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Molecular characterization of MMP-9 gene in Murrah buffaloes 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 as well as pathological process including ovulation, fertilization, implantation and parturition. A study was conducted to confirm the presence of MMP-9 gene in blood samples of Murrah buffalo cows at different reproductive physiological stages. Healthy Murrah buffalo cows (18), aged about 3–6 years was divided into 3 groups, each group consisting of 6 animals, viz. pregnant (group 1), oestrous (group 2) and anoestrous (group 3) buffalo cows were selected. Experimental animals were managed and fed as per the farm schedule. Blood samples were collected in vacutainer containing sodium citrate and the blood samples were transferred immediately to the laboratory and were stored at –20°C in deep freezer till further use. Total RNA was isolated from the blood samples and reverse transcription- polymerase chain reaction (RT-PCR) was carried out. Primers targeting catalytic domain of the MMP-9 was designed. The total cellular RNA was obtained from 500 µL of blood was 0.108 µg and the concentration of the RNA was 0.216 µg/mL. The ratio of A260/A280 was 1.81 indicating that isolated RNA was reasonably pure. 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 RT-PCR products were subjected to 1% agarose gel electrophoresis and the expected sizes of 1080 bp for catalytic domain was observed in all the three groups of Murrah buffalo cows.

Key words: Anoestrus, MMP-9 Gene, Murrah buffalo, Oestrus, Pregnant, RT-PCR

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 Zn^{2+} atom) held in a catalytic pocket. This large group of proteases was classified into eight clans, each consisting of several families. Till 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) consisting of (i) a signal peptide, (ii) a propeptide, which is essential for maintaining the proMMP in a latent form, (iii) a catalytic domain containing the highly conserved Zn^{2+} binding site (HExGHxxGxxHS/T), (iv) a proline-rich hinge region that links the catalytic domain to (v) the hemopexin-like domain, which determines the substrate specificity of the MMP. In addition, the catalytic domain of gelatinases contains three repeats of the fibronectin-type II domain, involved in binding of these enzymes to gelatin.

These were involved in many normal remodelling processes such as ovulation, embryonic development and 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 (Amãlinei *et al.* 2010)

Domestic buffaloes are considered to have low reproductive efficiency, characterized by late attainment of puberty and maturity, seasonality of calving, long postpartum anoestrus, poor expression of oestrus signs, low conception rate and long calving intervals (Barile 2005, Perera 2011). Furthermore, there is some evidence of a high rate of embryonic loss in particular during the critical phase of embryonic attachment (Campanile *et al.* 2008). The organs of the adult reproductive system can undergo extensive remodelling, experiencing rapid changes in tissue mass and function. Much of this matrix remodelling is

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attributed to the action of matrix metalloproteinases. Matrix metalloproteinase family members are expressed in a highly-regulated manner in many reproductive processes, including menstruation, ovulation, implantation and uterine, breast and prostate involution. Metalloproteinase concentrations and activity can be regulated by various reproductive hormones as well as by various growth factors and cytokines that participate in reproductive events. 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 (Hulboy *et al.* 1997). This present study focuses on the expression of matrix metalloproteinases in reproductive tissues and discusses the evidence supporting a role for these enzymes in modulating the structure and function of reproductive organs in bubaline species.

MATERIALS AND METHODS

Healthy Murrah buffalo cows (18), aged 3–6 years with good body condition (score 5–6) were selected from the herd of organized farm, Orathanadu, India and were maintained under uniform feeding, housing and managemental conditions. The experimental animals were divided into 3 groups and each group consisting 6 animals (n=6) of pregnant (group 1), oestrous (group 2) and anoestrous (group 3) buffaloes were selected. Animals were vaccinated and dewormed as per the farm schedule. Blood samples were collected in vacutainer containing sodium citrate and were transferred immediately to the laboratory and stored at –20°C in deep freezer 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 primers were supplied as desalted oligonucleotides by Imperial Biomed (USA).

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. 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.

Table 1. Gene transcripts, primer sequences and resulting fragment size

Target	Primer sequence	Product
Catalytic domain	Forward primer: 5'-CGGCGGATCCTGGCACCACAAC GACATCACTTA-3 Reverse primer :5'-CGGCGTCGACTCTAGAGGGAGG ACCAGTAGCGCAGA-3'	1080bp

Table 2. The thermocyclic conditions

Reaction	PCR	Number of cycles
Initial denaturation	9C° 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	101 cycles

The amplified PCR product was checked by submarine gel electrophoresis using 1.5% agarose mixed with ethidium bromide at 100 volts for 1 h with 100 bp plus DNA ladder ran simultaneously on a parallel well. Agarose gel electrophoresis of the PCR product was carried out.

