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MATRIX METALLOPROTEINASE -9 (MMP-9) ACTIVITY IN URINE SAMPLES OF DOGS AFFECTED WITH MAMMARY TUMOR

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ABSTRACT

Matrix metalloproteinases (MMPs) are a large group of proteases activated by Zn^{2+} metal ion. A positive correlation between MMP-9 expression and high grade breast carcinoma was reported. Cancer cells appeared to be the main source of MMP-2 and MMP-9 in canine neoplasia. Three classes of urinary MMPs (Mr 72,000, Mr 92,000 and a high Mr 150,000) were detected and they were identified as MMP-2 and MMP-9 respectively by western blot analysis. All the three forms of MMP-9 (92, 135 and 220 kDa) were detected in the urine samples collected from the normal as well as tumor bearing dog urine samples. The level of MMP-9 activity correlated positively with the disease status of cancer patients.

Key Words: *Mmp-9, Urinary Matrix Metalloproteinases, Gelatin Zymography, Western Blotting*

INTRODUCTION

Matrix metalloproteinases (MMPs) are a large group of proteases activated by Zn^{2+} metal ion. They exert their hydrolytic action on the matrices of the basement membrane. MMPs are involved in many normal tissue remodeling processes such as embryonic development, post-partum involution of the uterus, bone and growth plate remodeling, ovulation and wound healing, as well as in some important disease processes such as joint destruction and in tumor invasion. MMP-2 and MMP-9 expression is more in malignant tumors of dogs (Lana *et al.*, 2000). Overproduction of MMPs by a tumor communicating with the vascular and lymphatic system might A positive correlation between MMP-9 expression and high grade breast carcinoma was reported result in the increased levels of MMP activity in other body fluids such as blood or urine (Davies *et al.*, 1990). MMP-9 plays an important role in the progression of canine mammary tumor and the assay of serum MMP-9 is helpful in early diagnosis in progression of adenocarcinoma (Yokota *et al.*, 2001)MMP-9 activities in canine neoplasm has been reported to be very high than that in surrounding unaffected surrounding structures (Lana *et al.*, 2000). Cancer cells appeared to be the main source of MMP-2 and MMP-9 in canine neoplasia (Loukopoulos *et al.*, 2003). Moses and co-workers (1998) reported that three classes of urinary MMPs (Mr 72,000, Mr 92,000 and a high Mr 150,000) were detected reproducibly and correlated with the disease status in a variety of cancers like prostate, renal and breast and they were identified as MMP-2 and MMP-9 respectively by western blot analysis. The present study was undertaken to identify the presence of MMP-9 activity in the urine samples collected from the clinical cases of dogs affected with mammary tumor.

MATERIALS AND METHODS

Collection Of Urine Samples

A total of twelve samples, each 5 ml of random urine samples were collected in a clean cryovial from the female dogs affected with mammary tumor, brought to the Polyclinic, IVRI, Izat Nagar. Samples were frozen immediately after collection and stored frozen at -20°C . prior to analysis, specimens containing blood and leucocytes were excluded by testing for the presence of blood and leucocytes under light microscopy. After thawing, the urine samples were centrifuged at 4°C at 4000 rpm for 5 minutes and the supernatants were collected. 30 μl of the urine sample was mixed with equal volume of 2X SDS-PAGE sample loading buffer(0.0625 M Tris –HCl, 2% SDS, 10% glycerol and 0.01% Bromophenol blue, pH 6.8)as suggested by Lamelli, 1970. Samples were applied without boiling into the wells of the Lamelli

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SDS-PAGE system, co-polymerized with gelatin. Gelatin zymography was performed as per the method of Heussen and Dowdle (1980) with some modifications.

