



Comparative studies of gelatinase activity in cattle and buffalo bull seminal plasma through gelatin zymography

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ABSTRACT

A comparative study was undertaken to study the gelatinase activity in seminal plasma of Murrah buffalo and Jersey crossbred cattle bull through gelatin zymography. In gelatin zymography, the presence of 3 prominent bands at 220 KDa, 92 KDa and 72 KDa for both buffalo and cattle seminal plasma were observed. All these 3 forms are proteolytically active, degraded the gelatin in gelatin zymography. The 220 KDa homodimer of MMP-9 was very prominent in buffaloes semen compared to cattle semen. On the contrary, 135 KDa heterodimer of MMP-9 was observed only in cattle seminal plasma. The 72 KDa band was very feeble in both the cases. The level of expression of 220 KDa and 92 KDa bands was constant compared to that of 72 KDa. The relative amount of 92 KDa band to that of 72 KDa band was at least 3–5 times higher. The difference between buffalo and cattle seminal plasma was very obvious by the presence of 135 KDa band. On gelatin zymography, the distinguished feature of cattle seminal plasma was the presence of 135 KDa heterodimer of MMP. It is concluded that the 135 KDa band was observed only in cattle seminal plasma. Further, more upregulation of MMP-9 mediated through MMP-2 activity was observed in buffalo seminal plasma.

Key words: Buffaloes, Gelatin zymography, Gelatinase, MMP, Seminal plasma

Matrix metalloproteinases (MMPs) belong to a large family of enzymes (the metzincins) that are zinc-dependent proteinases. They are known as the main enzymes degrading the extracellular matrix (ECM) at physiological pH (Nagase and Woessner 1999). They have been involved in cell differentiation and connective tissues remodeling (Woessner 1991). Matrix metalloproteinases and their tissue inhibitors play a key role in many physiological processes, including ovulation, fertilization and implantation (Hulboy *et al.* 1997). These enzymes participate in embryonic development, morphogenesis, blastocyst implantation, angiogenesis and tissue resorption and in diseases such as arthritis, cancer cell invasion and metastasis (Nagase 1996). They are involved in release and activation of growth factors and cytokines (Fowlkes *et al.* 2002). So far 5 subgroups, the collagenases, gelatinases, stromelysins, matrilysins and membrane type MMPs are known. Common substrates

of the gelatinases MMP-2 MMP-9 are type I, IV, V, VII and X collagens, elastin, fibronectin and tumour necrosis factors- α . Their activity is co-regulated and inhibited by the tissue inhibitors of metalloproteinases (TIMP) (Woessner 1994).

MMPs are secreted as latent forms which are activated through cleavage of the inhibitory pro-peptide. Metallo-proteases like MMP-2, MMP-9 and serine protein-ases have been detected in turkey and human seminal plasma (Kotlowska *et al.* 2005) and their latent forms in canine seminal plasma (Tentes *et al.* 2007). Seminal plasma contains many proteinases originating either from testicular cells or from prostate and other accessory sex glands (Yin *et al.* 1990). There are a limited number of studies focused on the presence of MMPs in semen (Baumgart *et al.* 2002). In this study, we report on the existence of 2 gelatinases: MMP-2 and MMP-9 in seminal plasma and compared the gelatinase activity in Murrah and Jersey crossbred bull through gelatin zymography.

MATERIALS AND METHODS

Collection and evaluation of semen

Four Murrah buffalo and Jersey crossbred apparently

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healthy bulls of approximately 4 to 6 yr of age 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 managerial conditions. Semen was collected using artificial vagina as per the standard practice. Immediately after collection, the samples were kept in a water bath at 37°C and evaluated for volume, colour, consistency, mass activity and pH. After the preliminary evaluations, samples were subjected to the initial dilution with pre-warmed (37°C) Tris egg yolk citrate extender (TEYC). The partially diluted samples were then brought to the laboratory in an insulated flask containing warm water (37°C) for further processing.

