Innate immune proteins as biomarkers for mastitis and endometritis

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ABSTRACT

Subclinical infections of the mammary gland and reproductive tract of dairy cattle cause significant production losses through decreased milk yield and lowered fertility. In the absence of overt signs of infection, there are no simple, reliable, diagnostic tools to accurately identify cows with these conditions, some of which may progress to a clinical stage at a later date. A search was undertaken to identify proteins in milk and reproductive tract secretions that could serve as the basis for a biomarker-based diagnostic test. A 2D gel-based proteomic analysis of milk and of uterine secretions revealed a number of proteins whose abundance was altered with mastitis or endometritis. Western blotting and ELISA indicated that the levels of two of these proteins, the cathelicidins and S100A12 were increased 25 ± 3 fold, and 29 ± 6 fold, respectively, in milk from 8 cows with clinical mastitis, compared with milk from uninfected quarters. The levels of these proteins correlated strongly with somatic cell count in the milk (r=0.85 and 0.93 for cathelicidin and S100A12, respectively). Changes in milk lactoferrin were less dramatic (7.9 ± 0.2 fold). In the uterine secretions, cathelicidins and S100A9 were increased (2 and 3-fold, P> 0.001; respectively) in secretions from cows with uterine infections, as assessed by uterine cytology, and were correlated (r = 0.87 and 0.64, respectively) with the percent of neutrophils in uterine cytological samples. Cathelicidins and S100 proteins may have value as a basis for screening cattle for mucosal infections.

Keywords: Mammary; uterine; bovine; infection; inflammation; lactoferrin; cathelicidin; S100;

INTRODUCTION

Mastitis and post-partum endometritis are two of the most significant infectious diseases of cattle. Mastitis is a significant economic cost to dairy industries worldwide (Viguier et al. 2009), while endometritis causes reduced fertility, with consequent loss of genetic gain through culling of empty cows (LeBlanc 2008). Mastitis is normally first detected through clinical signs and confirmed through a positive Rapid Mastitis Test, or through elevated Somatic Cell Count (SCC) in a herd test sample. Delayed detection can lead to a more prolonged course of treatment and reduced milk production. Similarly, sub-clinical endometritis can be difficult to diagnose but results in delayed return to oestrus, reduced conception rates, and failure to conceive if unresolved. There are currently no sensitive and reliable tests suitable for screening herds for “pre-clinical” mammary gland or reproductive tract infections (i.e. infections that are asymptomatic but may progress to more serious disease if host natural defences are inadequate to control them). There is, therefore, a need for improved methods to screen herds for early onset or pre-clinical mastitis (Viguier et al. 2009).

The presence of pathogens on mucosal surfaces results in the induction of a range of responses by the host aimed at controlling the incipient infection. These include the production of molecules that mediate local and systemic responses as well as stimulation of secretion of a range of antimicrobial proteins and peptides (Smolenski et al. 2007; Boehmer et al. 2010; Ibeagha-Awemu et al. 2010; Wheeler et al. 2012). These responses have only been partially characterised (Wheeler et al. 2012). A proteomics-based approach was used to identify proteins secreted into milk or in reproductive tract secretions during an infection, together with a preliminary evaluation of their merit as biomarkers.

MATERIALS AND METHODS

Five Holstein-Friesian dairy cows in mid-lactation were experimentally infected in two of their udder quarters by inoculation with Streptococcus uberis via the teat, using previously described procedures (Smolenski et al. 2011). Approval was obtained from the Ruakura Animal Ethics Committee. Milk was collected from all quarters prior to inoculation and from the nine infected quarters at the first milking at which clinical signs of mastitis first appeared, which ranged from 24-48 h after inoculation. A sub-sample from each quarter was analysed for SCC, using a commercial service, and a second sample was collected aseptically for bacteriological analysis to confirm infection status. A sub-sample of each was also stored for protein analysis. The remainder of the samples were processed to create separate pools of whey and milk fat globule membrane proteins, as previously described (Smolenski et al. 2007). Milk
samples were also collected from the infected quarter in eight cases of naturally-occurring mastitis at the time of first diagnosis. These samples were analysed for SCC and the presence of mastitis pathogens, and a subsample analysed for protein variables. Quarters were infected with a range of pathogens, including *S. uberis*, *P. aeruginosa*, *S. aureus*, *S. dysgalactiae*, coagulase-negative *Staphylococcus* and *E. coli*. The uterine status of each of 46 multiparous cows without overt clinical endometritis was assessed approximately 3 wk post-partum by taking samples within the uterus using cytobrushes. The recovered material was spread on microscope slides, fixed and stained with Giemsa stain, and the proportion of polymorphonuclear neutrophils (%PMN) amongst the somatic cells determined by a trained cytologist. The cytobrush was then washed with 1 ml of buffered saline and the cells pelleted. The supernatants were subjected to western blot analysis.