RESULTS AND DISCUSSION

Total RNA was isolated from the blood samples using Genei easy RNA isolation kit. The concentration and purity of RNA was determination by 260 and 280 nm in a spectrophotometer. The total cellular RNA obtained from 500 µL of blood was 0.108 µg and the concentration of the RNA was 0.216 µg/mL. The ratio of A260/ 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 are shown in the expected size of 1080 bp for catalytic domain (Fig. 1). The products

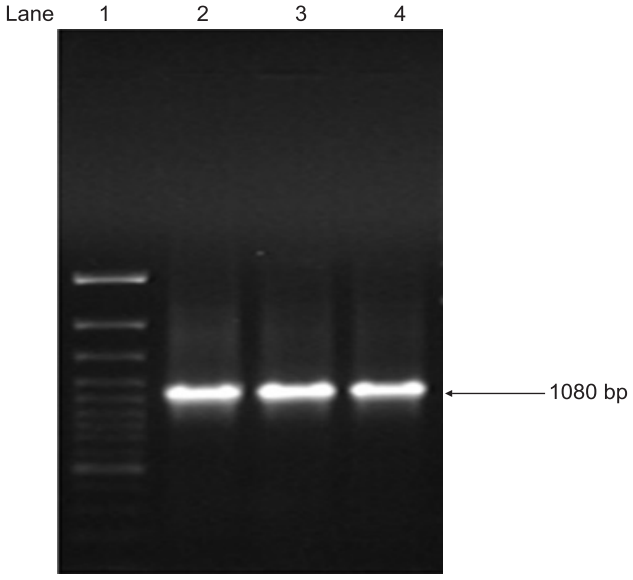

Fig 1. RT-PCR analysis of catalytic domain of MMP-9 in blood of Murrah buffaloes. Lane 1: 100 bp markers; lane 2: Catalytic Domain of MMP-9 gene of pregnant buffalo cows; lane 3: Catalytic Domain of MMP-9 gene of oestrous buffalo cows; lane 4: Catalytic Domain of MMP-9 gene of anoestrous buffalo cows.

were very specific and devoid of any spurious amplification. The concentration of the amplicons appeared to be very high as shown in the purified amplicons, products from 100 µl PCR reactions for the domain was run in low melting point (LMP) agarose. The bands of interest were cut and eluted with the gel extraction kit. Two microlitres of this gel eluted product was electrophoresed to check the presence of specific band. Specific discrete band was observed and the gel- purified product was used for cloning of further study.

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 extracellular matrix (ECM) of the fetal membranes and facilitating the rupture under both physiological and pathological conditions was reported Vu and Werb (1998). Components of the ECM can be changed through the process of cleavage by MMPs and whose activities are also inhibited by enzymes called such as tissue inhibitors of metalloproteinases (TIMPs) (Nagase and Woessner 1999). Moreover, during implantation of embryo, MMPs are expressed in both by the trophoblast cells as well as by the endometrium especially the endometrial stromal cells in the pregnant animals have been found to express high levels of gelatinase A (MMP2) as well as gelatinase B (MMP9). Expression of these enzymes is very much important for maintenance of the endometrial structure as well as for the early phases of implantation, decidualization as well as the neovascularization, occurred during placentation (Novero *et al*. 2002). Normal maintenance and continuation of pregnancy depends upon the cyclical activity of the various hormones as well as pregnancy related genes and the correct and precise timing of their expression during pregnancy is very crucial to maintenance of pregnancy and for successful completion of reproduction. In addition to playing an important role in the loss of connective tissue mass, the MMPs can also influence the expression of phenotype of the cellular components of the tissues and alteration of the basic cellular functions includes cell proliferation, differentiation and apoptosis.

Earlier reports by Lei *et al.* (1995) suggested 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. Similarly, Yokota *et al.* (2001) reported that the cDNA of MMP 9 was isolated from canine adenocarcinoma at 1080 bp and it was having 79.6% homology with human 80.6% with rabbit and bovine 82.3%. 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 to human 92 KDa gelatinase activities. Gene expression profile of MMP-2 and MMP-9 mRNA was detected in the endometrium during the estrous cycle as well as at day 35 of pregnancy and mRNA of MMP-2 was significantly higher after ovulation and decreased at mid-cycle and day 35 of pregnancy, but slight expression was found in the fetal membrane (Mishra *et al.* 2010). Similarly at day 19 of gestation, MMP-2 was slightly expressed in the stroma but moderately at the site of placentation at day 30 of pregnancy. The expression levels of MMP-9 mRNA were very low, whereas its localization remained below the detection limit at each stage in the cyclic and pregnant endometrium (Mishra *et al.* 2010). It has been reported that the major MMP activity in the preimplantation uterus is to originate from the proMMP - 9-bearing neutrophils attracted by seminal plasma and the pro form of MMP - 9 is converted to MMP - 9 by various proteases such as MMP - 1, MMP - 2, MMP - 3, and MMP - 7, tissue kallikrein and plasminogen activator (Vu and Werb 1998), but MMP - 9 is not always found in tissues (Niu *et al.* 2000). Knock out of the seminal vesicles from male leads to absence of the post-coital increase of MMP - 9 in the female mice. This results indicate that the major MMP activity in the preimplantation uterus to originate in proMMP - 9 bearing neutrophils attracted by seminal plasma. Moreover, it has been suggested that proMMP - 9 acquires its activity by binding to ligands or to substrates without proteolytic activation (Bannikov *et al.* 2002). In addition, binding to the plasma membrane of neutrophils enables proMMP - 9 to evade inhibition by TIMPs. Therefore, proMMP - 9 in the endometrium would be active in its latent form even in the presence of abundant TIMPs in the uterus.

