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Research Article

CLONING AND EXPRESSION OF 1080 BP CATALYTIC DOMAIN OF MMP-9 FROM CANINE MAMMARY TUMOR

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ABSTRACT
204 bp propeptide domains, 1080 bp catalytic domain and 178 bp hemopexin domain were cloned and sequenced from the mammary tumor of dogs. All the three cloned domains had close relationship with the full length MMP-9 sequence already reported. The cloned sequences had higher sequence homology with the MMP-9 sequences already reported from other species. The catalytic domain was cloned in the expression vector and the transformation was confirmed by culture PCR and plasmid isolation. The medium level of expression of catalytic domain recombinant protein was observed through SDS-PAGE and western blot.

Key Words: *MMP-9, Catalytic Domain, Expression Canine Mammary Tumour*

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that play important roles in morphogenesis, tissue remodelling, reproduction and control of cell behaviour under physiological conditions (Hansen *et al*., 1993; Jeziorska *et al.,* 1996 and Dubois *et al.,* 2002). In excess, they may participate in accelerated pathological destruction of extracellular matrices associated with various connective tissue diseases and cancer cell invasion and metastasis (Declerck, 2000 and Steen *et al*., 2002). Matrix metalloproteinae-9 (MMP-() is the largest member among the at least 18 mammalian matrix metalloproteinase enzyme family. The role of MMP-9 in malignancy has been well documented in several tumours affecting human beings (Davies *et al.,* 1993). The level of expression of MMP-9 is higher in benign mammary tumor than in normal canine mammary tissue, but it is present in highest in malignant mammary tumor (Hirayama *et al.,* 2002). Higher levels of proMMP-9 and proMMP-2 and active MMP-2 were detected in most canine tissues (Loukopoulos *et al.,* 2003). Several reports are available about the cloning of human MMP-9 (Wilhelm *et al.,* 1989 and Huhtala *et al.,* 1991). Kroger and Tschechn (1991) reported the cloning of the catalytic domain (AA residue 113-450) of the human 92 kDa gelatinase in pET-12b vector and expressed in E.coli. Kridel *et al.,* (2001) reported cDNA encoding for the catalytic domain of human MMP-9 by PCR and cloned into pCDNA3 and transfected the HEK 293 cells by electroporation and the catalytic domain of MMP-9 was purified from the conditioned medium by gelatin sepharose chromatography.

A full length cDNA of MMP-9 was obtained from canine mammary adenocarcinoma (Yokota *et al.,* 2001). Campbell *et al.,* (2001) reported the molecular cloning of the canine MMP-9 promoter. The present study was aimed to clone and expression of the catalytic domain of the canine MMP-9 from the RNA isolated from the mammary tumor tissue, collected from the clinical cases of canine mammary tumor.

MATERIALS AND METHODS

A total of 10 fresh canine tissue samples, each weighing about 50 g were collected at the time of surgery carried out in the polyclinic, IVRI, Izatnagar. Tissue samples included tumor and tissues surrounding the tumor. Samples were carried out hygienically in ice and were stored at -70^0C, until processed. Part of each tissue sample was fixed in 10% neutral buffered formalin, routinely processed and embedded in

paraffin blocks. The sections of the tissue were stained with hemotoxylin and eosin (H & E) for the purpose of diagnosis and sent for histopathological examination.

Identification of MMP-9

The method suggested by Lana and Coworkers (2000) was followed to identify the presence of MMP-9 activity in the tumor tissue. Samples were homogenized using a mechanical homogenizer in 4 ml of cold 50 mM Tris-buffered saline. Samples were centrifuged for 10 minutes at 1500 x g at 4^0C and the supernatants were harvested and preserved at -70^0C until further use. Protein estimation was done by the method of Lowry *et al*., (1951).

