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Original article

Expression of the bovine neutrophil *β*-defensins4 (*BNBD4*) and 10 (*BNBD10*), and *β*-defensin 1(*DEFB1*) in the bovine mammary gland with chronic mastitis by *Staphylococcus aureus*

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A R T I C L E   I N F O

A B S T R A C T

Bovine mastitis is the most costly infectious disease of dairy cattle worldwide and is characterized by inflammation of the mammary gland. The etiology of the pathogens influences the severity of the inflammation and the immune response, and specifically some elements of the innate immunity, like the antimicrobial peptides, are known to display some pathogen specificity. The antimicrobial peptides play an important role in the innate immune defense mechanisms of the mammary gland against mastitis causative microorganisms. In the present study the expression of three *β*-defensins was estimated across 5 localizations of the mammary gland infected with *Sthapylococcus aureus*, by quantitative real-time PCR. Copies of mRNA for *BNBD10* and *DEFB1* were detected in Lymph node, parenchyma, streak canal, cisterna, and Rosette of Furstenberg, with the highest level observed in lymph node and the lowest in Rosette of Furstenberg, and the level of expression was higher for *BNBD10*. The level of mRNA expression for *BNBD4* was very low in all localizations, with respect to *BNBD10* and *DEFB1*. Therefore, *BNBD10* and *DEFB1* could play a key role in the mammary gland

with mastitis by *S. aureus*.

1. Introduction

Mastitis is the most common infectious disease that affects dairy cows causing great economic losses. Bacteria are the main causative agents of mastitis, where *Escherichia coli* and *Staphylococcus aureus* are the most common pathogens. The severity of the inflammation and the immune response in the udder varies significantly between these two pathogens (Petzl et al., 2008; Whelehan et al., 2011). While *E. coli* causes acute mastitis with severe clinical signs, *S. aureus* may cause persistent, almost chronic infections, with pathogens surviving inside host cells,which leads to a low response to conventional antibiotic therapy and the establishment of subclinical mastitis (Whelehan et al., 2011; Alva-Murillo et al., 2013). To prevent and treat this disease is important to understand the immune function of the mammary gland. Innate immunity is a non-specific mechanism that precedes the long-term immunity, where antimicrobial peptides (AMP) play an important role (Ganz, 2003; Swanson etal., 2004) and they expression has been shown to vary depending on the pathogen involved (Alva-Murillo et al., 2013).

Antimicrobial peptides (AMP)are a family of approximately 900 molecules belonging to the primitive innate immune system in vertebrates, insects and plants (Tomasinsig et al., 2010). Defensins are substantial components of these defense mechanisms in epithelial cells, effective against a variety of microorganisms and constitute a family of small cationic peptides(3–6 kDa), classified as *α-, β-* and y *ϑ* defensins. The expressionof *β*-defensins varies in epithelial cells, with the highest levels being in those tissues that are constantly exposed to, and colonized by, microorganisms (Swanson et al., 2004). Defensins can be expressed either constitutively or inducibly, as is the case for most epithelial *β*-defensins (Yang et al., 2004).

In bovines only *β*-defensins have been found and includes: the lingual antimicrobial peptide (*LAP*) (Schonwetter et al., 1995; Isobe et al., 2011); enteric *β*-defensin(*EBD*), also known as *β*-defensin1 (*DEFB1*) (originally identified in bovine small intestine where was highly inducible during *Cryptosporidium parvum* infections) (Tarver et al., 1998); bovine neutrophil *β*-defensins (*BNBD1-13*) (Selsted et al., 1993; Goldammer et al., 2004); tracheal antimicrobial peptide (*TAP*) (Diamond et al., 1991) and other bovine *β*-defensins (Cormican et al., 2008).

Several *β*-defensins, including *BNBD4*, *BNBD10* and *DEFB1*, have shown a constitutive expression in the healthy bovine mammary gland (Tetens et al., 2010; Goldammer et al., 2004). Also, *BNBD4*, *BNBD10*, and *DEFB1*, have been expressed in an inducible manner in response to a challenge with *Escherichia coli* in cultured mammary epithelial cells as well as in vivo (Gunther et al., 2009). The expression of the *β*-defensin *LAP*, *BNBD5*, *BNBD4*, *BNBD10* and *DEFB1* genes (except TAP), have been shown to be constitutively expressed in healthy mammary gland parenchyma, but their expression (*except BNBD10*) was significantly higher in *S. aureus* infected tissue (Knsciuczuk et al., 2014). Another study reported that bovine mammary epithelial cells (bMEC)showed a basal expression of *TAP*,*LAP*,*BNBD5*, *BNBD10*, *BNBD4* and *DEFB1*, but also all of them were induced by *S. aureus* in vitro showing higher levels of expression (Alba-Murillo et al., 2013).

