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Identification of three novel myeloid cathelicidin cDNAs and their predicted peptides in buffalo (*Bubalus bubalis*)

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Antimicrobial peptides (AMPs) are broad spectrum antibiotics, which mostly act without specific receptors. Identification of AMPs is important in the current scenario of emerging multi-drug resistant bacteria. In the present study, in an attempt to identify new AMPs, myeloid cathelicidin cDNAs were synthesized from buffalo (***Bubalus bubalis)*** bone marrow and were amplified using specific primers. Sequence analysis of cloned cDNAs revealed three novel myeloid cathelicidins. They were named based on the number of active amino acids in the C-terminal region of their predicted peptide sequences as BuMAP-28 (having an additional Gly at position 22nd), BuMAP-29 (having an additional IIe at position 27) and BuMAP-34, compared to BMAP-27, BMAP-28 and BMAP-34 of cattle. The BuMAPs showed 93%, 95% and 87% homology respectively with that of its cattle counterpart. Predicted number of amino acids of the cDNAs was 159, 155 and 157 residues, with cationic C-terminal sequences of 28, 29 and 34, respectively, which correspond to putative antimicrobial domains. Several amino acid substitutions were observed in all the three cathelicidins. The conformation of the peptides was predicted to be alpha helical, having total net positive charge and hydrophobicity, similar to that of BMAPs in cattle. Comparative analysis of the predicted peptides suggested potential antimicrobial activity and the sequence variations detected might enable the peptides to act as effective broad spectrum antimicrobial agents.

Keywords: Antimicrobial peptide, buffalo, ***Bubalus bubalis,*** BuMAPs, Cathelicidin, cDNAs.

Antimicrobial peptides (AMPs) are evolutionarily ancient weapons to fight against a wide spectrum of microorganisms and are widely distributed among living organisms. They show a marked inter-species diversity in their spectrum of activity and structure. AMPs are relatively short, positively charged molecules of 12-100 amino acids in length. Several AMPs have been isolated from mucosal epithelia and macrophages of mammals[1]. In phagocytes, the AMPs originate from the precursors that are synthesized in bone marrow cells. Antimicrobial action of these molecules is mainly due to two common structural characteristics – net positive charge and the propensity to fold into amphipathic structure. These features enable them to insert into the microbial membranes by binding to negatively charged surface of microorganisms, thereby disrupting the membrane integrity[2].

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Abbreviations: AMPs, antimicrobial peptide; Bac, bactenecin; BMAP, bovine myeloid antimicrobial peptide; BuMAP, buffalo myeloid antimicrobial peptide; CAP, cathelicidin family of antimicrobial peptide; PMAP, porcine myeloid antimicrobial peptide.

In mammals, AMP families are grouped into two categories – defensins and cathelicidins. Defensins comprise of small cationic peptides exhibiting broad spectrum antimicrobial activities through membrane permeabilization. The β-sheet structure, stabilized by three disulfide bonds differentiate defensins from other antimicrobial peptides[3]. Myeloid antimicrobial peptides originate from bone marrow and belong to the cathelicidin group. Precursors of these peptides have highly identical N-terminal prosequences and a variable C-terminal sequence. The latter sequence harbors the antimicrobial activity on proteolytic removal of the propiece. The propiece is connected to the antimicrobial domain *via* amino acid residues that are recognized by specific processing enzymes. The most frequently occurring amino acid is Val, but in certain instances, Ala, IIe and Thr are also seen.

In pigs and cattle, the myeloid-derived cathelicidins are cleaved by the azurophilic elastase in coincidence with nucleophilic degranulation, so as to liberate the antimicrobial peptides at the site of infection[4,5]. Cathelicidin group of antimicrobial peptides include the Cys-rich dodecapeptide[6], protegrins[7], Pro- and Arg-rich Bac5 and Bac7[8], prophenin[9],

PR-39[10], Trp-rich indolicidin[11], PMAP-23[12], α-helical PMAP-36[13], PMAP-37[14] and CAP 18[15].

