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Characterization of defensin gene of Asom hill goat and *in-silico* designing of novel antimicrobial peptides

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ABSTRACT

Tissue samples of tongue were collected from apparently healthy Asom hill goat from local slaughter house. Total RNA was extracted and reverse transcribed to cDNA. Size of the amplified products of defensin gene with specific primer were 266 bp. Positive clones were sequenced and analyzed using DNA Star software. At nucleotide level, the Asom hill goat LAP cDNA showed a variation of two nucleotides at 37 and 194 with *Capra hircus* LAP. Nucleotide sequence of Asom hill goat LAP showed highest similarity of 99% with *Capra hircus* LAP followed by 95.4% with *Capra hircus* EBD and 91.3% with sheep BD2. The predicted peptide of Asom hill goat LAP comprised 64 amino acids. Analysis of mature domain of Asom hill goat LAP revealed the presence of 13 basic, 20 hydrophobic, 3 proline and 1 histidine amino acid. Support Vector Machine (SVM) algorithm was used for designing and prediction of antimicrobial potency and amino acids in between 25–39 of mature domain of Asom hill goat LAP predicted to be most potent among all the designed peptide.

Key words: Asom hill goat, cDNA, Defensin, Lingual antimicrobial peptide, RNA

Antibiotics are commonly used for treatment, prevention and control of diseases. Besides these, in meat industry, the antibiotics are used at low concentration as growth enhancers. The use of antibiotics as growth enhancers is a common practice and extensive use of antibiotic in meat industry causes an alarming increase of antibiotic resistance in microbes across the world (Gorbach 2001). Antibiotic resistance has been posing increasingly serious concern to the public, health specialist and milk and meat industry. New strategies are required for synthesis of novel antimicrobial agents to deal with the threat of bacterial resistance (Ravi *et al.* 2011). Antimicrobial peptides are prevalent throughout the nature as part of the intrinsic defenses of most organisms. Antimicrobial peptides hold promise as broad-spectrum alternatives to conventional antibiotics (Gee *et al.* 2013). The Asom hill goat is an important meat type animal with high prolificacy and resistance to different diseases from the North Eastern region of India. However, there is no information available pertaining to defensin antimicrobial peptides in Asom hill goat. Therefore, the present study was undertaken to characterize the defensin gene from tongue epithelium of Asom hill goat and *in-silico* designing of novel antimicrobial peptides from the predicted mature functional peptide.

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MATERIALS AND METHODS

Tongues were collected from Asom hill goat immediately after slaughter in sterilized phosphate buffer saline (pH 7.4) and RNA was extracted from tongue epithelium. The purity and integrity of RNA was judged on the basis of optical density (OD) ratio at 260:280 nm and 1% agarose gel electrophoresis respectively.

The cDNA synthesis was carried out using Revert Aid™ First Strand cDNA Synthesis Kit (Thermoscientific, USA). Specifically designed primer sequences forward (F:5´-CCARCATGAGGCTCCATCA-3´) and reverse (R:5´-CGAAGGCBGCAGTTTCTG-3´) was used for amplification of the targeted cDNA. PCR reaction was carried out using initial denaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 45 sec with a final extension step at 72°C for 5 min. Amplified PCR product was confirmed in 1.5% agarose gel electrophoresis and the specific product was purified by gel purification kit (Qiagen).

Purified PCR product was ligated to Ptz57R/T cloning vector and transformed into competent cell (DH5α). The cells were plated on LB agar plate containing IPTG (100 mM), X-gal (20 mg/ml) and ampicillin (50 mg/ml).The

atg	agg	ctc	cat	cac	ctg	ctc	ctc	gtg	ctc	ttc	ttc	gtg	gtc	ctg	
M	R	L	H	H	L	L	L	V	L	F	F	V	V	L	15
tct	gct	ggg	tca	gga	ttt	act	aca	gga	ata	aga	agt	cgt	cga	agc	
S	A	G	S	G	F	T	Q	G	I	R	S	R	R	S	30
tgc	cat	agg	aat	aaa	ggc	gtc	tgt	gcg	ctg	acc	agg	tgc	cct	aga	
C	H	R	N	K	G	V	C	A	L	T	R	C	P	R	45
aac	atg	aga	cag	att	ggc	acc	tgt	ttc	ggg	ccc	cca	gta	aaa	tgc	
N	M	R	Q	I	G	T	C	F	G	P	P	V	K	C	60
tgc	aga	aag	aag	taa											
C	R	K	K	Stop	64										

