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Detection of cysteine proteinase gene *hmcp*5 of *Haemonchus contortus* by reverse transcription-polymerase chain reaction

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Abstract
Barber's pole worm cysteine proteinases are considered as the important candidates in the immunological control of haemonchosis in sheep and goats. The control of *Haemonchus contortus* is largely based on pasture management and the antihelmintics utilization. Due to indiscriminating use of drugs, occurrence of resistant to anthelmintics and it is need for the development of alternative control methods i.e. vaccine development. Cysteine proteases are considered as the excellent vaccine candidates due to their role in maintenance of worm nutrition as well as immune evasion. Cysteine proteases have been implicated for development of the vaccination because of their affinity purified detergent soluble extracts of *Haemonchus contortus*. The aim of the present study was to standardize the reverse transcription-polymerase chain reaction of Cysteine Proteinase gene hmcp5 of *Haemonchus contortus*. The characterization of cysteine protease genes in South Indian isolates may give ideas for control of *Haemonchus contortus* in future. It is essential for the further research protocols in development of the diagnostic or vaccination modalities.

Keywords: *Haemonchus contortus*, RT- PCR, Cysteine protease, *hmcp*5 gene

Introduction
Haemonchus contortus cysteine proteases are considered important candidates for control of haemonchosis in sheep. Very limited information is available regarding the immune protective properties of the molecules in small ruminants (Bakker *et al.*, 2004) [1]. *Haemonchus contortus* is one of the pathogenic nematode in small ruminants throughout the world including in India. During the course of infection, it causes development of the anaemia, weakness, progressive loss of weight and stunted growth in young animals. Prevalence of different gastro intestinal parasites was recorded and reported the antihelmithic resistance in the YSR Kadapa district of Andhra Pradesh in small ruminants (Sivajothi and Reddy, 2017) [8]. Estimates of the average blood loss per worm per day range from approximately 0.003 to 0.07 ml of blood/day and the cost of anthelmintic treatment against *H. Contortus* alone in India has been estimated to be $103 million per annum (McLeod, 2004) [4]. There is an alarming condition for development of the alternative strategies for control of infection (Wolstenholme *et al*. 2004) [15]. Many efforts have been directed towards the development of vaccines against *Haemonchus* spp. and panel of promising vaccine candidates have been identified (Smith 1999) [13] without expected degree of success till today. Significant among these antigens are H11, cysteine proteases (Sajid and James, 2002) [6] and H-gal-GP (Smith *et al*. 1997) [12]. Cysteine proteases are vaccine candidate molecules in *H. contortus* which had the capability to digest nutrients, haemoglobin, fibrinogen, collagen and immunoglobulin G (Knox *et al*., 2005) [2]. Therefore, it is in great need to know the presence of cysteine protease genes and its characterization in Indian isolates, which may give ideas for control of *H. Contortus* in future.

Materials and Methods
Collection of *Haemonchus contortus* adult worms
Gastro intestinal tract of sheep and goats were collected from local abattoirs of Proddatur, Y.S.R Kadapa District, Tirupati, Hyderabad and Chennai. The contents of abomasum were thoroughly examined for presence of parasites and subsequently worms were collected by hand picking method (Figure -1). The parasites were washed repeatedly with physiological saline to remove the debris. Then the male and female worms were separated.

Male and female worms were identified as per morphology described by Soulsby (1982) [14]. Finally, the collected worms were washed separately in 0.1 M phosphate buffered saline (PBS pH 7.2).

Adult worms were homogenized in 1XPBS, centrifuged at 10 000 *g* at 4 °C and the supernatant was collected, filtered (0.22 µm) and stored at -20 ℃ for subsequent analyses. A spectrophotometric assay was carried out to determine the protein concentrations of the homogenates.

