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K Bharali
Department of Animal Biotechnology, Indian Veterinary Research, Institute, Izatnagar, Bareilly, Uttar Pradesh, India

DJ Kalita
Department of Veterinary Biochemistry, CVSc, AAU, Guwahati, Assam, India

SN Yadav
Department of Veterinary Clinical Medicine, Ethics and Jurisprudence CVSc, AAU, Guwahati, Assam, India

DP Saikia
Department of Animal Biotechnology, CVSc, AAU, Guwahati, Assam, India

B Devi
Department of Animal Biotechnology, CVSc, AAU, Guwahati, Assam, India

Correspondence
K Bharali
Department of Animal Biotechnology, Indian Veterinary Research, Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Molecular characterization of cathelicidin gene in Assam hill goat

K Bharali, DJ Kalita, SN Yadav, DP Saikia and B Devi

Abstract
Cathelicidin antimicrobial peptides constitute a large family of endogenous peptide antibiotics with broad-spectrum activity against various microbes. RNA was extracted from bone marrow samples and reverse transcribed to cDNA. The PCR amplified product of 414 bp size cloned in Topo-TA cloning vector and confirmed by restriction enzyme digestion. Positive clones were sequenced and the nucleotide sequence of cathelicidin gene was BLAST (Basic Local Alignment Search Tool) with other published sequences. The sequence was further analyzed using DNA Star software. At nucleotide level of Assam hill goat cathelicidin gene showed 187 conserved bases with other sequences. The mature peptides of cathelicidin comprises of 11 amino acids from 127-137 and has two glycine, two arginine and one each of lysine, alanine, serine, isoleusine, leucine, phenylalanine and proline residues. Presence of proline, lysine and arginine in the mature domain might provide more resistance to these breed to fight against diseases in comparison to other breeds. The predicted peptide of Assam hill goat cathelicidin gene is composed of 137 amino acids. Phylogenetic tree revealed a closer relationship of Yunnan goat (CATH2) with Assam hill goat cathelicidin which suggested, these might have diverged from the same ancestral gene.

Keywords: Assam hill goat, antimicrobial peptide, cathelicidin, cDNA, RNA

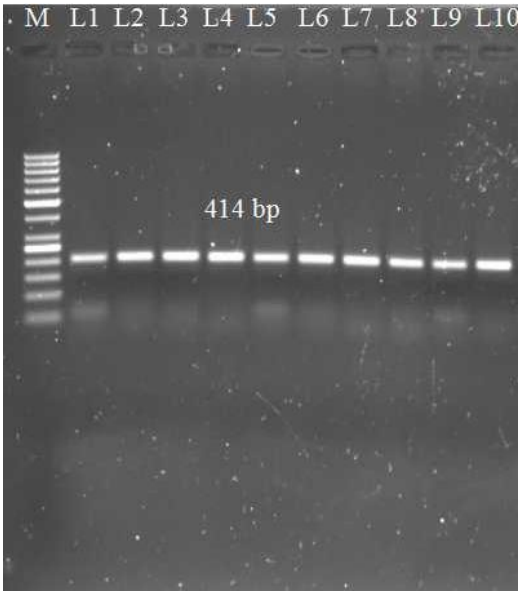
1. Introduction
Antimicrobial peptides are the ancient host defense effector molecules that stimulates innate immune response against a broad array of infectious agents, such as bacteria, virus, fungi, parasite and cancerous cells [1]. The widespread uses of antibiotics in the biomedical and agricultural fields are responsible for the emergence of multidrug-resistant (MDR) organisms [2]. Now a day, bacterial resistance to many classes of antibiotics is becoming a major clinical problem. Therefore, the search continues for new antibiotics that are active *in-vivo*, are fast acting and broad spectrum in activity, do not induce bacterial resistance and have limited side effects. To achieve this goal, antimicrobial peptides have been considered as an alternative to conventional drugs. Antimicrobial peptides could be used as mucosal adjuvant to augment or direct favorable immune responses to co-administered antigens in vaccine for prophylactic prevention of disease [3]. Antimicrobial peptides are conserved in their structure, function and mechanisms of action and thus the synthetic antimicrobial peptides can be used to prevent or treat different microbial infections [3]. Cathelicidin are mostly synthesized in the bone-marrow progenitor cells of mammalian species. Precursors of the cathelicidin family possesses a N-terminal signal peptide of 29-30 amino acids, a pro-sequence of approximately 99-114 amino acids which is highly conserved both intra-species as well as inter-species and in the C-terminal region there is substantial heterogeneity which encode the mature peptide, containing 12-100 amino acids. More than 20 congeners of Cathelicidin with molecular mass of 16-26 kDa have been identified [4]. Resistance of animals to different pathogenic exposure can be increased through introduction of these antimicrobial peptide genes to animal population via production of transgenic animals.Moreover different synthetic analogues can be produced from the sequences of the antimicrobial peptides. So, antimicrobial peptides have dual potential as they can be used either as antibiotics (synthetic peptides) or through transgenic approaches to decrease the dependence of animal population on the use of traditional antibiotics. They are called “new generation antibiotics” for their potential use in preventive and therapeutic medicine [5]. To date there is no information available pertaining to cathelicidin antimicrobial peptides of Assam hill goat. Keeping that point in view the present study was under taken to characterize the cathelicidin gene of this species.