This is the first report of confirmation of the presence of cDNA specific for MMP 9 gene in blood of Murrah buffaloes at different reproductive physiological 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.

REFERENCES

Amălinei C, Căruntu I D, Giuºcă S E and Bălan R A. 2010. Matrix metalloproteinases involvement in pathologic conditions. *Romanian Journal of Morphology and Embryology* **51** (2): 215–28.

Bannikov G A, Karelina T V, Collier I E, Marmer B L and Goldberg G I. 2002. Substrate binding of gelatinase B induces its enzymatic activity in the presence of intact propeptide. *Journal of Biological Chemistry* **277**: 16022–27.

Barile V L. 2005. Improving reproductive efficiency in female buffaloes. *Livestock Science* **92** (3): 183–94.

Campanile G, Del Vecchio D, Di Palo R, Neglia G, Gasparrini G, Prandi A, Zicarelli L and D'Occhio M J. 2008. Delayed treatment with GnRH agonist, hCG and progesterone and reduced embryonic mortality in buffaloes. *Theriogenology* **70**: 1544–49.

Hulboy D L, Rudolph L A and Matrisian L M. 1997. Matrix metalloproteinases as mediators of reproductive function. *Molecular Human Reproduction* **3** (1): 27–45.

Johansson N, Ahonen M and Kahari V M. 2000. Matrix metalloproteinases in tumor invasion. *Cell and Molecular Life Sciences* **57**: 1–15.

Lana S E, Ogilvie G K, Hansen R A, Powers B E, Dernell W S and Withrow S J. 2000. Identification of matrix metalloproteinases in canine neoplastic tissue. *American Journal of Veterinary Research* **61** (2): 111–14.

Lei H, Vadillo-Ortega F, Paavola L G and Strauss III J F. 1995. 92–kDa gelatinase (matrix metalloproteinase-9) induced in rat amnion immediately prior to parturition. *Biology of Reproduction* **53**: 339–44.

Mishra B, Kizaki K, Koshi K, Ushizawa K, Takahashi T, Hosoe M, Sato T, Ito A and Hashizume K. 2010. Expression of extracellular matrix metalloproteinase inducer (EMMPRIN) and its related extracellular matrix degrading enzymes in the endometrium during estrous cycle and early gestation in cattle. *Reproductive Biology and Endocrinology* **8**: 60.

Nagase H and Woessner J F (Jr.). 1999. Matrix metalloproteinases. *Journal of Biological Chemistry* **274** (31): 21491–94.

Niu R, Okamoto T, Iwase K, Nomura S and Mizutani S. 2000. Quantitative analysis of matrix metalloproteinases-2 and -9, and their tissue inhibitors-1 and -2 in human placenta throughout gestation. *Life Science* **66**: 1127–37.

Perera B M A O. 2011. Reproductive cycles of buffalo. *Animal Reproduction Science* **124**: 194–99.

Tanaka H, Hojo K and Yoshida H. 1993. Molecular cloning and expression of the mouse 105 kDa gelatinase cDNA. *Biochemical and Biophysical Communications* **190** (3): 732–40.

Vadillo-Ortega F, Gonzalez-Avila G, Furth E E, Lei H, Musche R J, Stetler-Stevenson W G and Strauss III J F. 1995. 92 kDa type IV collagenase (matrix metalloproteinase-9) activity in human amnio chorion increases with labor. *American Journal of Pathology* **146** (1): 148–56.

Vu T H and Werb Z. 1998. Gelatinase B: structure, regulation and function. (Eds) Parkes W C and Mecham R P. Matrix Metalloproteinases. San Diego, Academic Press. pp. 115–48.

Yokota H, Kumata T, Taketaba S, Kobayashi T, Moue H, Taniyama H, Hiryama K, Kagawa Y, Itoh N, Fujita O, Nakade T and Yuasa A. 2001. High expression of 92 kDa type IV collagenase (matrix metalloproteinase-9) in canine mammary adenocarcinoma. *Biochimca et Biophysica Acta* **1568**: 7–12.