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Influence of sex and age on Serum Matrix Metalloproteinases expression in bovine species

T. C. Balamurugan[1], R. Prakash Krupakaran[1], S. Anandhi[1], C. Senthamil Pandian[1] and P. Perumal[2]
[1]*TANUVAS-Veterinary College and Research Institute, Salem-636 112, Tamil Nadu, India*
[2]*ICAR-Central Island Agricultural Research Institute, Port Blair, Andaman and Nicobar Islands, India*
***Corresponding author's E-mail:** tcbalamurugan@gmail.com*

Abstract

A comparative study was conducted to find out the existence of Matrix Metallo Proteinases (MMPs) in the serum of cattle breed of Tamil Nadu (Umblachery; n=24) and Jersey crossbred cattle (n=24). Umblachery breed is a recognized and registered breed of cattle in the home tract of Cauvery delta region of Tamil Nadu, India. Based on their age and sex, these experimental cattle were divided into four groups for each breed and each group consisted of six animals viz., Group I: Male (1 - 3 years), Group II: Male (4 - 6 years), Group III: Female (1 - 3 years) and Group IV: Female (4 - 6 years). Blood samples were collected in the morning prior to concentrate feeding. Blood samples were centrifuged and separated the serum. These serum samples were subjected to gelatin zymography. Major bands at 220, 135 and 92 kDa of MMP-9 and at 72 kDa of MMP-2 were observed in different experimental groups in both breeds. In Umblachery breed, two prominent bands were observed at 92 and 72 kDa and they represent the latent forms of MMP-9 and MMP-2, respectively. Further, in each experimental group, two lytic bands were observed at 220 and 135 kDa and they represent as the proforms of MMP-9. Intensity of latent form of MMP-2 (72 kDa) was 1.5 times higher in groups of aged as compared to younger cattle. Similarly, aged cattle had showed thicker bands at 135 and 220 kDa; indicating that the expression of pro-enzymatic forms of MMP-9 was clear and higher. In context of sex, intensity of latent form of MMP-2 (72 kDa) was 1.5 times higher in male than female cattle in Umblachery breed. On the other hand, intensity of latent form of MMP-9 was lower in male than the female cattle in Umblachery breed. Similarly, male cattle expressed two more prominent bands at 220 and 135 kDa; these are lytic band and pro-enzymatic form of MMP-9, respectively. The study concluded that MMPs were expressed in both cattle breeds. MMP-2 expression was increased and MMP-9 expression was decreased as age advanced in both breeds and both sexes. Similarly, MMP-2 expression was higher in male than female cattle whereas MMP-9 expression was higher in female than male cattle in both breeds. Therefore, results indicated that breed, age and sex modulate the expression of MMPs profiles in bovine species because MMPs are involved in extracellular matrix accumulation and are connected with concentric remodeling of tissues.

Key words: *Matrix Metallo proteinase, gelatin zymography, serum, cattle*

Introduction

Tissue matrix homeostasis is a complex process which is essential for normal growth, development and wound healing processes (Hingorani *et al*., 2018). MMPs are members of the metzincins family; a family of zinc-dependent proteases that degrade the components of the extracellular matrix (ECM) (Nagase and Woessner, 1999). Several types of proteinases are involved in the degradation of matrix proteins; however, MMPs, also known as matrixins, are the most important proteinases involved. Most connective tissue remodeling processes are accomplished by degradation of extracellular components by MMPs. These proteins are involved in the release and activation of growth factors and cytokines and to regulate the apoptosis in the human reproductive system (Riccioli *et al*., 2005). MMP-2 and MMP-9 are members of a family of more than 25 zinc-dependent endopeptidases that degrade or cleave many extracellular proteins including the extracellular matrix (ECM) components.

MMPs are classified as (i) gelatinases (ii) stromelysins (iii) collagenases and (iv) membrane-type (MT) MMPs (transmembrane enzymes that cleave ECM

components and activate other MMPs) (Nagase and Woessner, 1999). MMPs degrade various extracellular matrix components such as collagen, elastin, laminin, and proteoglycans (Visse and Nagase, 2003). Members of the MMP family are widely expressed in many reproductive processes including menstruation, ovulation and embryo implantation as well as the processes of uterine, mammary gland and prostate gland involution (Jezierska and Motyl, 2009). In many organs, MMP activity affects the cell behavior by altering the key functions such as proliferation, differentiation, migration and apoptosis (Jezierska and Motyl, 2009). Currently, more than 25 different types of MMPs have been identified in vertebrates and most of them are expressed and have similar functions in both humans and dogs (Aresu *et al.,* 2011).