Fig. 1. Nucleotide and predicted amino acid sequence of lingual antimicrobial peptide gene of Asom hill goat.

plates were incubated overnight at 37°C and screened for the blue and white colonies. Single white colony was streaked on another LB agar ampicillin plate and kept for 12 h at 37°C. The bacterial clones were inoculated in LB-ampicillin broth and kept for 16 h in shaking incubator. Plasmids were isolated following Sambrook and Russel (2001). The plasmids were screened by digesting with *Eco*RI in order to release the insert. The positive recombinant plasmid was sequenced and analysed using Lasergene Software (DNA Star, USA) and compared with other published sequences.

Expasy (http://web.expasy.org/translate) protein prediction tool and DNA star (Lasergene) was used for prediction of peptides from complete cDNA. The predicted amino acid sequences and retrieved homologous sequences were aligned to know the amino acid distribution pattern as well as to find out the different domain namely signal, pro and functional peptide. From the functional peptide, support vector machine (SVM) algorithm was used to design peptide with antimicrobial potency. Segment showing the highest antimicrobial potency was used for synthesis in subsequent experiment.

RESULTS AND DISCUSSION

The yield of isolated RNA, extracted from epithelium of tongue was 393.7 ng/µl. OD ratio (260:280) ranged from 1.89 to 1.93 and upon 1% agarose gel electrophoresis yielded two high intensity ribosomal RNA bands of 28S and 18S and a faint band of 5S RNA. PCR amplification at optimum annealing temperature i.e. 56°C yielded a specific product of 266 bp upon 1.5% agarose gel electrophoresis. Open reading frame (ORF) of Asom hill goat lingual antimicrobial peptide (LAP) comprised 195 bases (Fig. 1). Nucleotide sequence of Asom hill goat LAP was aligned with ten (10) other published sequences of defensin gene of different species. Among ruminants, 149 nucleotides were found to be conserved. Asom hill goat LAP cDNA sequence had highest similarity of 99% with *Capra hircus* LAP (DQ836129) followed by 95.4% with *Capra hircus* EBD (DQ532360) and 91.3% with sheep BD2 (NM_001198545) (Fig. 2).

Amino acid deduced from ORF of Asom hill goat LAP cDNA contained 64 amino acids (Fig. 1). Predicted

Per cent identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1		88.7	91.3	95.4	99.0	85.1	86.2	64.6	91.3	91.3	72.8	1	Asom Hill Goat LAP
2	12.3		96.4	89.2	88.7	85.6	87.8	62.6	87.2	87.2	74.9	2	Buffalo LAP
3	9.4	3.7		91.8	91.3	88.7	88.9	61.5	88.7	88.7	72.8	3	Buffalo EBD
4	4.8	11.7	8.8		95.4	84.6	86.8	64.1	88.2	88.2	72.8	4	*Capra hircus* EBD
5	1.0	12.4	9.5	4.8		85.1	86.8	63.6	90.3	90.3	72.3	5	*Capra hircus* LAP
6	16.7	15.9	12.2	17.3	16.7		84.7	60.0	84.1	84.1	69.2	6	Cattle BD5
7	15.3	12.7	11.4	14.6	14.6	16.5		63.5	84.7	84.7	68.8	7	Cattle BD7
8	53.0	52.7	55.2	53.6	55.2	61.7	53.7		61.5	61.5	67.7	8	Equine BD1
9	9.3	14.1	12.2	12.9	10.5	17.9	16.5	55.7		100.0	70.3	9	Sheep BD2
10	9.3	14.1	12.2	12.9	10.5	17.9	16.5	55.7	0.0		70.3	10	Sheep tan BD
11	33.0	30.6	32.9	32.8	33.8	38.5	39.7	47.1	36.8	36.8		11	Swine BD1
	1	2	3	4	5	6	7	8	9	10	11		

