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Cloning and Expression of a 15 kDa Excretory-Secretory Protein of the nematode *Haemonchus contortus* Bareilly isolate

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Abstract
Haemonchus contortus is considered as a major pathogenic nematode of small ruminants, due to its blood feeding habit causing severe anemia and death. The infestation is controlled by using anthelmentics traditionally, but the emergengence of resistant strains and restrictions for drug residues for export purposes pose a major challenge now a days. Alternate control strategies like biological control, pasture management and vaccines are being employed to overcome these limitations. Excretory–secretory proteins are considered to be effective targets for vaccine development, since most of them are recognized by host immune systems. The current study describes the characterization and expression of a 15 kDa ESP in a heterologus system for its possible use as vaccine canditate. The gene of interest was amplified from cDNA template by PCR. The amplicon was cloned into an *E.coli* expression vector. The protein was expressed and purified to homogeneity by column chromatagraphy. But it showed less reactivity to antibodies against infected animals serum.

Keywords: Excretory-secretory protein (ESP), *Haemonchus contortus*, Nematode, Vaccine canditate

Introduction
The small ruminant livestock industry suffers huge economic loseses in terms of production due to various gastro intestinal nematodes [1]. *Haemonchus contortus* is a gastrointestinal nematode parasite which inhabits the abomasum of sheep and goats, causing severe anemia due to its blood feeding nature. It causes nutritional disturbances by altering digestive process and decreased absorption [2]. In order to survive inside the host, the parasite produces molecules collectively referred as excretory/secretory products (ES products) [3]. ES molecules perform diverse functions such as host tissue penetration, aid in digestion, modulation of the host immune response etc. [4, 5]. Although the composition of ESP is largely unknown, it is a source of potential vaccine components because ESPs are able to induce up to 90% protection in sheep. Thus, targeting ESP molecules is an attractive proposal as a control measure and also it could be used for diagnostic purposes. Many low molecular weight antigens were reported from the ESPs of *H.contortus*, but detailed studies on them is limited. A 15 kDa protein was identified in the E/S products of adult *H. contortus* that appears to be a natural antigen because of the presence of antibodies against the protein in the infected animals [6].In this context, this study was designed to clone and express the 15 kDa protein from an Indian isolate of *H.contortus* from Bareilly, U.P in a heterologus host system, since the yield of proteins from parasites will be too low. The recombinant forms of the protein could be used for vaccination studies or use in diagnostic tests.

Materials and Methods
Mo-MLV reverse transcriptase, RNase inhibitor were from Promega, USA. Restriction enzymes *Pfu* polymerase and T4 DNA ligase were from Fermentas Life Sciences, Lithuvania. PrepEase gel extraction kit was purchased from Affymetrix, Inc. USA. Primers were synthesized by Merck, India. pProEXHT™ prokaryotic expression system was from Life technologies, California, USA. All other reagents were of analytical grade.

RNA isolation and cDNA synthesis
Adult *H.contortus* worms were collected aseptically from the abomasum of goats from a local

abattoir at Izatnagar, Bareilly, U.P. Total RNA was extracted from the adult worms using TriZol reagent duly following the manufacturer's instructions. The RNA pellet after extraction was dissolved in DEPC treated water. The RNA preparation which gave A260/280 =1.8 -1.9 was used for cDNA synthesis.

First strand of cDNA was synthesized using Mo-MLV Reverse transcriptase. The reaction mixture consisted of RNA(3µg/µl)- 1µl, Oligo dT primers- 1µl, 5X RT buffer- 5µl, dNTP mix (10 mM each) - 5µl, RNASin- 1µl, Mo-MLV RT enzyme (200u /µl) - 1µl, DEPC treated water-13 µl.

Polymerase chain reaction

The PCR reaction was carried out in a 0.5 ml PCR tube, using the following reagents.

cDNA template (25 ng/µl) - 1.0 µl, dNTPs (2.5 mM each) - 1.0 µl, Forward primer (5pmoles) - 1.0 µl, Reverse primer (5pmoles) - 1.0 µl, 10X PFU buffer-2.5 µl, *Pfu* DNA Polymerase (2.5U/µl) - 1.0 µl and nuclease free water up to 25 µl.

