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Research Article

MOLECULAR CHARACTERIZATION OF MMP-9 GENE IN GIRIRAJA FOWL BY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

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ABSTRACT
Matrix metalloproteinase -9 (MMP-9) is one of the most important metalloproteinases involved in degradation of cellular matrix during several physiological and pathological conditions including ovulation, fertilization and parturition. In this study, 5 mL of blood samples were collected from Giriraja fowl and it was centrifuged at 10,000 rpm for 15 minutes at 4°C. Total RNA was isolated using standard protocols. The total cellular RNA was obtained from 500 µL of blood sample was 0.114 µg and the concentration of the RNA was 0.228 µg/mL. The ratio of A 260/ A280 was 1.81 indicating that isolated RNA was reasonably pure. 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. Primers targeting catalytic domain of the MMP-9 were designed. The RT-PCR products were subjected to 1% agarose gel electrophoresis and the expected size of 1080 bp for catalytic domain was observed.

Keywords: *Blood Samples, MMP-9 Gene, RT-PCR, Giriraja Fowl*

INTRODUCTION
Metalloproteinase (or metallopeptidases) are a large group of hydrolases in which the nucleophilic attack on the peptide bond in cellular matrix was mediated by a water molecule activated by a metal ion (usually a Zn^{2+} atom) held in a catalytic pocket. This large group of proteases was classified in eight clans, each consisting of several families. To date, at least 18 mammalian matrix metalloproteinases (MMPs) have been identified and according to structure and substrate specificity, they can be divided to subgroups of collagenases, stromelysins, gelatinase, membrane-type MMPs (MT-MMPs) and other MMPs. MMPs have a characteristic multidomain structure (Johansson *et al.*, 2000).

These were involved in many normal remodelling processes such as ovulation, embryonic development, postpartum involution of the uterus, bone and growth plate remodelling and wound healing as well as in some important disease process such as joint destruction in rheumatoid and osteoarthritis, tumor invasion and periodontitis. In addition to playing a role in the loss of connective tissue mass, the metalloproteinases can influence the phenotype of the cellular components of the tissues, altering basic cellular functions such as proliferation, differentiation, and apoptosis. The present study was carried out to assess the presence of MMP-9 gene in blood samples collected from Giriraja fowl.

MATERIALS AND METHODS
Blood samples were collected in vacutainer containing sodium citrate and the blood samples were transported immediately to the laboratory and were stored at -20 °C till further use. Total RNA was isolated from the blood samples by adopting the protocol given in the QIAGEN RNeasy Mini Handbook. Primers targeting catalytic domain of the MMP-9 was designed through the computer software "DNASTAR". The primer was supplied as desalted oligonucleotides by Imperial Biomed (USA).

The details of the primers used were as follows:

Target	Primer sequence	Product
Catalytic domain	Forward primer 5'-CGGCGGATCCTGGCACCACAACGACATCACTTA-3'	1080 bp
	Reverse primer 5'-CGGCGTCGACTCTAGAGGGAGGACCAGTAGCGCAGA-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 as 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	95 °C for 2 min	1 cycle
Denaturation	95 °C for 30 sec	32 cycles
Primer annealing	60 °C for 30 sec	32 cycles
Primer extension	72 °C for 90 sec	32 cycles
Final extension	72 °C for 10 min	1 cycle

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) which was run simultaneously on a parallel well. Agarose gel electrophoresis of the PCR product, was carried out.

RESULTS AND DISCUSSION

The total cellular RNA was obtained from 500 µL of blood sample was 0.114 µg and the concentration of the RNA was 0.228 µg/mL. The ratio of A 260/ A280 was 1.81 indicating that isolated RNA was reasonably pure. The integrity of RNA was assessed by agarose gel electrophoresis. The RT-PCR products were subjected to 1% agarose gel electrophoresis and the results were shown.

The expected sizes of 1080 bp for catalytic domain, was observed. 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 each of the domains were run in LMP agarose. The bands of interest were cut and eluted with the QIAquick gel extraction kit. Two microliters of this gel eluted product was electrophoresed to check the presence of specific band. Specific discrete bands were observed and the gel- purified product was used for cloning.

The presence of MMP-9 in pregnant animals is an indication of its role in several processes associated with the foetus- uterine interactions. An increased expression of MMP-9 by amnion epithelium, macrophages and chorion, resulting in the degradation of the ECM of the fetal membranes and facilitate the rupture under both physiological and pathological conditions was already noticed by Vadillo-ortega *et al.*, 1995. Lei *et al.*, 1995 demonstrated that there is a striking increase in MMP-9 expression in amnion and possibly the capsular region of the visceral yolk sac placenta approximately 12 h prior to delivery is responsible, in part of the alteration in the structure of the fetal membrane before parturition in rats.

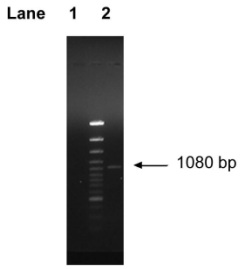

Figure.4.4. RT-PCR analysis for the presence of catalytic domain of MMP-9 gene
Lane 1- 100 bp DNA molecular marker
Lane 2- Catalytic domain of MMP-9 gene

Figure 1: RT-PCR analysis of Catalytic Domain of MMP-9 gene
Lane 1- 100 bp DNA molecular marker
Lane 2- Catalytic domain of MMP- 9 gene

Yokota *et al.*, (2001) reported the cDNA of MMP 9 isolated from canine adenocarcinoma 1080 bp and it was having 79.6 % homology with human 80.6 % with rabbit and 82.3% with bovine. 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) had 75 % homology human 92 KDa gelatinase activity.
This is the first report of confirmation of the presence of cDNA specific for MMP 9 gene in serum of murrah buffaloes at different reproductive stages. The levels of this enzyme are to be estimated in detail to correlate its role during several reproductive stages, which may form the future goals of the research dealing with MMP-9 in reproduction.

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