Umblachery breed is a recognized and registered breed of cattle in the home tract of Cauvery delta region of Tamil Nadu, India. Expression of MMPs between men and women have been previously identified and discussed (Mattey *et al*., 2012). However, no study was conducted and no report was available in bovine species with different age groups and sex on expression of MMPs. Therefore, objective of the present study was to examine the expression pattern of MMPs (MMP-2 and MMP-9) in serum of Umblachery and Jersey crossbred cattle in different age groups of both sexes.

Materials and Methods

The present study was conducted at Department of Veterinary Physiology and Biochemistry, TANUVAS-Veterinary College and Research Institute, Orathanadu, Tanjore, Tamil Nadu, India. The institute is located 30 meters above mean sea level with a latitude of 10.6°N and 79.3°W.

Experimental animals

Experimental animals (n=48) were selected from an organized farm. Umblachery breed (n=24) and Jersey Crossbred (n=24) cattle were divided into four groups and each group consisted of six animals, viz., Group I: Male (1 -3 years), Group II: Male (4 - 6 years), Group III: Female (1 - 3 years) and Group IV: Female (4 - 6 years). All the animals were vaccinated and feeding and watering was followed as per the farm schedule.

Collection and evaluation of serum

Blood samples were collected from the experimental animals in blood clot activator in the morning prior to concentrate feeding. The blood samples were centrifuged at 3000 rpm for 15 min at 4°C and serum was separated and labelled. The protein content of the serum samples was estimated by the standard procedure of the Lowry method (Lowry *et al*., 1951) with use of a spectrophotometer (Thermoscientific, Germany). A standard curve was constructed using different concentrations of bovine serum albumin as a standard. Serum samples were stored at −80° C for further analysis.

Gelatin zymography

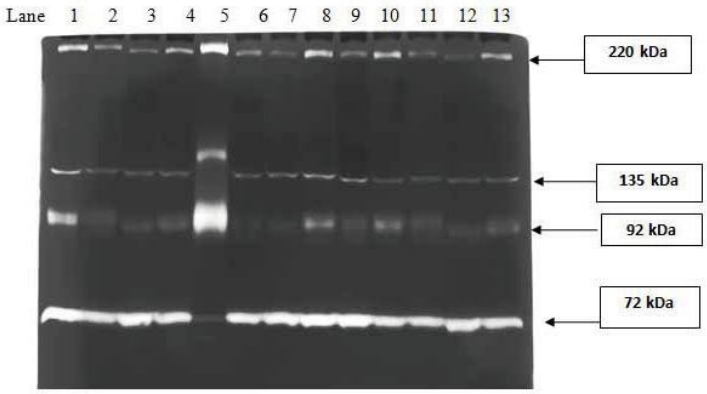
Serum samples were subjected to gelatin zymography by modified SDS-PAGE [a modified method of Laemmli (1970) as performed by Heussen and Dowdle (1980). In this method, gelatin (0.3%) was added as a copolymerization substrate to obtain (final concentration 0.15%) the resolving gel (8%). The samples were electrophoresed at 100 V for 20 min. Renaturation was performed with 2.5% Triton X-100 for 3 h on a mechanical shaker with gentle shaking. The gel was then incubated 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 a destaining solution for 1 h. Finally, the gel was washed with distilled water.

Analyzing the results of gelatin zymogram

Human capillary blood gelatinase served as the standard marker for evaluating the zymogram bands, following the protocol outlined by Makowski and Ramsby (1996). By performing a finger stick, blood was obtained from a capillary and measured using a precise analytical balance in a tarred polypropylene tube. Afterward, the samples were combined with 20× volume of Laemmli buffer and thoroughly blended. These aliquots remained stable for a duration of 3 months at -20° C.

Results and Discussion

Serum samples of four groups for both breeds were subjected to gelatin zymography. Serum samples were proteolytically active as all completely degraded the gelatin. Major bands at 220, 135 and 92 kDa of MMP-9 and 72 kDa of MMP-2 were observed in all four experimental groups in both breeds. In Umblachery breed, two prominent bands at 92 kDa and 72 kDa were observed and they represent the latent forms of MMP-9 and MMP-2, respectively. Further, in each experimental group, two lytic bands were observed at 220, and 135 kDa and they represent as the proforms of MMP-9. However, the active form of MMP-9 and MMP-2 is not observed in serum sample of different experimental groups of Umblachery breed. On the other hand, latent forms of MMP-9 (92 kDa) and MMP-2 (72 kDa) were observed in different experimental groups of Jersey crossbred cattle. In Umblachery breed, the proforms of MMP-9 was observed at 220 and 135 kDa in different experimental groups. MMP-2 (72 kDa) was a prominent proteinase in the bovine species as compared to human marker (lane 5). The results of present study were in agreement with the results of Bannikov *et al*. (2011), Newby *et al*. (2014) and Krupakaran *et al*. (2016) in bovine species and Krupakaran *et al*. (2015) in bubaline species. Similarly, Krupakaran *et al*. (2016) reported that the latent form of MMP-2 (72 kDa) was more prominent as compared to that of MMP-9 monomer (92 kDa) in serum of Jersey crossbred bull (Fig. 1).