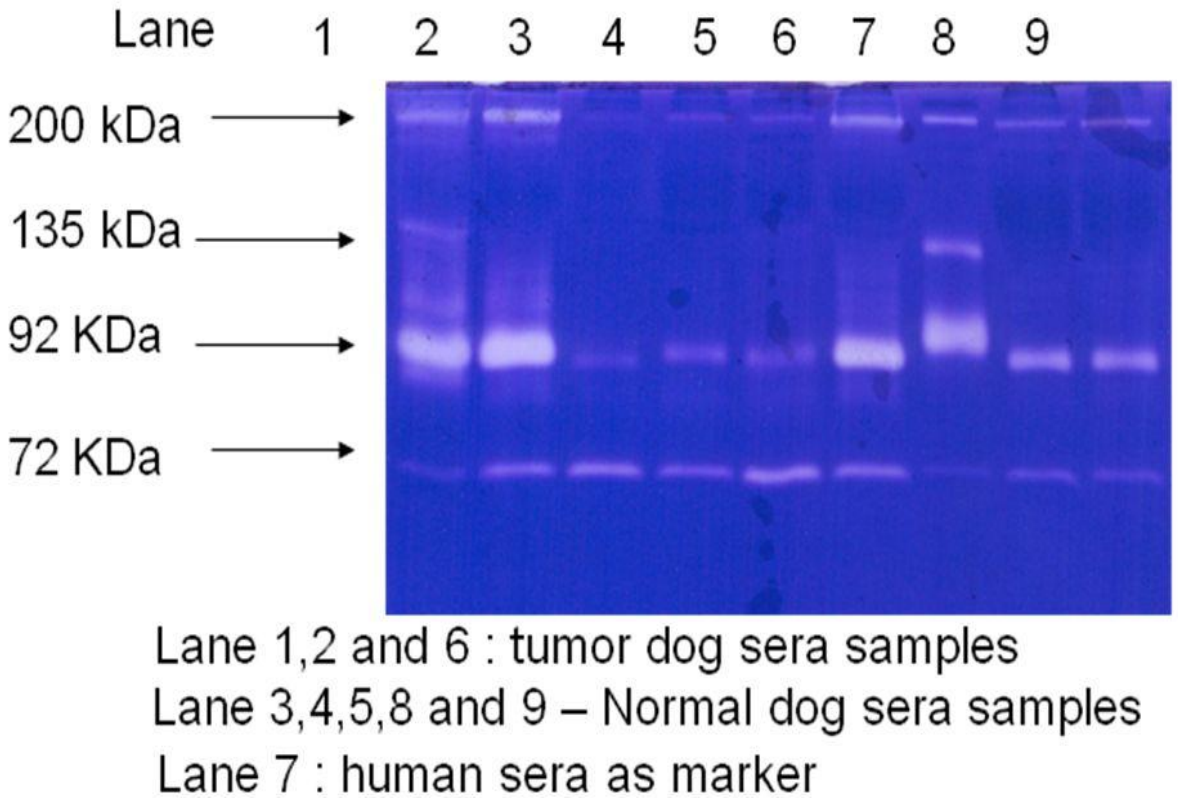


Figure 1: Gelatin zymography of matrix metalloproteinases of canine mammary tumour

Isolation of Total Rna from Canine Mammary Tumor

Total RNA was isolated from the mammary tumor tissue by following the protocol given by QIAGEN Rneasy mini Hand book. The reagents were supplied by QIAGEN.

Designing of Oligonucleotide Primers

1080 bp catalytic domain of the canine MMP-9 was aimed and the primers were developed and obtained from Imperial biomed (USA) as desalted oligonucleotides. The details of the primers used were, forward primers 5'-CGGCGGATC……3' and the reverse primer was 5'-CGGC…..3'. RE sites were incorporated for BamHI, XBAI and SalI along with appropriate overhanging sequences on forward and reverse primers respectively for directional cloning of the amplified product. The primers were diluted with Rnase free distilled to a concentration of 20 picomoles.

RT-PCR of RNA Isolated from Canine Marry Tumor

The first strand synthesis of cDNA was standardized as 25 microliters reaction mixture. The conditions and reagents used were as follows:

Reagents	**Volume (µl)**
Rnase free water	3.75
RNA	10
Reverse primer (20 picomoles)	1
The mixture was incubated at 70 ^{O}C for 10 minutes	
5 X RT buffer	5
Rnase inhibitor(40U)	0.25
10 mM dNTP mixture	4
The mixture was incubated at 37 ^{O}C for 5 minutes	
MMLV-RT enzyme (100U)	1
The mixture was incubated at 42 ^{O}C for 1 hour	

MMLV was inactivated by heating the tube at 70O C for 10 minutes. The cDNA thus obtained was used for amplification by PCR.

Polymerase Chain Reaction

The PCR was carried out to amplify the catalytic domain in a final volume of 25 microliters mixture by using Taq DNA polymerase and the thermocyclic conditions are as follows

Initial denaturation	95^{O}C for 2 min	1 cycle
Denaturation	95^{O}C for 30 sec	32 cycles
Primer annealing	60^{O}C for 30 sec	32 cycles
Primer extension	72^{O}C for 90 sec	32 cycles
Final extension	72^{O}C for 10 min	1 cycle

The amplified PCR products was checked by submarine gel electrophoresis using 1.5% agarose mixed with ethidium bromide at 100 volt for 1 hour with 100 bp plus DNA ladder (MBI fermentas) ran simultaneously on a parallel well.

