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Research Article

MOLECULAR CHARACTERIZATION PROPEPTIDE DOMAIN OF MMP-9 GENE IN *GASTROTHYLAX CRUMENIFER* THROUGH REVERSE TRANSCRIPTION- POLYMERASE CHAIN REACTION(RT-PCR)

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ABSTRACT
Collection of live, adult *Gastrothylax crumenifer* flukes was made from local abattoirs and was washed thoroughly with phosphate buffer saline. Total RNA was isolated from adult flukes using standard protocol. Reverse Transcription Polymerase Chain Reaction was carried out to detect the presence of catalytic domain of MMP-9 gene in *G.crumenifer*. By RT-PCR analysis, the propertied domain of MMP-9 was amplified and the presence of propertied was confirmed as 204 bp product. Hence this is the first report of confirmation of the presence of cDNA specific for MMP-9 gene in *Gastrothylax crumenifer* infection.

Keywords: *Gastrothylax Crumenifer, MMP-9 Gene, RT-PCR, Buffalo*

INTRODUCTION
Host invasion and tissue migration of several helminths have been linked to the expression and release of parasite-derived proteases. The synthesis of proteolytic enzymes and their release as excretory and secretory (ES) products have been reported in various parasitic helminths.
In nematodes, MMPs are the proteases which are thought to play an important and essential role in these migratory and invasive phenomena (Kerrow *et al.*, 1990).
According to their substrate specificity MMPs can be categorized as collagenases, gelatinases, elastases, Stromelysins and membrane-type MMPs. Nematode MMPs generally include collagenases, gelatinases and elastases (Healer *et al.*, 1991).
Several authors provided information related with the multiple enzyme activities of MMPs with various molecular weights in different helminthiasis and MMP mediated histolysis of skin and intestinal walls through substrate impregnated zymographic analysis of extracts and ES products of different nematode parasites and degradation of ECM proteins (Tort *et al.*, 1999).
Most of these works assessed MMPs activity from excretory/secretory (ES) products as well as the extracts of parasitic nematodes (Lai *et al.*, 2005). But the work on MMPs in parasitic amphistomes is very limited.
Hence, the present study was undertaken to characterize the MMP-9 gene in *Gastrothylax crumenifer* by Reverse Transcription Polymerase Chain Reaction (RT-PCR).

MATERIALS AND METHODS
Mature *Gastrothylax crumenifer* were collected from the rumen of Indian water buffaloes (*Bubalus bubalis)*, slaughtered at the local abattoirs of Pattukkottai and Thanjavur.
Worms were thoroughly washed in Phosphate Buffer Saline without glucose, pH 7.4 and pre maintained at 37ºC. After careful preservation in PBS, the worms were immediately transferred to the laboratory for further processing (Saifullah *et al.*, 2011).
Total RNA was isolated from the *Gastrothylax crumenifer* as per the protocol given in the QIAGEN RN easy Mini Handbook. Primers targeting propertied domain of the MMP-9 was designed through the computer software "DNASTAR".

The primers were supplied as desalted oligonucleotides by Imperial Biomed (USA).
The details of the primers used were as follows:

Target	**Primer sequence**	**Product**
Propeptide domain	Forward primer 5'-CGGCGGATCCCCCAGACCACACAAGCCCACC-3'	204 bp
	Reverse primer 5'- CGGCGTCGACTCTAGATTTGTCCAGCTCTCCAGTCTCAG-3'	

RE sites were incorporated viz. BamHI, XbaI and SalI along with appropriate overhanging sequences on forward and reverse primers respectively for directional cloning of the amplified product. After incorporation of the RE sites the expected size of the amplicon was at 204 bp. RT-PCR was carried out in 2 steps and the first step was to synthesize cDNA and the second step to amplify the desired genes from cDNA by PCR. The first strand synthesis of cDNA was standardized in a 25 µL reaction mixture containing the following reagents Merck genei, Mumbai. The cDNA thus obtained was used for amplification by PCR in the next step. PCR was carried out to amplify the desired genes from the synthesized cDNA in a final volume of 25 µL. The thermocyclic conditions were set as follows

Reaction	PCR	No. of cycles
Initial denaturation	95ºC for 2 min	1
Denaturation	95ºC for 30 sec	32
Primer annealing	60ºC for 30 sec	32
Primer extension	72ºC for 90 sec	32
Final extension	72ºC for 10 min	1

The amplified PCR product was checked by submarine gel electrophoresis using 1.5% agarose mixed with ethidium bromide at 100 volts for 1 hour with 100 bp plus DNA ladder (MBI fermentas).

RESULTS AND DISCUSSION

Total RNA was isolated from *Gastrothylax crumenifer* using QIAGEN easy RNA isolation kit. The concentration and purity of RNA was determined at 260 and 280 nm, in a spectrophotometer. The total cellular RNA obtained from 500 mg of parasite was 0.131 µg and the concentration of the RNA was 0.262 µg/g .The ratio of A_{260}/ A_{280} was 1.78 indicating that isolated RNA was reasonably pure.

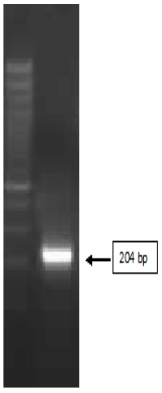

Figure RT-PCR analysis for the presence domain of MMP-9 gene in E/S antigens of Gatrothylax crummier

Lane 1-100 bp DNA molecular marker
Lane 2- Propeptide domain of MMP-9 gene

The RT-PCR products were subjected to 1% agarose gel and propeptide domain was observed (Figure Lane 2). The products were very specific and devoid of any spurious amplification. The concentration of the amplicons appeared to be very high, as shown in figure. To purify the amplicons, products from 100 µL PCR reactions for the domain was run in LMP agarose. The bands of interest were cut and eluted with the QIA quick gel extraction kit. Two microliters of this gel eluted product was electrophoresed to check the presence of specific band.

Our results corroborated entirely with the earlier reports suggested by Lana *et al.*, (2000) and Tanaka *et al.*, (1993). The PCR products reported by Tanaka *et al*., (1993) were having 75 % homology human 92 KDa MMP-9 gene sequences. Yakota *et al.*, (2001) reported the presence of 1080 bp catalytic domain of MMP 9 gene in canine adenocarcinoma and it was having 79.6 % homology with human, 80.6 % with rabbit and 82.3% with bovine MMP-9 gene sequences.

So far, the RT- PCR of propeptide domain of MMP-9 gene was not carried out in any of the parasites of domestic animals viz. cattle, buffaloes, sheep, goat and equines. Hence, this is the first report of confirmation of the presence of cDNA specific for MMP-9 gene in *Gastrothylax crumenifer* infection. Still more quantification of specific gene responsible for remodelling can be explored. Moreover, these studies will augment for immunodiagnostic tool to identify the *Gastrothylax crumenifer* infection at the earliest stage itself.

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