Lane	Breed	Sex	Group	Age (Years)	No. of animals
1.	Umblachery	Male	I	0-3	6
2.	Umblachery	Female	III	0-3	6
3.	Umblachery	Male	II	4-6	6
4.	Umblachery	Female	IV	4-6	6
5.	Human capillary blood MMP marker				
6.	Jersey Crossbred	Male	I	0-3	6
7.	Jersey Crossbred	Male	II	4-6	6
8.	Jersey Crossbred	Male	I	0-3	6
9.	Jersey Crossbred	male	II	4-6	6
10.	Jersey Crossbred	Female	III	0-3	6
11.	Jersey Crossbred	Female	III	0-3	6
12.	Jersey Crossbred	Female	IV	4-6	6
13.	Jersey Crossbred	Female	IV	4-6	6

Fig. 1. Comparative Gelatin Zymogram of Umblachery (n=24) and Jersey Crossbred (n=24) cattle of male and female

Effect of age on the expression of gelatinase activity

Gelatin zymogram of different age groups in both breeds were compared to find out the expression pattern of gelatinase with respect to age. In Umblachery breed, the intensity of latent form of MMP-2 (72 kDa) was 2-3 times higher than that of the latent form of MMP-9 (92 kDa) in different age groups. Similarly, expression intensity of latent form of MMP-2 (72 kDa) was 1.5 times higher in aged (G II: lane 3 & IV: lane 2) as compared to younger experimental cattle (G I: lane 1 & III: lane 4). Similarly, two more prominent bands at 220 and 135 kDa were observed in younger groups as they are lytic bands of MMP-9 which indicated that they are the pro-enzymatic forms of MMP-9.

In Jersey crossbred, intensity of the latent form of MMP-2 (72 kDa) was 2-3 times higher than the latent form of MMP-9 (92 kDa) in different age groups. Similarly, the intensity of the latent form of MMP-2 (72 kDa) was 1.5 times higher in aged groups (G II: lane 7, 9 & G IV: lane 12,13) than younger groups (G I lane 6,8 & III: lane 10,11). Similar to Umblachery breed, the younger groups (G I lane 6,8 & III: lane 10,11) exhibited thicker bands at 135 and 220 kDa indicating that they are the lytic bands of MMP-9 and they are the pro-enzymatic forms of MMP-9 was very prudent and clear.

Results of the present study were in agreement with the results obtained by Bonnema *et al*. (2007), McNulty *et al*. (2005) and Cancemi *et al*. (2020). Similar to the present study, the expression pattern of MMP-2 is age dependent as expression was increased as age advanced; higher expression was noticed in aged groups than younger groups. Similar result was obtained in human subjects that ageing process is associated with higher activities of MMP-2 (McNulty *et al*., 2005). Similarly, Bonnema *et al*. (2007) conducted a study on human subjects from age groups of 20 to 90 years and the result revealed that MMP-2 concentration was increased (from 1188 ± 99 to 1507 ± 76 ng/mL) as age increased. Further, Cancemi *et al*. (2020) reported that the serum activity of MMP-2 was higher in long-living individuals as compared to younger individuals. Yu *et al*. (2013) also reported that the activities of both MMP-2 and MMP-9 were higher in the tendons of aging than in younger rats.

In the present study, the expression of MMP-9 was decreased in aged groups than younger groups of same sex. This results clearly indicated that the concentration of MMP-9 was decreased as age advanced and in younger animals showed marked higher concentration. Similar to the results of the present study, Paczek *et al*. (2008) and Bonnema *et al*. (2007; from 29±7 to 8±2 ng/mL) reported that the concentration of active MMP-9 decreased as age advanced in human. Therefore, alteration in expression and concentration of MMPs is age-dependent as because MMPs are involved in extracellular matrix accumulation and are connected with concentric remodeling of tissues. Moreover, MMPs play a key role in regulating the matrix remodeling as they are responsible for the degradation of collagens and proteoglycans (Sharma and Maffulli, 2006).

Effect of sex on the expression of gelatinase activity

Gelatin zymogram of male and female cattle in both breeds were compared to find out the relationship between the MMPs expression and sex. In Umblachery breed, the intensity of latent form of MMP-2 (72 kDa) was 1.5 times higher in male (G II: lane 3; G I lane 1) as compared to female groups (G III: lane 2; G IV: lane 4). The intensity of latent form of MMP-9 was lower in male (G II: lane 3; G I lane 1) compared to female cattle (G III: lane 2; G IV: lane 4). The female cattle (G III: lane 2; G IV: lane 4) had thicker bands at 135 and 220 kDa as compared to male groups indicated that the expression of pro-enzymatic forms of MMP-9 was very clear and they were lytic bands of MMP-9.