2. Material and Methods

Bone-marrow samples were collected from Assam hill goat immediately after slaughter in sterilized phosphate buffer saline (pH 7.4) and total RNA was extracted from bone-marrow myeloid cells using TRIzol (Invitrogen, U.S.A.). The purity and integrity of the extracted RNA was judged on the basis of optical density (OD) ratio at 260:280 nm and as well as in 1% agarose gel electrophoresis respectively. cDNA synthesis was carried out using Revert Aid^{TM} First Strand cDNA Synthesis Kit (Thermo scientific, U.S.A.) from the extracted total RNA as per manufacturer's guidelines. A pair of specific designed primers (F:5´-GGACCATGCAGACCCAGA-3´and R:5´-TGTCCAGAAGCCCGAATC-3´) were used for amplification of the cathelicidin gene. The PCR reaction was carried out using initial denaturation at 94°C for 5 mins, followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min with a final extension step at 72°C for 5 mins. PCR amplification was confirmed by running the product in 1.5% agarose gel electrophoresis and the specific product was purified by QIAquick Gel Extracion kit (Qiagen, Germany). The purified PCR product was ligated to PCR 2.1-TOPO (Thermo Fisher Scientific) cloning vector as per manufacturer's protocol with vector and insert DNA fragments in the ratio of 1:3 respectively. The ligation mix was incubated overnight at 16^0C and used for transformation of *E.coli* DH5α competent cells by heat shock method. The cells were then plated on LB plate containing IPTG (100mM), Xgal (20 mg/ml) and ampicillin (50mg/ml).The plates were incubated overnight at 37°C and screened for the blue and white colonies. Single white colony was streaked on another LB-amplicillin plate and kept for 12 hours at 37°C. The bacterial clones were inoculated into LB-ampicillin broth and kept for 16 hours in shaking incubator at 37°C. Plasmids were isolated using the alkaline lysis method as described by Sambrook and Russel (2001). Extracted plasmids were screened by digesting with *EcoR*I (NEB) according to the manufacturer's protocol. The restriction digestion mixture was electrophoresed on agarose gel and release of the expected size DNA fragment confirmed the presence of insert in the recombinant plasmid. The recombinant plasmid was sequenced and the sequence results were analysed using Lasergene Software (DNA Star, USA).

3. Results

The yield of isolated RNA was 332.2 ng/µl on an average. The purity of RNA was judged on the basis of the Optical Density (OD) ratio (260:280) which ranged from 1.92 to 1.97 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 of the cathelicidin gene at optimum annealing temperature of 55^0C yielded a specific 414 bp amplicon upon 1.5% agarose gel electrophoresis (Figure 1).The purified PCR product was cloned in pCR 2.1-TOPO (Thermo Fisher Scientific) cloning vector and sequenced. Sequence analysis revealed that the Open Reading Frame (ORF) of Assam hill goat cathelicidin gene comprised of 414 bases and the translated region has 87 A (21.01%), 139 G (33.57%), 69 T (16.67%) and 119 C (28.74%) residues. The predicted molecular mass of the translated precursor sequence was 15.05 kDa. The amino acid sequence deduced from ORF of Assam hill goat cathelicidin contains 137 amino acids (Figure 2). The mature peptides of Assam hill goat cathelicidin comprised of 11 amino acids from 127-137 and has 2 glycine, 2 arginine and 1 each of lysine, alanine, serine, ileisoleusine, leucine, phenylalanine and proline residues. Presence of proline, lysine and arginine in the mature domain of Assam hill goat cathelicidin of bone-marrow myeloid tissue might provide more resistance to Assam hill goat to fight against different diseases with compare to other breeds of goats. Analysis of percent divergence and identity at nucleotide level of Assam hill goat cathelicidin gene sequence showed identity of 58.9% with buffalo indolicidin followed by 58.2% with Yunnan goat CATH2 and 57.2 % with sheep CATH2.Others like, cattle CATH4, goat MAP 28 and equine CATH2 showed the identity of 56.5%, 53.1% and 51.2% respectively with Assam hill goat cathelicidin (Figure 3). At amino acid level, Assam hill goat cathelicidin and Yunnan goat CATH 2 showed highest identity (52.2%) among the different species. The other cathelicidin like buffalo indolicidin, cattle CATH4, sheep CATH2 and equine CATH2 showed the identity of 51.4%, 47.8%, 42.0% and 41.3% respectively with Assam hill goat cathelicidin (Figure 4). Phylogenetic analysis of Assam hill goat cathelicidin with different other cathelicidin sequences revealed close evolutionary relationship with Yunnan goat CATH2 at nucleotide (Figure 5) and amino acid levels (Figure 6)



