t Method using the formula $2^{-\Delta\Delta C_T}$. Bars show the mean and standard deviation of triplicates

found in the sonicated bacterial supernatant. After purification from the supernatant by chromatography on the Ni-NTA, the protein was seen as a single band with the molecular mass of 18 kDa on the SDS-PAGE gel (Fig. 4). Because of the 3.7 kDa fused protein in the vector, the recombinant protein's molecular weight was more than the value of 14300 Da calculated based on the deduced amino acid sequence.

Western blot analysis: Western blot analysis showed that rat anti-HcMSP serum bound to a band at about 14 kDa in the somatic extract of *H. contortus* adult worms (Fig. 5, lane 1). However, recombinant HcMSP could not be recognized by sera from goat naturally infected with *H. contortus* (Fig. 5, lane 2). This may indicate that HcMSP was not excreted or secreted by the parasite during the infection.

Differential mRNA transcriptions: Quantitative RT-PCR was used to examine the mRNA expressions of HcMSP in different genders and life stages of *H. contortus*. The relative mRNA of HcMSP in adult male worms, females, L3s and eggs were 7.88, 0.013, 0.014 and 1, respectively. The result indicated that the *HcMSP* gene transcription in male adults was 600 time higher than that of females. It seems that this gene was gender (male) specific (Fig. 6).

Most research of nematode MSP was focused on *A. suum* and *C. elegans* (Ma *et al*., 2012). In this study, researchers reported the cloning, expression and characterization of the homology gene from parasitic nematode *H. contortus* for the first time. Results of multiple sequence alignment and MSP domain analysis indicated that *HcMSP* gene belongs to the family of major sperm proteins.

Spliced Leader (SL) sequence was commonly seen in nematode genes this special sequence tends to be spliced very close to the translation start site. It plays major roles in the formation of the optimal length of 5' UTR in mature mRNA and in the formation of nucleotide constructs that benefit the translation of genes (Stover and Steele, 2001; Williams *et al*., 1999). However, no SL sequence was found in the mRNA of HcMSP. And the same phenomenon was found in *msp* gene of other nematodes (Blaxter and Liu, 1996). This may indicated that MSP mRNA use another mechanism to form mature mRNA. In the present research, the HcMSP was devoid of a typical signal peptide cleavage site at its N-terminal end indicating that HcMSP is an intracellular protein or structural protein. This was consistent with the earlier reports where MSP was functioned as intracellular cytoskeletal proteins (Roberts *et al*., 1998; Haaf *et al*., 1996).

The results of the quantitative RT-PCR showed that the HcMSP transcription in adult male worms was much higher (600 times) than that in female adults. This may indicated this gene was gender specific. The similar result was found in *D. viviparous* (Laabs *et al*., 2012). The earlier study also supported this observation. In that study, the whole proteins of *H. contortus* from adult male and females were separated by 2D gel electrophoresis and the differential expressed proteins were identified by Matrix-Assisted Laser Desorption/Ionisation (MALDI)-Time of Flight (TOF) Mass Spectrometry (MS) or MALDI-TOF-TOF MS. A protein homology with *C. elegans* MSP-49 was found in the males but not in the females. In this research, the HcMSP transcriptions in different life stages were also compared. Results indicated that this gene was over expressed in adult males, moderate in eggs while rarely in L3.

In *C. elegans*, sperm secrete MSP to induce oocyte maturation and ovarian sheath contraction which together facilitate fertiliazation (Tarr and Scott, 2004; Pall *et al*., 2004; Yang *et al*., 2010). This may indicated that MSP could act as a hormone and play important roles in the egg productions. Further research on the functions of MSP of *H. contortus* should be done in the future and that may be helpful to provide new method for reducing the generation of eggs.

CONCLUSION

Results showed that the ORF of HcMSP was 381 bp long and encoded a putative protein with 126 amino acid residues. Homology analyzing showed that HcMSP protein was highly similar (94%) with that of *Oesophagostomum dentatum*. The native protein extracted from *H. contortus* adults could be detected by the serum from rats immunized with the recombinant HcMSP. The expression profiles of HcMSP mRNA in two genders and three different life stages of this parasite were quantitated by real time PCR. It was found that *HcMSP* gene was over expressed in adult males while that in adult female was 1/600 times of the male worms.

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