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Original article

Expression of the lingual antimicrobial peptide (*LAP*) in the bovine mammary gland with *Staphylococcus aureus*

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ABSTRACT

Mastitis is one of the most important infectious diseases in dairy cows around the world that causes great economic loses. To prevent and treat mastitis is important to understand the immunology of the mammary gland. Mammalian *β*-defensins are peptides of the local innate host defense with a potential as therapeutic agents. With the aim to better understand the role of these local antimicrobial peptides in the prevention of bovine mastitis, in the present study the mRNA expression of the lingual antimicrobial peptide (*LAP*) was determined in 5 localizations of the bovine mammary gland with mastitis applying a quantitative real time PCR. The results showed that *LAP* was mainly expressed in the cisternal tissue with an average of 1.36×10^7 copies of mRNA, followed by 9.7×10^6 copies in parenchyma, and the lowest expression was obtained streak canal. Therefore, *LAP* could be involved in the local defense of the mammary gland since is over expressed in these regions where *S. aureus* mainly invades and multiplies.

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 et al., 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 expression of *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).The constitutive expression of *LAP* has been shown in the healthy bovine mammary gland (Tetens et al., 2010; Goldammer et al., 2004). Also, LAP has 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). Moreover, Knsciuczuk et al. (2014) confirmed the constitutive expression of LAP in healthy mammary parenchyma but the expression was significantly higher in *S. aureus* infected tissue. Another study reported that bovine mammary epithelial cells (bMEC) showed a basal expression of *LAP*, but also was 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 the *β*- defensing *LAP* 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^{o}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 (1×10^{2}). 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 (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 mRNA expression levels for *LAP* are shown in Figure 1. The highest expression was detected mainly in cisternal tissue (1.36×10^{7}), followed by parenchyma (9.75×10^{6}). Lower levels of expression were observed in rosette of Furstenberg (4.72×10^{6}), inguinal lymphnode (9.52×10^{5}) and streak canal ($2.0x\ 10^{5}$), respectively.

The *β*- defensins are a group of multifunctional peptides with antimicrobial activity against a broad spectrum of microbes including bacteria, viruses and fungus (Ganz, 2003). *LAP* representsa non-oxidative microbicide mechanism to contain pathogenic microbes(Das *et al.*, 2010). The highest levels of expression of *LAP* in cisternal tissue and parenchyma suggest that mRNA *LAP* is inducible at the site of *S. aureus* infection, since these tissues are the first exposed or invaded by microorganisms(Molenaar et al., 1996). These results are consistent with previous studies where by in situ hybridization analysis found the predominant expression of *LAP* in cisternal tissue from mammary gland with mastitis (Singh et al., 2004; Das et al., 2010), and confirms the involvement of *LAP* as a weapon against chronic mastitis, either as an antimicrobial defense or as mediators of inflammation.

The high expression of *LAP*suggests that this *β*-defensin could play an important role as local mechanism of defense in the mammary gland with mastitis.

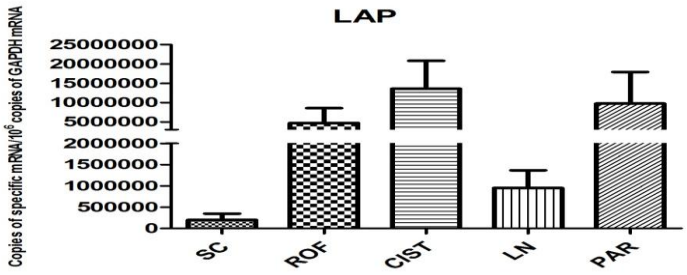

Fig. 1. Levels of mRNA for *LAP*in different localizations of the bovine mammary gland with mastitis by *S. aureus*.

4. Conclusion

The present study shows the pattern and level of expression of LAP in 5 localizations of the mammary gland, confirming its high expression especially in cisternal tissue and parenchyma of the bovine mammary glandwith chronic mastitis by *S. aureus*. This might be the regions of the gland where *S. aureus* internalize the cells and remains unable to be cleared resulting in a chronic mastitis, and it is possible that *LAP* function as a mediator of inflammation.

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The authors declare that they have no conflict of interest.

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