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Existence of gelatinases in Kilakarisal sheep of Tamil Nadu

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Abstract
This study was carried out to identify the presence of gelatinases viz MMP-2 and MMP-9 in serum of Kilakarisal sheep, a native sheep breed of Tamil Nadu. Four adult male and female native sheep breed Kilakarisal sheep was selected. On gelatin zymogram reveals that the presence of four prominent bands at 220, 92 kDa of MMP-9 and 72 and 62 kDa of MMP-2 were clearly observed for all the animals. All the four forms of MMP are proteolytically active, completely degraded the gelatin. Both latent forms of MMP-2 and MMP-9 were exhibited and the latent bands were observed as thicker bands than the active form. The intensity of 62 kDa of MMP-2 was 2-4 times higher than 82 kDa of MMP-9. The level of expression of 72 kDa of MMP-2 was constant as compared to 92 kDa of MMP-9. In all the groups, 220 homodimer or MMP-9 was also observed. Further, in Kilakarisal sheep showed maximum gelatinolytic activity as compared to marker by showing more intensity in 62 kDa of MMP-2. In addition, the 82 kDa of MMP-9 was also observed. It was concluded that there was no significant difference between the expression of MMP-9 and MMP-2 in both the sexes. But Further, there is more up regulation of MMP-2 mediated through MMP-9 activity observed in all animals. It was inferred that the expression of MMP-2 and MMP-9 were to be correlated with physiological and reproductive status of individual animal since MMP played extensive role in tissue remodeling and extra cellular degradation.

Keywords: Gelatinase, Kilakarisal sheep, MMP-2, MMP-9

Introduction
Sheep is an important economic livestock species contributes greatly to the agrarian Indian economy. Sheep is a multi facet animal known for its utility like wool, meat, milk, skin and manure, forms an important component of rural economy particularly in the arid and semi-arid and mountainous areas of country. As per the 20th Livestock census, the population of sheep declined by 6.36 per cent to 45 lakh from 48 lakh [1].

Matrix metallo peptidases (MMPs), also known as matrix metalloproteinases or matrixins, are metalloproteinases are that are calcium-dependent zinc-containing endopeptidases [2]. MMPs belong to a large family of enzymes (the metzincins) that are zinc-dependent proteinases. MMPs and their regulators – Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) – participate in extracellular matrix remodeling, fibrosis, and semen liquefaction, as well as to inflammatory activity. The MMPs or matrixins were first described in 1962 by Gross and Lapiere and are mostly known by their ability to degrade components of the ECM. MMPs need zinc ion for both their physiological and pathological roles. They participate roles in cell migration, tissue remodeling, cell proliferation and destruction of extracellular matrix. MMP-2 and MMP-9 are members of gelatinase group and are able to degrade gelatin and collagen. In vertebrates, MMP's includes 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 [3]. 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 [4]. In reproduction, MMP's plays a foremost role in menstruation, folliculogenesis, pregnancy and parturition where the extracellular remodeling is predominant. This family of endoproteases has been considered essential in a number of normal physiologic processes as well pathological events. Hence, the present study was conducted to find out the presence of gelatinases in Kilakarisal sheep a native sheep breed of Tamil Nadu.

Materials and Methods
This plan of work was conducted at the Department of Veterinary Physiology and Biochemistry, TANUVAS - Veterinary College and Research Institute, Orathanadu, Tamil Nadu, India.

Collection and evaluation of serum

Four healthy male and female animals of Kilakarisal breed of sheep were selected. In early morning before feeding the animals, blood samples were collected from all the eight animals in heparinised vacutainer hours. After collection, immediately the samples were transported to the laboratory and evaluated for its protein content by using standard procedure of Lowry's method (Lowry *et al.* 1951) [5]. The blood samples were centrifuged at 3000 rpm for 15 min. Serum was separated and analyzed for its protein content 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 into the method of modified SDS-PAGE (modification of Laemmli's method, 1970) [6] carried out by Heussen and Dowdle (1980) [7] 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 hrs on a mechanical shaker with 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 hrs at 37 °C. The gel was stained with 0.25% coomassie brilliant blue for 2 hrs, followed by destaining with destaining solution for 1 hr and finally the gel was washed with distilled water.