Separation and preparation of seminal plasma

Semen (1 ml) from fresh ejaculate was aliquoted immediately after collection. This was subjected for centrifugation at 4,000 rpm for 20 min at 4°C to separate sperm pellet and seminal plasma. The seminal plasma was stored at –80°C for further analysis. The supernatant containing seminal plasma proteins was separated for SDS-PAGE analysis. The pellet containing DNA samples was preserved separately for polymerase chain reaction to identify the presence of MMP gene.

Gelatin zymography

The method of Heussen and Dowdle (1980) was followed with some modifications as 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. Renaturation was carried out with renaturation solution for 3 h on a mechanical shaker with mild agitation. Developing was carried out by incubating the gel in developing buffer for 18 h at 37°C and then stained with 0.25% coomassie blue for 2 h, followed by destaining for 1 h with destaining solution and then further destaining was carried out with distilled water.

Calibrating gelatin zymograms with human gelatinase standards

The procedure suggested by Makowski and Ramsby (1996) was followed. 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 (–20°C) and the preparation were found to be stable for at least 3 months.

RESULTS AND DISCUSSION

The physico morphological seminal attributes of Murrah buffalo bulls and Jersey crossbred cattle bull semen are compared and depicted in Table 1. The mean semen volume

Table 1. Mean values of physical and morphological attributes of neat Murrah buffalo bull and Jersey crossbred bull semen

Parameter	Buffalo	Cattle
Volume (mL)	2.62±0.13 ^a	3.24±0.12 ^b
pH	6.46±0.12	6.82±0.15
Colour	White	Creamy Yellow
Mass activity (grade)	2.82±0.09 ^a	4.32±0.22 ^b
Initial motility (%)	69.53±0.42 ^a	78.24±0.36 ^b
Sperm concentration (×10 ⁶ /mL)	992.85±10.56 ^a	1044.65±18.56 ^b
Livability (%)	87.52±0.53	84.23±0.56
Total morphological abnormality (%)	5.89±0.29 ^a	4.24±0.68 ^b

Means bearing superscripts differ significantly ($P < 0.05$) in rows.

of Murrah bulls (2.62 ± 0.13 ml) was significantly ($P < 0.05$) lower than in Jersey crossbred bull (3.24 ± 0.12 ml). Similar results were observed by other workers in buffaloes (Kumar *et al.* 1993) and cattle (Patel *et al.* 2000). However, higher results were observed by Gokhele *et al.* (2003) and Kanchan and Singh (2005) in Murrah buffaloes.

Jersey crossbred bull has significantly higher mass activity (0 to 5 scales) (3.86 ± 0.22) than Murrah bull semen (2.84 ± 0.09). Similar report was observed in Gir bulls (Sori *et al.* 2006) and Jersey bulls (Singh *et al.* 2000). But lower values are observed in Jersey crossbred bull by Fiaz *et al.* (2010). In buffaloes, similar observation was reported by Bhakat (1994). The pH of Murrah bull semen was 6.46 ± 0.32 as compared to 6.82 ± 0.05 in Jersey crossbred bull semen. The colour of buffalo bull semen was observed in the present study was white, as reported by Kumar (1993) in Indian buffalo bull semen. The colour of Jersey crossbred bull semen was yellow which is accorded to the report of Singh *et al.* (2000) and Fiaz *et al.* (2010). The yellow colour of semen may be due to lipochrome pigment derived from the epithelium of ampulla during seminal secretion and considered to be a normal colour (Singh *et al.* 2000).

Mean sperm concentration was significantly ($P < 0.05$) higher in Jersey crossbred bull (1044.65 ± 28.56 million/ml) than in Murrah buffalo semen (992.85 ± 10.56 million/ml). Similar reports were observed in Murrah bulls (Bhakat *et al.* 2011) and Jersey crossbred bulls (Singh *et al.* 2000). But in buffalo, higher value was observed by Ravimurugan *et al.* (2008). This might be due to individual variations of bull ability to produce semen used in their respective studies.

