

GELATIN ZYMOGRAPHIC ANALYSIS OF MATRIX METALLOPROTEINASE (MMP) ACTIVITY IN MASTITIS MILK OF JERSEY CROSS-BRED COWS

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ABSTRACT

Gelatin zymography was carried out to assess the presence of matrix metallo proteinase (MMP) activity in both mastitis and control milk samples. In control milk samples, MMP-9 (210 kDa and 82 kDa forms) and MMP-2 (72 kDa) were observed. In mastitis milk of cows, 210 kDa, 82 kDa, 72 kDa, 62 kDa and 42 kDa bands were observed. A faint band at 160 kDa was also observed in the mastitis milk. Among these bands, the 82 kDa band was showing the greatest gelatinolytic activity. In mastitis milk, the degree of expression of 82 kDa MMP-9 to that of 62kDa MMP-2 (active form of the 72kDa pro MMP-2) was very high when compared to both the control milk sample and also the human marker sample. A 42 kDa band was only observed in mastitis milk samples. But, it was absent either in the control milk or in human marker sample. The pasteurized cow's milk sample was also subjected to gelatin zymography and it was observed that all the bands of MMP-9 and MMP-2 were missing. The gelatinolytic activity of MMP-9 and MMP-2 was inhibited by 10mM of EDTA and 5mM of 1,10- phenanthroline.

Keywords: MMP, Gelatinase, Gelatin Zymography, Mastitis Milk, Jersey Cross-bred Cow

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of enzymes, comprising at least 18 members of enzymes, capable of degrading Extracellular matrix (ECM) during several physiological and pathological conditions (Hu *et al.*, 2007). Among others, MMP-2 and MMP-9 are endopeptidases of the MMP family produced by neutrophils, macrophages and monocytes. Milk polymorphonuclear neutrophils (PMN) release many proteases that have the potential of degrading extracellular matrix proteins and milk proteins. Mastitic milk proteases hydrolyzed casein, gelatin, collagen, hemoglobin, mammary gland membrane proteins, and lactoferrin. Activated bovine PMN can express matrix metalloproteinase (MMP)-9. Milk PMN usually have lower total proteolytic enzymatic activities than peripheral blood PMN indicating that PMN release many proteases during migration into the mammary gland. The enzymes involved in bovine mammary tissue destruction are the mastitic milk proteases, which can hydrolyze casein, gelatin, collagen, hemoglobin, mammary gland membrane proteins, and lactoferrin, indicating that mastitic milk proteases have a broad spectrum of activities. Further, the direct involvement of proteases in epithelial cell damage was demonstrated by the fact that co incubation of normal mammary tissue with mastitic milk, but not normal milk, caused tissue degradation. Therefore, proteases released by PMN is likely involved in mammary tissue damage during mastitis. Sub-clinical mastitis does not lead to visible changes in milk or the udder, although it is characterized by reduced milk yield, altered milk composition and the presence of inflammatory components and bacteria in milk. Hence, the present study was carried out to evaluate the role of Matrix Metallo Proteinase in the event of mastitis through gelatin zymography.

MATERIALS AND METHODS

A fresh mastitis milk sample was collected from the affected cow from a nearby village Orathanadu. By following strict aseptic measures, 20 mL of mastitis milk sample was collected in a clean 50mL centrifuge tube and it was carried to the laboratory in ice Box. The milk samples were centrifuged at 10

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minutes for 3000 rpm and the clear solution was collected and preserved at -70°C until further use. The protein content of the samples was estimated. Gelatin zymography of milk samples was carried out as per the method of Heussen and Dowdle (1980) with some modifications. And the procedure was as follows. SDS-PAGE was carried out, as described by the method of Laemmli, (1970). The resolving gel (8%) was co-polymerized with 0.3% gelatin solution (final concentration of gelatin in gel was 0.15%) and the electrophoretic run was carried out at 100 V until tracking dye reaches the bottom. Then, renaturation was carried out with renaturation solution (2.5% Triton X -100) for 3 hours on a mechanical shaker with mild agitation. Then, developing was carried out by incubating the gel in developing buffer (10 mM CaCl₂,

0.15 M NaCl and 50 mM Tris (pH 7.5)) for 18 hours at 37 °C and then stained with 0.25% Coomassie blue for 2 hours, followed by destaining for 1 hour with destaining solution and then, further destaining was carried out with distilled water. Then, calibration of the gelatin zymogram was carried out with human capillary blood gelatinases, as per the procedure suggested by Makowski and Ramsby (1996). A drop of human capillary blood (15-20 µL) was obtained by fingerstick puncture and placed in a tarred polypropylene tube. The weight of the blood was determined in an analytical balance and 20 volumes of non-reducing Laemmli buffer was immediately added. The sample was then vortex mixed (30s) and aliquots stored at -20°C.

RESULTS AND DISCUSSION

The presence of MMP activity in the mastitis milk of Jersey cross-bred cow was assessed by gelatin zymography, shown in the following figure.