Calibration of gelatin zymogram

Human capillary blood gelatinase was used as the standard marker for comparing the zymogram bands based on the procedure of Makowski and Ramsby (1996) [8]. The blood was collected from a capillary and weighed in a tarred polypropylene tube using analytical balance by using a fingerstick puncture. Samples were added with 20X volume of Laemmli buffer and thoroughly mixed. Then, the aliquots were stable for 3 months at -20 °C.

Results and Discussion

By gelatin zymography, it was confirmed that the existence of gelatinases *viz* MMP-2 (gelatinase A), MMP-9 (gelatinase B) were present in the serum samples of all the animals, and the results were depicted in Fig -1. Further gelatin zymogram suggests, four prominent bands at 220, 92 kDa of MMP-9 and 72, 62 kDa of MMP-2 were evidently present in both the sexes of all animals. All the four forms MMP-9 and MMP-2 are proteolytically active, and fully degraded the gelatin. In gelatinase A (MMP-2), the active form 62 kDa of MMP-2 is thicker than the latent form (72 kDa of MMP-2) where as in gelatinase B (MMP-9) the latent bands (92 kDa of MMP-2) were observed as thicker bands than the active form (82 kDa of MMP-9). Several authors reported the existence of gelatinases in various domestic animals [9, 10, 11].

In our earlier study with sheep and goats, we observed 220, 92, and 135 kDa of MMP-9 and 72 of MMP-2 was observed in all the sheep and goat breeds of Tamil Nadu. But in sheep and goats, Mecheri and Pattinam sheep breeds and Kodi and Tellicherry goat breeds showed the active form of 62 kDa MMP-2 [9, 10]. This might be due to physiological status of the individual animals. Further, MMP-2 (62 kDa) was very prominent in all the animals as compared to human markers (lane 6, 7 and 11). The intensity of 62 kDa of MMP-2 was 2-4 times higher than 82 kDa of MMP-9. The level of expression of 72 kDa of MMP-2 was constant as compared to 92 kDa of MMP-9.

In all the animals, 135 homodimer or MMP-9 was also observed. But the active form of MMP-9 (82 kDa) is observed as a faint band in all the animals. In addition, the 62 kDa of MMP-2 band was observed. The level of expression of 72 kDa band was constant compared to that 92 kDa. Similarly, in 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 by Cummings *et al.* (2012) [12]. The elevated level of MMP-2 and MMP-9 was observed due to remodeling of tissues during implantation. Gelatinases plays a significant role in tissue remodeling and implantation. In another study by Ilhan *et al.* (2012) MMP-9 expression was increased in sheep during *Listerial meningoencephalitis* infection as compared to normal sheep. MMP-9 expression was increased in goat during Listeria meningoencephalitis infection as compared to normal goat [13]

Further, the active form of MMP-9 was observed as a fainter band. This might be due to gelatinase B might be expressed lightly in normal animals as compared to pathological conditions. To concord with the present results, Chegeni *et al.* (2015) [14] observed that total MMP-9 was higher in dogs affected with dilated cardiomyopathy (DCM) than the control group and further concluded that the active form of MMP-9 was detected only in patients with DCM. Similarly, Sakalihasan *et al.* (1996) [15] carried out studies in abdominal aortic aneurysm (AAA) patients and healthy patients, the predominant form was active MMP-9, active MMP-2 found higher in AAA patients than the normal population. Thus, gelatinases present in physiological level and it is increased during any internal physiological changes. Moreover, in pathological conditions like cancer, there was enormous increase in the gelatinase expression.

Wilson *et al.* (2003) [16] 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 another study Bannikov *et al* (2011) [17], serum gelatinase (MMP-9) activity was increased in acute septicemic and chronically ill animals as compared normal animals. They further concluded that the level of MMP-9 in acute and chronic metabolic disease was identical, and normal in healthy animals.

In the present study, the latent form of 72 kDa MMP-2 and active form 62 kDa MMP-2 band were observed was in concordant to Daniele *et al.* (2010) [18] where the expression of MMPs, in serum of patients with metastatic and non metastatic breast cancer, and compared with controlled group and reported that MMP-2, MMP-9 were significantly higher in metastatic breast cancer than non metastatic breast cancer, and normal control.