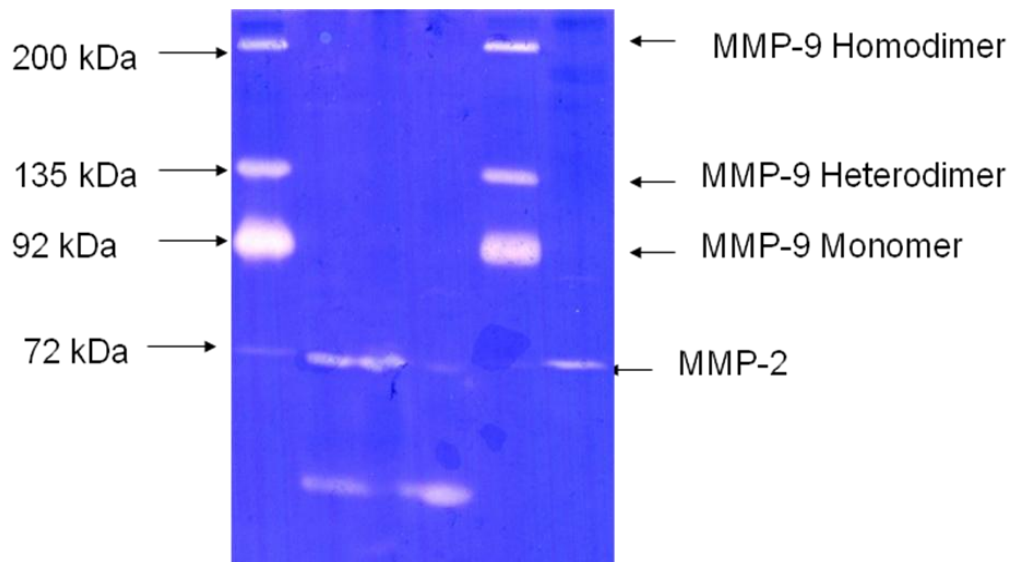


Figure 1: Gelatin zymography of human capillary blood matrix metalloproteinases

Gelatin zymography

The presence of MMP activity in the homogenized tissue sample was confirmed by gelatin zymography (Heussen and Dowdle, 1980). All the reagents were similar to the Laemmli method of polyacrylamide gel electrophoresis (SDS-PAGE, non-reducing, denaturing). Gelatin (0.15% final concentration) was co-polymerized with non-reducing, denaturing SDS-PAGE (Laemmli, 1970).

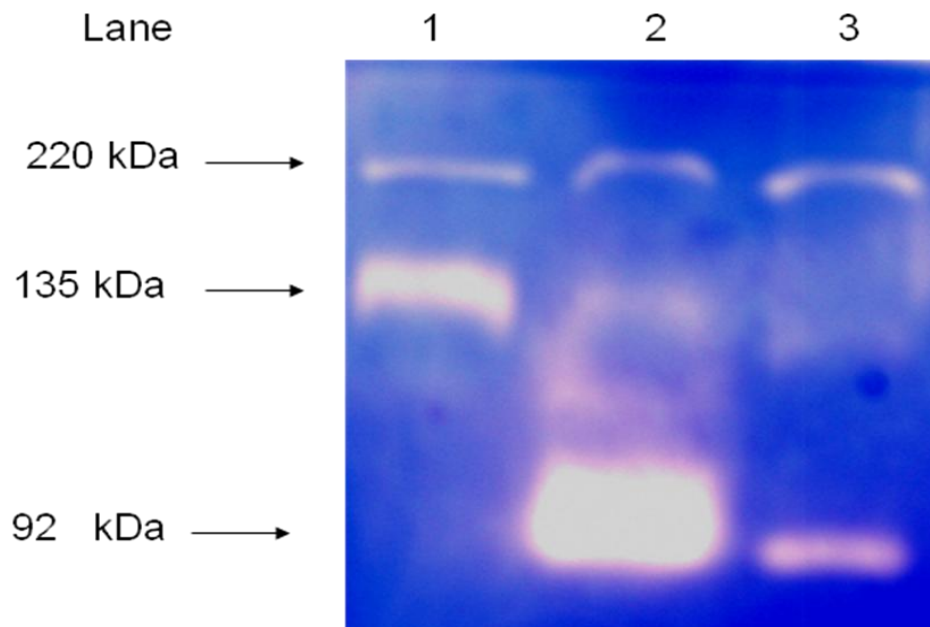


Figure 2: Gelatin zymography of human capillary urinary matrix metalloproteinases of dogs

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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 200 ml of renaturation solution (2.5% Triton –X 100) for 3 hours on a mechanical shaker with mild agitation. Then, the gel was developed by incubating the gel in developing buffer (10 mM CaCl₂, 0.15 M NaCl, 50 mM Tris, 0.02% Brij-35 pH 7.5) for 18 hours at 37°C and the gels were 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, the bands appear clear.