The constitutive and inducible expression of *β*-defensins in the healthy and mastitic bovine mammary gland has demonstrated significant importance of these antimicrobial peptides in the immediate innate response to invading pathogens. Therefore the aim of this study was to determine the expression level of *β*- defensinas *BNBD10*, *BNBD4* and *DEFB1* in different localizations of the bovine mammary gland with chronic mastitis by *S. aureus*.

2. Materials and methods

2.1. Collection of mammary tissue

Mammary tissues were collected from two Holstein Friesian dairy cows from a local abattoir, diagnosed with chronic mastitis by the California mastitis test and somatic cell count (Calvinho, 1998). The presence of *S. aureus* was detected by bacteriological analysis of milk samples and confirmed by commercial biochemical tests following

manufacturer's instructions (API Staph, bioMérieux, U.S.A). Immediately after the animals were slaughtered tissue samples of the gland cisterna, parenchyma, the Rosette of Fuerstenberg, the streak canal and the inguinal lymph node were removed from all the quarters. Samples collected were of an approximately 1 cm^{3}size each, and were placed in RNAlater solution (Ambion, Austin, Texas) and stored at -20°C until isolation of RNA.

2.2. RNA extraction and cDNA isolation

Total RNA from the tissues was isolated using the RNeasy Mini Kit (Qiagen, Germany) with the DNAse digestion step according to the manufacturer´s protocol. The RNA was quantified by spectrophotometry (260/280) and its quality checked by gel electrophoresis on a 1% agarose gel.

To obtain cDNA 250 ng of each total RNA template was subjected to reverse transcription using 200 U of Superscript III reverse transcriptase (Invitrogen, Carlsbad, California), according to manufacturer´s instructions. For gene expression of each of the peptides the specific forward and reverse primers previously described by Tetens et al. (2010), were used. In addition to target genes, PCR primers for bovine GAPDH were included as reference genes.

2.3. Quantitative Real-Time PCR

The qRT-PCR was carried out on a CFX96 real time PCR (Bio- rad) with the SYBR Green technique (Invitrogen) following the manufacturer´s instructions. The reactions were set up in a final volume of 20 µL containing 25µmol/L of dNTPs and 0.5 µM of each primer using the Platinum SYBR Green qPCR Super Mix UDG Kit (Invitrogen). The following amplification protocol was used: initial denaturation at 95°C for 3 min, followed by 40 cycles consisting of denaturation (95ºC for 15 s), annealing (58° C for 30 s), and elongation (72°C for 30 s). This protocol was followed by a melting curve analysis. All runs included a negative control (water + primers; primers + dNTPs).

A standard curve was built with the dilutions of the final point PCR product obtained for each gene, utilizing dilutions from 1x10^{10} to 1x10^2, as well as the negative controls (water + primers; primers + dNTPs). The amounts of the specific mRNA of each gene and each anatomical region were determined according to the standard curve. Also, since the mRNA of GAPDH is constitutively expressed, was used as a reference gene (Infante et al., 2005). The target genes measurements were normalized against GAPDH to make the results comparable between the different localizations. The qPCR results were reported as the ratio of the amount of mRNA of each of the specific peptides genes upon 1x10^6 copies of the GAPDH gene.

3. Results and discussion

3.1. Performance of qPCR

The limit of detection for the qPCR was 100 copies of mRNA according to the last dilution of the standard curve (1x10^2). The melting curve analysis revealed no signs of additional unspecific PCR products, indicating that the primer pairs were specific. As an example, Figure 1 shows the melting curve for LAP.The stability of the housekeeping gene (GADPH) appeared to be good with a mean threshold cycle (C$_t$) standard deviation between repeated measures below 0.5 cycles.

3.2. Expression of AMP in the mammary gland with mastitis

The transcripts of all studied *β*-defensins genes were found in all localizations of the udder but at different levels. The expression levels for *BNBD4*, *BNBD10* and *DEFB1* are shown in figure 2. *BNBD10* and *DEFB1* displayed the same pattern of expression in the 5 localizations of the mammary gland evaluated, with the highest number of mRNA copies in inguinal lymph node (*BNBD10* 1.78x10^{7}and *DEFB1* 3.14 x 10^6, respectively), followed by parenchyma (*BNBD10* 5.13 x 10^6 and *DEFB1* 2.17 x 10^6, respectively), streak canal (*BNBD10* 2.8 x 10^6 and *DEFB1* 1.31 x 10^6, respectively), cisterna (*BNBD10* 1.36 x 10^{6}and *DEFB1* 6.27 x 10^5, respectively), and the lowest level detected in Rosette of Furstenberg (*BNBD10* 7.11 x 10^5 and *DEFB1* 5.58 x 10^5, respectively), although the levels of expression were higher for *BNBD10* with respect to *DEFB1*. The expression pattern of *BNBD4* was found to differ from that of the other two β-defensins, except for the inguinal lymph with the highest level of expression (3.62 x 10^5), and the Rosette of Furstenberg with the lowest expression (8.3 x 10^1). Moreover, mRNA expression in