Fig. 2. Per cent divergence and similarity of lingual antimicrobial peptide gene of Asom hill goat at nucleotide level with different â-defensin gene.

molecular mass of the translated precursor sequence was 7.24 KDa. Six cysteine residues at C^{31}, C^{38}, C^{43}, C^{53}, C^{60}, and C^{61} of Asom hill goat LAP were conserved with β-defensin molecule of other species. All these cysteine residues involved in forming three intermolecular disulfide bridges to provide well-defined three dimensional tertiary structures to β-defensin and this unique fold and conformation is essential to exert its biological activities (Kalita and Kumar 2009). The disulfide bridges of the defensin peptide have been conserved from the period of evolution of complex multicellular organisms (Yount and Yeaman 2006). In all β-defensins, including Asom hill goat, glycine at 20 was conserved which might be the probable site for proteolytic cleavage to release the signal sequence from the pro-sequence. The signal sequence of Asom hill goat β-defensin comprised of N- terminal 1–20 amino acids which corroborated with the other defensins (Kalita and Kumar 2009, Sharma *et al*. 2010, Kumar *et al*. 2010). Immediately after the signal sequence, 2 neutral amino acids i.e. FT at position 21 and 22 of Asom hill goat β-defensin were conserved in all ruminants, which probably act as pro-sequence. Presence of pro-segment ($FT^{21–22}$) in *Capra hircus* LAP and *Capra hircus* EBD was also reported by Sharma *et al.* (2010) and Kumar *et al.* (2010) respectively. The mature peptides of Asom hill goat LAP comprised 42 amino acids from 23–64. It has been reported that mature active peptides are released quickly from precursor by proteolytic processing during microbial invasion (Leher *et al.* 1991, Ganz 2003). Presence of 13 strongly basic (K,R) and 20 hydrophobic (A,I,L,F,W,V) amino acids and 3 proline residues and 1 histidine in the mature peptide might make the Asom hill goat LAP a highly potent endogenous antimicrobial peptide compared to other β-defensins of different species and probably this might make this species more resistant to different diseases as compared to others. The cationic nature of this peptide helps to pass through the anionic phosholipid rich bacterial membranes and act by disrupting the physical integrity of the bilayer, by transient poration (Roberts *et al*. 2006). One histidine residue was present at 32, which has the high cationic charge density make the β-defensin to bind and insert into the microbial membrane, leading to the killing of the microorganism by forming multiple membrane pores (Yang *et al.* 2011). Three proline residues in Asom hill goat LAP present at 44, 56 and 57 enhances the antimicrobial activity forming flexible helical kink (Suh *et al.* 1999, Park *et al.* 2002). Moreover, proline rich peptide can enter the cells without membrane lysis and can inhibit the activity of specific molecular targets in cytoplasm essential to bacterial growth (Genaro *et al.* 2002). The per cent divergence and similarity at amino acid level showed highest similarity (96.9%) between Asom hill goat LAP and *Capra hircus* LAP followed by 86.2% with *Capra hircus* EBD (Fig. 2). This high degree of similarity demonstrates the remarkable conservation of defensin gene (Josheph and More 2011). Construction of phylogenetic tree of Asom hill goat LAP cDNA with other defensin sequences revealed close evolutionary relationship with *Capra hircus* LAP (Fig. 3).

