BRIEF COMMUNICATION: Cathelicidin levels in milk from cows infected with a range of mastitis causing pathogens

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INTRODUCTION

Mastitis is an inflammatory condition of the udder that is highly prevalent in cows on New Zealand dairy farms. It results in reduced production, increased treatment costs and lower profitability (Petrovski *et al.,* 2006). An elevated somatic cell count (SCC) in the milk, mainly due to recruitment of immune cells to the site of infection, is the most widely accepted standard for diagnosis of subclinical infection whereas bacteriological culturing is typically used for identification of the causative pathogen from clinical cases (Moyes *et al.,* 2009; Schukken *et al.,* 2003). At present, both tests require laboratory analysis and are not routinely performed on individual cows throughout lactation. Recent research has focused on identifying new biomarkers and developing cow-side tests that the farmer can perform rapidly, with high accuracy and with minimal cost (Viguier *et al.,* 2009). We recently reported that cathelicidin levels are elevated in mastitic milk (Smolenski *et al.,* 2010). The cathelicidin family of proteins are expressed predominantly in neutrophils and have potent antimicrobial activity (Tomasinsig & Zanetti, 2005). In this paper, we compare cathelicidin levels across a range of mastitic milk samples in order to assess whether the pattern of cathelicidin protein expression correlates with the causative pathogen.

MATERIALS AND METHODS

Sample collection

Foremilk samples were collected aseptically from a total of 35 dairy cows with clinical signs of mastitis according to guidelines provided by the National Mastitis Council (USA) together with a sample from an uninfected quarter. Standard laboratory methods were used for the culture and identification of bacteria (Hogan *et. al.,* 1999) and SCC was measured by electronic fluorescence-based cell counting (DeLaval cell counter; DeLaval, Tumba, Sweden). Bacteriology and SCC were performed by The Veterinary Centre (Te Awamutu, New Zealand). Phenylmethysulfonyl fluoride (2 mM) was added to all aliquots of skim milk before storage (-20°C) and subsequent western blot analysis.

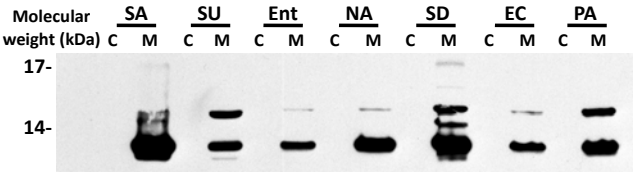
Cathelicidin western blot analysis

For gel electrophoresis, all milk samples were solubilised and diluted 10-fold in SDS-electrophoresis sample buffer. A 10 µL portion of each diluted milk sample was loaded onto pre-cast 12% Bis-Tris polyacrylamide gels (Criterion XT, Bio-Rad, California, USA) and subjected to electrophoresis in MES (2-[N-Morpholino] ethanesulfonic acid) running buffer according to the manufacturer's instructions. Resolved proteins were transferred to nitrocellulose membrane using the iblot™ system (Invitrogen, California, USA). Membranes were blocked with 4% non-fat milk powder in Tris-buffered saline with 0.1% Tween-20 and 0.1% bovine serum albumin (TBST/BSA) for 2 hours before being probed with polyclonal antibodies raised against a region common to all of the cathelicidin proteins (pan-cathelicidin) for 16 hours at 4°C or specific to cathelicidin-1, cathelicidin-2 and -4 for 2 hours at room temperature in TBST/BSA. These antibodies were produced by immunising rabbits with the following keyhole limpet hemocyanin coupled cathelicidin peptides (pan-cath: CNEQSSEPNIYRLLELDQ, cath-1: CIRITKQPWAPPQAA, cath-2: TPNDDLDPGTRKC, cath-4: CPPKDNEDLGTR) using a previously described procedure. The secondary antibody, horse radish peroxidise-conjugated goat anti rabbit IgG (Sigma, Missouri, USA), was applied to blots for 2 hours before visualisation using enhanced chemiluminescence. The immunoblot signal was captured on a ChemiDoc XRS (Bio-Rad) and quantified by Quantity One (v4.6.5) analysis software (Bio-Rad) using purified recombinant cathelicidin-1, -2 or -4 as internal standards.

Statistical analysis

The proportion of cathelicidin positive and cathelicidin negative samples for *Streptococcus uberis* and *Staphylococcus aureus* samples were compared using a chi-square test of association. The mean log transformed SCC values for the two groups were compared using a two-tailed *t*-test, assuming equal variance.

FIGURE 1: Abundance of cathelicidin in milk by western blot analysis. Skim milk collected from control (C) and mastitic (M) quarters of cows with naturally occurring *Staphylococcus aureus* (SA), *Streptococcus uberis* (SU), *Enterococcus* (Ent), *Nocardia asteroides* (NA), *Streptococcus dysgalactiae* (SD), *Escherichia coli* (EC) and *Pseudomonas aeruginosa* (PA) infections were probed with the pan-cathelicidin antibody.