Mean percentage initial motility of Jersey crossbred bull (78.24 ± 0.36) was significantly ($P < 0.05$) higher than in Murrah buffalo semen (69.53 ± 0.42). Similar reports were observed in buffalo (Kumar *et al.* 1993, Ravimurugan *et al.* 2008) and in cattle bull semen (Sori *et al.* 2006). Mean percentage of live sperm count in buffalo bull semen was 84.23 ± 0.56 as compared to 87.52 ± 0.53 in Jersey crossbred bull semen. The results of buffalo bull semen were similar to the results of Mondal *et al.* (2000). The average abnormality (%) in Murrah bull semen was (4.24 ± 0.68)

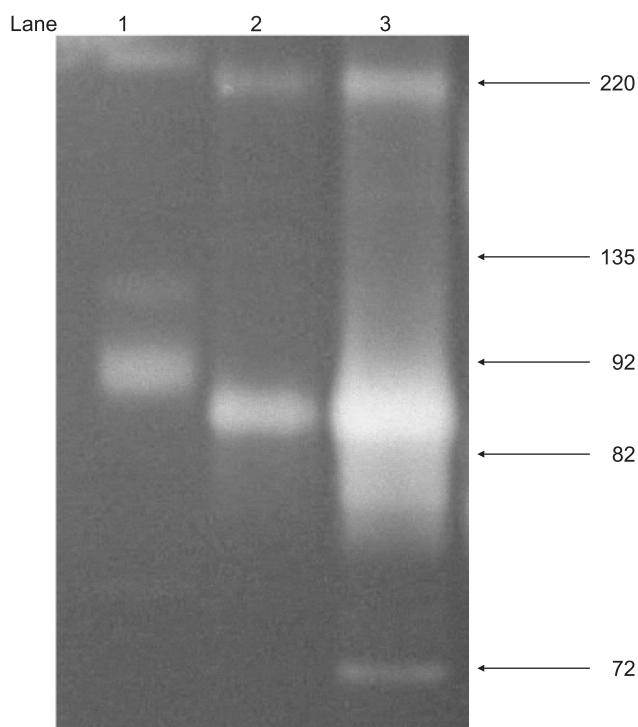


Fig. 1. Gelatin zymography analysis of seminal plasma of cattle and buffalo; Lane 1– Cattle seminal plasma; Lane 2– Buffalo seminal plasma; Lane 3– Human capillary blood markers.

significantly lower ($P < 0.05$) than in Jersey crossbred bull semen (5.89 ± 0.29). Similar results were reported in buffalo bull (Mondal *et al.* 2000) and in Jersey crossbred bull (Singh *et al.* 2000).

Based on the present study, it was concluded that the physico morphological attributes of buffaloes was comparatively lower than Jersey crossbred bull semen. The variation in semen quality parameters recorded in the present study, which were well supported by earlier reports, may be due to individual variations (Saxena and Tripathi 1978), ejaculation rate (Nath 1988), differences in age, genetic makeup of the bulls (Tomar *et al.* 1966), season of study, degree of sexual excitement, method of semen collection, and agro climatic conditions (Koivisto *et al.* (2008).

The presence of MMP activity in the cattle and buffalo seminal plasma was assessed by gelatin zymography (Fig. 1). Prominent bands were observed at 220 kDa, 92 kDa and 72 kDa in both buffalo and cattle semen. All the 3 forms are proteolytically active, as degraded the gelatin. The 220 kDa homodimer of MMP-9 was very prominent in buffaloes semen compared to cattle semen. On the contrary, 135 kDa heterodimer of MMP -9 (Containing NGAL complex) was observed only in cattle seminal plasma. The relative amount of 92 kDa band to that of 72 kDa band was at least 3–5 times higher. The difference between buffalo and cattle seminal plasma was very obvious by the presence of 135 kDa band. The ratio of MMP-9 and MMP-2 was higher comparing to the human marker ratio. The 220 kDa enzymes were found to be catalytically active and TIMP-1 free.