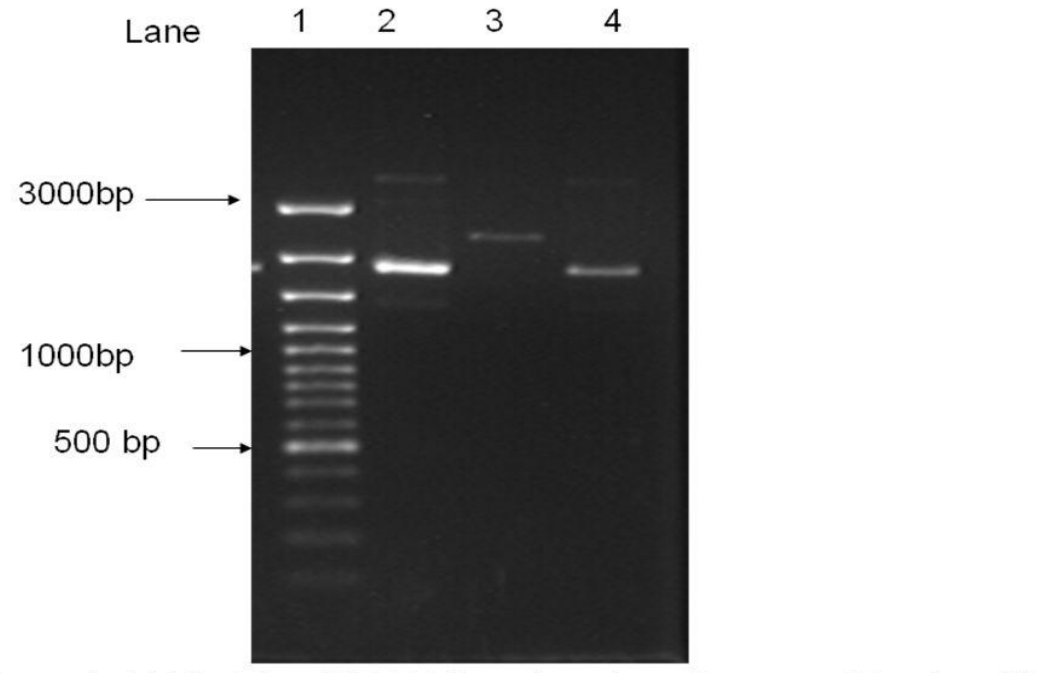


Figure 2: PCR products of matrix metalloproteinase-9 (MMP-9) catalytic domain

The PCR product of 1080 bp catalytic domain was mixed with 6x gel loading buffer and loaded in a preparative 1% agarose gel. The electrophoretic process was continued at 50 V for 2 hours in 1x TAE buffer. The gel piece containing 1080 bp catalytic domain was marker using UV light and cut with a sharp blade and taken into a 1.5 ml microfuge tube and the PCR product was eluted from the agarose gel by adopting the procedure and chemicals supplied by QIAGEN.

The gel eluted PCR product was ligated to pTZ57R cloning vector in a total volume of 15 microliters mixture and incubated at 22OC for overnight in water bath shaker and transformation of the recombinant plasmids were carried out into the *E.coli* DH5α competent cells. The transformants were selected by blue-white screening and transformed cells were checked for the presence of recombinant plasmids by plasmid isolation. Plasmids were isolated by the protocol given by omega biotech EZNA plasmid miniprep kit.

The recombinant plasmids were checked for the presence of the desired insert by RE digestion using BamHI and XbaI. The digested product was checked for their respective size of 1080 bp by running 1.5% agarose gel at 50V for 2 hours with 100 bp plus ladder and visualized under uv light on a transilluminator. Recombinant clones with insert of the expected size were selected for sequencing and sent to Delhi University South campus for sequencing.

cDNA sequence of the catalytic domain were aligned by clustal method with the earlier reports of MMP-9 sequences of different species using megalign programme of Lasergene software (DNA star Inc., USA). The sequences used for comparison were collected form EMBL database as well as from NCBI website (Human: NM_004004; Rat:NM-03155; Mouse: NM-013599; Bovine NM_174744; Rabbit :D26514; Pig:DQ132879; Canine : AB006421). The amino acid sequence was deduced and aligned for analysis with the MMP-9 transcripts of other species using the same computer software.

Directional Cloning of the Catalytic Domain into pPROEX HTB Expression Vector

The catalytic domain insert (1080bp) was released from the cloning vector by double digest with BamHI and XbaI. The released product was gel purified and ligated with the expression vector pPROEX HTb which was already digested with the same pair of restriction enzymes. The protocols for ligation and transformation were as same as for cloning process, as described earlier. The transformants were checked for the presence of recombinant pPRO EX HTb plasmids by plasmid isolation and agarose gel electrophoresis. The presence of catalytic domain in the transformed cells was checked by culture PCR and the insert release by restriction enzyme BamHI and XbaI.

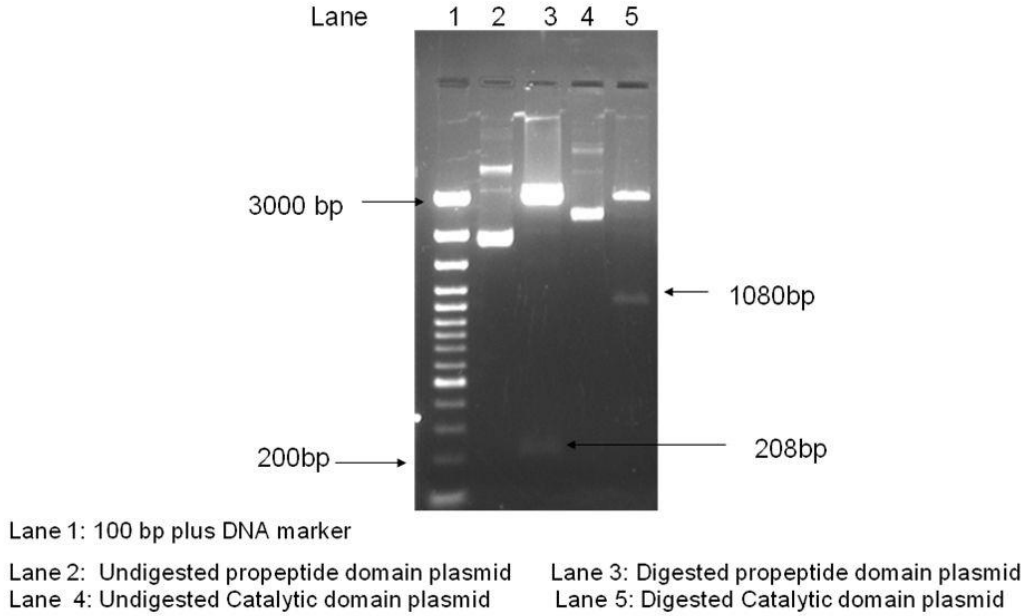


Figure 3: Digested PCR products of matrix metalloproteinase-9 (MMP-9) catalytic domain