Primers were self-designed and had sites for *Eco*RI and *Hind* III restriction enzymes to facilitate cloning and had the sequence as given below.

1. P15 forward: 5' CTCG *GAA TTC* GGT AAT CAA GTA ATG TTC G 3 ' (29bp)
2. P15 reverse: 5' TGA ATT GCA TTG GTC TCA GTT *AAG CTT* GGG 3' (30bp)

The following conditions were standardised for amplification: Initial denaturation: 94ºC for 5 mins, denaturation: 94ºC for 1min, annealing: 53ºC for 30 secs and elongation: 72 ºC for 1min. Steps from denaturation to elongation were repeated 29 times and then final elongation was done at 72 ºC for 10 min. The amplified product was visualized by agarose gel electrophoresis on 0.8% agarose gel using 1Kb DNA ladder as marker. Electrophoresis was performed at 90 Volts for 1 hour. The PCR products were sent for sequencing to Bio Serve technologies, Hyderabad for sequencing.

Cloning of P15 gene into the expression vector

The PCR amplified fragment & expression vector were double digested with *Eco* RI and *Hind* III and then gel purified with PrepEase TM gel extraction kit. The double digested PCR product was ligated to the vector and the ligation mixture was used to transform DH5α competent cells. The cells were incubated at 37ºC overnight. In order to identify the positive clones, colonies were picked up randomly and were inoculated in 5 ml LB broth containing 100 mg/ml ampicillin and grown overnight at 37ºC with constant shaking at 140 rpm. Plasmids were isolated following the standard protocol [7] and then digested with restriction enzymes to check for the insert release. Colonies with the insert were considered as recombinant.

Expression of recombinant P15 protein

The selected positive clones were checked for protein expression. The recombinant clones were grown in LB medium in the presence of ampicillin and incubated at 37°C overnight. This was subcultured at 1/100 dilution in LB broth with ampicillin till $O.D_{600}$ reached 0.6. About one ml of the culture was saved in a microfuge tube and labeled as uninduced. The remaining culture was induced with IPTG (1mM final concentration). Both uninduced and the induced cultures were kept at 37°C with vigorous shaking for 3hrs.The bacteria were pelleted by centrifugation and stored at -20°C. To one ml of bacterial pellet (both uninduced and induced), 20µl of normal saline and 30µl 5X sample buffer was added. This was kept at room temperature for 15 minutes and then analyzed on a 5-15% linear gradient SDS-polyacrylamide gel for protein expression.

Purification of recombinant protein

One of the clones expressing the protein was bulk cultured and the bacterial pellet was stored at -40 °C. The pellet was thawed and resuspended in 4 volumes of the lysis buffer (20mM of sodium phosphate (pH 7.4), 1 M NaCl and 1mg/ml Lysozyme), mixed and kept in ice for 30min. It was then sonicated at 10 Hz for 5sec, the process repeated twice with 1 min interval. The sonicated bacterial pellet was centrifuged at 12,000 rpm for 15 min at 4 ºC. The supernatant was then passed through a nickel-agarose column.

The column was washed with wash buffer (20 mM sodium phosphate (pH 7.4), 0.1M Tris Cl
(pH 7.4)). The bound proteins were eluted with stepwise increasing gradients of imidazole (2.5, 5, 7.5, 10 and 20 mM) prepared in wash buffer and fractions of 2 ml were collected.

Western blot analysis of the recombinant protein

The reactivity of the recombinant protein with the serum obtained from infected animals was checked by Western blot [8]. About 1-2 µg of the purified recombinant protein was electrophoresed on a SDS-gel and subsequently transferred to a nitrocellulose membrane (NCP) at 150 V constant current for 2 hr.The free sites on the NCP were blocked by incubating in 4% skimmed milk at 4°C overnight. The paper was washed with PBS-Tween and incubated at room temperature (25°C) with primary antibody (1:250 dilutions in PBST) for 3 hrs. The paper was then washed several times with PBST and incubated with secondary antibody conjugated to peroxidase (1:500 dilutions in PBST) for 90 min. After several washings, the peroxidase activity was measured by adding diaminobenzidine.