Table 1. Amino acid sequences of most potent five designed peptide using SVM algorithm derived from the lingual antimicrobial peptide of Asom hill goat

Most potent positions of the mature peptides	Amino acids sequences of the designed peptides	Predicted antimicrobial potency (%)
25–39	RQIGTCFGPPVKCCR	81.4
6–20	RSCHRNKGVCALTRC	68.4
12–26	KGVCALTRCPRNMRQ	66.4
5-19	RRSCHRNKGVCALTR	60.8
19–33	RCPRNMRQIGTCFGP	54.8

Support Vector Machine (SVM) was used for designing and prediction of antimicrobial peptide from the mature segment prevailing from 23–64 of the predicted peptide. Five most potent designed peptides are presented in Table 1. The predicted antimicrobial potency of the peptide varied from one another. This was due to the distribution of the different amino acids with different property across the peptides (Xiao *et al.* 2006). The most potent peptide has been searched and designed with shortest length to make it cost effective, to increase the probability of the amino acid coupling, reduce the time and labour in synthesis process. The lower efficacy of the longer peptides might be due to their low capacity to diffuse to the media and inability or

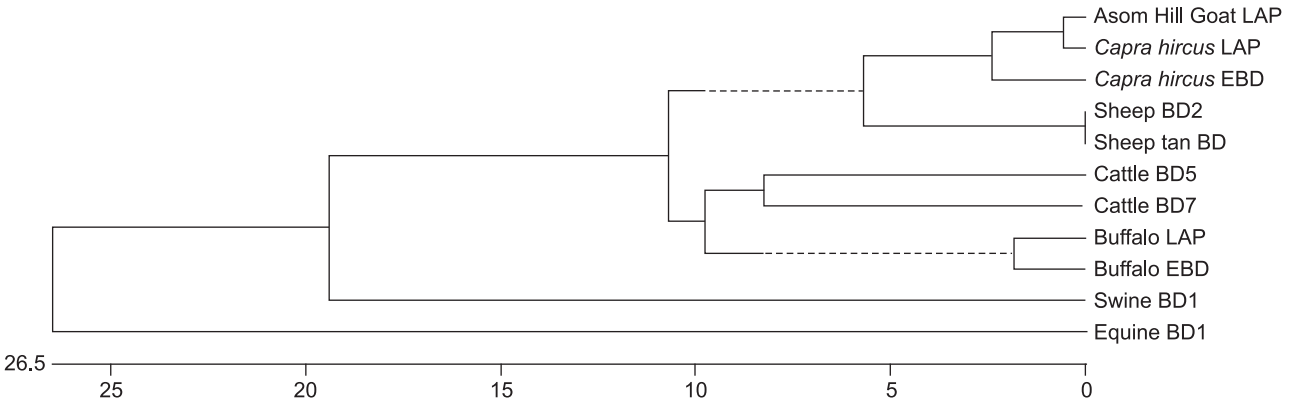

Fig. 3. Phylogenetic tree of lingual antimicrobial peptide gene of Asom hill goat at nucleotide level with different β-defensin.

reduced ability to penetrate through the bacterial membrane (Tew *et al.* 2002). Moreover, hydrophobic clustering and electrostatic interactions accompanied by the relative flexibility in peptide molecules also provides appropriate juxtaposition for its action (Thomas *et al.* 2010), which is certainly higher in short length peptides. Besides, artificial synthesis of long peptide may interfere in the deprotection reactions, accumulation of by-products and aggregation of fragmented peptide (Saranya *et al.* 2013). The C-terminal region of 25–39 of mature domain (Table 1) of Asom hill goat LAP was predicted to be most potent fraction among all the designed peptide. Designed peptides were also compared with different peptides already available in the AMP databases for its functional activity using antiBP2 server (Lata *et al.* 2009) and C-terminal region of 25–39 showed highest similarity with a antimicrobial peptide originated from a amphibian (Yang *et al.* 2012) and sheep (Bagella *et al*. 1995). Distribution of amino acids, cationicity, hydrophobosity are the major criteria for functional diversity of the different designed peptide (Van Hoek 2013). From the present study, it can be concluded that the tongue epithelia of Asom Hill Goat have a very potent antimicrobial peptide and this can be used as template for *in-silico* designing of short novel antimicrobial peptides.

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