It was inferred that the expression of gelatinases MMP-2 and MMP-9 observed in both sexes of Kilakarisal sheep. Further, there was no significant difference between the expression of MMP-9 and MMP-2 in both the sexes. were to be correlated with the reproductive status of individual animal as MMP played extensive role in tissue remodeling and extra cellular degradation. Further, there is more up regulation of MMP-2 mediated through MMP-9 activity observed in Kilakarisal sheep serum.

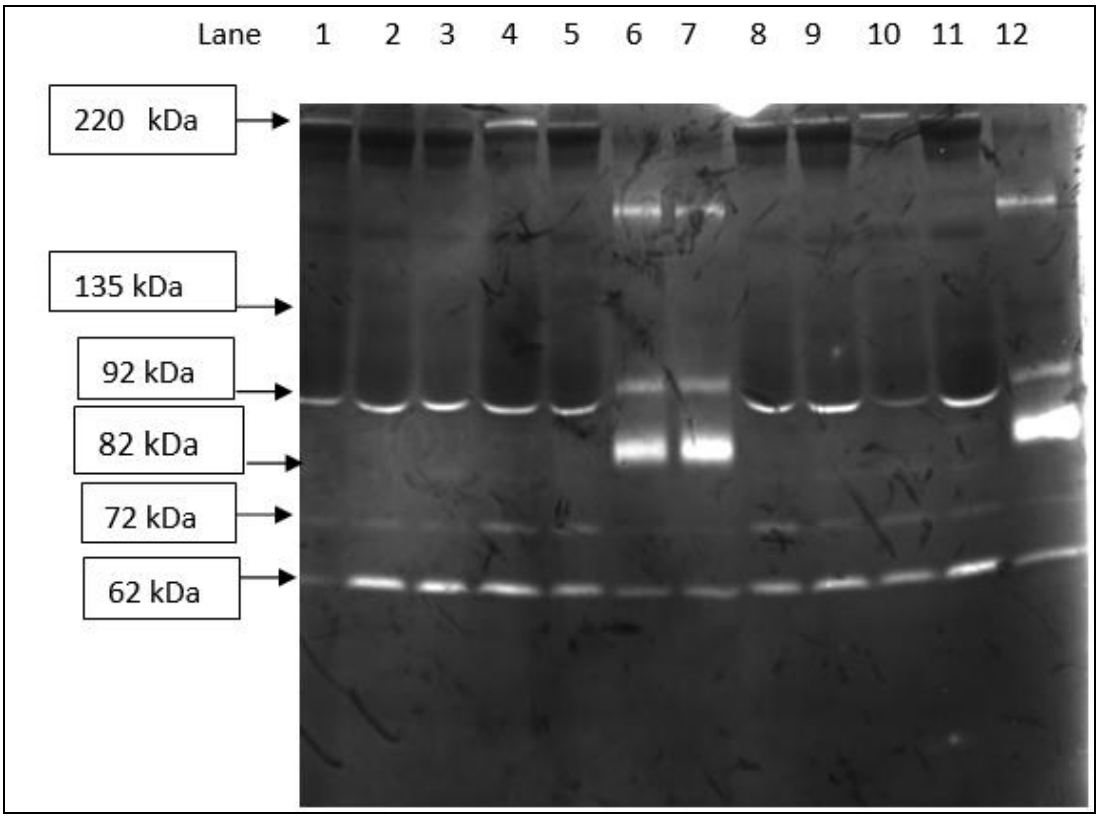

Fig 1: Gelatin zymography of MMPs in serum of Kilakarisal sheep (15 microliters of serum in each well)
Lane 1-5. Serum of Kilakaraisal male animals
Lane 6, 7 & 12-. Human capillary blood gelatinases as marker
Lane 8-11. Serum of Kilakaraisal female animals

Conclusion

It was concluded that there was no significant difference between the expression of MMP-9 and MMP-2 in both the sexes. Further, there is more up regulation of MMP-2 mediated through MMP-9 activity observed in sheep serum. Since, gelatinases might have important functions in various reproductive activities, they have to be targeted as a therapeutic tool to augment animal production.

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