The spatial expression pattern of antimicrobial peptides across the healthy bovine udder

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ABSTRACT

Antimicrobial peptides are key molecules in local host defense. With the aim to better understand the possible involvement of these peptides in the prevention of bovine mastitis, we determined, for the first time to our knowledge, the spatial pattern of constitutive expression of 5 bovine β -defensins and bovine psoriasin (*S100A7*) across 5 localizations of the bovine mammary gland applying a quantitative real-time PCR approach. We observed 3 different expression patterns in the healthy udder: 1) constitutive expression of the lingual and tracheal antimicrobial peptides (*LAP* and *TAP*), as well that of bovine neutrophil β -defensins 4 and 10 (*BNBD4* and *BNBD10*), is essentially restricted to the mammary lymph node; 2) bovine β -defensin 1 (*DEFB1*) is mainly expressed in the cisternal epithelium and the Rosette of Fürstenberg; 3) strong constitutive mRNA expression of the calcium-binding protein *S100A7*, which is also known as psoriasin and which has been reported to be highly active against *Escherichia coli*, was detected in the streak canal. These results indicate a crucial role of *S100A7* in the early-stage prevention of coliform mastitis, and the analyzed β -defensins might be regarded as inducible weapons against already invading pathogens. **Key words:** antimicrobial peptide, β -defensin, psoriasin, mastitis

INTRODUCTION

Antimicrobial peptides (**AMP**) are substantial components of the innate immune system in a broad variety of organisms including mammals. They display direct antimicrobial activity against a wide range of pathogens and play a major role in the modulation of innate immune response (Mookherjee and Hancock, 2007). The relative importance of direct killing versus immunomodulatory function is, however, unclear (Bowdish et al., 2005).

Defensins are an important class of the vertebrate AMP divided into 3 sub-families: α -, β -, and θ -defensins (Ganz and Lehrer, 1998; Lehrer and Ganz, 2002). The small cyclic θ -defensins are only found in nonhuman primates (Tang et al., 1999; Selsted, 2004), whereas the α -defensins have been identified in several groups of mammals (Patil et al., 2004, 2005; Xiao et al., 2004), including the horse (Looft et al., 2006; Bruhn et al., 2007). The only sub-family found in bovines are β -defensins (Fjell et al., 2008), a large family of cationic peptides with characteristic disulfide bridging between cysteine residues (Bauer et al., 2001). Defensins can be expressed either constitutively, as seen in neutrophil- and Paneth cell-derived defensins, or inducibly, as is the case for most epithelial β -defensins (Yang et al., 2004).

Several bovine β -defensins have been found to be expressed at possible sites of microbial invasion, often in an inducible manner. The tracheal antimicrobial peptide (**TAP**) was identified in bovine tracheal epithelial cells (**TEC**; Diamond et al., 1991). It is expressed in the entire airway epithelium (Diamond and Bevins, 1994) and is induced in response to injury and infection (Schönwetter et al., 1995). Another AMP expressed in TEC is the lingual antimicrobial peptide (**LAP**), originally discovered in squamous epithelial cells of the bovine tongue (Schönwetter et al., 1995; Russell et al., 1996). Both genes show inducible expression in cultured TEC in response to bacterial LPS and tumor necrosis factor- α (Diamond and Bevins, 1994; Russell et al., 1996). Unlike in TEC, the β -defensin expression in bovine alveolar macrophages is not inducible by LPS (Ryan et al., 1998).

As a part of their crucial role in local host defense, bovine β -defensins might also be involved in the prevention of udder infections (mastitis). Because of the immense economic importance of mastitis in dairy cattle, different studies have been aimed at the determination of β -defensin expression in the udder. Constitutive expression of β -defensins in the bovine mammary gland has been shown for *LAP* and *TAP*, as well as for bovine neutrophil β -defensins (*BNBD*) 3, 4, 5, 9, and 12, β -defensin 401, and β -defensin 1 (*DEFB1*), also

