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Comparative study on serum matrix metalloproteinases in various species of domestic animals

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ABSTRACT

A study was conducted to evaluate the presence of matrix metalloproteinases (MMP) in the serum of domestic animal species. The serum samples were collected from four healthy male animals of each species, viz. goat, cattle, horse, rabbit, sheep, pig and 4 tumor affected dogs in a heparinzed vacutainer, during early morning before feeding the animals. All the serum samples were subjected to gelatin zymography. The major bands were observed at 220, 92 kDa of MMP-9 and 72 kDa of MMP-2 in all the species with minor variations in rabbit and goat. It was observed that these bands indicated the normal physiological state of the animals and in tumour samples, the intensity of both MMP-9 and MMP-2 was 2–3 times higher. The level of expression of latent form of MMP-9 band was comparable in goat, cattle, horse, sheep and pig and also they were as expressed in human, on contrast there was low level of expression in rabbit as it clearly indicated these MMP proteins were in low concentration in the serum of rabbit. The thickness of Pro-MMP-9 (92 kDa) band in horse serum was alike as in the human marker and it might be related to human protein. There was a faded band below 72 kDa band in all the species but it was absent in human serum as it could be the active form of MMP-2 (62 kDa). MMP-2 band in cattle and horse serum were correlated. The concentration of the MMP-2 band in sheep serum was higher than in the other species used in this study but it was lesser than the activity of protein isolated from canine tumor. It was concluded that MMP plays a significant role in normal physiological functions of every species and its activity was 4–5 times higher in tumor samples due to greater gelatinolytic activity. Thus, it was concluded that tumor samples exhibit greater gelatinolytic activity because of higher concentration of MMP proteins.

Key words: Domestic animal species, Gelatin zymography, Matrixmetallo proteinase, Serum

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases, function in the extracellular environment of cells and capable of degrading extra cellular matrix (ECM) proteins (Nagase *et al.* 2006, Iyer *et al.* 2012). There are many types of proteinases involved in the degradation of matrix proteins, but the MMPs also known as matrixins are the major proteinases involved. They are majorly engaged in morphogenesis, wound healing, tissue repair and remodeling in response to injury (Lu *et al.* 2011). Their expressions are constantly regulated by inflammatory cytokines, growth factors, hormones under normal physiological conditions in all the species, but when they are not regulated, there will be an outcome of many diseases such as arthritis, nephritis, cancer, encephalomyelitis, chronic ulcers, fibrosis, etc (Shah 1997, Spinale 2002, Newby 2005,). MMP's activities are also regulated by precursor zymogens and tissue inhibitors of metalloproteinases (TIMPs) (Nagase *et al.* 2006). In vertebrates, MMP's include 23 endopeptidases having gelatinases (MMP-2 and MMP-9), collagenases (MMP-1, -8, -13 and -18), stromelysins (MMP-3, -10 and -11) and other MMPs (Page-McCaw *et al.* 2007). Of these gelatinases, MMP-2 and MMP-9 are the chief proteinases concerned in a number of cardiovascular diseases, including atherosclerosis, stroke, heart failure, ischemic heart disease and aneurysm (Kupai 2010). In reproduction, MMP's play a foremost role in menstruation, folliculogenesis, pregnancy and parturition where the extracellular remodeling is predominant.

Most of the studies were carried out in female animals and there were limited studies in male domestic animals. Ferrer *et al.* (2012) found that MMP-2 along with acrosin plays an important role in fertilization process. Hence the present study was conducted to find the existence of gelatinases (MMP-2 and MMP-9) in various species of

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domestic animals. The presence of these gelatinases can be acknowledged by gelatin zymography as they readily digest gelatin with the help of their three fibronectin type II repeats that binds to gelatin/collagen (Page-McCaw *et al.* 2007). The objective of this study was to examine the presence of MMP-2 and MMP-9 gelatinases in the serum of healthy animals under normal physiological conditions. Then, it was compared with pattern of expression with serum sample of tumor affected canine by using gelatin zymography.

