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Molecular characterization of exon-2 of buffalo mammocytes defensin for exploring its potency for synthesis of novel antimicrobial agents

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Abstract
Antibiotic resistant is a serious concern for both veterinary and medical practices and to trounce these problems there is a need to search alternate group of drugs for prevention and treatment of different diseases. Mammalian defensin and cathelicidin are the two broad classes of antimicrobial peptides expressed by different epithelial lining of the living organisms. Present study was designed to characterize the buffalo mammocytes defensin to find out the template for synthesis of novel antimicrobial agents. Genomic DNA was isolated from buffalo mammocyte. The amplified PCR product was purified cloned and sequenced. The size of the PCR product was 263 bp and cloned DNA after sequencing revealed that exon-2 of the mammocyte defensin is comprised of 129 bases. The total number of predicted amino acids was 42 and the aligned amino acid sequences of buffalo mammocyte defensin with other defensin peptides showed six conserved cysteine residue at different position. The mature peptide had six strongly basic and eleven hydrophobic amino acids including one tryptophan in all most at middle of the peptide. From the present study, it can be concluded that the buffalo mammocyte defensin can act as blue print for synthesis of new antimicrobial agents.

Keywords: Antimicrobial peptide, mammocyte, cationic peptide, defensin

Introduction
Antimicrobial peptides are effective components of host defense that can be explored as possible alternative to conventional antibiotics. Traditional antibiotics usually have single or limited types of target molecules, which can be mutated easily by bacteria to gain resistance (Ganz, 2003) [3]. The action of antimicrobial peptide involve the physical disruption of the microbial membrane by direct electrostatic interaction due to negative charge of the membrane without involving any specific receptors. This special character makes them an attractive candidate as next generation therapeutic agents for treating multi-drug resistant bacterial infections. Mammalian defensin and cathelicidin are the two broad classes of antimicrobial peptides constitute a large family of endogenous peptide antibiotics with broad-spectrum activity against various bacteria, fungi and viruses expressed by different epithelial lining of the living organisms (Nannette *et al*., 2009) [10]. Expression of human cathelicidin (hCAP-18) is demonstrated in the male and female reproductive system along with bone marrow progenitors cells (Malm *et al.*, 2000) [3]. Several β-defensin namely cryptidin from mouse sertoli cells and Bin1b from rat epididymis has been isolated (Li *et al*., 2001) [8]. Antimicrobial peptide gene from the tongue of buffalo has been sequenced and characterized (Kalita *et al*., 2009) [6]. Synthesis of different length of natural analogue of buffalo lingual antimicrobial peptide and functional study revealed its potency against both gram positive and negative bacteria (Kalita *et al*., 2009) [7]. Present study was carried out to characterize the defensin from buffalo (*Bubalus bubalis*) myoepithelial cells to elucidate its potency to use as blue print for novel antimicrobial agents.

Material and methods
Genomic DNA was extracted following phenol and chloroform-isoamylalcohol extraction (Sambrook and Russell, 2001) [13] from myoepithelial cells of mammary gland collected from freshly slaughtered buffalo. The purity of extracted DNA was checked by determining the OD at A_{260}/A_{280} and integrity of the DNA was checked by 1.0% agarose gel electrophoresis. Primer (forward5'CGCTCAGAGGGGACACAGAT3' and reverse 5'GCAGTTTCTATCTCCGCATCAG 3') was designed using conserved sequences of cattle defb403 (β-defensin 401, AJ567354)

