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Two β Defensin Cationic Peptides from Mastitic Milk of *Bubalus bubalis*

[1]H. Das, [2]S.U. Ahmed, [3]S.K. Shukla, [3]S. Shukla, [1]A. Latif and [3]D. Sharma
[1]Department of Physiology and Biochemistry,
College of Veterinary Science and Animal Husbandrary,
SDAU, Sardarkrushinagar, Gujrat-385506, India
[2]Indian Veterinary Research Institute, Izatnagar-243122, U.P., India
[3]Central Avian Research Institute, Izatnagar-243122, U.P., India

Abstract: Mastitis represents a failure of innate immune mechanism of udder teat canal epithelial cells and peripheral PMN cells, whose cationic peptides constituted innate immunity. An attempt was made to examine in the year 2007, the actual presence of β-defensins peptides in milk of mastitis-affected buffalo (*Bubalus bubalis*), due to lack of such information. AU-PAGE revealed higher frequency and density of the peptides in case of mastitis milk as compared to normal milk sample, particularly in the most cationic zone with highest electrophoretic mobility or lower molecular weight region. These low molecular peptides were also separated out using 10 kDa cut-off membrane ultra filtration, which was subsequently examined for *in vitro* antibacterial sensitivity and subjected to SDS-PAGE and low molecular weight zone was further subjected to MALDI-TOF mass spectrometry, which identified the most anodic two peptides of about 5 kDa as β-defensin viz. The LAP (lingual antibiotic peptide) and BNBD-2 (bovine neutrophil beta-defensin-2) based on amino acid sequences unlike that of healthy buffalo milk. Sequence homology studies also supported mass spectrometry data as both the peptides shared 100 and 98.5% identities with cattle β-defensins, respectively. Antibacterial assay against *S. aureus* and *E. coli* revealed significant activity of these peptides against both gram-positive and gram-negative organisms at 10 μg concentration. It is concluded that expression of β-defensin peptides in ductal epithelium were induced in mastitis and these defensin peptides were also released into the milk.

Key words: Beta-defensin, buffalo, innate immunity, mastitis

INTRODUCTION

Mastitis is an inflammatory condition of mammary gland caused by wide range of bacteria a nd fungi and its prevalence is alarming. *Staphylococcus aureus* and *Escherichia coli* are the two most contagious pathogens responsible for bulk of the clinical cases of bovine mastitis (Bannerman *et al*., 2004; Barkema *et al*., 1998). Innate immune system of mammals contributes significantly to counteract mammary infections (Goldammer *et al*., 2004). The β-defensins, an integral component of innate defense mechanism, are secretary cationic peptides that exhibit antibiotic and cytotoxic properties (Kagan *et al*., 1994; Rainard and Riollet, 2006). These are multifunctional small peptides of 30-40 amino acid

Corresponding Author: Sanjeev Kumar Shukla, Department of Biochemistry,
Indian Veterinary Research Institute, Izatnagar-243122, U.P., India

residues and effective against a variety of microbes such as Gram-positive and Gram-negative bacteria, mycobacteria, fungi, yeast and some enveloped viruses (Klotman and Chang, 2006; Yeman and Yount, 2003). Besides, β-defensins also link both innate and adaptive immune response due to their chemo attractant property on both immature dendritic cells and memory T cells (Yang *et al*., 1999, 2001) as well as for monocytes (Garcia *et al*., 2001).

Buffaloes (*Bubalus bubalis*), a unique species contributing considerably to farm economy in the form of milk and meat, remains poorly understood in terms of innate immunity. Preliminary reports from this laboratory have characterized cationic antimicrobial peptides from myeloid cells (Das *et al*., 2006) neutrophil granules (Sahoo *et al*., 2000, 2004; Singh *et al*., 2004) and various epithelial tissues like trachea, tongue (Anbu *et al*., 2002) and intestine (Das *et al*., 2005) of buffaloes; however, no studies have been carried out to investigate expression of β-defensin peptides in mastitis milk buffalo. Further, buffaloes are known to be relatively more resistant to mastitis than other species (Joshi and Gokhale, 2006). The arsenal of antimicrobial peptides also differs from one animal species to another (Ganz and Lehrer, 1995). Hence, it will be interesting to characterize β-defensins from milk obtained from infected mammary quarter of buffalo. Present manuscript describes purification, primary structure determination and antibacterial activity of two novel β-defensin peptides secreted through mastitic milk of buffaloes. It has been studied with regard to effects of variations in pH, time of reaction and concentration of reactants, permissible levels of reagents and interfering substances (Lowry *et al*., 1951).

MATERIALS AND METHODS

Buffaloes

Institutional dairy farm buffaloes suffering from mastitis showing visible signs of clinical mastitis were selected for the study.

Milk

Milk samples from all the affected quarters were collected aseptically in sterile bottles and brought to the laboratory on ice immediately for further processing.