MATERIALS AND METHODS

The proposed study was carried out at the Department of Veterinary Physiology and Biochemistry, TANUVAS - Veterinary College and Research Institute, Orathanadu, Tamilnadu, India. The institute is located at an altitude of 30m feet above the mean sea level, at a latitude of 10.6° north and a longitude of 79.3° east.

Collection and evaluation of serum: Healthy male animals, four from each species, viz. goat, Jersey bull, horse, rabbit, sheep, pig and 4 tumor affected male dogs were selected for the study. Blood samples from each animal were collected in a heparinised vacutainer during early morning before feeding the animals. The samples were transported to the laboratory immediately and evaluated for protein content using standard procedure of Lowry's method (Lowry *et al.* 1951). The blood samples were centrifuged at 3,000 rpm for 15 min and the separated serum was analyzed for protein content by photometric estimation of blue color by using spectrophotometer. The standard curve was built by using various concentrations of bovine serum albumin (BSA) as standard. The serum samples were stored at -20°C for further analysis.

Gelatin zymography: The serum samples were subjected to modified SDS-PAGE (modification of Laemmli's method 1970) carried out by Heussen and Dowdle (1980) by the addition of co-polymerizing substrate of gelatin (0.3%) (final concentration was 0.15% to the resolving gel (8%). The samples were electrophoresed at 100V for 20 min. Renaturation was carried out with 2.5% Triton X-100 for 3 h on a mechanical shaker with a mild agitation. Then developing was done by incubating the gel in 10 mM $CaCl_2$, 0.15 M NaCl and 50 mM Tris pH 7.5.for 18 h at 37°C. The gel was stained with 0.25% coomassie brilliant blue for 2 h, followed by destaining with destaining solution for 1 h and finally the gel was washed with distilled water.

Analyzing the results of gelatin zymogram: Human capillary blood gelatinase was used as the standard marker for comparing the zymogram bands as per Makowski and Ramsby (1996). Using a fingerstick puncture, the blood was collected from a capillary and weighed in a tarred polypropylene tube using analytical balance. Samples were added with 20×volume of Laemmli buffer and thoroughly mixed. Then the aliquots were kept stable for 3 months at –20°C.

RESULTS AND DISCUSSION

It was confirmed that MMP-2 and MMP-9 were present in the serum samples of all the species used in the present study (Fig. 1). On gelatin zymography, all the lanes revealed the presence of major bands at 220, 92 of MMP-9 and 72 kDa of MMP-2. MMP-2 (72 kDa) band was very prominent and its activity was higher than that of MMP-9 (92 kDa). MMP-2 (72 kDa) was very prominent in all the species as compared to human markers (lane 7 and 8). All the three forms of MMP proteins were proteolytically active as they completely degraded the gelatin.

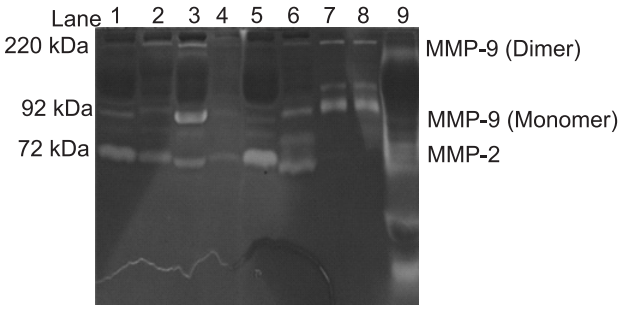

Fig. 1. Comparative gelatin zymogram of domestic animals. L1, Goat serum; L2, Cattle serum; L3, Hourse serum; L4, Rabbit serum; L5, Sheep; L6, Pig; L7, Marker; L8, Marker; L9, Tumor sample.