Antibiotic resistance is increasing at a rate that far exceeds the pace of development of new drugs. One of the major strengths of AMPs is their ability to kill multi-drug resistant (MDR) bacteria and hence such molecules are gaining importance in clinical research as a treatment modality to tackle the global issue of MDR bacteria[16]. Buffalo (*Bubalus bubalis*) is well-known for their natural resistance to microbial infection and the information generated from the mRNAs of buffalo bone marrow may give an insight into the types of active antimicrobial peptide motifs expressed in phagocytes.

In the present study, we report three novel myeloid cathelicidins having structurally different C-terminal putative antimicrobial domains, which are identified by the sequence analysis of cloned cDNAs from buffalo bone marrow.

Materials and Methods

RNA extraction and cDNA synthesis

Total RNA was extracted from buffalo bone marrow cells with guanidinium thiocyanate[17]. First strand cDNA was synthesized using 10 µl of the extracted RNA using RevertAid cDNA synthesis kit (Fermentas, Lithuania). The enzyme M-MuLV reverse transcriptase and oligo $(dT)_{18}$ primers were used to obtain the full-length cDNAs. Since the 5' end region of all cathelicidins are highly conserved, a single forward primer 5'- ATGGAGACCCAGAGGG CCAGC-3 was designed for the cDNA amplification[4]. Specific reverse primers were designed from the cathelicidin cDNA sequences of cattle available in the Genbank database. The primers were 5'-GCCTGAGCCAGGGAGGACACAC-3' (BMAP-27), 5'-GGCCGGCAGACCCTTAGGACTC3' (BMAP-28) and 5'GGACTTTATCCCCTGAAGATATC3' (BMAP-34).

Using the common forward and specific reverse primers, three cDNAs were amplified. Except for annealing temperature, all other parameters were same for amplifying the three cDNAs. Each reaction mix contained 10 µl of cDNA, 200 µmol of each dNTP, 25 pmols of each primer and 1.5 units of *Taq* polymerase in a total volume of 50 µl. PCR was performed for 35 cycles in a gradient thermal cycler (PTC-200, MJ research, USA) with the cycle parameters: denaturation at 92°C for 1 min, annealing at 59.2°C (for BMAP-27), 58.2°C (for BMAP-28) and 53°C (for BMAP-34) for 1 min and extension at 72°C for 2 min.

cDNA cloning and sequence analysis

Amplified cDNAs were cloned in JM107 cloning vector (InsTAclone PCR cloning kit, Fermentas, Lithuania). Both the strands were sequenced and sequences were analyzed using Gene Tool Lite software (Bio Tools, Inc.). Homology searches of cDNA sequences were performed using the BLAST network service of the National Centre for Biotechnology Information. Secondary structure of the peptide was predicted using antimicrobial peptide database generated by Wang *et al*.[18]. Homology search, total net charge and hydrophobicity of predicted peptides were carried out using antimicrobial peptide database[18]. Helical wheel projection analysis of the peptides was also carried out using the software helical wheel projections-RZ lab (rzlab.ucr.edu/scripts/wheel/wheel.cgi).

Results

cDNA sequence analysis

Three novel myeloid cathelicidin cDNAs were amplified and sequenced. They possessed a size of 556, 508 and 484 bp compared to BMAP-27 (580 bp, Accession no. NM-174832), BMAP-28 (585 bp, NM-174510) and BMAP-34 (588 bp, NM-174831) of cattle, respectively (Fig. 1A, B, C). The cDNAs were named based on the number of active amino acids in the C-terminal region of the predicted peptide sequence as BuMAP-28 (JX909278), BuMAP-29 (JX909279) and BuMAP-34 (JX961615), which showed 93%, 95% and 87% homology with that of BMAP-27, BMAP-28 and BMAP-34 of cattle, respectively. Peptide sequences of the three cDNAs were predicted and analyzed using Gene Tool Lite software (Bio Tools, Inc).