Separation of Acellular Milk from Whole Milk

Whole milk was centrifuged at 200 g for 20 min at 4°C. The upper lipid layer and cell pellet, if any, were discarded and the remaining lipids and sediments etc., were removed by filtration through cotton wool. The resulting solution was again centrifuged at 27000 g for 30 min at 4°C. The supernatant comprised the acellular milk, which was collected in sterile tubes, the pellet being discarded (Pickering *et al*., 1983). The acellular milk was stored at 4°C after adjusting the pH to 7.0-7.5 with 1 M NaOH.

Peptides Purification by Chromatography

The method described by Valore *et al*. (1998) as followed to purify the cationic proteins and peptides with slight modification 20 mL of the acellular milk was mixed with 4 mL slurry of the cationic exchanger, Macro-Prep CM (Biorad,USA) equilibrated with 4-5 volume of 0.01% acetic acid, pH 7.0-7.5. The mixture was kept at room temperature for 2 h with intermittent stirring. The Macro-Prep CM beads were then allowed to settle overnight at 4°C, poured into the column and washed with 25 mM ammonium acetate, pH 7.5, until stable absorbance at 280 nm was achieved. The adsorbed proteins and peptides were eluted with 5% acetic acid. The column effluent was continuously monitored at 280 nm and 1.5 mL fractions were collected. The protein fractions so obtained were pooled and stored at -20°C.

Separation of Less than 10 kDa Peptides

Further, the eluted proteins were subjected to Centricon concentrators YM-10 (Amicon Inc, USA), that is of 10 kDa cut-off membrane filters, which were subsequently centrifuged for 30 min, 4000 g at 4°C as per the manufacturer's instructions. The filtrates containing the peptides of <10 kDa were lyophilized and stored at -20°C, after estimating the protein concentration.

Bactericidal Assay

The bactericidal activity of the peptide fraction below 10 kDa was assessed against *Escherichia coli* and *Staphylococcus aureus* by disc diffusion test (Cruickshank *et al*., 1975).

Polyacrylamide Gel Electrophoresis

The cationic proteins and peptides of <10 kDa fraction were identified by their electrophoretic mobility on acid urea-polyacrylamide gel electrophoresis (AU-PAGE). The purity and molecular mass of the peptides was determined by Sodium-Dodecyl Sulfate Electrophoresis (SDS-PAGE).

Maldi-tof Mass Spectrometry

Two peptide bands from the lowermost region in Coomassie Blue-stained SDS-PAGE gel were excised and digested with mass spectroscopy grade trypsin (Promega). The resulting peptides were mixed with matrix and subjected to MALDI-TOF analysis for peptide mass fingerprinting and protein sequencing using Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). The resulting m/z spectra were used to search for matching proteins in the NCBI database using the MASCOT search program (www.matrix Science.com) and significant matches were identified. Scores were calculated by the molecular weight search score algorithm (MOWSE) and significant ($p<0.05$) if greater than 35 in case of LAP and 27 in case of BNBD-2. Thus, a search result would be expected to occur at random with a frequency of less than 5% (Pappin *et al*., 1993).

Sequence Analysis

Interpreted amino acid sequences are then used for sequence homology search using National Center for Biotechnology Information (NCBI) BLAST network server (http://www.ncbi.nlm.gov/BLAST). Phylogeny analysis was carried out for both the sequences with the help of 'MegAlign program' of Lasergene software (DNAstar Inc., USA). The signal peptide and propeptides were determined by SignalP 3.0 Server using neural network (NN) and hidden Markov models (HMM) trained on eukaryotes (http://www.cbs.dtu.dk/services/SignalP-3.0/).

RESULTS AND DISCUSSION

On comparing the AU-PAGE pattern of cationic proteins from milk samples of both healthy and mastitis-affected buffaloes, it is observed that expression of cationic peptides (most cathodic) is enhanced in mastitis (Fig. 1). The cationic proteins and peptides were subjected to ultra filtration with 10 kDa cut-off membrane that resulted into retentate and filtrate fraction. The concentration of the filtrate containing lower than 10 kDa polypeptides was 0.452 mg mL^{-1}, whereas that of retentate was 0.321 mg mL^{-1}. Both the fractions exhibited significant antibacterial activity against *E.coli* and *S. aureus* grown in Muller Hinton Agar

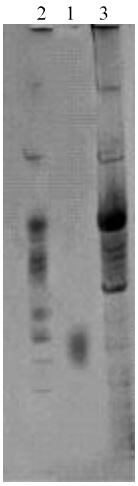

Fig. 1: AUPAGE pattern of normal and mastitis milk cationic proteins and peptides; lane 1: Lysozyme reference; lane 2: Normal milk; lane 3: Mastitis milk