In Jersey bull serum (lane 2), latent form of MMP-2 (72 kDa) was more prominent compared to that of MMP-9 monomer (92 kDa). The intensity of MMP-2 band was more than that of 220 kDa of MMP-9 band. The results were in agreement with the results of Bannikov *et al.* (2011) and Hinds *et al.* (2014) in bovine species. The intensity of the latent form of MMP-2 (72 kDa band) in cattle sample was matched with the intensity of the same band in regular cyclic buffaloes demonstrated by Prakash *et al.* (2015). Similarly, the level of MMP-9 (220 kDa) expression was matched in both the studies. Bannikov *et al.* (2011) in their work scrutinized the concentration of MMP–19 gelatinase was present in healthy cattle and estimated its concentration was as 330 ng/mL in serum. In another study, MMP-9 found as a diagnostic tool/marker in the diagnosis of bovine respiratory disease. When the lipopolysaccharide was injected, the concentration of HP-MMP-9 present in the serum gets increased and it is easy to predict the diagnosis compared to traditional acute phase protein markers as elucidated by Hinds *et al.* (2014).

Comparing the MMP levels in ruminants, viz. goat and sheep**,** (lane 1 and 5), it was prudent that MMP-2 (72 kDa) band was broader in sheep than in goat, but the expression of MMP-9 (92 kDa) was overriding in goat. The level of 220 kDa (MMP-9) expression was parallel in both the cases. Analogous to this, result of low level expression of MMP-9 (92 kDa) band in normal sheep demonstrated that MMP-9 expression was minor in normal condition and increased during Listerial meningoencephalitis (Ýlhan *et al.* 2012). In another study of using lamb model, elevated level of MMP- 9 (220 kDa; Dimer), pro-MMP- 9 (92 kDa; Monomer) and pro MMP- 2 (72 kDa) were detected after the implantation of tissue engineered vascular graft. These MMP proteins help in the remodeling of tissues (Cummings

et al. 2012). Wilson *et al*. (2003) examined the regional levels of MMP's in post-MI (Myocardial Infarction) sheep model and found that there was a significant induction of MMP expression above the normal level with respect to pathological remodeling. Hence, the MMP's were present in normal level under normal conditions but increased during external pressure or during internal physiological changes.

In lane 3 of horse serum, all the three bands 220, 92 kDa of MMP-9 and 72 kDa MMP-2 were prominent. The 92 kDa band was more predominant than 220 kDa band. The intensity of 92 kDa band was 3–4 times higher and appeared as discrete band than 72 kDa band of MMP-2 as compared to all the other species. The presence of MMP-2 and MMP-9 in equine was confirmed by various authors (Abu Bakr *et al.* 2014, Li *et al.* 2015). MMP-9 and MMP-2 were used as a diagnostic tool/marker as described by Li *et al.* (2015).

The active form of MMP-2 (62 kDa) has greater activity in colic horses than in healthy horses. Further, MMP-9 plays a major role in the pathogenesis of kidney damage. Abu Bakr *et al.* (2014), demonstrated the gelatinolytic activity of pro MMP-9 and pro MMP-2 at 92 and 72 kDa respectively with broad bands, analyzed in synovial fluid of horses affected with osteoarthritis. Other studies carried out by Li *et al.* (2015) explained that MMP-2 plays an important role during early acute development phase of oligofructose induced laminitis and inhibition of MMP-2 was a treatment for laminitis.

In lane 4 of rabbit serum, the latent form of MMP-9 (220kDa) was absent, but a faded band of MMP-9 (monomer; 92 kDa) and a latent form of MMP-2 (72 kDa) were confirmed. Matsumoto *et al.* (1998) and Yamada *et al*. (2008) observed the expression of matrix metalloproteinase -12 in the aorta of cholesterol-fed rabbits and there was no expression of other three gelatinases. Sang *et al.* (2006) studied the computational sequence analysis of matrix metalloproteinases and found that MMP-9 has 75–85% sequence homology among rats, mice, rabbits, humans and cattle.

In pig serum (lane 6), all the three major bands of MMP-9 and MMP-2 were present and the latent form 72 kDa of MMP-2 was prominent than the other two bands. The latent form of MMP-9 (220 kDa) and pro MMP-9 (92 kDa) bands were faded. Similar results were obtained by Kiczak *et al.* (2013) in skeletal muscles from both diseased and healthy animals in non-reducing and non-denaturing conditions. In this study also, there was no high molecular weight complexes (220, 170, 130 and 92 kDa), and thus the proteolytic activity was associated with the presence of 72 and 68 kDa bands (proMMP-2 and MMP-2).