Calibrations of gelatin zymograms were carried out with human capillary blood gelatinases as standards (Makowski and Ramsby, 1996).

Calibration of Gelatin Zymograms with Human Capillary Blood Gelatinase Standards

The procedure suggested by Makowski and Ramsby (1996). A drop of human capillary blood (15-20 µl) was obtained by finger stick puncture and placed in a tarred polypropylene tube and mixed with 20 volumes of non-reducing Lamelli sample loading buffer (0.0625 M Tris–HCl, 2% SDS, 10% glycerol and 0.01% Bromophenol blue, pH 6.8). The mixture was then vortexed for 30 seconds and stored at -20°C. This preparation was found to be stable for at least 3 months. 5 µl of this solution was loaded to 8% resolving gel during gelatin zymographic procedure, as described earlier. The MMP-9 and MMP-2 present in this human capillary blood is used as standards, to calibrate the gelatinases present in canine mammary tumor tissue and blood plasma collected from the dogs affected with mammary tumor.

RESULTS

Determination of Total Mmp-9 Activity in Gelatin Zymography

Total MMP-9 activity was determined by the ability of the enzyme to degrade a known amount of gelatin co-polymerized with the resolving gel in SDS-PAGE. 150 mg% final concentration of gelatin was used in the resolving gel. The actual amount of gelatin present in the resolving gel could be determined by multiplying the concentration of the gelatin solution used by the volume of the gelatin solution taken in the resolving gel. From the total amount of gelatin present in the resolving gel, amount of gelatin present in q square centimeter of the gel could be calculated by dividing the total amount of gelatin present in the resolving gel by the total area of the resolving gel. When the MMP-9 present in the sample was made to separate in SDS-PAGE and subsequently renatured and developed, the gelatinases present in the resolving gel was degraded by the different forms of MMP-9 viz., 92 kDa, 135 kDa, and 220 kDa forms. This degradation of gelatin resulted in a clear zone visible on staining with Coomassie blue. The total area, degraded by individual forms of MMP-9 was individually measured in centimeters. From the area degraded by the individual enzyme, total amount of gelatin degraded by the individual enzyme could be calculated, then, total activity of MMP-9 was determined by summing up of the individual enzyme activities and multiplying the value by the total volume of the sample taken and the dilution factor.

Preparation of Polyclonal Antibodies against Canine Mmp-9 and Immunoblotting

The solutions containing MMP-9 activity were pooled from the gelatin sepharose chromatography and lyophilized at -80°C. 600 mg of the lyophilized, purified MMP-9 from canine mammary tumor tissue was subjected to SDS-PAGE on a preparative 6% polyacrylamide gel. The Coomassie brilliant blue stained protein bands were identified as MMP-9 with help of human capillary blood MMP-9 used as standard. The MMP-9 bands were cut from the gel and homogenized in a buffer solution of 0.05 M Tris-HCl pH 7.6 containing 0.15 M NaCl and 0.08% SDS. The homogenized material was centrifuged at 12000 rpm and the supernatant was collected and used for immunization. 0.5 ml of the clear homogenate was emulsified with an equal volume of complete Freund's adjuvant and injected intradermally into Newzealand white rabbits. Three boosters were given with Freund's incomplete adjuvant on day 14, 21 and 30th day of first injection. Test bleeding was collected on 42nd day and the presence of antibody was checked by AGPT. Then, the blood was collected from heart venepuncture of rabbits and serum was separated and used as polyclonal serum for ELISA and western blotting.