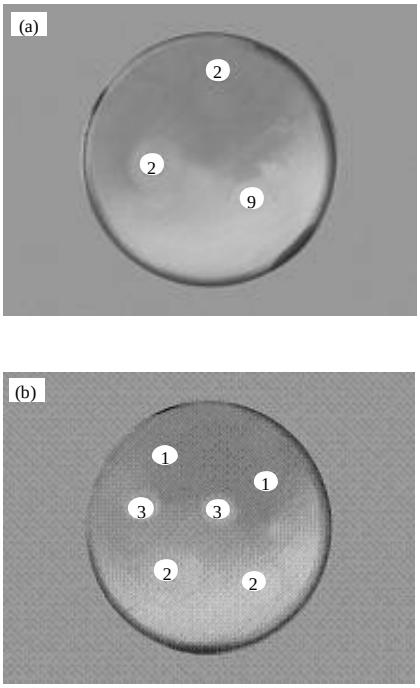

Fig. 2: (a) *E.coli* and (b) *S.aureus* zone of inhibition. 1: Control; 2: Filtrate fraction and 3: Retentate

(MHA) as indicated by clear inhibitory zone around the discs treated with 10 μg mL^{-1} concentration of each fraction (Fig. 2a, b). However, filtrate fraction demonstrated greater antimicrobial activity as it produced wider zone of inhibition. The AU-PAGE of this fraction

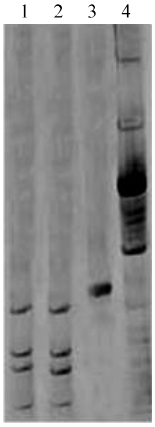

Fig. 3: AUPAGE pattern of <10 kD fraction from mastitis milk: lane 1 and 2: <10 kD filtrate fraction; lane 3: Lysozyme reference; lane 4: Proteins and peptides in retentate

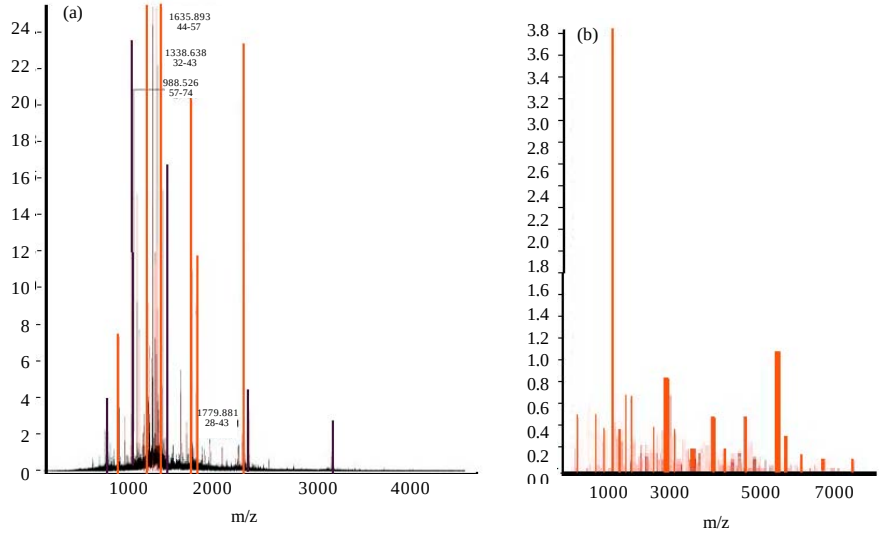

Fig. 4: (a, b) MALDI-TOF-MS {m/z} spectra

subsequently revealed presence of 4 major peptide bands in lower than 10 kDa fraction with greater cathodal migration as compared to reference lysozyme as well as higher molecular weight proteins (Fig. 3).

This finding indicates the presence of more cationic low molecular polypeptides released in mastitis milk. Molecular weight and homogeneity of all these four peptides were assessed by 5-20% gradient SDS-PAGE and found to be in between 3.5 kDa to 10.0 kDa (Fig. 6). Conversely, above 10 kDa fraction represents a pool of proteins and peptides with higher range of molecular weights and lower cationicity than the polypeptides below 10 kDa. The SDS-PAGE profile further reveals that out of the four peptides detected in AU-PAGE, two peptides possess molecular weight in the range of 3.5 to 6.5 kDa (Fig. 6). These two peptide bands with the lowest molecular weights were excised and subjected to MALDI-TOF-MS analysis to determine the amino acid sequence. The resulting m/z spectra have been shown in Fig. 4a and b. MASCOT search results revealed a top score of 101 for Lingual

