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Molecular characterization and *in-silico* analysis of myeloid cathelicidin gene in Swamp buffalo

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ABSTRACT

Mammalian cathelicidin is the one of the important antimicrobial peptides expressed by different tissues. Present study was undertaken to characterize the cathelicidin gene of swamp buffalo (*Bubalus carabanensis*) to know the potency of the predicted peptide for *in-silco* designing of antimicrobial peptides. Total RNA was isolated from the bone marrow myeloid cells and reverse transcribed the cathelicidin gene by specific primers. The amplified PCR product was purified, cloned and sequenced. The size of the PCR product was 520 bp and cloned cDNA after sequencing revealed the open reading frame (ORF) of 447 bases. The total number of predicted amino acid in the pre-pro-peptide was 148. Alanine at 29 was found to be conserved in most of the congeners and might be the probable site for proteolytic cleavage of the signal sequence. Valine at 130 was common in all most all congeners which revealed the point of termination of pro-sequence from the mature peptide. The antimicrobial activity exists only in C-terminal mature domain from 131–146. Presence of 6 arginine, which inferred more cationicity as well as 3 proline and 5 tryptophan may make this congener more potent antimicrobial peptide. Support vector machine algorithms showed the antimicrobial potency of different segments of the mature peptide. From the present study, it is concluded that the mature domain of the swamp buffalo cathelicidin can be used as template for synthesis of novel antimicrobial agents.

Key words: Antimicrobial peptide, Cathelicidin, Open reading frame, Swamp buffalo

Antimicrobial peptides are prevalent throughout the nature as part of the intrinsic defenses of most organisms. These antimicrobial peptides represent a unique and quite complex host defense tool, having many blades with overlapping function (Tomasinsig and Zanetti 2005). Most antimicrobial peptides are cationic molecules with spatially separated hydrophobic and charge residues and have evolved as integral components of strategic and carefully regulated mechanism of immunity to infection (Yang *et al*. 2002). 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. Cathelicidins are mostly synthesized from the bone marrow progenitor cells of mammalian species and other tissues like reproductive tract. Precursors of the cathelicidin family possesses a N-terminal signal peptide, a pro sequence which is highly conserved for both intra and inter species and the substantial heterogenous C-terminal region represent the mature peptide (Lehrer *et al*. 1991). Present study was designed to characterize the cathelicidin gene of bone marrow myeloid cells of swamp buffalo to elucidate the potency of the predicted peptide for designing of the antimicrobial peptide to use as blue print for synthesis of novel antimicrobial agents.

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MATERIALS AND METHODS

Collection of samples and extraction of RNA: Bone marrow myeloid tissues were collected from freshly slaughtered apparently healthy swamp buffalo. Approximately 100 mg of tissues were taken individually and total RNA was isolated using TRI Reagents™ following manufacturer protocol.

PCR amplification and cloning of cathelicidin gene: The oligo nucleotide sequences of forward and reverse primers were 5'CGGCACCGACAGCATGAG3' and 5'GCCACGTCTTCGCCTTCT3' respectively. The cDNA synthesis was carried out by Revert Aid™ First strand cDNA synthesis kit using total RNA as template. The different annealing temperatures, viz. 55°C, 54°C, 53°C, 52°C and 50°C were employed initially to find out the most suitable annealing temperature for optimum amplification. Subsequently optimum amplified temperature was employed in Reverse Transcriptase Reaction. The 50 µl reaction mixtures was prepared using 5X QUIAGEN one step RT-PCR buffer 10 µl, dNTP mix (10mM) 2 µl, forward and reverse primer (25 pmoles) 2 µl each, QUIAGEN one step RT-PCR enzyme Mix 2µl, template RNA 4 µl and RNase free water 28 µl. The reaction mixtures were mixed

properly by pipetting up and down and after brief centrifugation incubated in the thermal cycler. The amplification cycle conditions were: reverse transcription 50°C for 30 min initial PCR activation 95°C for 15 min, denaturation 94°C for 1 min, annealing 1 min and extension 72°C for 45 sec and in these conditions 32 cycles were run.

Agarose gel (1.0%) electrophoresis was done to confirm the size of the amplified PCR product and specific product were eluted and purified. The purified PCR product was cloned using pGEM-T Easy cloning vector and cloned products were transformed. Positive clones were cultured and plasmids were isolated from overnight grown culture by alkaline lysis method. For screening of the recombinant clones, plasmids were isolated from different clones and were treated with *Eco*R1. Positive clones were sent for sequencing to Delhi University, South Campus.