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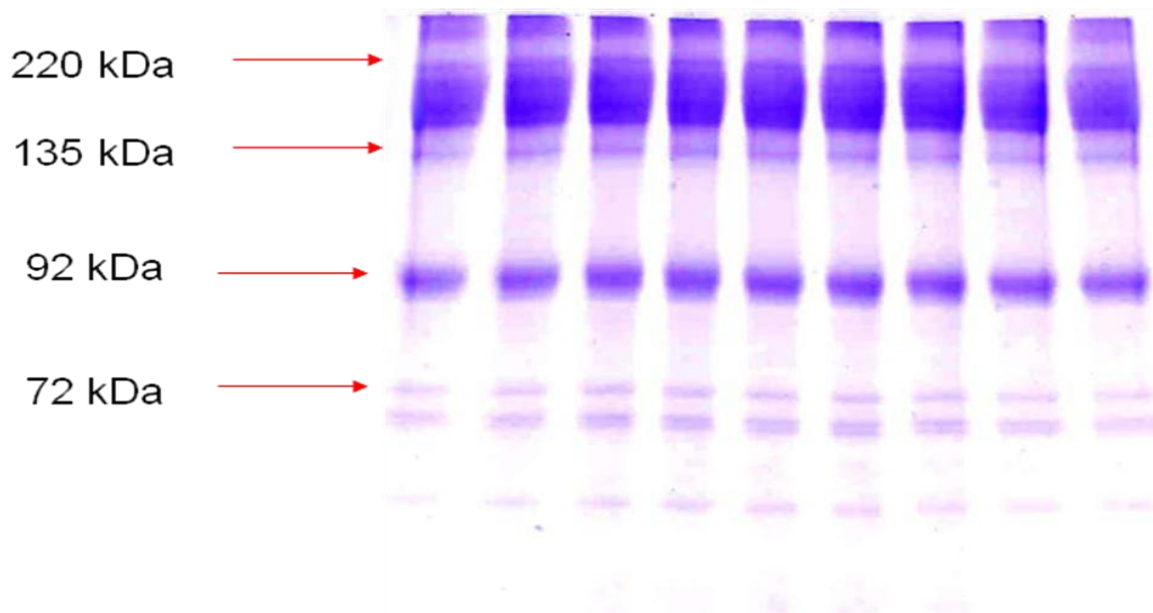


Figure 3: Preparatory SDS-PAGE of matrix metalloproteinases of dogs

Immunoblotting

Immunoblotting was carried out by the method suggested by Towbin *et al.*, (1979). The samples collected from the three chromatographic processes were subjected to SDS-PAGE, as described earlier. Then, the gel containing the separated proteins was equilibrated with transfer buffer for 5 minutes. Then, transfer of proteins to the 0.45 μ m Nitrocellulose membrane (Sigma) was carried out in 1x transfer buffer (0.025 M Tris, 0.192 M glycine pH 8.3 and 20% methanol) at 210 mA current for a period of two and half hours.

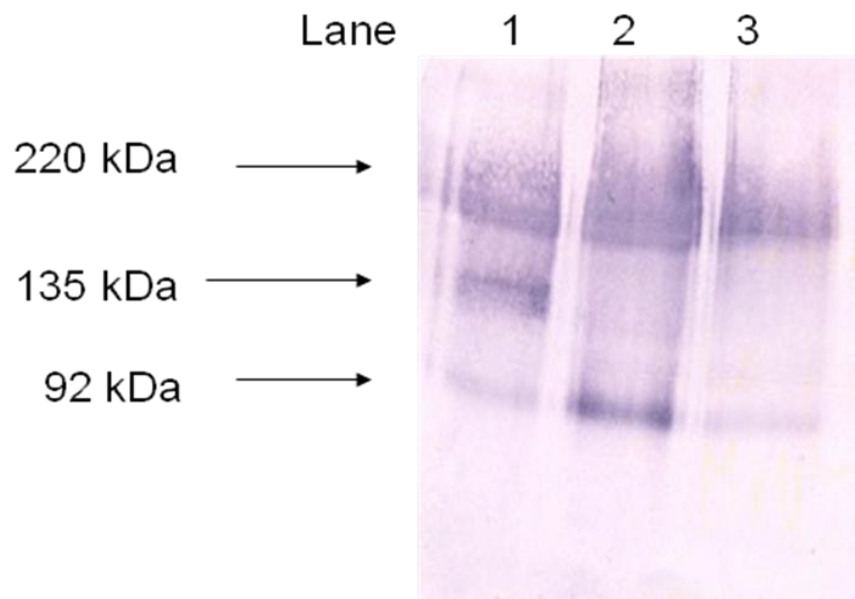


Figure 4: Western blotting of matrix metalloproteinases of dogs

Then, the membrane was put in 5% skimmed milk powder solution at 37⁰C for 2 hours with gentle constant agitation. 3 to 4 washings were given with each 25 ml of PBS- Tween (137 mM NaCl, 2.7 mM

