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Research into the importance of antimicrobial peptides in the bovine mammary gland

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Introduction

Bovine mastitis is one of the most expensive dairy-based diseases worldwide. It is caused by a broad spectrum of bacterial and fungal pathogens and is resulting in an increased amount of somatic cells in milk. Because of the activation of the immune defense through the invading pathogens. Hence, it is negatively influencing both, the milk yield and the processing quality (KING, 1978; MEIJERING *et al*., 1978). Next to an improvement of the environmental issues, it is possible to improve the udder genetically, because the heritability for the trait of mastitis is around 0.1 (HINRICHS *et al*., 2005). In this context, β-defensins are an interesting group of genes, not at least because they are located in a cluster on chromosome 27 (GALLAGHER *et al*., 1995), a genomic region with influence on the udder health (KÜHN *et al*., 2003). β-defensins belong to the larger group of antimicrobial peptides, which are known as evolutionary old components of the innate immunity of vertebrates, insects and plants. After the invasion of pathogens antimicrobial peptides cause the permeabilization of the membrane of the invading pathogens and therefore their lysis. β-defensins are multifunctional peptides with antimicrobial activity against gram-positive and gram-negative bacteria, some viruses and fungi. In addition to their antimicrobial activity they act as chemoattractants for T-lymphocytes, monocytes and immature dendritic cells (CHERTOV *et al*., 1996; RISSO, 2000; YANG *et al*., 1999). Their existence in the bovine mammary gland and their antimicrobial activity against *Staphylococcus aureus, Escherichia*

coli, Pseudomonas aeruginosa, and *Candida* spp. (DIAMOND *et al*., 1991; SCHONWETTER *et al*., 1995), causal agents of bovine mastitis, lead to the suggestion that they have an important role in the mechanisms of resistance to infectious diseases of the bovine mammary gland and therefore seem to be of particular importance to the udder health of dairy cattle.

The aim of this study is to show differences in the expression of antimicrobial peptides, particularly of the β-defensin genes in abundance of the different mastitis causing pathogens and to show via immuno-histochemical research the accurate location of the expression of antimicrobial peptides within the bovine mammary gland and therefore reveal the basic importance of antimicrobial peptides for the udder health of dairy cattle.

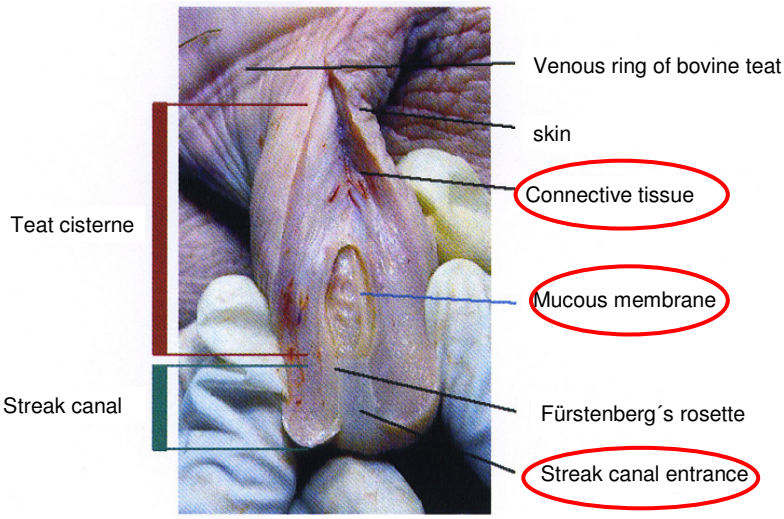
Material and Methods

In order to find defensin-genes, defensin-gene containing BACs were isolated by screening the bovine BAC-libraries RZPD No. 750 and 754 using defensin-consensus primers. 20 BACs containing defensin genes were identified. These clones were used to create two different contigs, which was highly labour-intensive, because some genes within the β-defensin-gene-region exist as duplicated genes.

The expression of β-defensin-genes in the tissue of the bovine mammary gland was demonstrated using RT-PCR-techniques and cDNA-sequencing.