Sequence analysis of cathelicidin gene: The nucleotide sequence of swamp buffalo myeloid cathelicidin was aligned with other published sequences and compared both at nucleotide and amino acid level using 'MegAlign' programme of Lasergene software (DNA Star Inc., USA) to know the per cent divergence and similarity with other reported sequences. Phylogenetic tree was constructed to know the evolutionary relationship with other available genomic sequences. Sequences used for comparisons were retrieved from EMBL data bank as well as from NCBI. Mature peptides were deduced from predicted amino acids and a support vector machine (SVM) algorithm was used to design and predict the antimicrobial peptide from the mature functional peptide.

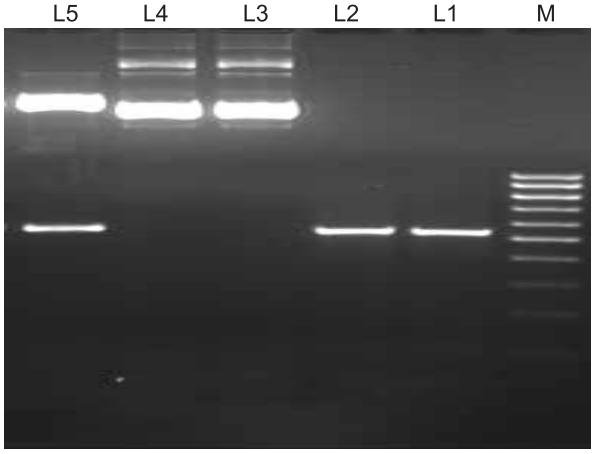

Fig. 1. 1. 0% Agarose gel electrophoresis of cathelicidin cDNA of swamp buffalo (M: 100bp^{+} ladder, L1: 520 bp PCR product, L2: purified PCR product, L3 and L4: undigested recombinant plasmid, L5 *Eco*R1 digested recombinant plasmid showing the release of 520bp insert).

RESULTS AND DISCUSSION

PCR amplification of swamp buffalo (*Bubalus carabanensis*) myeloid cathelicidin at 53°C annealing temperature yielded a specific product of 520 bp (Fig. 1). The ORF region of swamp buffalo myeloid cathelicidin cDNA has 447 bases and was from 6–453. The number of different bases in the coding region are 93 adenine (20.81%), 145 guanine (32.44%), 90 thymine (20.13%) and 119 cytosine (26.62%).The comparison of per cent similarity revealed that swamp buffalo myeloid cathelicidin had highest similarity with water buffalo testis cathelicidin (98%) followed by uterine cathelicidin (96.9%) (Fig.2). It is reported that genes involved in immunity and host defenses are rapidly diverged to fight competitively in anti germ war (Emes *et al*. 2003) and probably that might be the cause of variation of cathelicidin gene of different tissues. Phylogenetic tree drawn at nucleotides level exhibited the close evolutionary relationship of swamp buffalo myeloid cathelicidin with water buffalo testis and uterus cathelicidin as they formed single cluster (Fig.3). The deduced peptide of myeloid cathelicidin congener (Fig.4) comprised 148 amino acids containing 20 strongly basic, 19 strongly acidic, 50 hydrophobic and 42 polar amino acids. The predicted molecular weight and isoelectric point (P^{I}) for the peptide were 17.16 kDa and 7.75 respectively.

The deduced pre-pro-peptides of cathelicidin cDNA of

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11		
1		92.9	89.9	89.0	90.6	73.8	75.9	74.9	81.4	73.6	77.0	1	*B taurus* CATHL4
2	7.0		91.0	89.4	91.0	70.8	74.9	75.4	79.8	72.2	75.9	2	Buffalo Indolicidin
3	8.6	7.8		95.7	96.9	70.7	71.8	72.3	79.2	71.4	74.7	3	Buffalo Uterine CATHL
4	9.6	9.1	4.1		98.0	68.2	70.9	70.7	77.6	69.1	73.4	4	Buffalo Testis CATHL
5	8.0	7.5	3.2	1.8		70.0	72.3	72.0	79.0	70.5	73.8	5	Swamp Buffalo Myeloid CATHL
6	25.4	27.7	29.1	31.2	29.5		75.0	71.2	70.5	70.1	71.8	6	*B taurus* CATHL 1
7	20.8	21.7	25.0	26.3	24.4	19.8		73.3	72.7	77.6	74.7	7	*B taurus* CATHL 5
8	23.2	23.1	24.5	26.4	24.5	27.7	25.9		67.7	67.9	71.3	8	*B taurus* CATHL 7
9	16.1	18.2	19.2	21.2	19.4	30.8	25.2	29.0		69.6	73.8	9	*B taurus* CATHL 2
10	25.5	26.5	28.3	31.1	29.4	30.8	21.0	34.5	30.1		73.6	10	Goat MAP 28
11	19.2	20.5	20.0	22.5	21.6	28.9	24.0	25.2	23.1	24.9		11	Swine Protegrin 1
	1	2	3	4	5	6	7	8	9	10	11		

Fig. 2. Percent divergence and similarity of cathelicidin cDNA of swamp buffalo at nucleotide level with other cathelicdin congeners at nucleotide level.