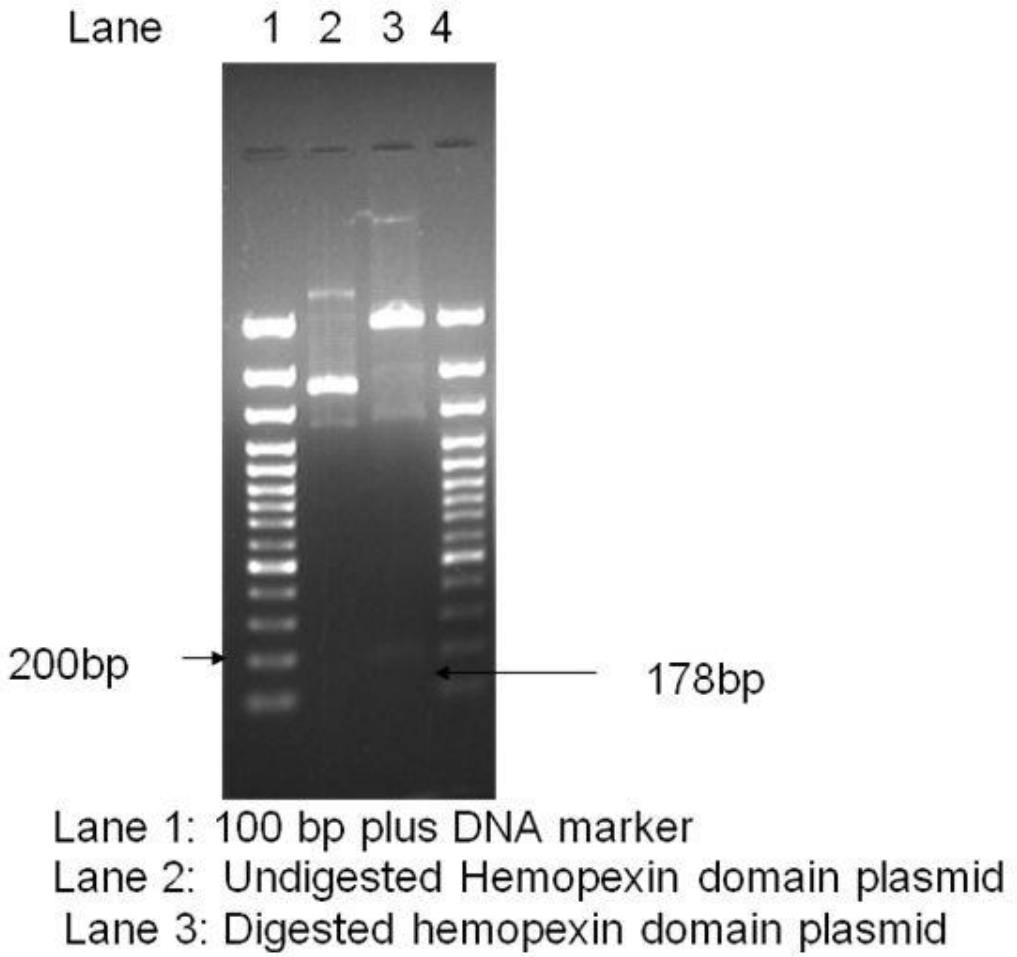


Figure 4:

GGTACCTCGCGAATGCATCTAGATTCGGCGGATCCTGGCACCACAACGACATCACTTACTGGAT
ACAAAACTACTCGGAAGACTTGCCCCGCGACGTGATCGACGACGCCTTTGCCCGAGCCTTCGCG
GTCTGGAGCGCGGTGACGCCGCTCACCTTCACTCGCGTGTACGGCCCCGAAGCCGACATCATCA
TTCAGTTTGGTGTTAGGGAGCACGGAGATGGGTATCCCTTCGATGGGAAGAACGGGCTTCTGGC
TCACGCCTTTCCTCCCGGCCCGGGCATTCAGGGAGACGCCCACTTCGACGACGAGGAGTTATGG
ACTCTGGGCAAGGGCGTCGTGGTTCCGACCCACTTCGGAAACGCAGATGGCGCCCCCTGCCACT
TCCCCTTCACCTTCGAGGGCCGCTCCTACTCGGCCTGCACCACGGACGGCCGCTCCGATGACAC
GCCCTGGTGCAGCACCACGGCCGACTATGACACCGACCGTCGGTTCGGCTTCTGCCCCAGCGAG
AAACTCTACACCCAGGACGGCAATGGGGACGGCAAGCCCTGCGTGTTTCCGTTCACCTTCGAGG
GCCGCTCCTACTCCACGTGCACCACCGACGGCCGCTCGGACGGCTACCGCTGGTGCTCCACCAC
CGCCGACTACGACCAGGACAAACTCTACGGCTTCTGCCCAACCCGAGTCGATTCCGCGGTGACC
GGGGGCAACTCCGCCGGGGAGCCGTGTGTCTTCCCCTTCATCTTCCTGGGCAAGCAGTACTCGA
CGTGCACCAGGGAGGGCCGCGGAGATGGGCACCTCTGGTGCGCCACCACTTCGAACTTTGACAG
AGACAAGAAGTGGGGCTTCTGCCCGGACCAAGGATACAGCCTGTTCCTTGTGGCCGCCCATGAG
TTCGGCCACGCGCTGGGTTTAGATCATTCATCGGTGCCAGAAGCGCTCATGTACCCCATGTACA
GCTTCACCGAGGGCCCCCCCCTGCATGAAGACGACGTGAGGGGCATCCAGCATCTGTACGGTCC
TCGCCCTGAACCTGAGCCACAGCCTCCAACCGCCCCGCCCACCGCCCCGCCCACCGTCTGCGCT
ACTGGTCCTCCCTCTAGAGTCGACGCCGAATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGC
ATGCAAGCTTTCCCTATAGTGAGTCGT