RESULTS AND DISCUSSION

As a first step in assessing the utility of cathelicidin proteins as a potential marker for mastitis, we examined the level of cathelicidin protein in mastitic milk samples associated with a range of pathogens. Seven milk samples representing a range of different pathogen micro-organisms were selected for an initial screen. These were all positive for at least one of the cathelicidin proteins (Figure 1) as determined by western blotting with the pan-cathelicidin antibody. The multiple bands are due to the different molecular weights of the various bovine cathelicidin proteins and the effect of neutrophil elastase cleavage during neutrophil degranulation (Shinnar *et al.,* 2003). Quantitative western blot analysis indicated that these mastitic milk samples had relatively high levels of cathelicidin protein (range: 113 to 690 µg/mL, median: 329 µg/mL, coefficient of variation: 1.1 to 9.9%) when compared with other alternative protein markers of inflammation such as serum amyloid A (median = 33 µg/mL) and haptoglobin (median = 19.9 µg/mL) (Grönlund *et al.,* 2003). In contrast, no cathelicidin could be detected in milk from uninfected quarters from the same cow.

Some variation was observed in the pattern of reactive bands between the different milk samples (Figure 1). We hypothesised that this pattern was the result of particular members of the cathelicidin family being induced and as such may reflect the species of pathogen present. To test this we examined cathelicidin protein in the remainder of the 28 mastitic milk samples which included multiple samples infected with the same pathogen. These were all positive for cathelicidin expression, as indicated by the pan-cathelicidin antibody, while the uninfected samples from the same cow were all negative. To determine the presence or absence of individual cathelicidin proteins, we analysed these

FIGURE 2: Presence (■) or absence (□) of total cathelicidin and cathelicidin-1, -2 & -4 (pan-Cath, Cath1, Cath2, Cath4) in skim milk from udder quarters infected with various pathogens. Values are expressed as SCC x 10^6 cells/mL. Samples for which the SCC could not be determined because of extensive clotting were assigned >20 x 10^6 cells/mL.

Pathogen	SCC	pan-Cath	Cath1	Cath2	Cath4
Streptococcus uberis	7.33	■	■	■	■
	9.43	■	■	■	■
	16.46	■	□	■	■
	17.21	■	■	■	■
	20.00	■	□	■	■
	>20	■	■	■	■
	>20	■	■	■	■
Staphylococcus aureus	1.39	■	■	□	■
	1.40	■	□	□	■
	2.32	■	■	■	■
	2.60	■	□	■	■
	2.98	■	□	□	□
	3.00	■	□	■	■
	4.93	■	□	□	□
	6.24	■	□	□	□
	19.34	■	□	■	■
Coagulase-negative *Staphylococcus*	1.24	■	□	□	□
	3.66	■	□	□	■
	4.03	■	□	□	■
	16.00	■	□	■	□
Pseudomonas aeruginosa	5.14	■	□	□	□
	9.24	■	□	■	■
	11.53	■	■	■	■
Escherichia coli	4.00	■	■	□	■
	20.30	■	■	■	□
Streptococcus dysgalactiae	2.86	■	□	■	■
Bacillus	>20	■	■	■	■
Corynebacteria	6.58	■	□	□	■

samples using a qualitative western blot approach utilizing anti-cathelicidin-1, -2 and -4 antibodies. These were previously shown to be specific for each particular cathelicidin using recombinant cathelicidin-1, -2 and -4 protein (B.J. Haigh, Unpublished data). Figure 2 indicates the presence or absence of cathelicidin-1, -2 or -4 in each of the samples. In five cases, despite having a positive result with the pan-cathelicidin antibody, cathelicidin-1, -2 and -4 were not detected (refer to rows 12, 14, 15, 17 and 21 of Figure 2). One possible explanation for this is that one or more of the other cathelicidin proteins , that is Cath-3, -5, -6 or -7, for which specific antibodies are not available, could be contributing to the pan-cathelicidin signal.

Both *S. uberis* (n = 7) and *S. aureus* (n = 9) were the most prevalent pathogens amongst the 28 samples collected. There was considerable variation in the pattern of cathelicidin expression within these two groups suggesting that cathelicidin expression alone will not enable the type of pathogen to be predicted by this method. It is possible that the variation observed for cathelicidin-1, -2 and -4

results from physiological factors such as SCC level or stage of infection. As a group, the *S. uberis* samples contained a higher proportion that are positive for cathelicidin-1 or -2 (P = 0.05, P = 0.02) compared to the *S. aureus* samples. The proportion positive for cathelicidin-4 on the other hand was found not to be significant (P = 0.09) between these two groups.

In conclusion, these preliminary results demonstrate that cathelicidin proteins are up-regulated in the milk of cows infected with a variety of Gram positive and Gram negative pathogens and that considerable variation exists in the pattern of cathelicidin-1, -2 and -4 expression between animals. The level of cathelicidin in milk using the pan-cathelicidin antibody shows promise as an accurate predictive marker. However the results also suggest the absence or presence of a particular cathelicidin is insufficient to provide an accurate test that discriminates between pathogens.

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