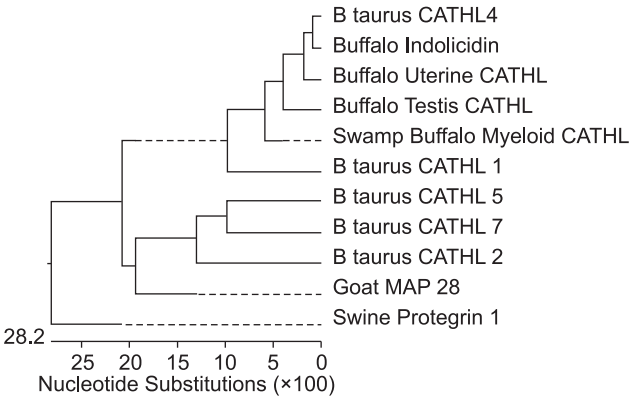

Fig.3 Phylogenetic tree drawn by comparing the nucleotide sequence of cathelicidin cDNA of bone marrow myeloid tissues of swamp buffalo with other congeners

buffalo myeloid cathelicidin has conserved N-terminal and diverse heterogeneous C-terminal, which is consistent with other cathelicidin congeners (Popsueva *et al*. 1996, Wu *et al*. 1999). Alanine at 29 is conserved in all the congeners might be the probable site of elastase mediated proteolytic cleavage to separate the signal sequence from the prosequence. The signal sequence comprised 1–29 hydrophobic stretch of amino acid residues and was corroborated with other congeners (Storici *et al*. 1992, Das *et al*. 2006). Valine at 130 was common to allmost all pre-pro-peptide indicating the common processing sites to yield the mature carboxyl terminal peptides (Storici *et al*. 1993, Skerlavaj *et al*. 1996). The prosequence comprised 101 amino acid residues from 30 to 130 residues which is highly identical to the cathelin motif, an inhibitor of thiol proteases (Ritonja *et al.* 1989). The cathelin like prosequence probably prevent the tissue injury and inflammation by neutralizing the high cationic charge of matured peptides by the presence of clusters of negatively charged amino acids (Selested *et al*. 1992; Storici *et al*. 1992, Ganz and Lehrer 1999). The mature peptide comprised 18 amino acid residues from 131–148. The glycine is the last residues for both cathelicidin and most of the congeners, might probably act as amide donor in post translation amidation of the C-terminus (Bradbury and Smyth 1991, Skerlavaj *et al.* 1996). The C-terminal amidation increases the lipolysaccharide binding ability and enhance the outer membrane permeabilization (Sitaram and Nagaraj 1999, Timothy and Roberte 1997). It is reported that C-terminal amidation of peptides provided an additional hydrogen bond for α-helix stabilization (Dennison *et al*. 2005). Presence of 4 conserved cysteine residues in allmost all congeners at proregion (C^{85}, C^{96}, C^{107} and C^{125}) provide possible structural conformity to all cathelicidin molecules (Storici *et al*. 1992). The pre-propeptide of swamp buffalo myeloid cathelicidin had 50 hydrophobic amino acids and sharing a high proportion of hydrophobic amino acids is one of the characteristic features

atg	cag	ccc	aga	ggg	cca	gcc	tct	cgt	tgg	ggc	ggt	ggt	39
M	Q	T	Q	R	A	S	L	S	L	G	R	W	13
cac	tgt	ggc	tac	tgc	tgc	tgg	ggc	ttg	tgg	tgt	cct	cga	78
S	L	W	L	L	L	L	G	L	V	V	S	S	26
cca	gcg	ccc	agg	acc	tca	gct	aca	ggg	aag	ccg	tgc	ttc	117
T	S	A ↓	Q	D	L	S	Y	R	E	A	V	L	39
gtg	ctg	tgg	atc	agc	tca	atg	agc	ggt	ctt	cag	aag	cta	156
R	A	V	D	Q	L	N	E	R	S	S	E	A	52
atc	tct	acc	gcc	tcc	tgg	agc	tag	aac	cac	ctc	cca	agg	195
N	L	Y	R	L	L	E	L	E	P	P	P	K	65
atg	atg	aag	atc	tgg	gca	ctc	gaa	agc	ctg	tga	gct	tca	234
M	M	E	D	L	G	T	R	K	P	V	S	F	78
cgg	tga	agg	aga	ctg	tgt	gcc	cca	gga	cga	ctc	agc	agc	273
T	V	K	E	T	V	C	P	R	T	T	Q	Q	91
ctg	cgg	agc	agt	gtg	act	tca	agg	agg	aag	ggc	ggg	tga	312
P	A	E	Q	C	D	F	K	E	E	G	R	V	104
agc	agt	gtg	tgg	gga	cag	tca	ccc	tgg	acc	cgt	cca	atg	351
K	Q	C	V	G	T	V	T	L	D	P	S	N	117
acc	agt	ttg	acc	taa	act	gta	atg	cgc	tcc	aga	gtg	tca	390
D	Q	F	D	L	N	C	N	A	L	Q	S	V ▲	130
gga	tac	gct	ttc	cat	ggc	cat	ggc	gat	ggc	cat	ggt	ggc ↑	429
R	I	R	F	P	W	P	W	R	W	P	W	W	143
gca	gag	tcc	gag	ggt	tga	447							
R	R	V	R	G	.	148							