Figure 5: DNA sequence of catalytic domain of matrix metalloproteinase-9 (MMP-9) catalytic domain

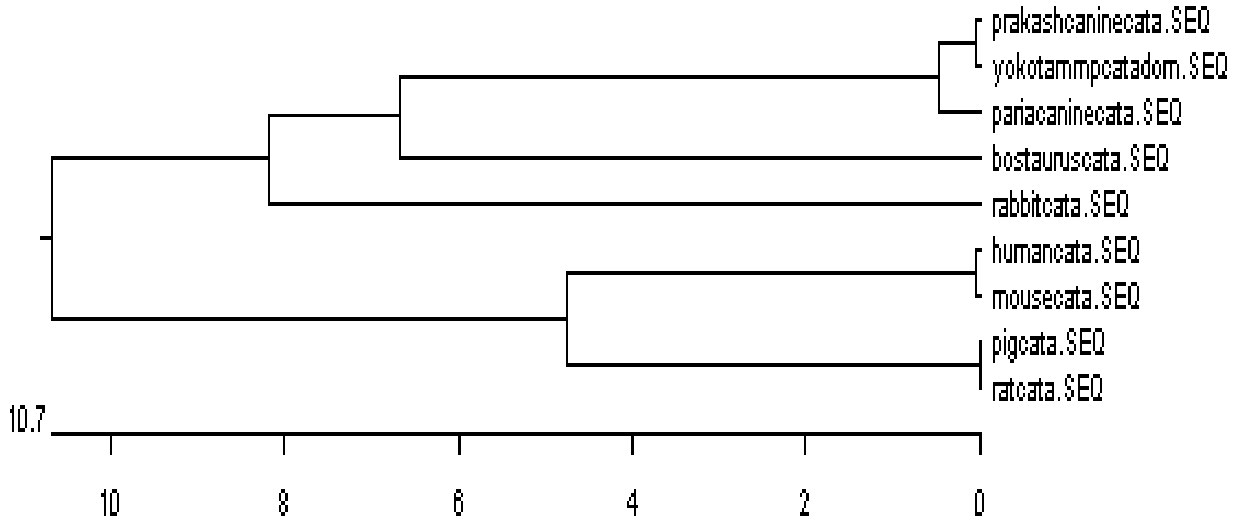


Figure 6: Phylogenetic relationship of matrix metalloproteinase-9 (MMP-9) catalytic domain