BuMAP-28

BuMAP-28 showed 159 amino acid residues with a predicted mass of 17893.08 Da and pI of 10.347. The signal peptide (29 amino acid residues) was followed by a cathelin-like domain and a cationic C-terminal region having 28 amino acid residues (Fig. 1A). Comparison of the C-terminal region with other mammalian antimicrobial peptides showed highest level of identity (75%) with BMAP-27. The peptide showed a predicted hydrophobicity of 39% and a total net charge of +11. On alignment with BMAP-27, presence of an extra amino acid Gly

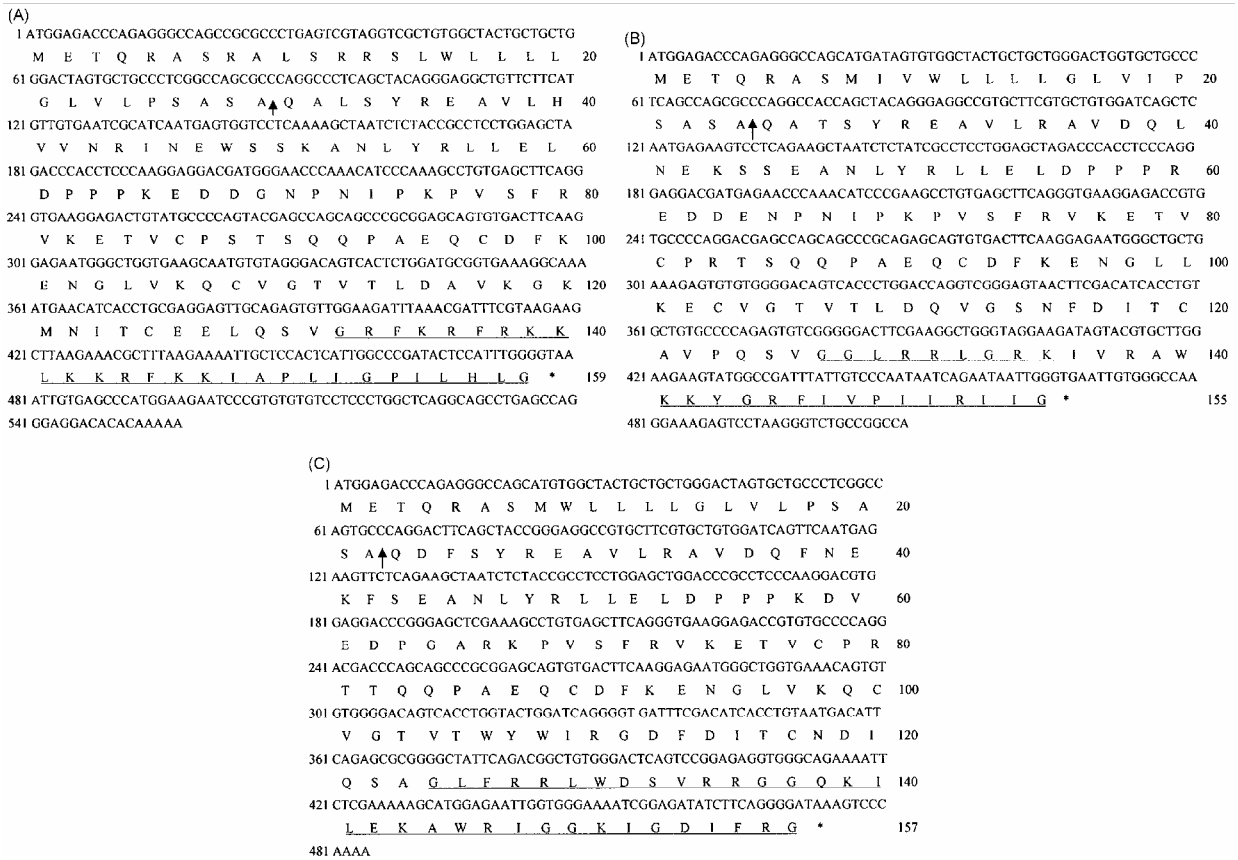

Fig. 1—Nucleotide and predicted amino acid sequences of the precursors of the buffalo myeloid antimicrobial peptides BuMAP-28 (A), BuMAP-29 (B) and BuMAP-34 (C) [The nucleotide sequences are numbered on the left and the amino acid sequences are numbered on the right. The arrows show the respective putative cleavage sites for the enzyme signal peptidase. Asterisks represent the stop codon]

at position 22nd and amino acid substitutions at various positions were also observed (Fig. 2A).