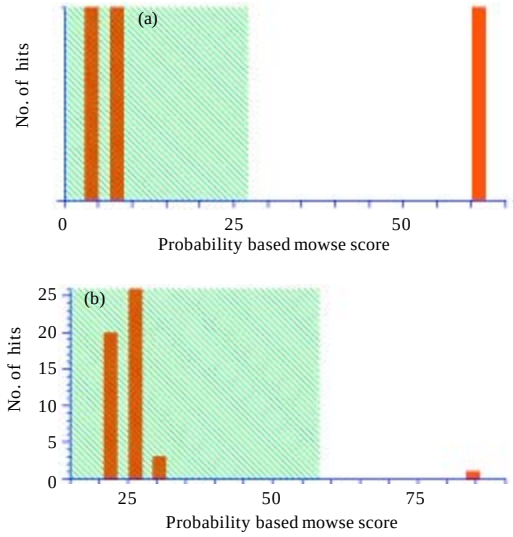

Fig. 5: (a, b) MALDI-TOF-MS-MASCOT search results

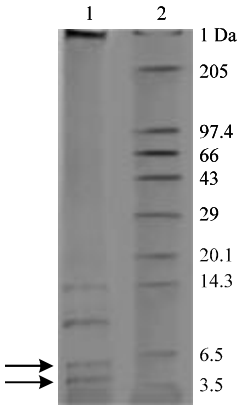

Fig. 6: SDSPAGE of <10 kD fraction from mastitis milk: lane 1,<10 kD and 2, protein molecular wt., markers (205, 97.4, 66, 43, 29, 20.1, 14.3, 6.5 and 3.5 kD). Horizontal arrows shows the bands subjected to MALDI-TOF

Antibiotic Peptide (LAP) (Fig. 5b), where probability based mowse score >35 are significant and indicates identity or extensive homology ($p<0.05$). The search also yielded a top score of 58 for beta-defensin-2 (BD-2), where scores >27 is significant ($p<0.05$) (Fig. 5a). Figure 7a and b show the primary structure of LAP and BD-2 of buffalo. The amino acid sequences of both LAP and BD-2 have been submitted to UniProt KB/Swiss-Prot database and the accession numbers are A3RJ36 and P85159 for LAP and BD-2, respectively. Amino acid compositions of these peptides as shown in Table 1 demonstrate presence of 6 cysteine residues in both the peptide. Furthermore, both LAP and BD-2 were found to be rich in basic amino acids. The LAP sequence contain 7 arginine residues and 3 lysine residues; whereas, BD-2 sequence possess 8 arginine residues, 1 lysine residue and 1 histidine residue (Table 1). The identity of the peptides examined by MALDI-TOF was further confirmed by

H-Val-Arg-Asn-His-Val-Thr-Cys-Arg-Ile-Asn-Arg-Gly-Phe-Cys-Val-Pro-Ile-Arg-Cys-Pro-Gly-Arg-Thr-Arg-Gln-Ile-Gly-Thr-Cys-Phe-Gly-Pro-Arg-Ile-Lys-Cys-Cys-Arg-Ser-Trp-NH_2

H-Val-Arg-Asn-Ser-Gln-Ser-Cys-Arg-Arg-Asn-Lys-Gly-Ile-Cys-Val-Pro-Ile-Arg-Cys-Pro-Gly-Ser-Met-Arg-Gln-Ile-Gly-Thr-Cys-Lys-Gly-Ala-Gln-Val-Lys-Cys-Cys-Arg-Arg-Lys- NH_2

Fig. 7: (a) Primary structure of β-defensin-2 and (b) Primary structure of lingual antibiotic peptide

Table 1: Distribution of amino acids in LAP and beta-defensin 2

Residues	Lingual antibiotic peptide	Beta-defensin 2
Cys	6	6
Asp	2	2
Ser	3	1
Gly	4	4
His	0	1
Arg	7	8
Thr	1	2
Ala	1	1
Pro	2	3
Val	3	3
Met	1	0
Ile	3	4
Leu	1	0
Phe	0	2
Lys	3	1
Try	0	1
Gln	3	1
Total	40	40
MW (Da)	4627	4444
SDS-PAGE	2	1

(order)^{r} r Relative order of migration with 1 being the highest Rf value

studying their sequence homology with those of other species retrieved from EMBL database. Amino sequence similarity search using BLAST algorithm revealed substantial identity of both LAP and BD-2 of buffalo with bovine β-defensin family peptide members. Percent homology search using MegAlign program of Lasergene software (DNAstar, Inc) also supported the result of BLAST search as BD-2 and LAP of buffalo shares 100 and 98.5% identity with those of cattle, respectively. Conversely, only moderate level (50-70%) of sequence similarities were noticed for both sequences with β-defensins of goat, sheep, horse, mouse and human. Dendogram constructed for phylogenetic analysis suggests common ancestral lineage of both BD-2 and LAP sequences of different species (Tomasinsig and Zanetti, 2005).