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known as bovine enteric β -defensin (Tarver et al., 1998; Roosen et al., 2004; Cormican et al., 2008). The latter was originally identified in the bovine small intestine and shown to be highly inducible during *Cryptosporidium parvum* infections (Tarver et al., 1998). Inducible expression has been shown for *BNBD5*, which is expressed predominantly in mammary epithelial cells and is strongly upregulated in mastitis (Goldammer et al., 2004). Another study has identified the induction of several bovine β -defensins including *LAP*, *BNBD4*, *BNBD10*, and *DEFB1* in response to a challenge with *Escherichia coli* in cultured mammary epithelial cells as well as in vivo (Günther et al., 2009). The abundance of induction seems to depend on the causative pathogen. Petzl et al. (2008) found *E. coli* to be a potent inducer of the innate immune defense including defensin expression, whereas *Staphylococcus aureus* was not able to trigger this early response.

Additionally, a broad spectrum of antibacterial and antifungal activities has been demonstrated for several bovine β -defensins: *LAP*, *TAP*, and *BNBD12* exhibit high activities against gram-negative *E. coli* and gram-positive *Staph. aureus*, which are characteristic mastitis-causing pathogens (Diamond et al., 1991; Schönwetter et al., 1995; Mandal et al., 2002). Consequently, β -defensins have also been studied at the genomic level with regard to mastitis and SCC in dairy cattle. Several studies have identified associations between β -defensin polymorphisms and SCC in Polish Holstein-Friesian and Jersey cows (Ryniewicz et al., 2002, 2003; Wojdak-Maksymiec et al., 2006; Bagnicka et al., 2007).

Another important antimicrobial agent present in bovines is the calcium-binding S100A7 protein (Hitomi et al., 1996), also known as psoriasin (Madsen et al., 1991; Broome et al., 2003; Gläser et al., 2005). It is highly active especially against several strains of *E. coli* in humans (Gläser et al., 2005) and cattle (Regenhard et al., 2009), and its expression is inducible by proinflammatory cytokines and bacteria (Gläser et al., 2005).

Constitutive and inducible expression in the bovine udder and antibacterial activity against pathogens indicate a crucial role of β -defensins and psoriasin in the local host defense against mastitis. Antimicrobial peptides able to establish an effective barrier should show a remarkable level of constitutive expression at the sites of bacterial invasion, whereas the expression of those acting as weapons against already invading pathogens should be highly inducible. The aim of this study was therefore to determine the constitutive expression level of β -defensins and psoriasin in different localizations of the healthy bovine udder to gather further evidence for the potential role of AMP in resistance to mastitis.

MATERIALS AND METHODS

Collection of Tissue Samples

Samples from different localizations of the healthy bovine udder were taken from 9 first-lactation German Holstein dairy cows. These animals were kept under the national guidelines for animal experiments and according to standard dairy farm practice without any intervention in the living animal. The study was approved by an institutional committee. To control for extraneous sources of variation, all animals were kept under the same environmental conditions. After parturition, udder health was controlled weekly by measuring SCC in milk samples and with clinical investigations. Milk samples from each quarter were tested for bacterial infection upon slaughtering at d 42 postpartum. All cows were milked 2 times a day with a 12-h interval. All animals included in the study were clinically healthy, the SCC was routinely <100,000 cells/mL, and cows did not show any indication of bacterial infection on the day of slaughter. Immediately after the animals were killed, tissue samples of the gland cisternal epithelium, the parenchyma (deep in the secretory part), the Rosette of Fuerstenberg, the streak canal, and the inguinal lymph node were removed from the left rear quarter, frozen immediately in liquid nitrogen, and stored at -80°C until isolation of RNA. Frozen tissues were ground with a mortar in liquid nitrogen. Homogenization of ground tissue samples was done with 1 mL of TRI Reagent (Sigma, Taufkirchen, Germany) using syringes and needles. For all tissues except inguinal lymph node, a proteinase K digestion was carried out. Then, RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol including an additional DNase treatment. The RNA was then quantified and its quality checked by gel electrophoresis on a 1% agarose gel. All RNA samples were checked for DNA contamination by performing an intron-spanning PCR using the reference gene primers.