Therefore, tissue samples of the bovine mammary gland were taken from different lactating cows with different clinical findings concerning mastitis, immediately after slaughter.

Picture 1: Samples taken from:



The teats were cut off the udder, opened inside their epithelial surfaces and then tissue samples were taken in the area of the teat cisterne and the streak canal. To gain further information about the infection status, microbiological research of some of the tissue was done by the Federal Ministry of Food, Agriculture and Consumer Protection in Kiel for other tissue samples histological-pathological research was done.

Tissue samples were stored in liquid nitrogen and total RNA was isolated. Then synthesis of complementary first strand DNA was performed. Afterwards complete cDNA was amplified using specific primers to give evidence to the expression of β-defensin-genes in the bovine mammary gland.

In addition to these expression studies, quantitative measurements were performed about the amount of expressed β-defensins and psoriasin (S100A7), another antimicrobial peptide, not belonging to the β-defensin-family, which is also expressed in udder tissue in abundance to the existence of mastitis causing pathogens, using real-time-PCR methods.

Next to the qualitative and quantitative analysis of udder tissue samples a MAC-T-Cell-line (Mammary Alveolar Cell with large T-antigen) is available, used to analyse different stimulation experiments, in order to show possible differences in the amount of the expression of individual β-defensins and their possible inducing period, subjecting to different mastitis causing pathogens. This MAC-T bovine mammary epithelial cell line is stable transfected with simian virus 40 large T-antigen, originally established as an *in vitro* model for bovine lactation (HUYNH *et al.,* 1991). Accessory to the MAC-T-cell-line,

samples from a primary cell culture were kindly allocated by B. Griesbeck from the University of Munich. This cell culture was established at the FBM in Dummerstorf.
The cells were incubated with *E. Coli*, *Staph. aureus* and the associated media for 1 h and 24 h, respectively.
To gain further information about the expression of β-defensin-genes subjecting to different mastitis causing bacteria we gained samples from a clinical infection trial. In this trial the udder of a healthy cow was intentional infected with *E. coli.* After 24 h of infection the cow was slaughtered and samples from the bovine mammary gland were taken as described.
For the recombinant protein production of the bovine psoriasin (S100A7), cDNA (amplified from bovine udder tissue RNA) was ligated into the bacterial vector pet 22b and expressed as a cytoplasmatic soluble protein in *E. coli* type BL21. Afterwards a purification using a cationic exchange was done. Next to that an activity-test was established.
Additionally, plasmids of the β-defensins LAP (lingual antimicrobial peptide, TAP (tracheal antimicrobial peptide), EBD (enteric antimicrobial peptide) , BNBD4,-5 (bovine neutrophil antimicrobial peptides) and the DEFB300 as well as the S100A7 (psoriasin) were produced from purified PCR products. Clones were streaked out on agar plates and tested by PCR, using gene-specific primers. Positive clones were grown in SOC-medium and then plasmids were prepared and sequenced.
Plasmids were used to establish dilution series in order to test the sample cDNA for the existence of the target β-defensin gene, for absolute quantification and in order to define a detection limit.

Results and Discussion

Antimicrobial peptides, a large family of evolutionary old molecules are part of the immune systems of vertebrates, insects and plants. They dispose a broad-range antimicrobial activity against gram-positive and gram-negative bacteria, some viruses and fungi and furthermore they have a function as signal molecules in the innate immune system as chemoattractants for T-lymphocytes, monocytes and immature dentritic cells. Therefore we hypothesised that antimicrobial peptides, particularly β-defensin genes might be candidate genes for the resistance to mastitis.

In previous research two contigs were established to show the progression of the β-defensin genes on BTA 27. Thereby 20 defensin genes were identified.

Of these 20 identified genes we proofed LAP (*lingual antimicrobial peptide*), TAP (*tracheal antimicrobial peptide),* EBD *(enteric bovine β-defensin)*, the bovine neutrophil β-defensins (BNBD) number 3, 4, 5, 9,10 and 12, the so called DEFB300 and DEFB401 and additionally two different versions of psoriasin (S100A7) and RNASE 7 to exist in infected and non infected udder tissue, using RT-PCR-techniques.