Mastitis is one of the most costly dairy-based diseases throughout the world affecting both yield and processing quality of milk (Boulanger *et al*., 2003). It is also a major cause of premature culling of dairy animals causing significant economic losses to dairy industry (Rainard and Riollet, 2006). Unrevealing the immune mechanism of the mammary gland is instrumental for developing effective therapeutic measures to control mastitis.

Polymorphonuclear neutrophils (PMNs) are the first immune effectors recruited to the antibacterial peptides like β-defensins (Tunzi *et al.*, 2000). The production of natural antibiotic peptides has emerged as an important mechanism of innate immunity in plants and animals (Ganz, 2003).

In this study, we report presence of two β-defensin antimicrobial peptides viz., LAP and BD-2 in mastitis milk representing the non-oxidative microbicidal mechanism for containing pathogenic microbes. Previous studies on cationic peptides from buffalo PMN cells (Bannerman *et al.*, 2004; Sahoo *et al.*, 2004; Singh *et al.*, 2004), tongue (Anbu *et al.*, 2002) evidenced MIC of these peptides to be in the range of 10-15 μg against *E.coli*, *S.aureus* and *P. multocida*. Present work has also demonstrated substantial *in vitro* bactericidal activity of the cationic peptides extracted from milk of mastitic buffaloes against both gram-positive and gram-negative organisms at a concentration of 10 μg. The observation that ultra-filtrate fraction lower than 10 kDa displayed higher activity against *S. aureus* and *E. coli* in terms of zone of inhibition implies more antimicrobial potency of the low molecular weight peptides and is in accordance with earlier reports (Roy and More, 2003; Sahly *et al.*, 2003; Singh *et al.*, 2004). The highly cationic peptides lower than 10 kDa in AU-PAGE indicates higher content of basic amino acids as compared to proteins and peptides of higher molecular weight with poor electrophoretic migration. Further, this low molecular weight peptide fraction showed absorbance maxima at 240 nm wave length around which amino acids like cysteine and phenylalanine have maximum absorbance.

Mammalian defensins represents a group of small antimicrobial peptides (3.5 to 4.5 kDa) characterized by the presence of six cysteine forming three disulphide bonds, whose ordered array defines the category as α or β defensins (Aono *et al.*, 2006). The α-defensins are found in human granulocytes (Fessler *et al.*, 2002) and paneth cells of small intestines (Jones and Bevins, 1992; Mallow *et al.*,1996). The β-defensins have been reported from leukocytes (Selsted *et al.*, 1993) and tongue and tracheal epithelial cells of bovines. Current study has attempted to identify the β-defensins present in mastitic milk of buffaloes by using MALDI-TOF.

Probabilistic scoring using MASCOT search tool indicated significant matches of the sequenced peptides with LAP and BD-2 of cattle. Accordingly, these two novel peptide of were designated as buffalo LAP and BD-2. Both the peptides posses the characteristic features of β-defensin family including predominance of basic amino acids like arginine and lysine in their sequence. Presence of basic residues imparts cationicity to the peptides at neutral pH and plays role in biological activity facilitating interaction of the peptides with negatively charged components of the microbial membrane and thus cause membrane disruption and lysis (Kamysz *et al.*, 2003). Further, buffalo LAP and BD-2 also possess six cysteine residues at positions that are invariably conserved among β-defensin family members, two of which are sequential and situated near the peptide carboxyl terminus (Selsted *et al.*, 1993). It is interesting to note that acidic residues were totally absent in both the sequences signifying total cationicity of the peptides. Sequence identity studies indicate high degree of similarity of buffalo LAP and BNBD-2 with other β-defensins reported from different species. However, maximum percentage of homology (80-100%) was noticed with β-defensins of bovine species reflecting more evolutionary closeness between the two species. Phylogeny analysis also suggests that these two novel â-defensin peptides originated from immediate round of gene duplication and subsequent diversification of the ancestor gene during the evolutionary process. MALDI-TOF data thus justify that antibacterial activity exhibited by lower than 10 kDa peptide fraction is mainly due to the presence of LAP and BNBD-2.

To our knowledge this is the first report that identifies presence of β-defensins in the mastitic milk of buffalo. Using routine protein purification methods (Sarmah *et al*., 1993; Singh *et al*., 2004) reported presence of four antibiotic peptides in buffalo PMN cells. Subsequently, RT-PCR approach revealed presence of Enteric Beta-Defensin (EBD) (Das *et al*., 2005) in this species. We also identified LAP, TAP, BNBD-4 and BNBD-9 from actual lining of buffalo mammary gland using similar approach and was cloned and sequenced. The nucleotide sequences of these peptide genes were submitted to NCBI database and the Accession Numbers are EF418028, EF418029, EF41830 and EF418031, respectively. However, only LAP with additional BNBD-2, have been identified in mastitic milk by MALDI-TOF and their presence in milk indicates severity of the udder infection. Nevertheless, it is reported that DNA microarray and proteomic analysis yields do not go hand in hand as there are different profiles in proteins and peptides and their gene expression. There is poor concordance between mRNA transcript and protein expression changes in case of activated human PMN cells (Fessler *et al*., 2002). Proteomics is complimentary tool for assaying actual cellular protein expression response, since cytikines released are dependent on microbial source of mastitis in cattle (Bannerman *et al*., 2004). A precise systematic study is required to correlate expression and induction of different β-defensins in milk at different stages of mastitis based on specific microbes. Till date, defensin genes EBD, LAP, TAP, BNBD4, BNBD9 and BNBD2 have been identified and characterized in buffaloes. Thus, there is further scope for searching remaining 12 beta defensins out of 18 reported in bovines, since there is 80-100% nucleotide sequence homology for these peptide genes between the two species.

