

# Equine laminitis: increased transcription of matrix metalloproteinase-2 (MMP-2) occurs during the developmental phase

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## Summary

**Reasons for performing study:** The dysadhesion and destruction of lamellar basement membrane of laminitis may be due to increased lamellar metalloproteinase activity. Characterising lamellar metalloproteinase-2 (MMP-2) and locating it in lamellar tissues may help determine if laminitis pathology is associated with increased MMP-2 transcription.

**Objectives:** To clone and sequence the cDNA encoding lamellar MMP-2, develop antibody and *in situ* hybridisation probes to locate lamellar MMP-2 and quantitate MMP-2 transcription in normal and laminitis tissue.

**Methods:** Total RNA was isolated, fragmented by RT-PCR, cloned into vector and sequenced. Rabbit anti-equine MMP-2 and labelled MMP-2 riboprobe were developed to analyse and quantitate MMP-2 expression.

**Results:** Western immunoblotting with anti-MMP-2 detected 72 kDa MMP-2 in hoof tissue homogenates and cross-reacted with human MMP-2. Immunohistochemistry and *in situ* hybridisation detected MMP-2 in the cytoplasm of basal and parabasal cells in close proximity to the lamellar basement membrane. Northern analysis and quantitative real-time PCR showed MMP-2 expression significantly ( $P < 0.01$ ) elevated in laminitis affected tissues.

**Conclusion:** The lamellar pathology of laminitis is associated with increased transcription of MMP-2.

**Potential relevance:** Real-time PCR analysis of lamellar MMP-2 accurately monitors laminitis development at the molecular level and can be used diagnostically and for testing preventive strategies. Controlling increased MMP-2 transcription may ameliorate or prevent laminitis in high risk clinical situations. Our findings represent a warning to clinicians that the basement membrane lesion of laminitis is insidious and well under way before clinical signs are apparent.

## Introduction

As laminitis develops, the distal phalanx detaches progressively from the lamellar hoof wall and descends into the hoof capsule

causing structural damage, pain and a characteristic lameness. Laminitis histopathology shows a characteristic loss and disorganisation of lamellar basement membrane (Pollitt 1996; Pollitt and Daradka 1998). Both zymogen and activated matrix metalloproteinase-2 (MMP-2) are increased in homogenates of laminitis affected tissue implying that MMP-2 activity causes the basement membrane degradation of laminitis (Pollitt *et al.* 1998). MMPs are a group of zinc-dependent enzymes that, when activated, degrade extracellular matrix (ECM) and basement membrane (BM) components and the activity of MMPs (particularly the gelatinases MMP-2 and MMP-9) correlates strongly with BM destruction and the associated degree of malignancy and invasiveness of tumours (Goldfarb and Liotta 1986; Stetler-Stevenson 1990).

At the lamellar dermal/epidermal interface adhesion between epidermal basal cells and the underlying BM depends on a large number of intact anchoring filaments bridging the *lamina densa* of the BM to the basal cell plasmalemma. Anchoring filaments are composed of the glycoprotein laminin 5, a known substrate of MMP-2 (Gianelli *et al.* 1997). Furthermore, cleavage of laminin 5 by MMP stimulates epidermal basal cells to lift from their BM and migrate (Koshikawa *et al.* 2000). During laminitis development cleavage of anchoring filaments by activated MMP-2 may play a role in the BM dysadhesion that characterises the disease.

As a first step towards understanding the molecular biology of MMP involvement in the pathogenesis of laminitis, we sought to clone, sequence and quantitate the cDNA encoding equine MMP-2 in normal and laminitis affected hoof tissue. An additional aim was to investigate the cellular location, within lamellar tissue, of MMP-2 protein and its m-RNA using immunohistochemistry and *in situ* hybridisation respectively.