Quantitative Real-Time PCR

To obtain cDNA, 250 ng of each total RNA template was subjected to reverse transcription using 200 U of Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). The reaction was carried out as described in the manufacturer's instructions in a final volume of 20 μL containing 25 $\mu\text{mol/L}$ (μM) of dNTPs and 0.5 μM of oligo-dT primers.

The quantitative real-time (qRT)-PCR primers (Table 1) were designed using the Primer3 software (<http://frodo.wi.mit.edu/primer3/>) and synthesized by biomers.

Table 1. Gene-specific primers (F = forward; R = reverse) used in quantitative real-time PCR

Gene ¹	Primer (5' to 3') ¹	Amplicon size, bp
<i>GAPDH</i>	F: GGAGAAACCTGCCAAGTATGATG R: CCAGGAAATGAGCTTGACAAAGT	193
<i>BNBD4</i>	F: CGTTCTTGTGCCGTGTAG R: AAATTTTAGACGGTGTGTTG	149
<i>BNBD10</i>	F: AGTTATCTAAGCTGCTGGG R: CGCTCTGTCAAAGGGTC	173
<i>DEFB1</i>	F: RD-ATCCTCTAAGCTGCCGTCT R: RD-AGCATTCTTACTGAGGGCGT	102
<i>LAP</i>	F: RD-GATTGGCACCTGTCTCGGAG R: RD-TATTCTGGTTTAAATTTTAACTCTCTGTCC	132
<i>TAP</i>	F: RD-GAGTAGGAAATCCTGTAAGCTGT R: RD-TGGCCTTCTTTTACTTCTTTCTA	135
<i>S100A7</i>	F: RD-AGAGCAGGCCATTACAGACT R: RD-CAAGGACAGGAAGTCTAGA	218

¹*BNBD4* = bovine neutrophil β -defensin 4; *BNBD10* = bovine neutrophil β -defensin 10; *DEFB1* = bovine β -defensin 1; *LAP* = lingual antimicrobial peptide; *TAP* = tracheal antimicrobial peptide; *S100A7* = bovine psoriasin.

²Primers appended with RD have a robust dosage tail (ggccaagtgt) attached to the 5' end.

net (Ulm, Germany). A robust dosage tail was added to the 5' end of some primers (Liu et al., 2003), because they were also used in subsequent experiments with respect to copy number variation of bovine β -defensins. To ensure gene specificity of primers in view of the high degree of sequence similarity between the analyzed β -defensin genes (all orthologs of the human *DEFB4* gene), sequences available in public databases (mRNA, expressed sequence tags, genomic) were aligned using the Multalin software (Corpet, 1988). Thereby, we were able to determine those sequence differences defining the most unambiguous gene-specific primer binding sites for at least one of the primers (Figure 1). In addition to the target genes, PCR primers for bovine *GAPDH* were designed that served as reference gene.

Quantification was done applying the standard curve method based on a dilution series of target gene plasmids. For construction of these plasmids, PCR products were purified from a preparative agarose gel using a PCR purification kit (Qiagen), cloned into a pDrive vector, and transformed into competent *E. coli* cells using the Qiagen PCR cloning plus kit according to the manufacturer's instructions. Plasmid DNA was prepared using the Fast Plasmid Mini Kit (Eppendorf, Hamburg, Germany), and each preparation was first subjected to a test PCR and subsequently sequenced to ensure the presence of the correct insert.

The qRT-PCR was carried out on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Munich, Germany). The reactions were set up in a final volume of 20 μ L containing 0.4 μ M of each primer using the Platinum SYBR Green qPCR Super Mix UDG Kit (Invitrogen). As template, 2 μ L of cDNA was used, corresponding to 1.56 ng of total RNA. The following protocol was used: after an initial denaturation at 95°C, 40 cycles were performed consisting of denaturation

(95°C for 15 s), annealing (58°C for 30 s), elongation (72°C for 30 s), and a high-temperature fluorescence acquisition step (79°C for 30 s) (Pfaffl, 2001). This protocol was followed by a melting curve analysis. The threshold cycle (C_t) of a sample was set at the cycle number at which the fluorescence signal intersected the threshold automatically determined by the iQ5 Optical System Software (Bio-Rad), so that the fluorescence signal exceeded the background noise. All reactions were done in triplicate and, on each reaction plate, a 1:10 dilution series of the target gene plasmid was included, corresponding to a theoretical range from 4.42×10^7 to 4.42 mRNA copies.