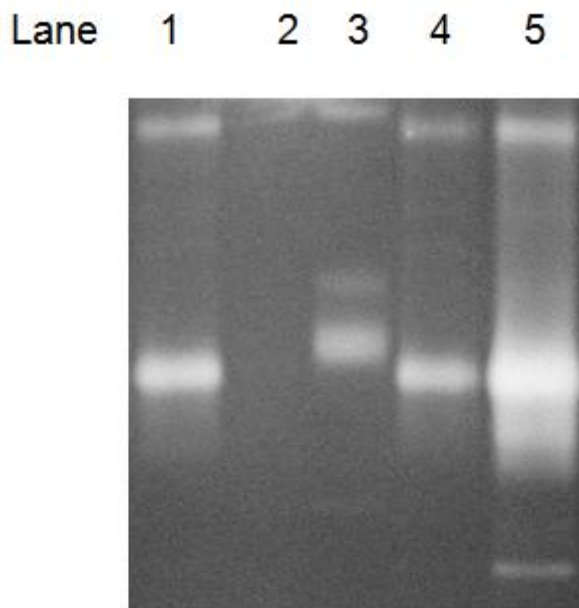


Figure Gelatin zymography of mastitis milk of Jersey cross-bred cow
Lane 1 & 4 - Healthy cow milk
Lane 2- Pasteurized cow milk
Lane 3- Human capillary blood gelatinases (MW in kDa)
Lane 5- Mastitis cow milk

On gelatin zymography (Figure), it was revealed that the 82 kDa and 72 kDa bands were found to overlapping. The degree of expression of 82 kDa MMP-9 for 62kDa MMP-2 (active form of the 72kDa pro MMP-2) was very high when compared to the control milk sample and also the human marker sample. It was observed that the 82kDa band was very prominent in both the control and mastitis milk

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samples. But the degree of expression of 82 kDa MMP-9 was at least three times as that of control milk. A 42 kDa band was only observed in mastitis milk samples' and was absent either in human milk or the normal milk. The normal milk contained a fainter band at 160 kDa which was observed in all the normal milk samples. As a comparison, pasteurized cow's milk was also subjected to gelatin zymography. Interestingly almost all the bands were missing, except the 210 kDa band. Notably the 210 kDa band was found to be very faint.

An increase in the activity of 92 kDa gelatinase by 10 and 7 fold at 24 hours and 72 hours after bacterial challenge, as compared to the controlled samples in cow's milk. Expression of MMP-9, and stromelysin-1 mRNA was increased in association with apoptosis during *E.coli* mastitis (Long *et al.*, 2001). MMP-9 can be produced by bovine Polymorpho nucleotide (Li *et al.*, 1999) and MAC-T cells (Long *et al.*, 2001). Stromelysin -1 contributed to the breakdown of most ECM components, including laminin and collagen type IV (Rudolph-Owen and Matrisian, 1998). Other proteases that have been reported to increase in milk from mastitic cows include MMP-2 and MMP-9 (Raulo *et al.*, 2002); (Lauzon *et al.*, 2006), as well as a 120kDa gelatinase (Lauzon *et al.*, 2006). As per the report of Raulo *et al.*, (2002), the presence of 72kDa MMP-2 and 92kDa MMP-9 by western immune blotting and the levels of milk MMP, were increased during the endotoxin challenge.

Both MMP-9 and MMP-2 were involved in the early proteolytic event. These increased MMP levels were associated with the penetration of small molecular weight proteins. Neutrophilic leucocytes also entered the site as they cause larger tissue damage in basal membrane and intestinal tissue structure than Bovine serum albumin and TIC to extra vasate. In naturally occurring disease, increased MMP-2 and MMP-9 levels were detected in milk. Thus, gelatinases especially MMP-2 was involved in the early degradation of the blood milk barrier which precedes the penetration of blood derived cellular components into milk in endotoxin induced mastitis. In the future measuring MMP in milk whey will be a useful tool for diagnosing early mastitis. Our results correlated with the earlier reports of Keer and Wellnitz (2003), suggesting the induction of MMP-9 activity was one of the ways adopted by the mammary gland to combat mastitis by expressing new genes. Further the mastitis was characterized by an influx of somatic cells, primarily polymorphonuclear neutrophils (PMN), to in the mammary gland and by an increase in milk protease. In the present study the result of gelatin zymography were in full accordance with the earlier reports of (Long *et al.*, 2001; Raulo *et al.*, 2002). The 72 kDa and 92 kDa bands of MMP-2 and MMP-9 were observed. In addition, 210 kDa, 160 kDa, 135 kDa. iso form 135 kDa heterodimer form at 92kDa monomeric forms of MMP-9 were also observed. MMP-9 exist in three different forms 220 kDa, homodimeric form (Kjeldsen *et al.*, 1992). The absence of MMP-9 and MMP-2 bands in pasteurized milk suggested that these enzymes got denatured permanently during the pasteurization process hence these MMPs could also be used as a biomarker for efficiency of pasteurization process. The appearance of a faint band of about 220 kDa in the pasteurized milk may be due to the post pasteurization developmental process in which there could be a development of thermophilic bacteria contributing for the slightest MMP activity. The variation in the number of bands of MMP-9 and MMP-2 between our study and the earlier reports may be due to the stage of lactation and the genotype of animal. Further the season has got an important influence on the expression and activity of MMP-9 (Shapiro *et al.*, 1995). Hence the additional band observed in the present study may reflect a specific case and further conformation is needed to continue the results authentically in any type of mastitis. The presence of 220kDa, 92kDa, and 72kDa MMP-9 and MMP-2 in lactating and involution group of goat milk was earlier reported by Wang *et al.*, (2008). Mehrzed *et al.*, (2005) observed the presence of gelatinolytic bands in from 161 kDa to 248 kDa in mastitis milk of dairy cows. And he suggested that the proteases contributed by PMNL (Polymorpho nuclear leucocytes were actively involved in udder tissue damage during mastitis).

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