## Materials and methods

Lamellar tissues were harvested from normal ( $n = 4$ ) horses and from horses with laminitis ( $n = 18$ ) induced by alimentary carbohydrate overload (C.C. Pollitt and A.W. van Eps, unpublished data). Experimentation on horses was conducted according to The University of Queensland Animal Ethics Committee guidelines and

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TABLE 2: Percentage similarity of vertebrate MMP-2 sequences

	Rat	Man	Horse	Rabbit	Chicken
Mouse	99	95	95	94	82
Rat		95	94	93	82
Man			96	95	81
Horse				94	81
Rabbit					80

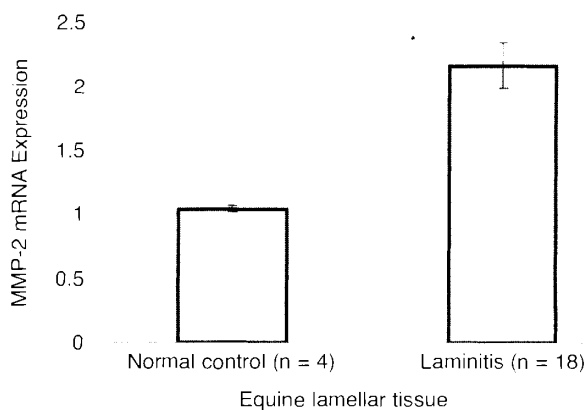


Fig 3: Graph showing the significantly different ( $P < 0.01$ ) mean values of MMP-2 expression between 4 normal hooves and 18 laminitis affected hooves.

tissues using *in situ* hybridisation. Protease treatment allowed probe penetration to the target nucleic acid. Tissue sections were hybridised over-night at 40°C with 50 ng/ml DIG-labelled equine-specific MMP-2 riboprobe. Following post hybridisation washes, MMP-2 RNA was detected with anti-DIG antibodies and chromogen<sup>†</sup>. For each assay, DIG-labelled sense probe, as well as 'no probe' reactions were negative controls.

## Results

The cDNA encoding the equine MMP-2 was cloned by the PCR cloning method. The entire coding sequence for the equine MMP-2 was derived using degenerate primers from highly conserved regions of known mammalian MMP-2 sequences. The open reading frame (ORF) of equine MMP-2 encoded a protein of 662 amino acids, including a segment of 29 amino acid residues at the amino terminus where cleavage at alanine results in enzyme activation (Fig 2). As in other MMPs, equine MMP-2 also had the conserved 'cysteine switch' (PRCGNPD), and 'catalytic zinc binding site' (HEFGHAMGLEHS), essential for protease activity (Springman *et al.* 1990; Vallee and Auld 1990). Furthermore, equine MMP-2 had the fibronectin *type II*-like domain seen only in gelatinases. Comparison of the deduced amino acid sequence of equine MMP-2 with other vertebrate MMPs showed that equine MMP-2 had high homology to its human and mouse counterparts: 96 and 95%, respectively. It was least similar to chicken MMP-2, sharing only 81% homology (Table 2). In contrast, equine MMP-2 showed only 38% similarity to its MMP-3 counterpart.

Southern analysis using the equine-specific cDNA clone confirmed that the RT-PCR products were from the equine MMP-2 gene. Constitutive expression of MMP-2 was observed in all samples from laminitic horses as well as normal horses. The expected RT-PCR product of 338 bp hybridised with the equine MMP-2 clone (data not shown).

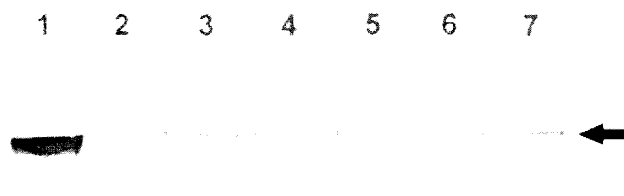


Fig 4: Detection of MMP-2 protein by Western blot analysis. Supernatants from normal hoof tissue homogenates were separated on a 4–20% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane. Equine (lanes 3–7) and human recombinant MMP-2 (lane 1) was detected using the rabbit anti-MMP-2 polyclonal antibody. There was no sample in lane 2.