BuMAP-29

The cathelicidin BuMAP-29 had 155 amino acid residues with a predicted mass of 17336 Da and pI of 8.64. Signal peptide comprised 24 residues, followed by a cathelin-like domain and a cationic C-terminal region containing 29 amino acids (Fig. 1B). Comparison of the C-terminal region with other antimicrobial peptides showed 82.75% identity with that of BMAP-28. It had a predicted total net charge of +9 and 44% hydrophobicity. On alignment with BMAP-28, presence of an additional amino acid IIe at position 27 and several amino acid substitutions were observed (Fig. 2B).

BuMAP-34

The third novel cathelicidin BuMAP-34 had 157 amino acid residues with a predicted mass of 17954 Da and pI of 8.047. The signal peptide (22 amino acid residues) was followed by

Fig. 2—Alignment of cationic C-Terminal region of antimicrobial peptides of buffalo with that of cattle [(A) BuMAP-28 with BMAP-27; (B) BuMAP-29 with BMAP-28; and (C) BuMAP-34 with BMAP-34]

cathelin-like domain and a cationic C-terminal region comprising 34 residues (Fig. 1C). Sequence comparison of the C-terminal region indicated 79.41% identity with that of BMAP-34. It had a predicted total net charge of +6 and 38% hydrophobicity. On alignment with BMAP-34, several amino acid substitutions were noted at various positions (Fig. 2C).

The helical wheel projection revealed that all the three peptides were having alpha helical structure (Fig. 3A, B and C)

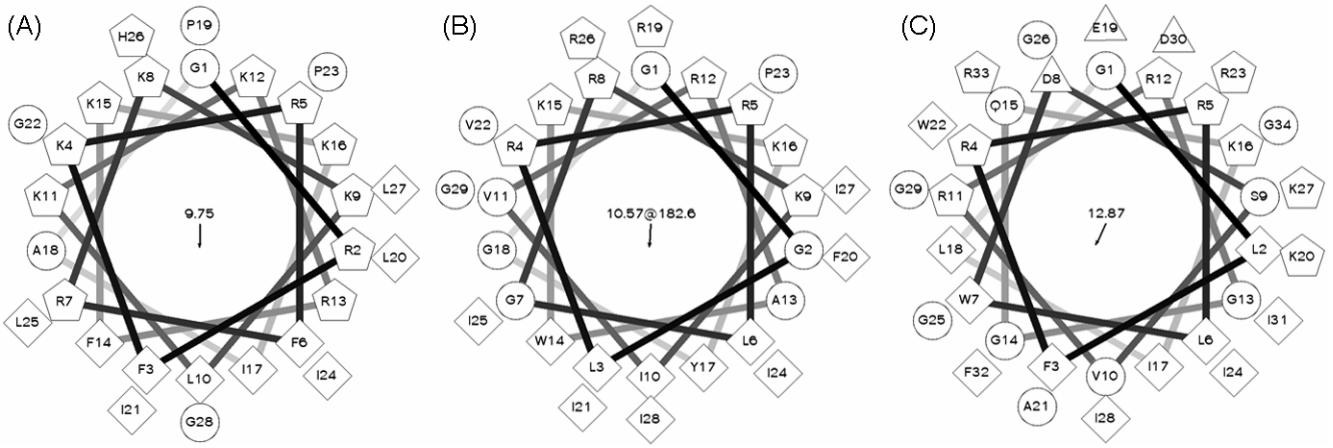

Fig. 3—Helical wheel projection of BuMAP-28 (A), BuMAP-29 (B) and BuMAP-34 (C) [Hydrophilic residues as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles and potentially positively charged as pentagons are shown]

Discussion

Highly conserved preproregion of cathelicidins was used as a base to obtain the transcripts encoding myeloid antimicrobial peptides in buffalo myeloid cells. The amplified cDNAs sizes were different from that of cattle (Genbank/EMBL database).