cisterna was moderate (2.17×10^4), whereas the parenchyma and streak canal showed a similar low expression level (7.2×10^3 and 4.7×10^3, respectively).
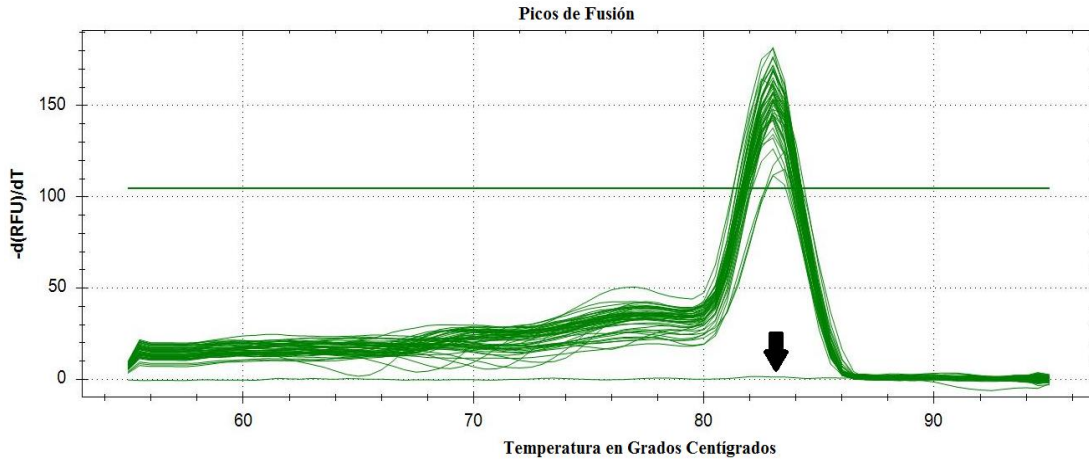

Fig. 1. Melting curve for BNBD10 showing that no amplification was observed in the negative controls (pointed by black arrow), demonstrating that the primers were specific. In addition, both the samples and positive controls showed the same melting temperature.
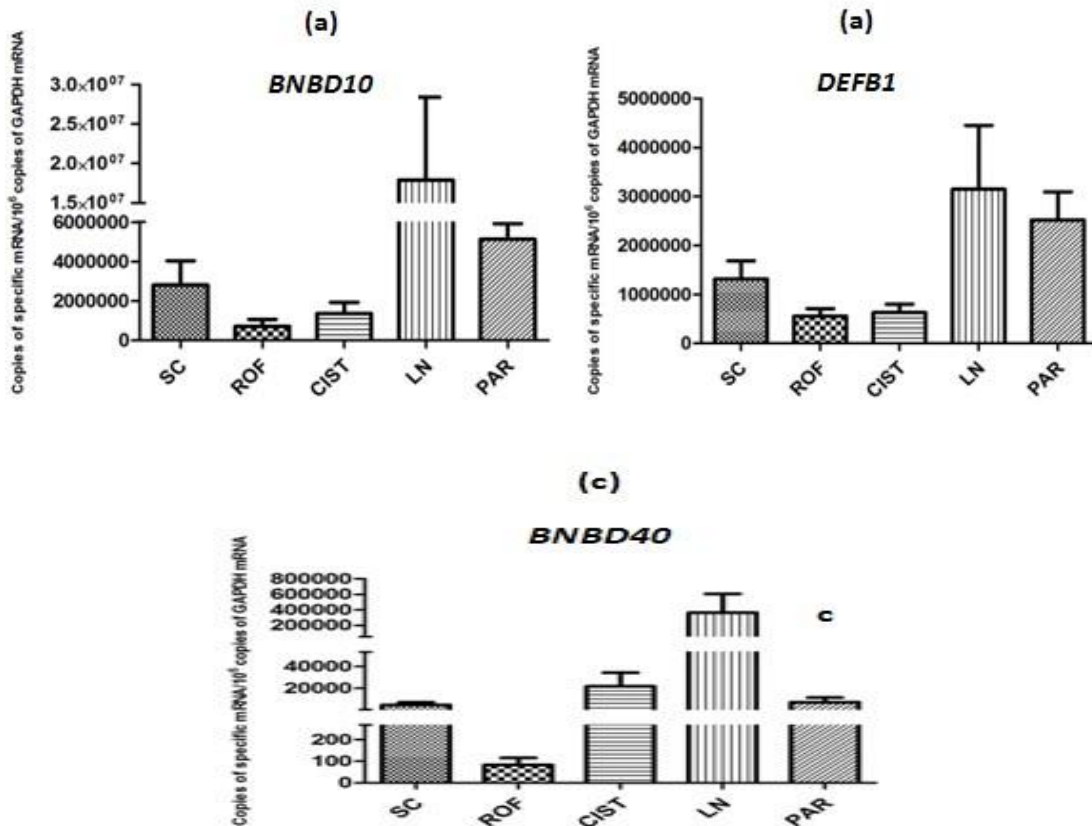