defb404 (AJ567358) and defb405 (AJ567360) by primer select prgromme of DNA star software (USA). PCR reaction mixture was prepared by adding Taq DNA polymerase buffer 5 µl, dNTP mix (10mM) 2 µl, forward primer and reverse primer (25 pmoles / µl) 2 µl each, Taq DNA polymerase 1µl, DNA 2 µl and nuclease free water 36 µl. The cycle condition in PCR was: initial denaturation for 5 minutes at 950C, followed by 32 cycles of each of denaturation at 950C for 1 minute, annealing for 1 minute, extension at 72°C for 45 seconds and final extension for 5 minutes at 72°C. Agarose gel electrophoresis (1%) was done for confirmation of amplified PCR product. PCR product was purified by 'DNA Elution Kit' (Sigma – Aldrich, USA). The purified PCR product was ligated to pGEM-T easy (Promega, Madison, USA) cloning vector and was transformed in *E. coli* DH5α competent cells (Chung *et al.*, 1989) [1]. Plating was done on LB agar containing ampicillin (50mg/ml), IPTG and X-gal (25 mg/ml). The plates were incubated for overnight at 37°C. White recombinant colonies were picked up for plasmids isolation (Sambrook and Russell, 2001) [13] and insert was confirmed by *Eco*R1 digestion. The recombinant plasmids were sequenced and analysed the nucleotides as well as amino acid distribution in predicted mature peptide.

Results and discussion

The total yield of genomic DNA per mg of mammocytes was 21.6µg.The A_{260}/A_{280} was recorded 1.83 indicating the high purity of DNA. The isolated DNA revealed intact quality upon 1.0% agarose gel electrophoresis. Optimum temperature for amplification was recorded at 52^0C. The amplified PCR product yield expected specific product of 263 bp (Fig. 1). The nucleotide sequence of the cloned PCR product revealed that exon-2 of the mammocyte defensin is comprised of 129 translated bases. The different nucleotides in the translated region were 32 adenine (24.81%), 35 guanine (27.13%), 35 thymine (27.13%) and 27 cytosine (20.93%). The buffalo mammocyte defensin was compared with other published β-defensin sequences. At nucleotide level this was varied by 33, 39, 17, 32, 38 and 26 bases from cattle defb401 (β-defensin 401), defb402, defb403, defb404, defb405 and cattle LAP like sequence respectively. Percent similarity at nucleotides level is presented in Fig. 2 and highest similarity (89.3%) was observed with cattle defb403. The nucleotide sequence of the mammocyte defensin was submitted to NCBI gene data bank (Accession no. DQ886701).

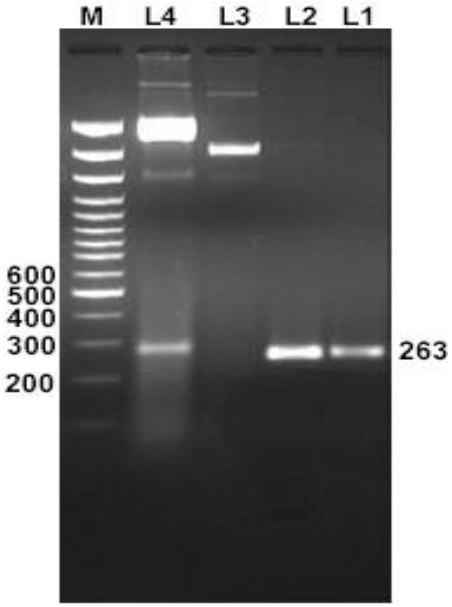

Fig 1: 1.0% Agarose gel electrophoresis of cloned mammocyte defensin gene. M: 100bp plus DNA ladder, L1: 263bp PCR product, L2: Purified PCR product, L3: Undigested recombinant plasmid L4: *Eco*R1 Digested Recombinant Plasmid showing the release of insert (263bp)

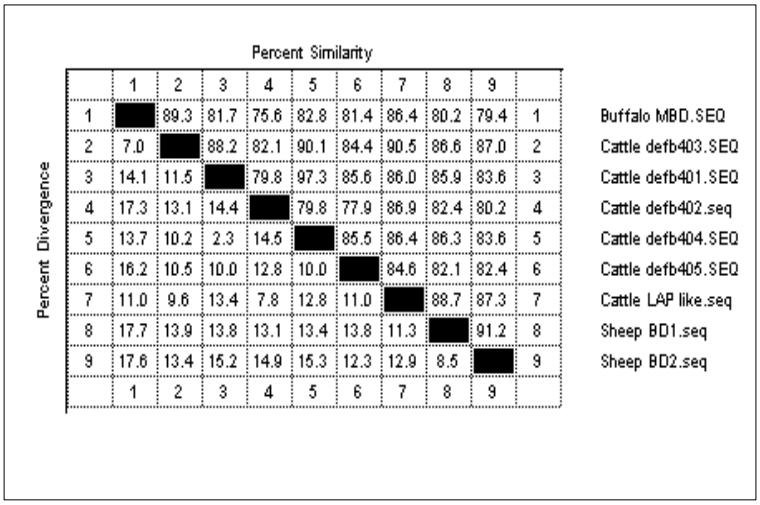

Fig 2: Percent divergence and percent similarity of buffalo mammocyte defensin at nucleotide level