The predicted polypeptides of the BuMAP cDNAs displayed the characteristic features of cathelicidins, which included a putative signal peptide and a cathelin-like prosequence containing four invariant cysteine residues[19]. A putative cleavage site for elastase commonly observed at the end of the cathelin-like domain was also present in the predicted peptides, which was followed by a cationic sequence of 28-34 residues. Elastase cleavage site of BuMAP-28 and 29 had a Val residue, while that of BuMAP-34 had an Ala similar to that of BMAPs.

Sequence comparison of the predicted BuMAPs-28, 29 and 34 with the mammalian antimicrobial peptides revealed highest identity (75 to 83%) with that of cattle[5,20], which were reported to have broad spectrum antimicrobial activity. Earlier, it is shown that an increase in the positive charge often enhances the antibacterial effect, extending the spectrum of action to Gram-positive bacteria without a parallel increase in cytotoxicity[21]. It could be due to the presence of large amounts of anionic lipids, such as phosphatidyl glycerol and cardiolipin and lack of cholesterol. Basic nature of the peptides contributes to initial interaction with negatively charged membrane components, while their amphipathic character favours their incorporation into bacterial membranes[22]. In the present study also, all the BuMAPs showed a high net positive charge and a comparable hydrophobicity. Increase of hydrophobicity generally correlates with enhanced activity against Gram-positive bacteria and hemolysis and is related to a reduction of peptide specificity to Gram-negative bacteria[23].

The rate of mutation in the sequence corresponding to the antimicrobial domain was higher than in the remaining part of the molecule in all the BuMAPs. In fact, a fast evolving antimicrobial domain is a feature, common to genes encoding defense peptides[10] and appears to be an important requisite for molecules that have to keep up with highly variable and rapidly changing pathogens[24].

As indicated by the structure prediction analysis, mature BuMAPs-28, 29 and 34 could adopt an amphipathic α-helical structure (Fig. 3A, B and C), which is a feature of most of the cathelicidin group of antimicrobial peptides. Since these peptides can take up an α-helical conformation, the peptides might be able to facilitate membrane interaction and disruption[25].

Analysis of the predicted amino acid composition showed that both BuMAP-28 and 29 lacked Trp residues, but contained more Lys residues and moderate number of Arg residues, whereas BuMAP-34 contained Arg, Trp and Lys residues. It is demonstrated that the ability of peptides of comparable conformation to induce phospholipid translocation is greater for those containing proportionately more Lys or His residues, compared with Trp[26]. Trp has a distinct preference for the interfacial region of lipid bilayers, while Arg residues provide positive charge to the peptides promoting hydrogen bonding properties necessary for interaction

with the anionic components of bacterial membranes[27]. In combination, these two residues are capable of facilitating enhanced peptide-membrane interactions[27]. It suggested that the predicted myeloid peptides of buffalo might have better antimicrobial activity compared to other mammalian myeloid cathelicidins. In addition, evolutionary changes might have also contributed to the structural diversity of the peptides, when compared to other myeloid cathelicidins. However, homology study of the cathelicidin proregions within and between species might provide valuable information on the evolutionary relationship between the peptides.

Nucleotide sequence comparison of the buffalo myeloid cathelicidins with that of cattle indicated that these genes were likely to have originated from a common ancestor. The C-terminal active amino acid region of predicted peptide sequences of BuMAP-28, 29 and 34 exhibited amino acid addition and substitution, which could be of value in their antimicrobial activity and might help to fight against rapidly evolving pathogens. Predicted peptide analysis suggested that the myeloid cathelicidins were specific to each species.

Conclusion

Cloning and sequence analysis revealed the presence of three novel myeloid cathelicidin cDNAs in buffalo, having several sequence variations in the antimicrobial domain. Amino acid variations, cationic charge and alpha-helical structure of the predicted active peptide motifs might be important for their antimicrobial activity. Further studies are required to establish the efficacy of these peptides as safe broad spectrum antimicrobial agents.

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