RNA isolation from *Haemonchus contortus*

Adult male *H. contortus* worms were used for RNA isolation on the day of collection. By using TRIZOL reagent, total RNA was extracted directly from the male *H. contortus* worms. Thirty number of adult male parasites was transferred to sterile RNase free 2 ml microfuge tube and centrifuged at 13,200 rpm for 10 min. After discarding the supernatant material, remaining pellet was dissolved in 750 µl of the Trizol reagent by vortex for 1 minute and incubated for 10 min at room temperature. Two fifty micro liters of the chloroform was added to the lysates followed by vortex for 1 min, kept at room temperature for 10 min and centrifuged at 13,200 rpm for 15 min. The clear aqueous phase was collected in to the fresh RNase free microfuge tube and equal volume of isopropanol was added, mixed thoroughly by inverting the tubes and kept at room temperature for 30 min. The nucleic acid was pelleted down by centrifugation at 13,200 rpm for 20 min and washed with 70% ethanol by centrifugation at 13,200 rpm for 5 min. The pellet was air dried and dissolved in 30 µl of the RNase free water and stored at -80ºC until use (Sivajothi *et al.*, 2018) [9].

Eluted RNA of *Haemonchus contortus* was tested for its concentration and purity with Nanodrop® and it was considered to be sufficient purity with the ratio of 2.0 by assessing the ratio of optical density value at 260 nm and 280 nm. The eluted RNA samples were stored at -80ºC for further use.

Synthesis of complementary DNA

Complementary DNA (cDNA) was synthesized using 3.0 µl of total RNA from male *Haemonchus 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 stored in -80°C till further use (Sivajothi *et al.*, 2019) [11].

Table 1: Reaction mixture

5X Prime Script Buffer	:	4.0 µl
10mM dNTPs Mix (1mM final conc.)	:	2.0 µl (1mM final conc.)
100 µM Oligo $(dT)_{18}$ Primer	:	1.0 µl (0.5 µg)
RNase Inhibitor (40U/ µl)	:	0.5 µl (20U)
Prime Script RT ase (200U/ µl)	:	1.0 µl (200U)
Total RNA (template)	:	3.0 µl
DEPC treated water	:	8.5 µl
Total volume	:	20.0 µl

Amplification of *hmcp5* gene of *H. contortus* by RT-PCR

The forward and reverse primers were designed based on published hmcp5 nucleotide sequences of *H. contortus* from NCBI using Primer-Blast programme http://www.ncbi.nlm.nih.gov/tools/primer-blast/)

Hmcp5-F: 5'-GGC CAT GGC GGG TTC TGG TGC CAC AG -3'

Hmcp5-R: 5'-GGA AGC TTT CAT GCG GAA ATA ACC TTC-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 25 µl EmeraldAmp Max Master mix, 20 pmol of forward and reverse primers and cDNA. The reactions were performed with the following cycling conditions; For amplification of hmcp5 gene, initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 63°C for 30 sec, elongation at 72°C for 1 min and final extension at 72°C for 10 min was followed. Obtained amplified end substances were run in agarose gel (1.5 %) and it was confirmed based on their size in the gel under UV gel-documentation system.

Table 2: The PCR conditions were as follows (Figure-3).

Step No.	Temperature	Time	No. of cycles	Remarks
1.	95° C	5 min	1	Initial denaturation
2.	95°C	1 min	35	Denaturation
	63°C	30 sec		Annealing
	72°C	1 min		Extension
3.	72°C	10 min	1	Final extension
4.	4°C	Soak	-	Hold

Results and Discussion

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 (Figure -2). Concentration of total RNA and the purity range was estimated in between 100. 50 - 120.60 n/µl and 1.8 - 2.0, respectively. The *Haemonchus contortus* hmcp5 gene (912 bp in length nucleotide length) was amplified with earlier amplified cDNA from RT. The PCR method standardized was found suitable for specific amplification of *H. contortus* cDNA. This amplified PCR product was checked in agarose gel and a single band of 912 bp was observed and compared against 1kb ladder (Figure-4). Concentration of the purified PCR amplicon of hmcp5 gene of *H. contortus* was 40 ng/µl.

Diagnosis of the *Haemonchus contortus* was done by demonstration of parasitic ova in faecal samples which is always confusing with other nematodes. Recent studies were carried out to record the prevalence of *Haemonchus contortus* by the antibody titers against the whole worm antigens. It is recommended the development of the specific diagnostic tool at field level which is unique for *Haemonchus contortus* parasites (Sivajothi and Reddy, 2018) [10]. It can be achieved by demonstration of the hmcp5 gene in *H. contortus* organisms. The most promising vaccine and drug targets identified in *H. contortus* till date are cysteine proteases (Knox *et al.*, 2005) [2]. The difficulty for the development of universally protective vaccine against *H. contortus* using cysteine proteases is the strain differences.