The total number of predicted amino acids in buffalo mammocyte exon-2 was 42 from the translated region of 83 to 211 bp of the sequence and the calculated molecular weight was 4.72 kDa. The aligned amino acid sequences (Fig. 3) of buffalo mammocyte defensin showed six conserved cysteine residues at position 11, 18, 23, 33, 40 and 41. Besides these other conserved positions were 1(F), 2(T), 16 (G), 29 (Q), 30 (I), 31(G), 32(T) and 42(R). Percent similarity and divergence (Fig.4) of deduced amino acid showed that buffalo mammocytes defensin and cattle defb403 shared highest (78.6%)similarity.

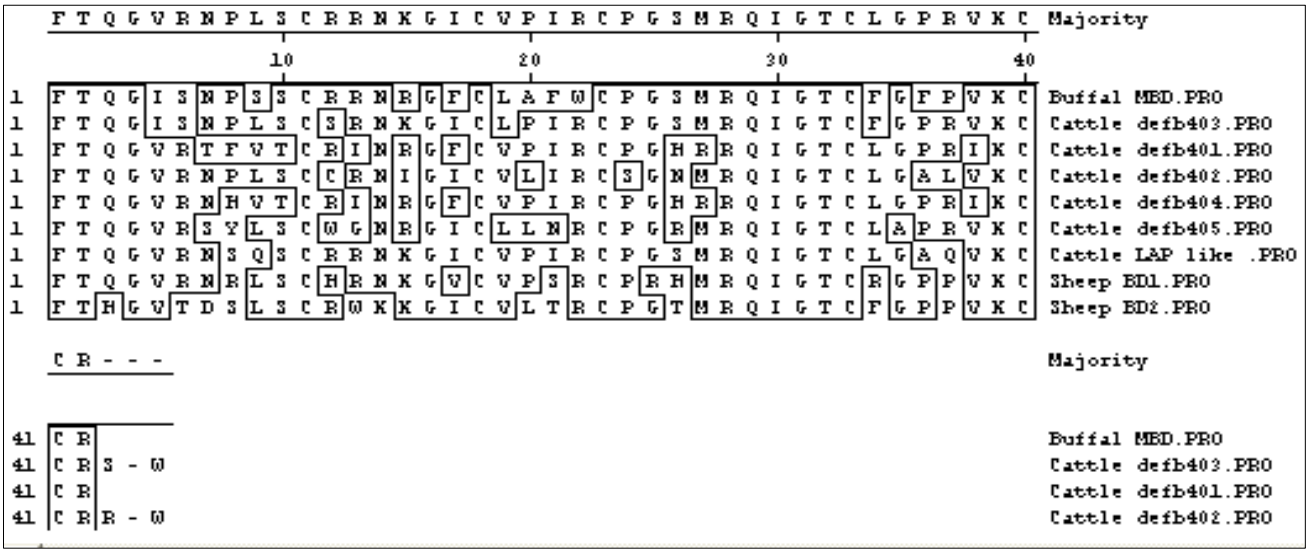

Fig 3: Alignment of amino acid sequences of buffalo mammocyte defensing with defensing of other species. The consensus residues were included within the same box