Data Analysis

The sequenced results were compared with other sequences available at GenBank database of NCBI using BLAST software programme (www.ncbi.nlm.nih.gov/blast).Signal peptide analysis was done using MitoProt and Signal P tools of the ExPASy website (http://expasy.org/tools/#ptm).

Results and Discussion

A specific amplification of the gene of interest by PCR was observed with an expected product size of approximately 455bp in the agarose gel electrophoresis. (Fig:1).The amplicon was sequenced and submitted to the Genbank of NCBI under accession no: JF826244.1 (Fig:2).It also matched with some of the already existing sequences from *H.contortus*, *Trichostrongylus spp.* but the putative identity remains unknown, which needs further experimentation.

The gene was succesfully cloned into the expression vector. The selected clones showed a good level of expression post induction especially after 3 hours (Fig:3).The protein was expressed in a soluble form and could be purified by Ni-agarose chromatography. The protein got eluted at 5mM and 10 mM imidazole concentrations. Presence of dimers and trimers was also evidenced at SDS –PAGE gel around 30 and 66 kDa regions (Fig:4) which was also obeserved in a similar study [9]. About 3-5mg of protein could be recovered from a

litre of the bacterial culture. But the recombinant protein did not react much with the serum obtained from infected animals (not shown).Hyperimmune sera obtained from naturally infected sheep reacted with the native 15 kDa E/S product differentially and strongly, suggesting a possible role in the infectious process [10]. The possible reasons for less reactivity in the present study could be due to variations in the expression levels under different enviroinmental conditions, folding defects of the protein produced from a prokaryotic expression system, batch variations, etc as reported earlier too [11]. Southern blotting studies indicated that the gene copies for 15 kDa protein were limited [9]. A recent study indicated that the expression of a 15 kDa protein was downregulated among many proteins which were differentially expressed after treatment with norepinephrine [12]. It was hypothesized that norepinephrine treatment induced oxidative stress, which was indicated by a upregulation in production of proteins like PDI. The above mentioned studies support our observation that some differences exist in the reactivity of native and recombinant proteins to the infected animal's serum and thus the varied response of this protein in western blot. Though there were some reports on the presence of a reactive spot in 15 kDa region in 2D PAGE [3, 13], The identity of the protein is yet to be established, to understand its biochemical nature for use as target canditate for vaccine and therapuetics. So expression in an eukaryotic system and raising of specific antisera against the protein may be prove helpful.