Nonetheless, the abundance of β-defensin mRNA in mammary gland and inducibility of some of these genes during infection as well as presence of β-defesin peptides in milk reveal their vital role in containing mastitis causing organisms (Roosen *et al*., 2004) detected expression of seven β-defensin genes including one novel gene viz., DEFB401, LAP, TAP, DEFB1, BNBD3, BNBD9 and BNBD12 in bovine mammary gland. They also noted that lactation status of the cow has no influence on gene expression (Regenhard *et al*., 2005) identified seven novel bovine β-defensin genes and four pseudogenes in bovine mammary glands through screening of two genomic bovine BAC-libraries using defensin consensus primers. Subsequent expression analysis revealed presence of LAP, TAP, EBD, BNBD3, BNBD5, DEFB300 and DEFB 401 in the mammary gland.

The expression of other bovine β-defensin peptides is tissue specific, with TAP in trachea (Diamond *et al*., 1991), EBD in intestinal epithelium (Tarver *et al*., 1998) and BNBDs in neutrophils (Selsted *et al*.,1993). Defensins are antimicrobial peptides that play a major role in innate immunity (Com *et al*., 2003). Real-time PCR quantification assay to measure the mRNA abundances of Toll-like receptor-9 (TLR-9), TLR-4, TLR-2 and β-defensin-5 in healthy and infected mammary gland indicated that mastitis strongly increased mRNA abundances of all of the genes except TLR-9 suggest significant contribution of the innate immune system to counteract mastitis (Goldammer *et al*., 2004). *In situ* hybridization revealed predominant expression of BNBD-5 and LAP in teat and cistern of mastitic quarters (Goldammer *et al*., 2004; Swanson *et al*., 2000), Furthermore, expression of defensin gene in bovine primary Mammary Epithelial Cell (MEC) culture is markedly (500 fold) increased by LPS (Strandberg *et al*., 2005). Thus, defensins are expressed in constitutive and inducible manner by cattle mammary gland. Broadly, similar expression pattern with some specific differences of β-defensin peptides in mammary gland is evident in cattle and buffaloes suffering from mastitis since, these species are extremely close evolutionary relatives. (Aono *et al*., 2006) Cloned and sequenced bovine β-defensin-1 (bBD-1) from mammary

papillary duct epithelia which is structurally much more similar to human β-defensin-1 (hBD-1) than other bovine defensins. The bBD-1 transcript was also detected in teat mucosa, kidney, vagina, ovary, oviduct and colon of cattle irrespective of health status. But the expression of LAP, TAP, DEFB 401 and EBD is induced by inflammation and infection (Diamond *et al*., 1991; Tarver *et al*., 1998; Kaiser and Diamond, 2000; Roosen *et al*., 2004). The high level sequence homology of these peptides among different species also indicates their role in inflammatory conditions to contain infection that is part of evolution of species. In conclusion, buffalo mammary epithelial cells play a pivotal role to combat mastitis pathogens. Upon stimulation with pathogenic insult, expression of β-defensin, LAP is induced and/or up regulated within the secretary epithelium of mammary gland, which are subsequently released onto epithelial surface and into the milk along with BNBD-2 from neutrophils. Hence, LAP and BNBD-2 may be the candidates for using them as the biomarkers of mastitis in mastitis.

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REFERENCES

Anbu, K.A., T. More and A. Kumar, 2002. Characterization of cationic proteins and peptides from buffalo tongue epithelium. Buffalo J., 18: 211-216.

Aono, S., C. Li, G. Zhang, R.J. Kemppainen and J. Gard *et al*., 2006. Molecular and functional characterization of bovine beta-defensin-1. Vet. Imm. Immunopat., 15: 181-190.

Bannerman, D.B., M.J. Paape, J.W. Lee, X. Zhao, J.C. Hope and P. Rainard, 2004. *Escherichia coli* and *Staphylococcus aureus* elicit different innate immune response following intra-mammary infection. Clin. Diag. Lab. Immun., 11: 463-472.