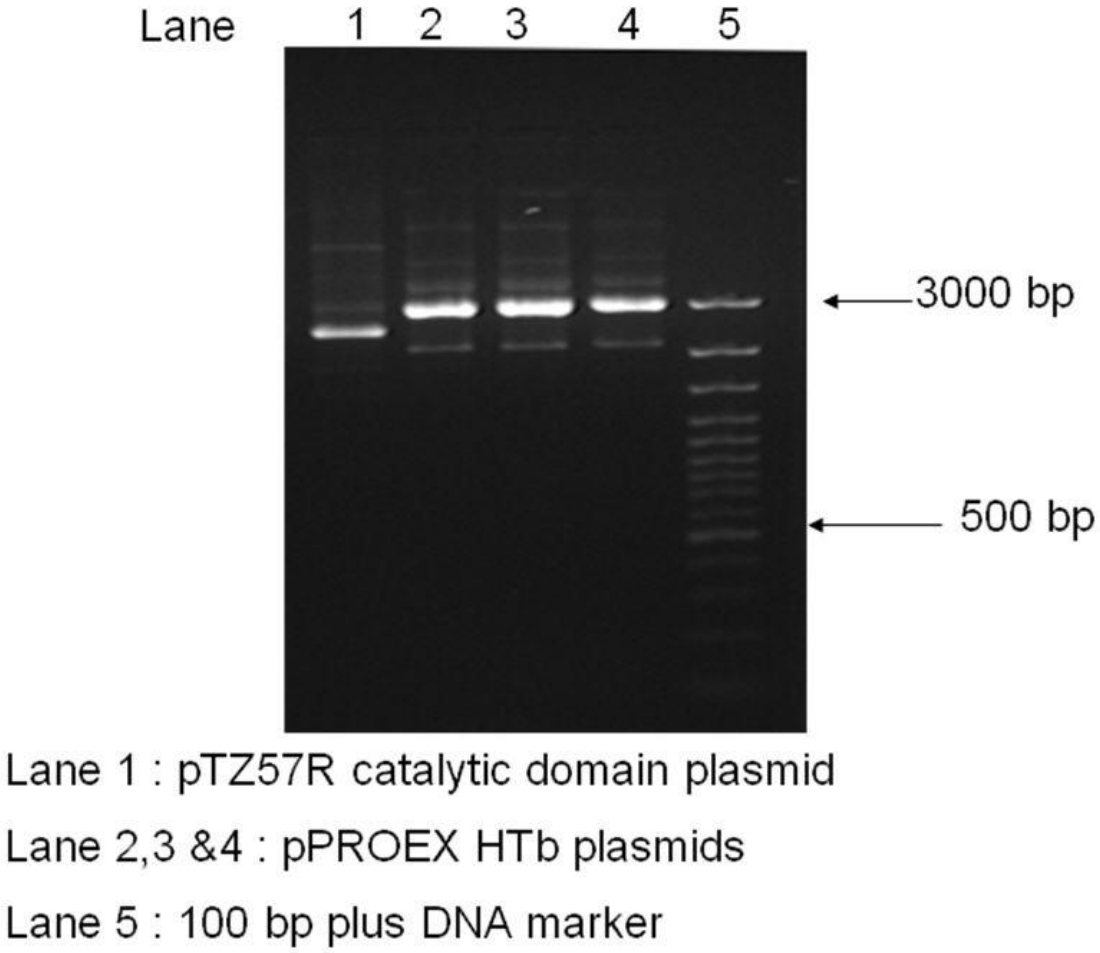
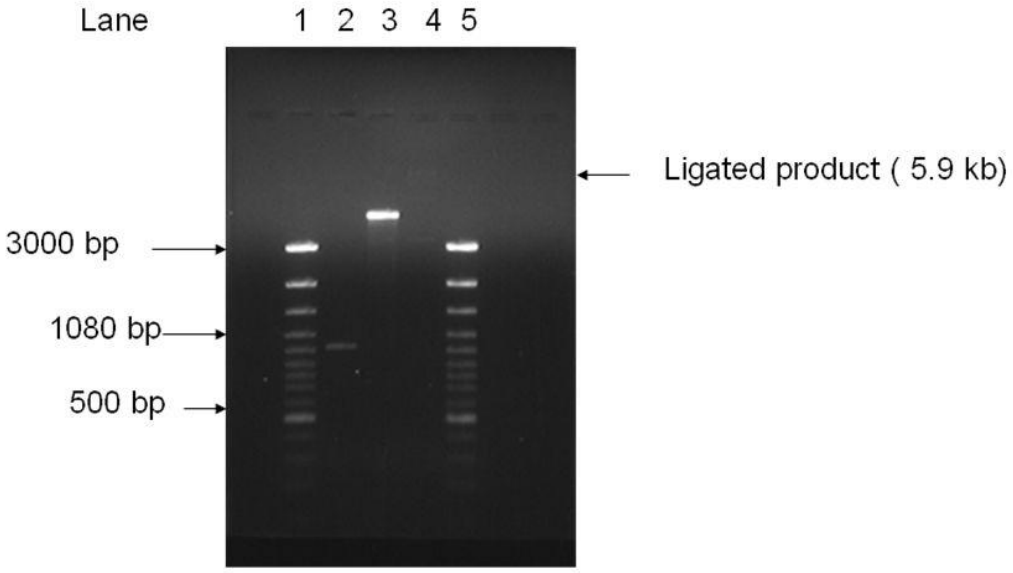


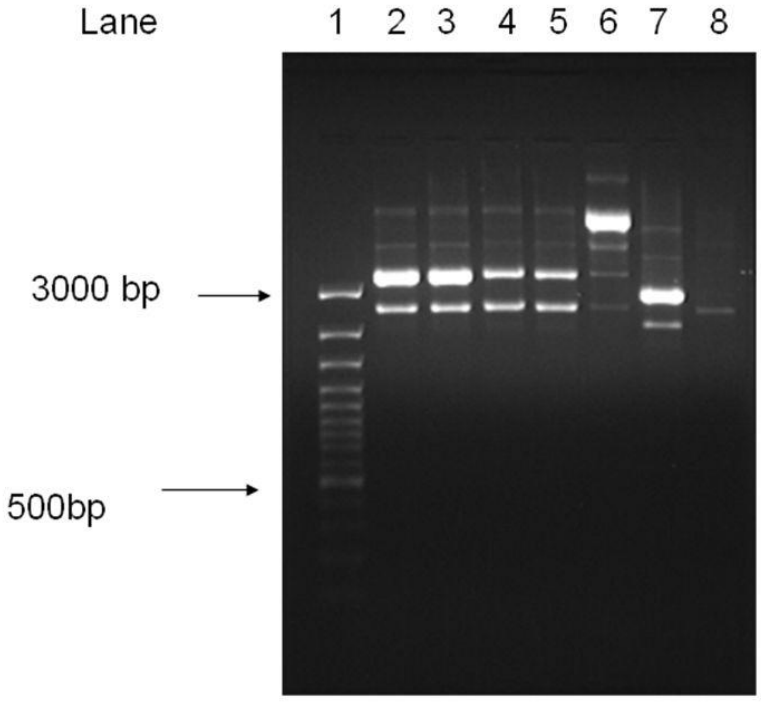
Figure 7



Lane 1 & 5 : 100 bp plus DNA marker Lane 2 : Catalytic domain DNA (1080 bp)