Analysis of Real-Time PCR Data

The statistical analysis of the real-time PCR data was carried out using the R statistical software (R Development Core Team, 2008). All triplicate measurements were checked for uniformity using the outlier test according to Nalimov (1963). If 1 of the 3 measurements was found to be an outlier with an error probability below 0.05, this value was rejected using the means of the remaining values for subsequent analyses. The plasmid dilution series was used to determine the range of linear dependency between the dyadic logarithm of the template concentration and the C_t value for each target gene. If deviation from linearity with no or close to no increase of the C_t value for the highest dilution steps was observed, a product-specific virtual detection limit was defined to avoid false-positive results or a massive overestimation of exceedingly low mRNA expression levels. The target gene measurements were normalized against *GAPDH* for all samples to make the results comparable between animals as well as between the different localizations. From these normalized values, the

Figure 1. Alignment of available bovine mRNA sequences orthologous to human β -defensin 4 (*DEFB4*). The alignment contains one representative sequence per gene and runs from the start codon to position 280 of the open reading frame. Start and stop codons are printed in bold letters, and the boundary between exon 1 and 2 is indicated by vertical dashes. The characteristic cysteine residues are marked within the consensus sequence. Within the sequences of the analyzed β -defensins, gene-specific PCR-primers are highlighted (forward primer = gray background, reverse primer = underlined).

number of mRNA copies was calculated based on the standard curve obtained from linear regression of the plasmid dilution series and scaled to the mRNA copy number per nanogram of total RNA initially subjected to reverse transcription.

RESULTS

Performance of qRT-PCR

For all target genes, the PCR efficiencies were calculated as the slope of the standard curve and found to be close to 1. The efficiencies of repeated measures of the same target gene were within a range of ± 1 standard deviation. For *LAP* and *S100A7*, a detection limit had to be set at 44.2 mRNA copies/ng of total RNA because of a deviance from linearity for the highest dilution step. The melting curve analysis revealed no signs of additional unspecific PCR products, indicating that the primer pairs were specific. The stability of the housekeeping gene appeared to be good with a mean C_t standard deviation between repeated measures below 0.5 cycles.

Constitutive Expression of AMP in the Healthy Udder

The mRNA expression levels for *LAP*, *TAP*, *DEFB1*, *BNBD4*, *BNBD10*, and *S100A7* are shown in Figure 2. The 2 epithelial β -defensins *LAP* and *TAP* as well as the neutrophil β -defensins *BNBD4* and *BNBD10* exhibited similar expression patterns with the highest amount of mRNA detected in the lymph node, followed by the cisternal epithelium, and no or close to no expression in the parenchyma. The abundance of *LAP* and *BNBD10* mRNA expression in the lymph node was at least an order of magnitude higher than that for *TAP* and *BNBD4*. The expression pattern of *DEFB1* was found to differ from that of the other β -defensins, because the highest abundance was found in the cisternal epithelium followed by the Rosette of Fürstenberg and only marginal mRNA expression in the lymph node and streak canal. The overall abundance of *DEFB1* was comparable to the levels of *TAP* and *BNBD4*. The level of *S100A7* mRNA expression was found to be very high in the streak canal and the Rosette of Fürstenberg with a level of 2.78×10^6 mRNA copies/ng of total RNA in the streak canal. In the other tissues no *S100A7* mRNA expression could be detected. As indicated in Figure 2, the standard errors were relatively high for all analyzed genes.