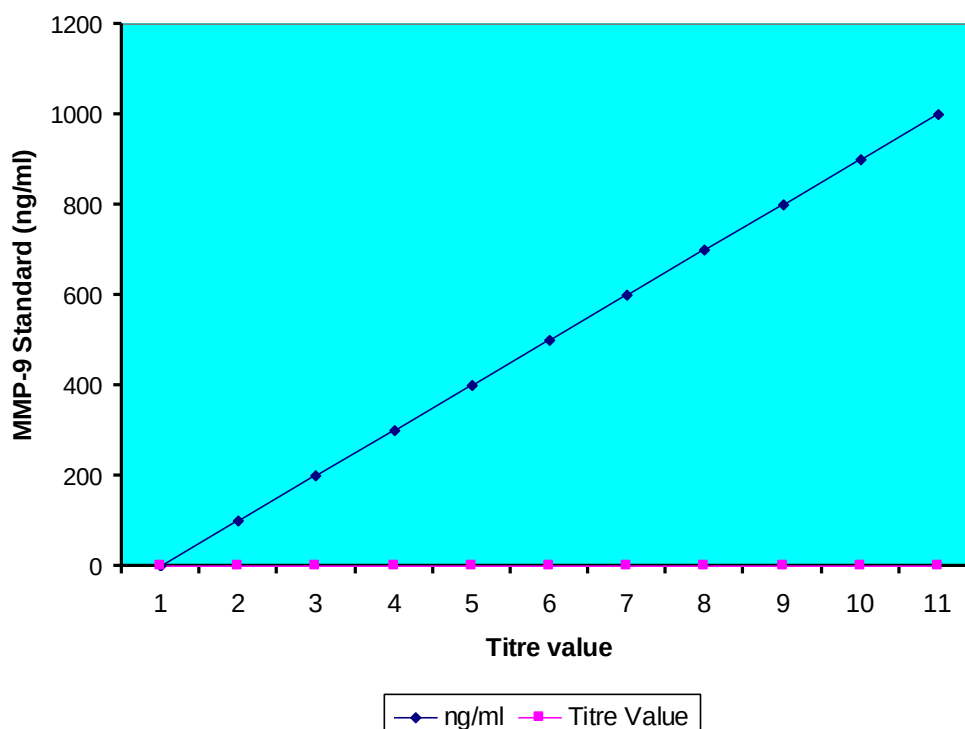
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KCl, 100 mM Na_2HPO_4 , 2 mM KH_2PO_4 and 0.05 Tween 20, pH 7.4) in a period of 30 minutes. Primary Antibody (antibody developed in rabbits against gelatin sepharose eluents containing MMP-9) was applied at 1:2000 concentration to the membrane and incubated overnight at 4°C . Then, washing with PBS was repeated. Secondary antibody at 1:2000 dilutions of goat anti-rabbit immunoglobulins fractions were applied to the membrane and incubated for one hour at 37°C with gentle and constant agitation. Then, washing with PBS was repeated. Rabbit anti-goat –alkaline phosphatase conjugate at a dilution of 1:2000 was added and incubated for one hour at 37°C with gentle agitation. Washing process was repeated. Then substrate buffer(0.1M Tris pH 9.5, 0.1 M NaCl and MgCl_2) containing 44 μl of 5% NBT and 88 μl of 5% BCIP was applied and the bands were allowed to develop and the reaction was stopped by adding distilled water. The membranes were air dried and stored at -20°C for future use.

Indirect Elisa of Mmp-9 in Sera and Tissue Homogenate Samples Collected From Dogs Affected With Mammary Tumor

The method suggested by Perlman and Engvall (1971) was followed with minor modifications. Optimum concentrations of conjugate and developing reagent were determined by cross dilution analysis. 50 μl of the serum and tissue homogenate samples were diluted optimally in coating buffer (0.05 M carbonate-bicarbonate buffer pH 9.6) and incubated at 4°C overnight in a humidified chamber. After washing the plates four times with PBS containing 0.05% Tween-20, blocking of unbound sites was carried out with 5% skimmed milk powder in PBS-T at 37°C for 3 hours with mild agitation. Washing procedures were repeated. The plates were incubated for 2 hours with appropriate dilutions of HRPO-conjugate antirabbit immunoglobulins (50 μl /well). Unbound conjugates were washed thoroughly with PBS-T and the color reaction was developing with O-Phenyl diamine at 37°C for 15 minutes. The reaction was stopped by adding 50 μl of 3 M sulphuric acid to each well. The intensity of color reaction was read at 492 nm using an ELISA reader. The human standard MMP-9 (sigma) was diluted serially and the absorbance was read at 492 nm.