Cysteine proteases of different helminths, belongs to the

cathepsin B like proteases and it is papain related enzymes further divided based on the amino acid sequence and affinity for synthetic substrates (Newton and Meeusen 2003) [5]. Enzymatic activity of the HCP (Helminth Cysteine Proteases) play important roles on helminthic parasitic host invasion, cuticle molting and blood digestion. It is recommended that making HCPs a potential target for new immunotherapeutic and immunodiagnostics strategies (Shompole and Jasmer, 2001) [7].

The present study identified the cysteine protease *hmcp*5 gene in male *H. contortus.* Presently we do not have any information on cysteine protease genes other than this, if any, need to be investigated in the South Indian isolates or strains. Molecular characterization, expression and functional studies of different cysteine protease genes in *Haemonchus contortus* are required for future studies.

Fig 1: Abomasum showing the adult *H. contortus*

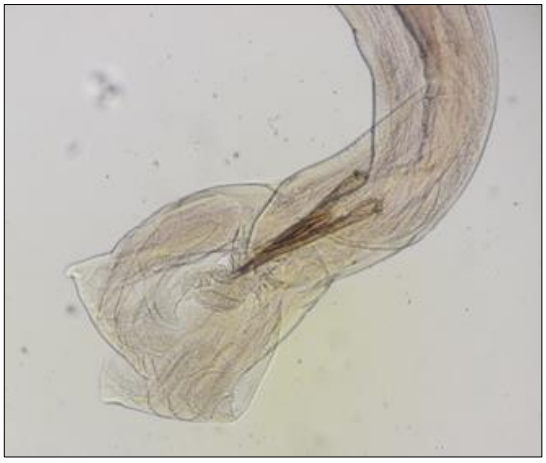

Fig 2: *H. contortus* male bursa with 'Y' shaped dorsal ray

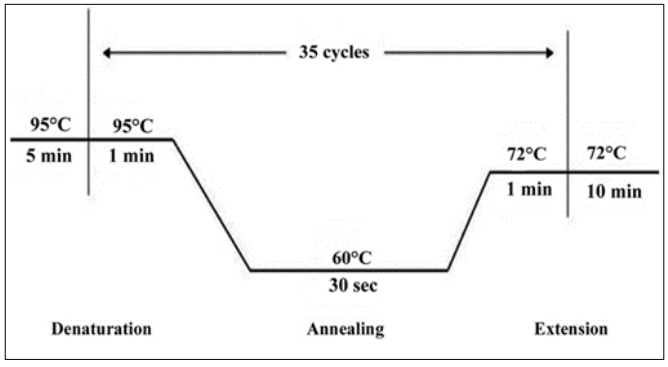

Fig 3: Cycling conditions for standardization of RT-PCR for amplification of *hmcp*-5 gene of *H. contortus*

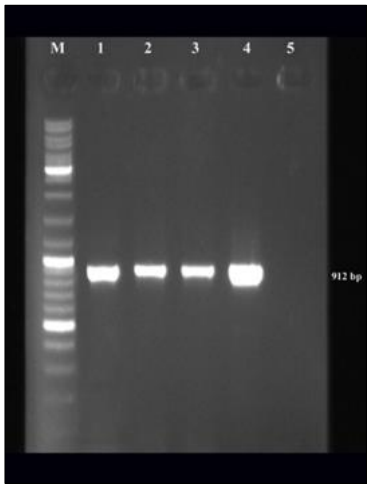

Fig 4: PCR amplification of *hmcp*-5 gene of *H. contortus* showing 912 bp amplicon
Lane M: 1kb DNA ladder
Lane 1 and 4: PCR amplification of *hmcp*-5 gene
Lanes 5: Negative control

Conclusion

In *Haemonchus contortus* cysteine proteinases are considered as important candidates in the immunity. In the present study, standardize the reverse transcription-polymerase chain reaction of cysteine proteinase gene *hmcp*5 of *Haemonchus contortus.*

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