DS cDNA converted from RNA extracted from each foot samples (4 normal and 18 laminitis) and subjected to the real-time PCR analysis provided accurate quantitation of MMP-2 expression. Each melting curve analysis showed only a single peak, indicating accurate PCR performance without nonspecific products (data not shown). The relative MMP-2 expression level of laminitic horses was computed with respect to the expression level of normal horses. Mean  $\pm$  s.e. of MMP-2 mRNA expression (Fig 3) in 18 laminitic hooves ( $2.26 \pm 0.20$ ) was up to 2.5 fold higher than the mean of 4 normal horses ( $1.03 \pm 0.02$ ); a significantly different result ( $P < 0.01$ ).

Western blot analysis using anti-MMP-2 raised in rabbits against a synthetic peptide derived from the amino acid sequence of our equine cDNA clones showed a single band from amongst the many electrophoresed proteins in the supernatants of the normal hoof tissue homogenates (Fig 4). The anti-MMP-2 cross reacted with 72 kDa human proMMP-2 and its co-alignment confirmed the MMP-2 identity and molecular weight of the equine bands.

Immunostaining of normal hoof sections with the MMP-2 antibody showed that MMP-2 was located in the cytoplasm of lamellar basal and parabasal cells (Fig 5). The keratinised axis of primary epidermal lamellae and the connective tissue of the dermal lamellae did not immunostain; some MMP-2 was present in the cytoplasm of vascular endothelial cells and fibroblasts. Sections incubated with rabbit serum instead of the primary antibody did not stain (data not shown). Immunostained MMP-2 in lamellar basal cells was concentrated in the basal cytoplasm adjacent to the basement membrane.

MMP-2 mRNA was located in formalin fixed, paraffin embedded tissue by *in situ* hybridisation (ISH) using DIG labelled MMP-2 antisense probe (Fig 6) in a pattern similar to MMP-2 immunostaining. ISH with an MMP-2 sense probe and no-probe showed no signal (data not shown).

## Discussion

RT-PCR has been used for cloning serine and cysteine-proteinases in many species (Sakanari *et al.* 1989; Marti *et al.* 1993) and was used successfully here to clone and sequence the cDNA encoding 72 kDa equine MMP-2. PCR primers were constructed in the overlapping manner from highly conserved regions specific to all MMP-2 genes. The equine MMP-2 genome encodes an amino-terminal propeptide, a catalytic domain, and a hemopexin-like domain at the carboxyl terminus, therefore resembling other MMP-2 genomes. The presence of a fibronectin