Fig. 4. Nucleotide (small letter) and predicted amino acid (single capital letter) sequence of cathelicidin cDNA of bone marrow myeloid tissues of swamp buffalo.

of cathelicidin (Zanetti *et al*. 1995).

The antimicrobial activity exists only in C-terminal domain of the mature peptide comprising 18 amino acids residues from 131–148. In this domain 6 arginine, 3 proline and 5 tryptophan are present. The positively charged arginine is essential to initiate interaction with negatively charged outer bacterial surface (Boman 1995, Sitaram and Nagaraj 1999). The tryptophan influences the localization of these peptides into membrane interfaces (Schiffer *et al*. 1992, Subbalakshmi and Sitaram 1998). Proline is an important amino acids to enhance the microbicidal activity by forming flexible helical kink and more ordered structure (Park *et al*. 2002). Peptide rich in arginine, tryptophan and proline have strong microbicidal activity against gram positive and negative bacteria (Selested *et al*. 1992) and fungi (Subbalakshmi *et al*. 2000) as well as anti-HIV–1 activity (Robinson Jr *et al*. 1998). Thus, expression of arginine, tryptophan and proline rich peptide in the swamp buffalo bone marrow myeloid tissue might be a factor to make this species more resistant to several diseases in comparison to other species.

Artificial synthesis of long peptide may included incomplete coupling and deprotection reactions, accumulation of byproducts and aggregation of fragmented peptide (Saranya *et al.* 2013). Besides synthesis of long peptide is laborious and economically may not be feasible. Hence, it is always suggested to go for small peptide synthesis, using most potent functional domain. Mature functional domain of swamp buffalo (*Bubalus carabanensis*) bone marrow myeloid cathelicidin has been identified from 131–148 of C-terminal, which had 18 amino acids. To reduce the length of the peptide template for synthesis, 6 numbers of peptides has been designed comprising 12 amino acids using support vector machine algorithms (SVM). Antimicrobial activity was also predicted and potency of the different segment of the mature peptides were presented (Table 1). Amino acid residues 5–16 and 6–17 of the mature domain *ie.* 135–146 and 136–147, respectively, of the c- terminal of the original pre-pro-peptide were predicted to be highly potent and have equal potency to that of the original mature peptide. Analysis of the designed peptide using peptide designed tool of the APD (anti microbial database) revealed that the sequences of these peptide resembles (52.6%) with tritrypticin (AP02640), a member of the tryptyophan rich cathelicidin family derived from neutrophil granules of water buffalo (Brahma *et al*. 2015). Antimicrbial activity of the mature peptide was also predicted using antiBP2: a server for antibacterial peptide prediction (Lata *et al*. 2009). From this study, it may be concluded that, the mature functional domain or segment of the swamp buffalo (*Bubalus carabanensis*) bone marrow myeloid cathelicidin can be used as template for synthesis of novel analogue of antimicrobial peptides. Further *in vitro* and *in vivo* experimental studies are needed to conclude the potent antimicrobial nature of the swamp buffalo bone marrow myeloid cathelicidin.

Table 1. Prediction and designing of antimicrobial peptides from the matured peptide using support vector machine (SVM) algorithms

Position	Sequence	Class	AMP Probability (%)
135-146	WPWRWPWWRRVR	AMP	100
136-147	PWRWPWWRRVRG	AMP	100
133-144	FPWPWRWPWWRR	AMP	99.9
134-145	PWPWRWPWWRRV	AMP	99.9
132-143	RFPWPWRWPWWR	AMP	98.7
131-142	IRFPWPWRWPWW	AMP	82.0

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