Barkema, H.M., Y.H. Schukken, T.G.M. Lam, M.L. Beiboer, H. Wilmine, G. Benediktus and A. Brand, 1998. Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts. J. Dairy Sci., 81: 411-419.

Boulanger, D., F. Bureau, D. Melotte, J. Mainil and P. Lekeux, 2003. Increased nuclear factor êB activity in milk cells of mastitis-affected cows. J. Dairy Sci., 86: 1259-1267.

Com, E., F. Bourgeon, B. Evrard, T. Ganz, D. Colleu, B. Jegou and C. Pineau, 2003. Expression of antimicrobial defensins in the male reproductive tract of rats, mice and humans. Biol. Repro., 68: 95-104.

Cruickshank, R., J.P. Duguid, B.P. Marimion and R.H. Swain, 1975. Medical Microbiology, the Practice of Medical Microbiology. 12th Edn., Vol. 11, Churchill Livingstone Ltd., Edinburgh, London and New York.

Das, D.K., B. Sharma, A. Mitra and A. Kumar, 2005. Molecular cloning and characterization of â-defensin cDNA expressed in distal ileum of buffalo (*Bubalus bubalis*). DNA Seq., 16: 16-20.

Das, H., B. Sharma and A. Kumar, 2006. Cloning and characterization of novel cathelicidin cDNA sequence of *Bubalus bubalis* homologous to *Bos taurus* cathelicidin-4. DNA Seq., 17: 400-414.

Diamond, G., M. Zasloff, H. Eck, M. Brasseur, W.L. Maloy and L. Bevins, 1991. Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: Peptide isolation and cloning of a cDNA. Proc. Nat. Aca. Sci. USA., 88: 3952-3956.

Fessler, M.B., K. Malcolm, M.W. Duncan and G.S. Worthen, 2002. A genomic and proteomic analysis of activation of the human neutrophil by lipopolysaccharide and its mediation by p38 mitogen-activated protein kinase. J. Biol. Chem., 277: 31291-31302.

Ganz, T. and R.I. Lehrer, 1995. Defensins. Pharmacol. Ther., 66: 191-205.

Ganz, T., 2003. Defensins: Antimicrobial peptides of innate immunity. Nat. Rev. Immunol., 3: 710-720.

Garcia, J.R., F. Jaumann, S. Schulz, A. Krause and J. Rodríguez-Jiménez *et al*., 2001. Identification of a novel, multifunctional β-defensin (hBD-3) with specific antimicrobial activity, interaction with oocyte membrane and inducing macrophage chemoattraction. Cell Tissue Res., 306: 257-264.

Goldammer, T., H. Zerbe, A. Molenaar, H.J. Schuberth, R.M. Brunner, S.R. Kata and H.M. Seyfert, 2004. Mastitis increases mammary mRNA abundance of β-Defensin 5, Toll-Like-Receptor 2 (TLR2) and TLR4 but not TLR9 in cattle. Clin. Diag. Lab. Immunol., 11: 174-185.

Jones, D.E. and C.L. Bevins, 1992. Paneth cells of human small intestine express antimicrobial peptide gene. J. Biol. Chem., 267: 23216-23225.

Joshi, S. and S. Gokhale, 2006. Status of mastitis as an emerging disease in improved and periurban dairy farms in India. Ann. N. Y. Acad. Sci., 1081: 74-83.

Kagan, B.L., T. Ganz and R.I. Lehrer, 1994. Defensins: A family of antimicrobial and cytotoxic peptides. Toxicology, 87: 131-149.

Kaiser, V. and G. Diamond, 2000. Expression of mammalian defensin genes. J. Leukoc. Biol., 68: 779-784.

Kamysz, W., M. Okruj and J. Lukasiak, 2003. Novel properties of antimicrobial peptides. Acta. Biochim. Pol., 50: 461-469.

Klotman, M.E. and T.L. Chang, 2006. Defensins in innate antiviral immunity. Nat. Rev. Immunol., 6: 447-456.

Lowry, O.H., N.J. Roseburg, A.L. Farr and R.J. Randall, 1951. Protein measurement by Folin-pheniol reagent. J. Biol. Chem., 193: 265-275.

Mallow, E.B., A. Harris, N.M. Salzman, J.P. Russel, R.J. de Berardomos, E. Ruchelli and C.L. Bevins, 1996. Human enteric defensins. J. Biol. Chem., 271: 4038-4045.

Paape, M.J., K. Shafer-Weaver, A.V. Capuco, K.V. Oostveldt and C. Burvenich, 2000. Immune surveillance of mammary tissue by phagocytic cells. Adv. Exp. Med. Biol., 480: 259-277.

Pappin, D.J., P. Hojrup and A.J. Bleasby, 1993. Rapid identification of proteins by peptide-mass fingerprinting. Curr. Biol., 3: 327-332.