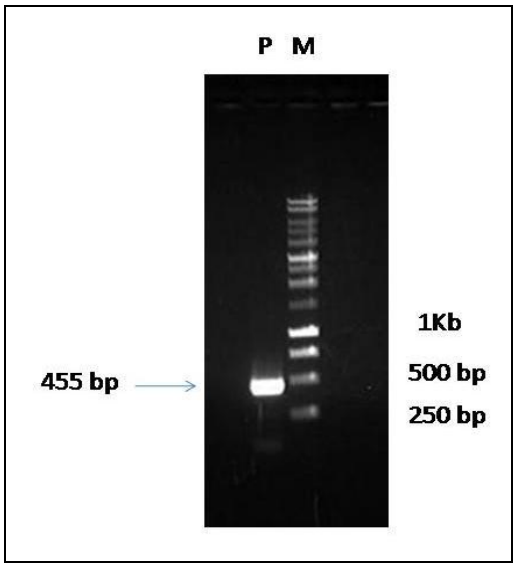

Fig 1: PCR amplification of P15 gene

Lane P- PCR product approx 455bp
Lane M – 1 Kb DNA ladder

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01 ATGTTCTTCG CTTTTGCAGT GCTACTCATC GCTCTAGCAA
41 CTCGTGAGGC TTATGGCGAG TCACAGCTCA ACACAGAATT
81 CATACTTGGC TCGGGTAATC AAGTAATGTT CGAGAACATC
121 AATAAGGAAT ACAACACACA TCTGAAGTGG GATGACGACT
161 TGGCGGCTAA GGCAATGGTG GAAGCTGTTA GGCCACACTA
201 TCGATTATTA TGGAACACCG GCGACTATTT CACGATAAGA
241 AACGACAAGC TCTTCACGAA GAGGTACGTA GGACCTCTGG
281 AGGAGAAAGT GCGCCTGGTT CTGCTGAACC CCTTCAAAAA
321 ATATGCAGAT AAACTTCGCC AACTTCCCGA AGGAACAACC
361 TACGGATGTA ATGGATTTTT CGACACTGAC ACGATGCCAA
401 ACGACAACTA CCTTTATGTG GCCGGTGTCT ACAATATCCC
441 CAACTGAGAC CAATG
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Fig 2: Nucleotide sequence of P15 gene

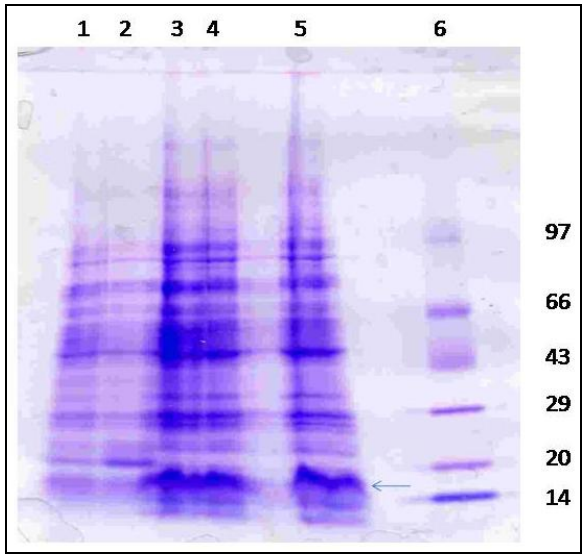

Fig 3: Induction of P15 protein- SDS PAGE

Lane 1 & 2- uninduced cultures
Lane 3 – 1 hour post induction
Lane 4 – 2 hour post induction
Lane 5 – 3 hour post induction
Lane 6 – Medium range protein molecular weight markers

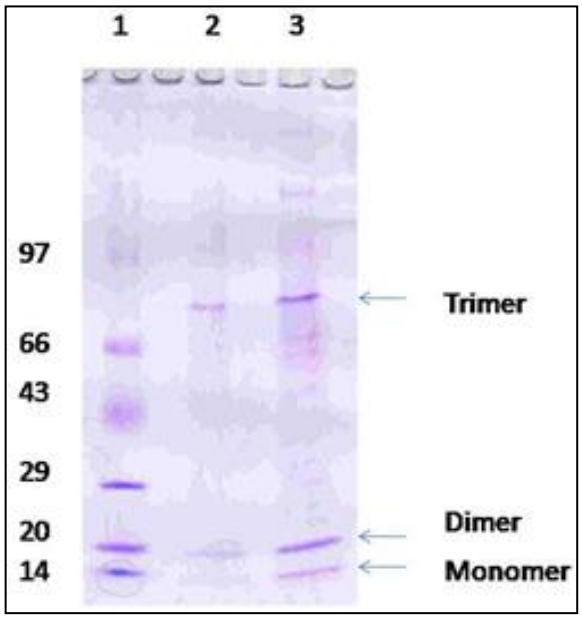

Fig 4: Purification of P15 protein- SDS PAGE

Lane 1 - Medium range protein molecular weight markers
Lane 2- 5mM imidazole eluted fraction
Lane 3 – 10mM imidazole eluted fraction

Conclusion

The p15 gene from bareilly isolate of *H.contortus* was succesfully cloned and expressed in a prokaryotic expression sytem in a soluble form. The recombinant protein could be used for further identifcation and biochemical characterisation, which could prove its therapuetic and vaccine potential in future.

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