The tissue distribution of the target gene mRNA expression is shown in Figure 3. The highest total abundance was seen in the streak canal and, to a lower extent, in the Rosette of Fürstenberg and was almost

solely because of the very high level of *S100A7* message detected. The lowest level of mRNA expression was found in the udder parenchyma, whereas the levels in the cisternal epithelium and the lymph node were higher with a total abundance of 2.69×10^3 and 2.13×10^4 mRNA copies/ng of total RNA, respectively. This can be assigned mainly to *LAP* and *BNBD10* transcripts.

DISCUSSION

Transcript analyses of bovine β -defensins are hampered by the problem of high sequence similarity. The β -defensins analyzed within this study are all orthologous to human *DEFB4*, and the similarity between them ranged from 81% (*TAP* vs. *BNBD10*) to 86% (*DEFB1* vs. *LAP/TAP* and *LAP* vs. *TAP*). The highest degree of similarity to one of the other bovine β -defensins listed in Figure 1 was found for *DEFB1*, which showed 97% identity with *DEFB405*. Especially in microarray studies, it is therefore not always possible to distinguish between different β -defensins based on the available probe sets. In comparative studies, this could lead to an overestimation of fold changes due to an admixture of several inducible β -defensins.

The results of transcript quantification in different localizations of the healthy bovine udder indicated the presence of 3 different patterns of constitutive expression among the analyzed AMP. The constitutive expression of *LAP*, *TAP*, *BNBD4*, and *BNBD10* was essentially restricted to the lymph node with different total abundances. The expression of *DEFB1* was located more distally in the cisternal epithelium and the Rosette of Fürstenberg, whereas *S100A7* expression was exclusively detected in the streak canal and the Rosette of Fürstenberg. The abundance of *S100A7* transcripts resulted in the highest overall AMP transcription level in these locations. Generally, abundance decreases with an increasing distance to the site of possible bacterial invasion (streak canal > Rosette of Fürstenberg > gland cisternal epithelium > parenchyma). An exception of this coherence is the lymph node, probably because of the β -defensin expression of neutrophils residing there. The biggest part of the total transcript level in the parenchyma can be ascribed to *LAP*, which is in concordance with other studies locating *LAP* expression to alveolar epithelial cells (Swanson et al., 2004; Isobe et al., 2009). Summarizing the patterns of constitutive expression, we hypothesize that *S100A7*, which is highly active against *E. coli* (Gläser et al., 2005; Regenhard et al., 2009) and inducible in skin (Gläser et al., 2005; Regenhard et al., 2009), forms an effective barrier against bacterial invasion and could be crucial in preventing coliform mastitis. Additionally, other calcium-binding *S100A*

Figure 2. mRNA expression level of 5 bovine β -defensins and bovine psoriasin (*S100A7*) in 5 different localizations of the udder (SC = streak canal, ROF = Rosette of Fürstenberg, CIST = gland cisternal epithelium, LN = inguinal lymph node, PAR = udder parenchyma). The mean numbers of mRNA copies per nanograms of total RNA normalized against *GAPDH* are shown. The error bars depict the standard error; no expr. = no expression, meaning that no transcripts were detectable above the detection limit. *BNBD4* = bovine neutrophil β -defensin 4; *BNBD10* = bovine neutrophil β -defensin 10; *DEFB1* = bovine β -defensin 1; *LAP* = lingual antimicrobial peptide; *TAP* = tracheal antimicrobial peptide.

proteins, namely S100A9 and S100A12, have been found to be induced in the udder by *E. coli* infections (Günther et al., 2009). Regarding the housing conditions of dairy cows, the exceedingly high transcript level could therefore also be the result of a permanent and recurrent induction by coliforms at the orifice of

the streak canal without any signs of infection. To further elucidate this hypothesis, future studies should investigate *S100A7* expression at different locations of the skin including sites far away from the galactogenic pathway of infection, probably in animals housed under different conditions.

Figure 3. Tissue distribution of the mRNA expression levels. The values represent the mean numbers of mRNA copies per nanograms of total RNA normalized against *GAPDH*. The diameter of the pie charts corresponds to the overall abundance at that localization, which is additionally given below each chart. *BNBD4* = bovine neutrophil β -defensin 4; *BNBD10* = bovine neutrophil β -defensin 10; *DEFB1* = bovine β -defensin 1; *LAP* = lingual antimicrobial peptide; *TAP* = tracheal antimicrobial peptide; *S100A7* = bovine psoriasin.