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RESULTS

In gelatin zymography, both tumor and normal dog urine samples possessed all the three MMP-9 forms as clear bands at Mr of 220, 135 and 92 kDa (fig. 1). The relative distribution among these three isoforms of MMP-9 varied between the tumor and normal dog urine samples. The tumor dog urine samples showed highest activity of 92 kDa form of MMP-9. The other 2 bands, 135 and 220 kDa were also more expressed in tumor dog urine samples than that of normal urine samples of dogs. There was 11.18 times increase in the specific activity of MMP-9 in urines samples of canine mammary tumor, comparing to the normal dog urine samples. The Western blot analysis confirmed these three forms were MMP-9 (fig. 2)

Table 1: MMP-9 activity in gelatin zymogram

	Nomral dog urine	Tumor dog urine
Total protein (mg%)	0.012±0.002	0.0213 ±0.006
Total activity (units)	2.123 ±0.010	42.126±0.113
Specific activity	176.91±5.0	1,977.75±18.83

The indirect ELISA revealed that the mean level of MMP-9 activity in normal dog urine sample was 0.016 ng/ml and it was 0.281 ng/ml in the case of mammary tumor affected dogs. The increase in the level of MMP-9 was more than 18 times in the urine samples of canine mammary tumor. Statistically, the difference was highly significant ($P < 0.0001$) between the level of MMP-9 in urine samples of tumor dogs and that of normal dogs.

DISCUSSION

In the present study, three forms of MMP-9 (92, 135 and 220 kDa) were detected in the urine samples collected from the normal as well as tumor bearing dog urine samples. The level of MMP-9 activity correlated positively with the disease status of cancer patients. Moses *et al.*, (1998) reported 150,000, 92,000 and 72,000 gelatinolytic enzymes present in the urine samples of human cancer patients. Yan and co-workers (2001) reported that apparent molecular masses of 65,000, 86,000 and 125,000 bands having major MMP activity were detected on gelatin zymogram and 65 kDa being the MMP-2 and 86 kDa and 125kDa could be MMP-9 NGAL complex and no detectable MMP-9 activity was found in the urine from normal controls.

REFERENCES

- Heussen C and Dowdle EB (1980).** Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Analytical Biochemistry* **102** 196-202.
- Laemmli UK (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680-685.
- Lana SE, Ogilvie GK, Hansen RA, Powers BE, Dernel WS and Withrow SJ (2000).** Identification of matrix metalloproteinases in canine neoplastic tissue. *American Journal of Veterinary Research* **61**(2) 111-114.
- Loukopoulos P, Mungall BA, Straw RC, Thornton JR and Robinson WF (2003).** Matrix metalloproteinase-2 and -9 involvement in canine tumors. *Veterinary Pathology* **40** 382-394.
- Makowski GS and Ramsby ML (1996).** Calibrating gelatin zymograms with human gelatinase standards. *Analytical Biochemistry* **236** 353-356.
- Moses MA, Wiederschain D, Loughlin KR, Zurakowski D, Lamb CC and Freeman MR (1998).** Increased incidence of matrix metalloproteinases in urine of cancer patients. *Cancer Research* **58** 1395-1399.
- Perlman P and Engvall E (1971).** Enzyme linked immunosorbent assay (ELISA): quantitative assay for immunoglobulin. *Immunochemistry* **8** 871.

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Towbin H, Staehelin T and Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitro cellulose sheets: procedure and some applications. *Proceedings of National Academic Sciences (USA)* **76**(9) 4350-435.

Yan L, Borregaard N, Kejeldsen L and Moses MA (2001). The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinaseB/MMP-9 and neutrophil gelatinase associated lipocalin(NGAL). *The Journal of Biological Chemistry* **276**(40) 37258-37265.

Yokota H, Kumata T, Taketaba S, Kobayashi T, Moue H, Taniyama H, Hiriyama K, Kagawa Y, Itoh N, Fujita O, Nakade T and Yuasa A (2001). High expression of 92 kDa type IV collagenase (matrix metalloproteinase-9) in canine mammary adenocarcinoma. *Biochimica et Biophysica Acta* **1568** 7-12.