Pickering, L.K., T.G. Cleary and R. Caprioli, 1983. Inhibition of human polymorphonuclear leukocyte functions by components of human colostrums and mature milk. Infect. Immunol., 40: 8-15.

Rainard, P. and C. Riollet, 2006. Innate Immunity of the bovine mammary gland. Vet. Res., 37: 369-400.

Regenhard, P., J. Reinhardt, S. Roosen, S. Paul, J.M. Schroeder, E. Kalm and C. Looft, 2005. Structural and functional analysis of antimicrobial peptides in the bovine mammary gland. Proceedings of the Plant and Animal Genome XIII Conference, Jan. 15-19, San Diego, CA., pp: 203-203.

Roosen, S., K. Exner, S. Paul, J .M. Schroder, E. Kalm and C. Looft, 2004. Bovine beta- defensins: Identification and characterization of novel beta-defensin genes and their expression in mammary gland tissue. Mamma. Genome., 15: 834-842.

Roy, S.C. and T. More, 2003. Antibacterial activity of buffalo polymorphonuclear cationic peptides on *Pasteurella multocida*. Indian J. Anim. Sci., 73: 114-1117.

Sahly, H., S. Schubert, J. Harder, P. Rautenberg, U. Ullmann, J. Schroder and R. Podschun, 2003. Burkholderia is highly resistant to human beta-defensin 3. Antimicrob. Agents Chemother., 47: 1739-1741.

Sahoo, G., T. More and V.K. Singh, 2000. Evaluation of oxygen dependent immuno-defenses of polymorphonuclear cells of some tropical ruminants. Vet. Res. Commun., 24: 505-515.

Sahoo, G., G.R. Reddy and T. More, 2004. *In vitro* effect of platelet activating factor and granulocyte cationic peptides on buffalo polymorphonuclear cell hydrogen peroxide and nitric oxide production and phagocytosis. Indian J. Anim. Sci., 74: 1203-1205.

Sarmah, S., G.R. Reddy and T. More, 1993. Cationic antibiotic granular peptide of buffalo polymorphonuclear cells. Indian J. Anim. Sci., 63: 618-618.

Selsted, M.E., Y.Q. Tang, W.L. Morris, P.A. McGuire and M.J. Novotony *et al*., 1993. Purification, primary structures and antibacterial activities of β-defensins, a new family of antimicrobial peptides from bovine neutrophils. J. Biol. Chem., 268: 6641-6648.

Singh, V.K., T. More and S. Kumar, 2004. Separation of cationic proteins and antibiotic peptides from buffalo polymorphonuclear cells. Buffalo J., 2: 173-182.

Strandberg, Y., C. Gray, T. Vuocolo, L. Donaldson, M. Broadway and R. Tellam, 2005. Lipopllyssacharide and lipoteichoic acid induce different innate immune responses in bovine mammary epithelial cells. Cytokine, 31: 72-86.

Swanson, K., S. Gorodetsky, L. Good, S. Musgrave, K. Stelwagen, V. Farr and A. Molen, 2000. Expression of a β-defensin mRNA, lingual antimicrobial peptide, in bovine mammary epithelial tissue is induced by mastitis. Infect. Immunol., 72: 7311-7314.

Tarver, A.P., D.P. Clark, G. Diamond, J.P. Rusell and H. Erdjument-Bromage *et al*., 1998. Enteric defensin: Molecular cloning and characterization of a gene with induced intestinal epithelial cell expression associated with *Cryptosporidium parvum* infection. Infect. Immunol., 66: 1045-1056.

Tomasinsig, L. and M. Zanetti, 2005. The cathelicidins–structure, function and evolution. Curr. Protein Pept. Sci., 6: 23-34.

Tunzi, C.R., P.A. Harper, B. Bar-oz, E.V. Valore, J.L. Semple, J. Watson-MacDonell, T. Ganz and S. Ito, 2000. Beta-defensin expression in human mammary gland epithelia. Pediatr. Res., 48: 30-35.

Valore, E.V., C.H. Park, A.J. Quayle, K.R. Wiles, P.B. Jr. McCray and T. Ganz, 1998. Human beta-defensin-1: An antimicrobial peptide of urogenital tissues. J. Clin. Invest., 101: 1633-1642.

Yang, D., O. Chertov, S.N. Bykovskaia, Q. Chen and M.J. Buffo *et al*., 1999. Beta-defensins: Linking innate and adaptive immunity through dendritic and T cell CCR6. Science, 286: 525-528.

Yang, D., O. Chertov and J.J. Oppenheim, 2001. Participation of mammalian defensins and cathelicidins in anti-microbial immunity: Receptors and activities of human defensins and cathelicidin (LL-37). J. Leukoc. Biol., 69: 691-697.

Yeman, R.M. and Y.N. Yount, 2003. Mechanisms of antimicrobial peptide action and resistance. Pharmacol. Rev., 55: 27-55.