On gelatin zymography, the serum samples of tumor affected dogs in lane 9 and 10, showed the greatest gelatinolytic activity by the presence of thickest MMP-9 band (220, 135 and 92 kDa) as compared to other groups. These bands were remarkably different from the bands of other healthy groups. The expression of different intensity bands could be used as biomarker for the detection of many diseases. The latent form of 72 kDa MMP-2 and active form 62 kDa MMP-2 were observed and also minor catalytic breakdown products of MMP-2 were also observed. Our results were in accordance with the results of Roomi *et al.* (2009), Daniele *et al.* (2010), Akkoc *et al.* (2011), Beltran *et al.* (2013) and Lotfi *et al.* (2015). MMP-2 and MMP-9 concentrations were increased in samples isolated from squamous cell carcinoma and MMP-2 was the significant marker used in evaluating malignancy. These studies were evaluated by Lotfi *et al.* (2015). Daniele *et al*. (2010) explained that MMP-9 had higher concentration levels in patients with breast cancer than in healthy volunteers. Thus, increased expression of these enzymes in the first lymph node involved in the metastatic process can predict a poor prognosis, survival and an unfavorable clinical course. Akkoc *et al.* (2011) observed that zymography was a useful tool for demonstrating MMP activities in tissue homogenates and found that MMP-9 may be an important prognostic tool for feline tubulopapillary carcinomas. Comparison of MMP-9 activity between tumor samples and control tissues explained a statistically significant increase ($P \leq 0.05$) in the activity and level of protein in tumor samples. MMP-2 and MMP-9 secretions are elevated in several types of human cancers and their elevated expression has been associated with poor prognosis. The expression of MMPs was highly regulated by cytokines and signal transduction pathways including those activated by phorbol 12-myristate 13-acetate (PMA) by Roomi *et al.* (2009). MMP-2 is not induced by PMA and that MMP-9 was induced by PMA but to a different degree depended upon the specific cell line. Beltran's retrospective study in 2013 investigated the expression of MMP subtypes 9 and 2 in canine intracranial meningiomas and their association with peritumoral edema. On comparing these results, the tumor affected dog's serum samples showed greater gelatinolytic activity than normal dogs.

On comparing the bands of three different forms of MMP's between each species, there were noteworthy differences between them. The bands of latent form of MMP 9 (220 kDa; Dimer), pro-MMP 9 (92 kDa; Monomer) and pro-MMP 2 (72 kDa) have similar intensities in lane 1 and 6. This means that serum from healthy animals of goat and pig has common proteins except the fact that there was an extended band below 72 kDa in pig serum. The level of expression of latent form of MMP 9 band was comparable in goat, cattle, horse, sheep and pig and also they were expressed as in human, on contrast there was a low level of expression in rabbit as it clearly indicated these MMP proteins were in low concentration in the serum of rabbit. The thickness of pro-MMP 9 (92 kDa) band in lane 3 was alike as in the human marker. Hence, the band of 92 kDa in horse serum was related to human protein. There was a faded band below 72 kDa band in all the species but it was absent in human serum as it could be the active form of MMP-2 (62 kDa). MMP-2 band in lane 2 and 3, i.e. in cattle and horse serum were correlated. The concentration of the MMP-2 band in sheep serum was higher than the other

species used in the present study but it was lesser than the activity of protein that isolated from canine tumor samples as shown in lane 9. On comparing these protein bands in the lanes 1 to 8 having healthy serum samples with lane 9 having tumor affected serum, there is an extended gelatinolytic activity in lane 9. Tumor samples exhibit greater gelatinolytic activity because of higher concentration of MMP proteins in disease conditions.

It was concluded that the gelatinases (MMP-2 and MMP-9) play a vital role in normal physiological state in all the species but their concentrations were increased during various pathological conditions. Further, these levels were correlated with their current physiological state for disease diagnosis.

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