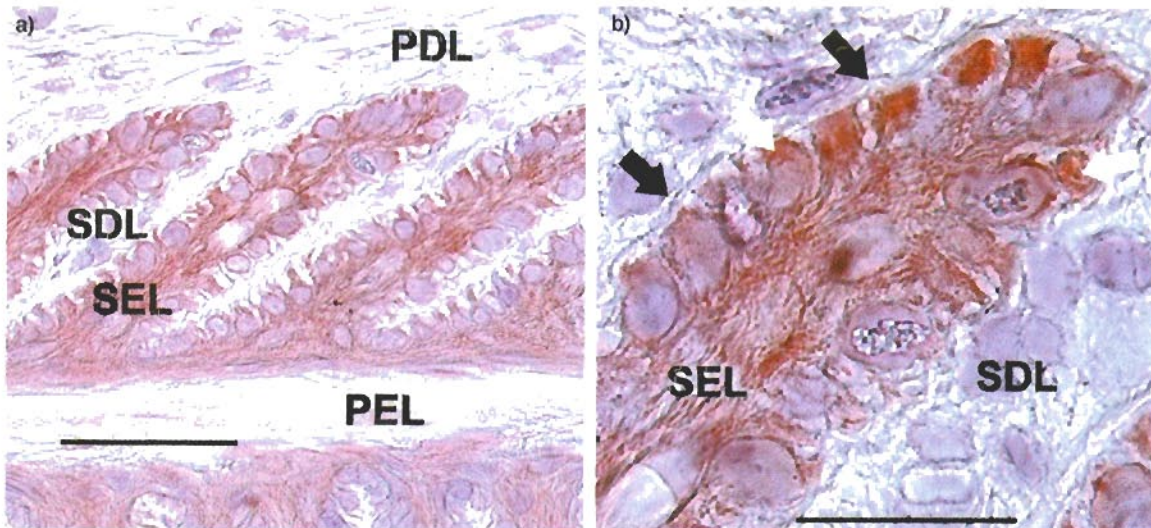


Fig 5: Immunostaining of normal hoof secondary epidermal lamella (SEL) with anti MMP-2. Dark brown, positive cytoplasmic staining was located mainly in lamellar basal and parabasal cells (a). Primary epidermal lamellae (PEL) and primary dermal lamellae (PDL) did not stain. Basal cell MMP-2 of SELs (b) was located in cytoplasm (white arrows) adjacent to the basement membrane (black arrows). Nuclei of epidermal cells and fibroblasts stained blue by the haematoxylin counterstain. Bar in a) = 50  $\mu$ m; Bar in b) = 20  $\mu$ m.

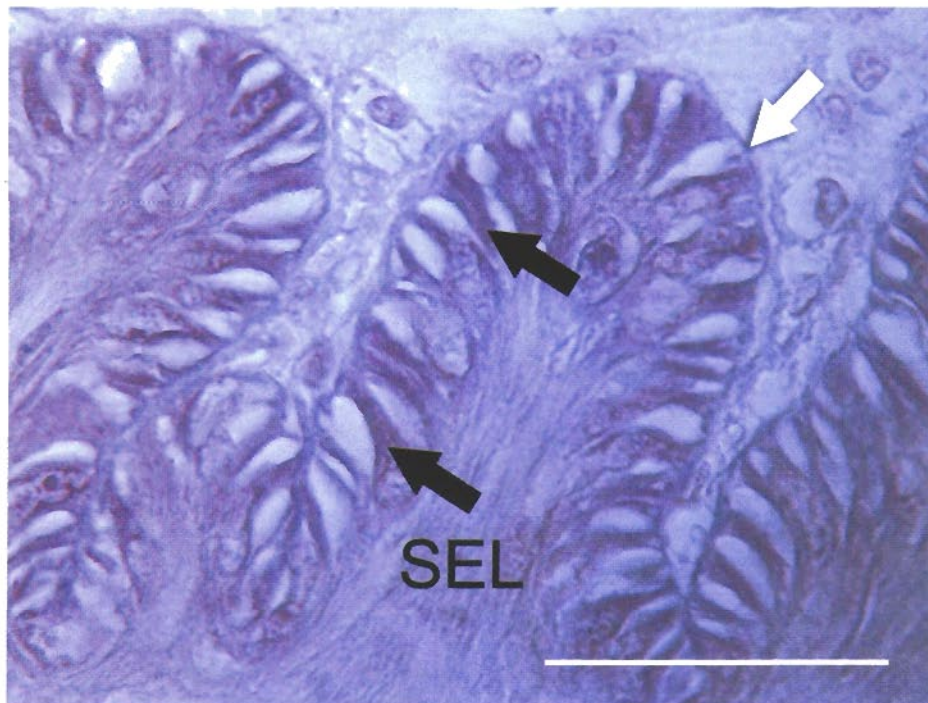


Fig 6: In situ hybridisation of MMP-2 mRNA in hoof tissue. Sections were hybridised with a DIG-labelled MMP-2 antisense riboprobe. MMP-2 mRNA is expressed in the cytoplasm of basal cells (black arrows) adjacent to the lamellar basement membrane (white arrow). Bar = 20  $\mu$ m.

type II-like domain within the genome of equine MMP-2, and its low identity (38%) to equine MMP-3, reflects the differences between gelatinases (MMP-2 and -9) and the stromelysins (MMP-3, -10, and -11). The ORF of equine MMP-3, the only other equine MMP identified so far, lacks fibronectin and contains only 477 amino acid residues. Equine MMP-2 has high homology to the human MMP-2 (96%) but is dissimilar to its chicken counterpart, a result commensurate with the evolutionary distance between birds and mammals.

Western blot analysis using anti-equine MMP-2 raised in

rabbits from deduced amino acid sequences of the cDNA clones confirmed both the presence of the MMP-2 proteins in hoof tissue homogenates and the specificity of our antibody.