Lame 3 : RE digested pPRPEX HTb Lane 4 : ligated product

Figure 8



Lane 1: 100 bp plus DNA marker

Lane 2 – 8 : Plasmid isolated from the transformants

Figure 9

Induction of the Target Recombinant Protein Expression

Single colony of the transformants was inoculated with LB medium containing 50 microgram/ml of ampicillin. Then, subcultures were made with 5 ml of LB medium containing 50 microgram /ml ampicillin and the cells were grown to A_{600} of 0.5 and the culture was induced with IPTG (1mM final concentration) and incubated at 30^{O}C in a shaking incubator at 225 rpm. After 6 hours of post-induction incubation, one ml of the culture was transferred into 1.5 ml eppendorf tube and the cells were pelleted by centrifuging at 12,000 rpm for 5 min and the pellet was stored at -20^{O}C. The samples of bacterial pellets collected at zero hour of induction and the DH5α cells containing no recombinant plasmids (grown for equal time) were taken in 50 microliters of 2X SDS-PAGE sample loading buffer (Lamelli, 1970) and 5 microliters of β-mercaptoethanol and urea at a final concentration of 8 M were added and the mixture was vortexed thoroughly and kept in a boiling water bath for 5 minutes. The bacterial cell proteins were separated in a SDS-PAGE gel containing 12% resolving and 3.5% stacking gel. Electrophoretic run was carried out at 100v, until the tracking dye reached the bottom of the resolving gel. The gel was stained with Coomassie blue and destained with destaining solution, as described by Lamelli, 1970.

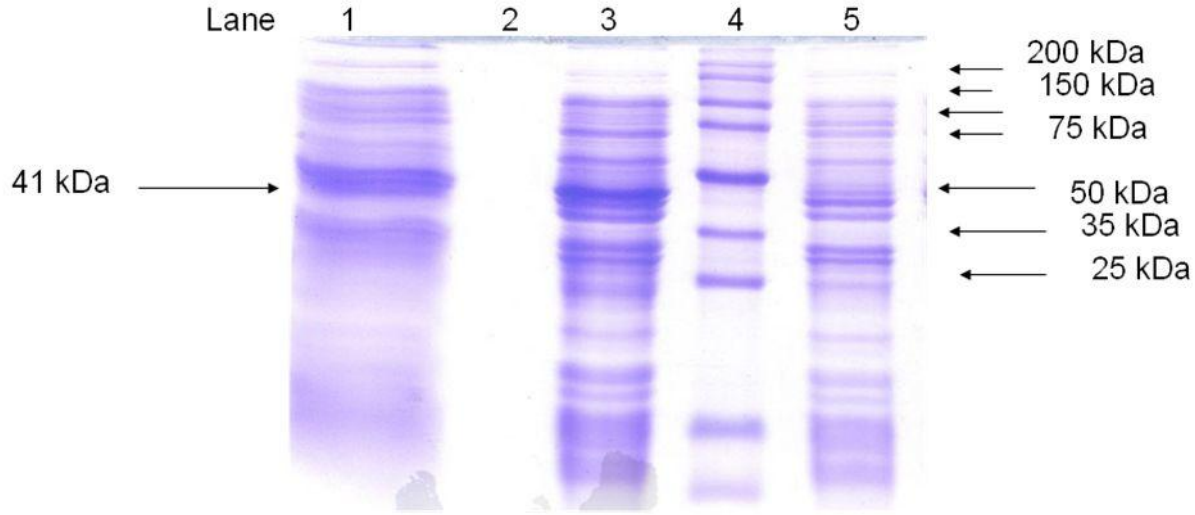


Figure 10: SDS-PAGE of over-expression of catalytic domain of matrix metalloproteinase-9 (MMP-9) catalytic domain

Western Blotting of the Recombinant Catalytic Domain Protein

The SDS-PAGE gel containing the induce cells and uninduced control cells was equilibrated in transfer buffer for 15 minutes. Then, electro transfer of the protein from the cell to the nitrocellulose membrane (NCM) was carried out at 2 mA per square cm of the gel for 1 hour at room temperature. Then the membrane was blocked by using blocking solution containing 5% skim milk powder, overnight at 4^{O}C. The blot was washed for 30 minutes with PBS-T and the presence of recombinant proteins containing catalytic domain of canine MMP-9 was detected by the antibodies raised in rabbits against the canine

MMP-9 isolated from the canine tumor tissue and also with the commercially available anti-human MMP-9 antibodies, procured form Sigma co., USA. After washing, the blot wa treated with anti-rabbit HRPO conjugate (1:4000) in PBS-T and incubated at 37^{O}C for 2 hours. The diamino benzidine (DAB) was added and the colour was allowed to develop in dark at room temperature. The reaction was stopped by washing the membrane with distilled water.