In Jersey crossbred, the intensity of latent form of MMP-2 (72 kDa) was 1.5 times higher in female (III: lane 10,11; IV: lane 12,13) than in male (G I: lane 6,8 & G II: lane 7, 9) cattle. The intensity of latent form of MMP-9 was lower in male (G I: lane 6,8 & G II: lane 7, 9) than the female cattle groups (G III: lane 10,11; G IV: lane 12,13). The female cattle (G III: lane 10,11; G IV: lane 12,13) exhibited thicker bands at 135 and 220 kDa than the male cattle indicated that the expression of pro-enzymatic forms of MMP-9 was prudent and clear and they are the lytic bands of MMP-9.

The present study results were in agreement with the results obtained by Bonnema *et al*. (2007), Vanessa

A. Belo *et al.* (2009), Kusnierova *et al.* (2015), and Cancemi *et al.* (2020). Similar to the results of the present study, the expression of MMP-2 was higher in male groups compared to female cattle groups. On contrary, expression of MMP-9 was higher in female than in male groups. To agree with our results, a human study on MMPs revealed that Pro-MMP-2 activity was increased as age advanced in male gender; but not in female gender (Cancemi *et al.,* 2020). This is because the expression of MMPs is also dependent on the hormonal status in women (Berg *et al.,* 2014). Similar to the results of the present study, Kusnierova *et al.* (2015) reported that the plasma MMP-2 level was significantly correlated with age in human as lower concentration was detected in persons ≤ 49 years of age. However, the plasma MMP-3 was significantly associated with both age and gender as lower concentration was detected in persons of ≤ 47 years of age and among the women. Plasma MMP-9 level was not age dependent; it was associated with gender, showed lower concentration of MMP-9 in women (Kusnierova *et al.,* 2015). Thus, Kusnierova *et al.* (2015) concluded that MMP-2 and MMP-3 levels were found to be age dependent and MMP-3 and MMP-9 levels were gender dependent. Similarly, Bonnema *et al.* (2007) reported that women had non-significantly lower value (MMP-2: 1352 ± 58 Vs 1300 ± 49; MMP-9: 16.5 ± 6.3 Vs 19.4 ± 3.8; TIMP-1: 1058 ± 54 Vs 867 ± 37; TIMP-2: 43 ± 6 Vs 43 ± 5) as compared to men with respect to MMPs (ng/mL).

On the contrary to the present study results, Belo *et al.* (2009) reported that non-significant difference was observed between boys and girls on different MMPs (MMP-8, pro-MMP-9, MMP-9, MMP-2, TIMP-1, and TIMP-2) and the concentration of Pro-MMP-9 (A.U) in girls (0.99) than boys (0.76) but the concentration of MMP-2 was equal in both sexes (1.59). Further, Andreas Jonsson *et al.* (2016) reported that MMPs (MMP-1, -2, -7, -8, -9 and -13) had shown non-significant difference between plasma and serum samples in men and women. However, Sathyamoorthy *et al.* (2015) reported that MMP-1, MMP-3, MMP-8 and MMP-9 were higher in men than women infected with tuberculosis. Further, Sathyamoorthy *et al.* (2015) found that plasma MMP-8 concentration was 1.51-fold higher in men than women with tuberculosis and this difference was not due to greater disease severity in men. Men mount a greater and often more damaging inflammatory response to infection compared to women of reproductive age (Guerra-Silveira Abad-Franch, 2013). Higher MMP level alteration observed in male patients as compared with female patients (Yadav *et al.,* 2018). These results of the present study clearly indicated that the expression of MMP-9 is more in female than in the male animals. It might be due to female sex hormones are protective (Marriott and Huet-Hudson, 2006) and female neutrophils have been found to express decreased MMP-9 during the period of the menstrual cycle when oestrogen levels are higher (Smith *et al.,* 2007). Hence, it might be inferred that the levels of gelatinases expression may be correlated with individual physiological status within the sex and age groups.

Conclusion

It was concluded that expression of gelatinase activity was confirmed in both breeds of Umblachery and Jersey crossbred. Expression of MMP-2 was higher as age advanced and aged animals had higher MMP-2 than the younger animals. Expression of MMP-9 was lower as age advanced and aged animals had lower MMP-9 than the younger animals in male and female cattle. Similarly, the expression of MMP-2 was higher in male than the female animals whereas the expression of MMP-9 was higher in female than in male animals. Therefore, the present study results indicated that breed, age and sex modulate the expression of MMPs profiles in bovine species because MMPs are involved in extracellular matrix accumulation and are connected with concentric remodeling of tissues.

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