Within the analysed samples (real-time-PCR) it was possible to show quantitative differences in the amount of expression, regarding to the infection status of the udder tissue (healthy tissue and infected tissue) but not to the causing pathogens. Differences were also found in individual animals used for sampling. This can possibly be ascribed to the existence of gene duplications within the β-defensin gene region on BTA 27, a genomic region containing a quantitative trait locus for udder health and to the different kinds of mastitis we found, for example, interstitial mastitis, acute and chronicle mastitis or even subclinical mastitis.

Working with the cell culture it was possible to show RNA-expression of β-defensin genes with the exception of RNase7 and the so called BNBDs (bovine neutrophil beta defensins) in cells stimulated with TNF-α, IL1-β, PMA, LTA, LPS*, E. coli, Pseudomonas spp.* and *Streptococcus spp.* BNBDs were identified in the udder tissue, but not in the MAC-T cell line. First quantitative outcomes tended to result in an advanced amount of expressed LAP, TAP, EBD and psoriasin (S100A7) in infected cells, confirming other findings that some bovine β-defensins are as well constitutively expressed as inducible. To ascertain that, meanwhile , time- and dose dependent stimulation experiments were going on with the cell culture, to make sure to narrow the time-frame of the induction of the expression of β-defensin genes and simultaneously to eliminate the chance of mistiming the harvesting of the cells in a moment were the expression of β-defensins is already down-regulated. Dilution series with the generated plasmids are done down to the level of 10^{-6} ng plasmid-DNA to be able to quantify the proper amount of the expressed β-defensin genes in udder tissue and cell culture in connection with the infection status and the proper amount of causing pathogens. Unfortunately these experiments showed that the amount of expressed β-defensin-gene RNA was so close to the detection limit that a further quantification was futile.

Concerning the primary cell culture, we found the following. The results show a significant increase of the amount of defensin-gene-RNA within those samples incubated with *Staph.*

aureus for 1 h compared to all the other samples. This leads to the suggestion that the expression of defensin-gene-RNA might be time-dependent.

In contrast to the results of the udder tissue samples, the results from the clinical infection trial showed different amounts of expression of β-defensin-RNA relating to mastitis causing pathogens. It was possible to show a significant increase of the amount of expressed β-defensin-RNA concerning udder tissue samples infected with *E. coli*. This indicates, that it seems to be of essential importance to have an identical genomical background, that is to say, infected and non-infected samples from one identical animal, in order to eliminate any individual differences in the amount of expressed defensin-genes by any reason. Aim of the protein biochemical work is to identify amino acid sequences of the purified peptides in the udder, which is already done in case of the psoriasin (S100A7). GLÄSER *et al.* (2005) found that human psoriasin is active against *E. coli* in human epithelia. The recombinant produced bovine psoriasin was tested for activity and showed antimicrobial activity against *E. coli.*

In further protein biochemical works it is planned to assign the spectrum of other sensitive causing pathogens and to compare them to the ones of the human psoriasin (S100A7). Additionally, it is planned to establish polyclonal antibodies to the bovine psoriasin in order to do some immuno-histochemical research.

Conclusion

It was possible to show differences in the expression of antimicrobial peptides in udder tissue, regarding to the individual animal the samples taken from as well as to the infection status of the udder (healthy tissue and infected tissue). In case of the clinical infection trial it was possible to show differences even concerning the causing pathogen. Further investigation in connection with mastitis causing pathogens should be done in form of clinical infection trials.

Working with samples from the primary cell culture, it was even possible to show different amounts of defensin-gene expression in dependence of the different stimulation periods, whereas the measured amount of defensin-gene expression within the MAC-T-Cell-line was too close to the detection limit to make a further quantification reasonable.

Additionally polyclonal antibodies to the bovine psoriasin have to be generated to show the accurate location of the expression of antimicrobial peptides within the bovine mammary gland.

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