Since the molecular components of desmosomes, hemidesmosomes and basement membranes are substrates for MMP activity the location of MMP-2 in hoof lamellar basal and parabasal cells implies that normal MMP-2 activation has a remodelling function, allowing the lamellar hoof wall to move past the stationary distal phalanx and its adnexa (Daradka and Pollitt 2004). Epidermal cells of the secondary epidermal lamellae (SEL)

and their adjacent basement membrane are constantly responding to the stresses and strains of growth and locomotion and we propose that it is the harmonious activation of MMP-2 that, in remodelling, these tissues accomplish the 'rips and tucks' required.

Increased transcription and activation of MMP has been implicated in various human cancers and degradation of extracellular matrix (ECM) components is an important early step in malignant metastasis (Birkedal-Hansen *et al.* 1993). Latent and active forms of equine MMP-2 are likewise increased in lamellar tissues from horses with laminitis (Pollitt *et al.* 1998) implying that enzymatic degradation of lamellar tissues is also occurring. Our quantitative real-time PCR results support, at the molecular level, the concept that increased MMP-2 transcription, in hoof tissues developing laminitis, promotes increased proteolytic degradation and structural failure of the hoof lamellar dermo-epidermal junction. Type IV collagen and laminin, key structural components of the lamellar BM, are known targets of activated MMP-2 (Takagi *et al.* 1998a; Nagase and Woessner 1999; Brew *et al.* 2000) and the molecular up-regulation shown here may be one of earliest events in the pathogenesis of laminitis.

Secreted from the same cells that produce MMPs are proteins capable of inhibiting protease activity; tissue inhibitors of metalloproteinase (TIMPs). However, TIMPs also form noninhibitory complexes with MMPs and recently, TIMP-2, a 21 kDa nonglycosylated MMP inhibitor, has been found preferentially to complex with proMMP-2 and play a pivotal role in the MMP-2 activation process. This process also involves a cell surface-anchored MMP known as membrane-type 1 MMP (MT1-MMP) to form a tri-molecular complex. There is evidence that the ternary MT1-MMP/TIMP-2/MMP-2 complex is involved in the connective tissue degradation resulting in failure of prosthetic hip joints (Takagi *et al.* 1998b). Furthermore, correlations between the MT1-MMP expression and proMMP-2 activation have been reported in various human cancer tissues (Fishman *et al.* 1996; Gilles *et al.* 1996; Kitagawa *et al.* 1998), suggesting the importance of this tri-molecular complex in tumour invasion and metastasis. The involvement of the MT-1, TIMP-2 and MMP-2 tri-molecular complex in laminitis is being investigated in our laboratory.

The new information on hoof MMP molecular biology reported here is the first step in understanding, with increased precision, the role of MMP activity in the pathophysiology of equine laminitis. Remarkably, the increase in MMP-2 expression occurred in the 48 h between the administration of a carbohydrate alimentary overload and the development of the first clinical signs of lameness. Our findings represent a warning to clinicians that the basement membrane lesion of laminitis (Pollitt 1996) is insidious and well under way before clinical signs are apparent. Any preventive strategies must be in place before overt foot pain develops if horses are to experience the developmental phase of laminitis without significant lamellar damage.

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### Manufacturers' addresses

- <sup>1</sup>Gibco BRL, Life Technologies Pty. Ltd., Mulgrave, Victoria, Australia.
- <sup>2</sup>Promega Corp., Annandale, New South Wales, Australia.
- <sup>3</sup>Applied BioSystems, California, USA.
- <sup>4</sup>Roche Diagnostics, New South Wales, Australia.
- <sup>5</sup>Amersham Pharmacia Biotech, Castle Hill, New South Wales, Australia.
- <sup>6</sup>Stratagene, California, USA.
- <sup>7</sup>Bio-Rad, New South Wales, Australia.
- <sup>8</sup>Chiron Technologies, South Australia.
- <sup>9</sup>DAKO Corp., Carpinteria, California, USA.

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