Similar results were observed by various workers (Tentes *et al.* 2007, Baumgart *et al.* 2002, Saengsoia *et al.* 2011, Pipan *et al.* 2010). Tentes *et al.* (2007) who was detected the latent and active forms of MMP-2 and MMP-9 in human seminal plasma. The latent forms were the predominant ones. MMP-2 and MMP-9 either in latent or active forms, were not correlated with semen parameters. ProMMP-9 levels were higher in semen samples with abnormally low concentration ($\leq 19 \times 10^6/\text{ml}$) compared with semen samples with concentration ($\geq 50 \times 10^6/\text{ml}$). This possibly implies impairment at the level of the activation of this enzyme.

In cattle seminal plasma, among 3 forms (220, 135 and 92 kDa), the 92 kDa was very prominent and exhibiting about 50% gelatinolytic activity, 220 kDa contributed about 35% and 135 kDa was for 15% of total gelatinolytic activity. In buffalo samples, 92 kDa exhibited 75% of total gelatinolytic activity and 220 kDa 25%. The relative distribution of these forms was found to be altered in both cattle and buffalo seminal plasma as compared to the human serum markers. This clearly indicated that the level of expression of MMP-9 and MMP-2 was different from that of blood serum. The ratio of MMP-9 and MMP-2 was higher in cattle seminal plasma (1.2) as compared to buffalo, which showed that the form of MMP present is very active and there in no regulation of MMP-9 mediated through MMP-2 activity in buffalo seminal plasma. In buffalo seminal plasma, the 72 kDa form of MMP-2 was absent as compared to human serum marker.

Metayer *et al.* (2002) identified gelatinases in the ram, boar and stallion. The inactive forms were inversely correlated with semen quality and active forms were positively correlated with semen quality traits and sperm functionality (Saengsoia *et al.* 2011). Similar results were recorded in the present study in which the active forms of MMP-9 was found in both cattle and buffalo seminal plasma. But, the presence of 135 kDa MMP-9 only in cattle seminal plasma indicated the regulation of semen function. In turn, this indicated the metabolic state of the particular semen samples in which, the activities were down-regulated as envisaged in the physico-morphological characters of semen.

But in cattle seminal plasma, the regulation of semen function activity was by MMP-9 alone, which was clearly observed by the presence of 135 kDa. The higher values of physico morphological seminal attributes were correlated with the absence of the regulatory MMP-9, 135 kDa in buffalo seminal plasma. The absence of MMP-2 bands in both cattle and buffalo seminal plasma could be attributed to the sperm function activity traits found in this study. In boars gelatinases (225, 78 and 66 kDa) MMP- 9, proMMP-2 and mature MMP-2 are identified in seminal plasma (Pipan *et al.* 2010). Negative correlation between semen indicators and the concentration of 72 kDa, 66 kDa means that higher values of semen indicators were correlated with lower concentrations of these metalloproteases in seminal plasma.

Baumgart *et al.* (2002) concluded that MMP-2 in seminal

plasma was strongly correlated to the sperm count in a linear fashion, whereas the MMP level was not significantly different in sperm of normozoospermic and azoospermic patients. In the present study, we observed that the absence of MMP-2 bands may be due to the low sperm count which was observed in non-reducing conditions.

Based on the present study, it was concluded that the physico morphological attributes of Murrah bull semen was comparatively lower than Jersey crossbred bull semen. On gelatin zymography distinguished feature of cattle seminal plasma was the presence of 135 KDa heterodimer of MMP. Further there is more regulation of MMP-9 mediated through MMP-2 activity in buffalo seminal plasma. Finally, in RT-PCR, catalytic domain of MMP-9 was observed in both cases of buffalo and cattle bull semen.

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