Fig. 2. Level of mRNA for the *β*-defensins *BNBD10* (a), *DEFB1*(b) and *BNBD4*(c) in 5 different localizations of the bovine mammary gland with mastitis by *S. aureus*. The number of copies of mRNA is normalized against the expression of the bovine gen GAPDH. *BNBD4* (bovine neutrophil *β*-defensin-4); *BNBD10* (bovine neutrophil *β*-defensin-10); *DEFB1* (*β*-defensin-1).

4. Discussion

In the present study the expression of the *β*-defensins *BNBD10*, *DEFB1* and *BNBD4* was confirmed in 5 localizations of the mammary gland with mastitis by *S. aureus*. The highest level of expression for the *β*-defensins *BNBD10*, *DEFB1* and *BNBD4* was restricted to the lymph node, and the lowest to the Rosette of Furstenberg, but with different total amounts. Except for *DEFB1*, these results match with those by Tetens et al. (2010) and Goldammer et al (2004) who reported the constitutive expression of these AMP´s in the healthy udder, which have been related to the *β*-defensin expression of neutrophils residing there (Tetens et al., 2010; Goldammer et al., 2004), therefore according to the results in this study these AMP´s might be also induced in the presence of *S. aureus* due to high affluence of neutrophils during the chronic inflammation. Furthermore, these results are similar to those obtained by Gunther et al (2009) who found that these AMP´s were inducible in the mammary gland with mastitis caused by *E. coli*, and therefore act as weapons against invading pathogens. Further, Kosciuczuk et al (2014) and Whelehan et al (2011) also found that expression of *BNBD4* and *DEFB1*, but not *BNBD10*, was significantly higher in *S. aureus* infected than healthy tissues. Hence, these *β*-defensins could be inducible with the presence of different infectious pathogens and the degree of expression might differ depending on the pathogen involved.

Comparing the pattern of expression obtained in this study for these 3 *β*-defensins with that in the healthy mammary gland we canconclude the following: for *BNBD10*: the highest expression was in the lymph node as in the healthy udder; could be inducible in Rosette of Furstenberg and parenchyma as it was not detected in healthy udder; and could be constitutive and inducible in cisterna and streak canal since was expressed in infected and non-infected tissue. For *DEFB1*: could be inducible in streak canal and lymph node, since high levels of expression were obtained compared with the healthy udder; could be constitutive and inducible in cisterna, Rosette of Furstenberg and parenchyma, since was expressed in healthy and diseased tissues, at different levels. Lastly, for *BNBD4*: the highest expression was in the lymph node as in the healthy udder; could be inducible in streak canal and Rosette of Furstenberg as it was not detected in healthy udder; could be constitutive and inducible in cisterna and parenchyma. Therefore, the results obtained in this study are consistent with those found in other studies including both healthy and mastitic tissues and that were inducible in the presence of *S. aureus*.

5. Conclusion

The present study shows the pattern and level of expression of *BNBD10*, *DEFB1* and *BNBD4* in 5 localizations of the mammary gland, confirming their role in the defense of the cow mammary glandwith chronic mastitis by *S. aureus*. The highest expression for the three *β*-defensins was limited to the lymph node and could be related to the *β*-defensin expression of neutrophils residing there; followed by parenchyma (for *BNBD10* and *DEFB1*) and cisterna (*BNBD4*), which might suggest that their expression could be increased once the pathogen is established in these regions of the mammary gland suggesting a chemiotactic role, due to the chronic inflammation induced by the persistence of *S. aureus*.

Acknowledgment

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