A conclusion regarding the role of the analyzed β -defensins cannot be clearly drawn from our data. Having been shown to be inducible in the mammary gland (Günther et al., 2009), *LAP*, *BNBD4*, *BNBD10*, and *DEFB1* might act as inducible weapons against invading pathogens. Considering the fact that *LAP* makes up approximately 70% of the total transcript level in cisternal epithelium in our study, an intermediate role could be suggested for this AMP.

The high individual variability of constitutive AMP expression (Figure 2) is a major finding of the current study that might indicate the presence of a genetically determined different expression level. Although management factors play an important role in mastitis control, resistance to udder infections has a substantial genetic component, giving the possibility of genetic improvement.

In many countries, selection for udder health is based on the indicator trait SCS. In the literature, selection based on SCS is controversially discussed. Weller et al. (1992) reported the genetic correlation between SCS and subclinical mastitis to be near unity, whereas in the same study the correlation between SCS and clinical mastitis was found to be only 0.3. This finding indicates

that SCS can be used to select for a lower incidence of subclinical, but not clinical, mastitis. On the other hand, Carlen et al. (2004) reported mean genetic correlations between mastitis and SCS in Swedish Holstein cows of 0.7, indicating the usefulness of SCS in breeding for a low incidence of clinical mastitis. However, the genetic improvement of udder health based solely on SCS bears the risk of impairing mechanisms related to the recruitment of neutrophils. Regarding the essential role of these cells in the immune response of the mammary gland (Burton and Erskine, 2003), this could lead to a decreased ability to fight arising udder infections. Effective breeding for udder health would therefore probably benefit from the discovery and utilization of mechanisms directly affecting the innate immune response such as the abundance of AMP expression in the mammary gland. Different QTL mapping studies have identified the 2 bovine β -defensin clusters on chromosomes 13 and 27 as candidate regions affecting SCS (Kühn et al., 2003; Rupp and Boichard, 2003; Ashwell et al., 2004), with the latter containing the β -defensins analyzed in the current study. The region of bovine chromosome 3 containing *S100A7* has also been identified as a QTL region for SCS (Klungland et al., 2001).

Considering the genomic structure of the human β -defensin cluster on human chromosome 8 (Groth et al., 2008; Hollox et al., 2008; Abu Bakar et al., 2009), it seems likely that copy number variation (CNV) might represent a major source of genomic variation related to individual differences in the innate immune response based on AMP. The available genomic tools in cattle, essentially the whole-genome BovineSNP50 SNP Chip (Illumina, San Diego, CA; Matukumalli et al., 2009), are not capable of covering this kind of variation. It is possible to detect CNV using SNP genotyping arrays, and the bovine 50k array has already been applied to the detection of large-scale CNV (Matukumalli et al., 2009), but reliable copy number detection for single genes requires a much higher SNP density (Carter, 2007). Additionally, the design of robust genotyping SNP assays is particularly difficult within regions containing segmental duplications and complex CNV (Carter, 2007). Because of this, the marker coverage of the BovineSNP50 Chip contains a gap spanning the entire bovine β -defensin cluster on chromosome 27. Further elucidation of the effect of CNV on the individual ability of local host defense, therefore, will benefit from the availability of advanced genomic tools.

CONCLUSIONS

For the first time to our knowledge, the data presented in this study provide a spatial expression pattern for different AMP across the healthy bovine udder. We demonstrated that the constitutive expression of *LAP*, *TAP*, *BNBD4*, and *BNBD10* is essentially restricted to the lymph node, whereas expression of *DEFB1* occurs in more distal regions of the mammary gland. Strong constitutive mRNA expression of *S100A7*, which is highly active against *E. coli*, was detected in the streak canal, suggesting this AMP to be crucial in the early-stage prevention of coliform mastitis. To identify genomic variation responsible for individual differences in AMP expression might, beyond current approaches, be useful in improving udder health.

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