The pools of individual whey and milk fat globule membrane samples from uninfected and infected quarters, and representative uterine samples with <5% and > 30% PMN were subjected to 2D electrophoresis (two gels per sample) and the protein spots visualised by Coomassie staining, as previously described (Smolenski et al. 2007). The 2D gel images were captured using a scanning densitometer (GS-800, Bio-Rad) and the spots quantified using the PDQuest software package (Bio-Rad). Spots that appeared by visual inspection to be altered in abundance between the low and high %PMN samples. Mass spectrometry identified 123 spots that appeared to be consistently altered greater than two-fold in abundance between the uninfected and infected milk samples. Mass spectrometry of these excised spots resulted in the positive identification of 41 distinct proteins. The change in abundance of lactoferrin, cathelicidin and S100 family members were particularly prominent. A similar 2D gel analysis of reproductive tract secretions resulted in ten spots that were apparently altered in abundance between the low and high %PMN samples. Mass spectrometry identified four of these proteins to be lactoferrin, S100A9, peptidoglycan recognition protein 1 (PGLYP1) and cathelicidin family members.

**RESULTS**

Visual inspection of the 2D gels of pooled milk fraction samples from experimentally infected quarters or from the same quarters before inoculation resulted in 123 spots that appeared to be consistently altered greater than two-fold in abundance between the uninfected and infected milk samples. Mass spectrometry of these excised spots resulted in the positive identification of 41 distinct proteins. The change in abundance of lactoferrin, cathelicidin and S100 family members were particularly prominent. A similar 2D gel analysis of reproductive tract secretions resulted in ten spots that were apparently altered in abundance between the low and high %PMN samples. Mass spectrometry identified four of these proteins to be lactoferrin, S100A9, peptidoglycan recognition protein 1 (PGLYP1) and cathelicidin family members.

Western blot analysis of the nine individual milk samples from inoculated quarters taken before and after the inoculation with *S. uberis*, confirmed that lactoferrin, cathelicidins, S100A9 and S100A12 were consistently increased in abundance in milk collected during a mastitis infection (Figure 1). Western blotting of the post-partum uterine proteins from the 46 animals revealed large variability in the abundance of lactoferrin, PGLYP1, cathelicidins, and S100A9 between the samples. Representative western blots of 15 samples are presented in Figure 2.
**Figure 1:** Western blots of selected mastitis-responsive proteins in cows' milk.

The gels were loaded with 30 mg of skim milk per lane, from the indicated quarter. The third and fourth quarters (M) of each cow were inoculated with S. uberis. The signals were visualised by chemi-luminescence using X-ray film.

Quantification analysis indicate that cathelicidin and S100A12 responded particularly robustly to infection (25 ± 3 fold, and 29 ± 6 fold, respectively). The abundance of cathelicidin concentrations in milk were highly correlated (r = 0.87) with SCC, for both naturally occurring and experimentally induced mastitis (Figure 3). Similarly, S100A12 levels were highly correlated with SCC (r = 0.88). Lactoferrin concentration, on the other hand, was increased to a much lesser extent (7.9 ± 0.2 fold) and was poorly correlated with SCC (r = 0.40). In the uterine samples, the cathelicidins and S100A9 were increased (2 and 3-fold, P> 0.001; respectively) in secretions with greater than 30% PMN. The levels of the cathelicidins (Figure 4) and S100A9 correlated with the abundance of neutrophils in the uterine cytological samples (r = 0.87 and 0.64, respectively). Changes in lactoferrin were not as marked and were not correlated with %PMN.

**DISCUSSION AND CONCLUSION**

Results confirm earlier studies, wherein milk lactoferrin and cathelicidin concentrations were increased during mammary infection (Smolenski et al. 2011; Harmon et al. 1975), and extend a previous report that the S100 family of proteins are present in milk during mastitis (Lutzw et al. 2008). Lactoferrin is also known to be expressed in the reproductive tract in response to steroid hormones of the oestrous cycle (Dixon and Gibbons 1979). However, this is the first report quantifying and comparing the responses of these proteins to infections in the mammary gland with those in the reproductive tract.

**Figure 3:** Relative abundance of cathelicidins plotted against SCC in milk

Cathelicidin abundance, estimated by ELISA, was plotted against the SCC. Samples from both naturally-occurring (natM) and experimentally-induced (indM) were analysed.

The responses of the individual proteins to infection are distinct between the mammary gland and uterus. This is most likely because they are secreted from different cell types in response to different signals. Cathelicidins and S100 family members are known to be present in neutrophils (Lippolis and Reinhardt 2005), explaining the high correlation of their abundance with SCC in milk and %PMN in reproductive tract secretions. On the other hand, lactoferrin is produced by a wider range of cell types (Teng 2002) and has a relatively high basal abundance in milk, lesser responsiveness to infection and possible responsiveness to steroid hormones in some tissues. This may explain the lack of a significant correlation with SCC in milk or %PMN in reproductive tract secretions in this study.

The data presented here provide candidate biomarkers that could be used in conjunction with SCC and conductivity (for mastitis), and cytological assessment (for endometritis) to better inform cow management decisions. It is possible that the use of several biomarkers in parallel may provide more
specific diagnostic information than is possible with a single biomarker. Such an approach could provide additional information on the stage of infection or type of pathogen involved. Further work could address this possibility. The commonality of responses between infections in the mammary gland and uterus suggests that diagnostic information could be produced for both mastitis and endometritis, using a single technological platform. Further research is warranted to evaluate the potential of the cathelicidins and S100 family members in the diagnosis of mammary and uterine infections.

**Figure 4:** Relative abundance of cathelicidins plotted against %PMN in uterine samples

Cathelicidin abundance, estimated by densitometry of western blot chemiluminescent signals, were plotted against the % PMN, as determined by cytological examination.

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