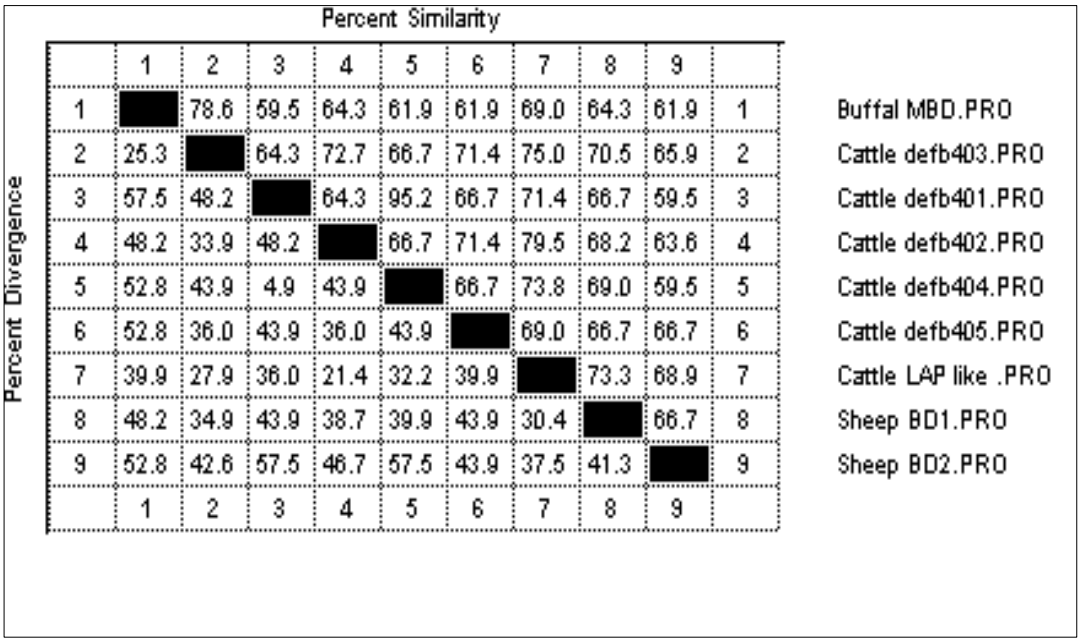

Fig 4: Percent divergence and percent similarity of buffalo mammocyte defensin at nucleotide level

Presence of six conserved cysteine residues at 11, 18, 23, 33, 40 and 41 of buffalo mammocytes defensin might provides the compact triple stranded β-sheet structure by forming three disulfide bonds which is essential for its activity (Roosen *et al*., 2004; Hill et al., 1991 and Yount *et al*., 1999) [12, 4, 16]. F and T at position 1 and 2 were conserved in all species and these two neutral amino acids comprised the prosegment. This prosegment is required to keep the peptide in an inactive form until it reaches the site where its activity is required, either outside or within the cell (Selested and Ouellette, 1995 and Kachel *et al*., 1995) [13, 5]. The mature peptide of mammocyte β-defensin is comprised of 40 amino acids from 3-42. It is reported that mature peptides are released by proteolytic cleavages at the time of microbial attack from precursor peptides (Ganz, 2003) [3]. Tryptophan at position 22, almost middle of the peptide was the unique residue in buffalo mammocyte defensin where it replaced arginine of all sequences used for comparisons. Tryptophan residues has high propensity to insert in to membranes and playing a role in the membrane positioning in several biologically active peptide(Kachel *et al*.,1995; Persson *et al*., 1998 and White *et al*., 1999) [5, 11, 15]. Presence of six strongly basic and elevan hydrophobic amino acids including one tryptophan in all most middle of the buffalo mammocyte β-defensin probably makes this molecule more potent endogenous antimicrobial peptide to resist or overcome the udder infection successfully as compared to cattle. It has been reported that the cationicity of defensin molecules favors the electrostatic interaction with

anionic microbial surfaces and hydrophobic residues interact with the surface of the membrane bilayer of the microbes (Crovella *et al*., 2005) [2]. In the present study the mammocyte defensin is characterized from the apparently healthy non-lactating animal and it can be state that β-defensin is expressed by the buffalo mammocytes, irrespective of the health and lactation status. From the pattern of amino acid distribution across the predicted mature peptide, it can be concluded that buffalo mammocyte defensin can be used as a blue print for synthesis of a novel antimicrobial agent to replace the conventional antibiotic.

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