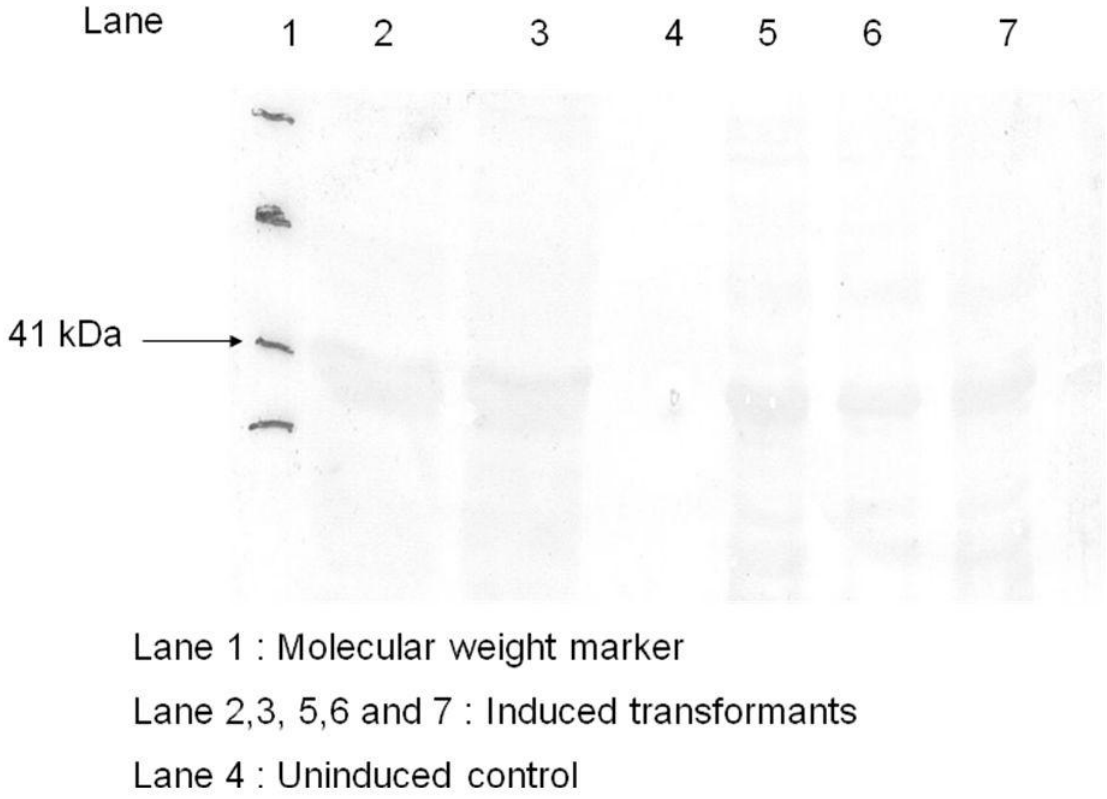


Figure 11: Western blot of matrix metalloproteinase-9 (MMP-9) catalytic domain

RESULTS

The total cellular RNA obtained from 30 mg of canine mammary tumor tissue was 0.236 micrograms and the concentration of RNA was 0.236 microgram/ml. The ration of A260/A280 was 1.86. The integrity was assessed by agarose gel electrophoresis. Two sharp bands corresponding to 28S and 18S and a fainter 5.8s ribosomal band were observed. There was no smearing towards the lower end of the gel. The intensity of 28S band was about two times brighter the 18S band. There was no smearing in the upper end of the gel also.

The RT_PCR product of expected size of 1`080 bp of catalytic domain was obtained. The product was very specific and devoid of any spurious amplification. To purify 100 microliters of the PCR reaction mix of the catalytic domain was run in LMP agarose. Then, the bands of interest were cut and elute in QIAquick gel extraction kit. Two microliters of the eluted product was checked and specific band of 1080 bp was observed. The gel purified PCR products were cloned in pTZ57R cloning vector (vector: insert ration as 1:3). Recombinant colonies appeared as white colonies. Plasmids from the recombinant clones were identified and the RE digestion of the recombinant plasmid yielded the release of 1080 bp catalytic domain on double digest with BamHI and XbaI.

The sequence analysis of the catalytic domain of canine mammary tumor tissue MMP-9 revealed that the present sequence had the following homologous percentage with the earlier reports as: canine (99.9%); rat

(77.5%); rabbit (81.3%); pig (77.5%); mouse (76.9); human (77.3) and cattle (86.0%). The phylogenetic analysis revealed that the catalytic domain sequence was very much closely related to the canine sequence of MMP-9, reported earlier.

The catalytic domain which was cloned in pTZ57R was isolated and directional cloning was carried out in pPROEXHTb vector. The positive clones were selected as whit colonies on LB agar plates containing ampicillin (50 microgram/ml). The presence of catalytic domain was confirmed by culture PCR and plasmids were isolated and subjected to RE digestion

In SDS-PAGE, thicker bands of expected size 40 kDa was observed in the induced cultures and the intense band was absent in the uninduced control cells. This suggested that the intense band could be of the recombinant catalytic domain of MMP-9, exhibiting a medium level of expression. In western blot, 40 kDa band was observed in all the induced cultures and it was absent in the control culture.

DISCUSSION

The identification of MMP-9 mRNA in mammary tumor tissue and the presence of MMP-9 transcript by reverse transcription PCR studies clearly demonstrated that biosynthesis of MMP-9 in tumor cells. Yokota *et al.,* (2001) reported that the amino acid sequence of the cDNA of the canine adenocarcinoma had homology with those of human (79.6%), rat (72.0%), rabbit (80.6%) and bovine (82.3%). MMP-9 has 75 to 85% sequence homology among rat, mice, rabbit, human and cattle. It is not unreasonable to assume that canine MMP would also have high sequence homology with human MMP-9 (Lana *et al*., 2000). Tanaka *et al*., (1993) reported that the entire length of mouse 105 kDa gelatinase was 3160 bp and it had 75% homology with human 92 kDa gelatinase activities. The level of expression of MMP-9 in the recombinant plasmids was found to be medium level, which may be due to the toxicity created by the catalytic domain in the *E.coli* system. Taken together, these results conclusively indicated that the purified enzyme is canine MMP-9 according to the following criteria: Molecular weight, specific inhibition by EDTA, pattern of activation products generated by APMA, binding to gelatin matrix and immunoreactivity. These studies suggest that MMP-9 might not only be good targets for antineoplastic therapy but may have clinical utility in identifying subgroups of patients at increased risk for recurrence. Although most studies have quantitated total enzyme levels to correlate with tumor invasiveness, the amount of activated enzyme present in a tumor sample may be important than evaluating the total enzyme.

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