M: 100 bp DNA Marker, L1-L10: PCR products

Fig 1: 1.5% Agarose gel electrophoresis of PCR products of cathelicidin gene.

atg	cag	acc	cag	agg	gcc	agc	ctc	tcg	ttg	ggg	cgg	tgg	tca	ctg	
M	Q	T	Q	R	A	S	L	S	L	G	R	W	S	L	15
tgg	ctt	ctg	ctg	Ctg	ggc	cta	gtg	gta	ccc	tcg	gcc	agc	gcc	cgg	
W	L	L	L	L	G	L	V	V	P	S	A	S	A	R	30
gat	ctg	ggg	agg	ggg	cca	gct	agt	tgg	ggg	gca	ggg	aga	cag	atc	
D	L	G	R	G	P	A	S	W	G	A	G	R	Q	I	45
aga	gga	aga	aga	aag	agc	cca	aat	ccg	gtt	tcc	cct	act	ttg	acc	
R	G	R	R	K	S	P	N	P	V	S	P	T	L	T	60
cgt	gac	cag	gag	gtc	gac	cca	ggc	acc	aga	aag	ccc	gtg	agc	ttc	
R	D	Q	E	V	D	P	G	T	R	K	P	V	S	F	75
acg	gtg	aaa	gag	acc	gtg	tgc	ccc	agg	acc	acc	cag	cag	ccc	ccg	
T	V	K	E	T	V	C	P	R	T	T	Q	Q	P	P	90
gag	gag	tgt	gac	ttc	aag	gag	aat	ggg	gtg	agg	ctg	ggg	tgg	ggc	
E	E	C	D	F	K	E	N	G	V	R	L	G	W	G	105
tgg	gcc	ata	acc	tcc	cgg	cct	cct	ccc	agg	agt	tgg	aaa	tca	tcc	
W	A	I	T	S	R	P	P	P	R	S	W	K	S	S	120
cca	agc	cca	ctg	tcc	gtg	ccc	ggg	aaa	gca	tct	aga	att	ctc	ttt	
P	S	P	L	S	V	P	G	K	A	S	R	I	L	F	135
aga	ggt	tga													
R	G	Stop	137												

Fig 2: Nucleotide and predicted amino acid sequence of Assam hill goat cathelicidin gene

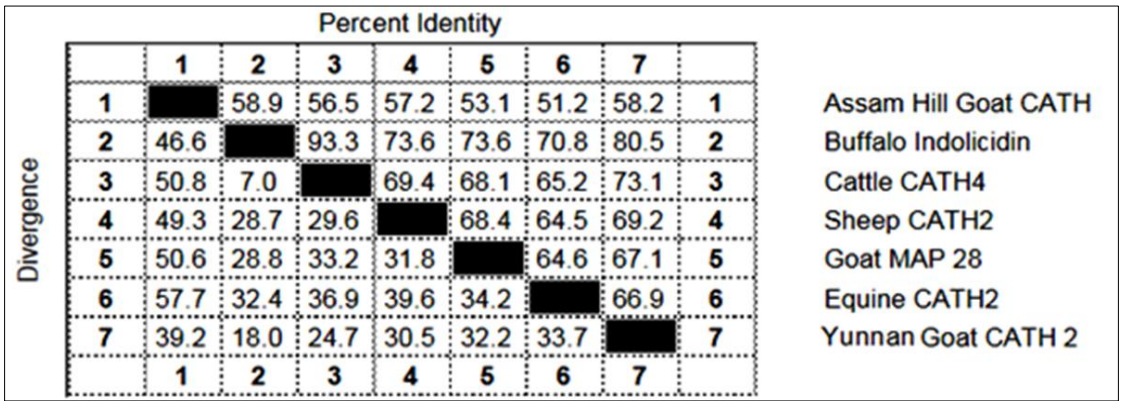


Fig 3: Percent divergence and percent similarity of cathelicidin gene of Assam hill goat at nucleotide level with different cathelicidin

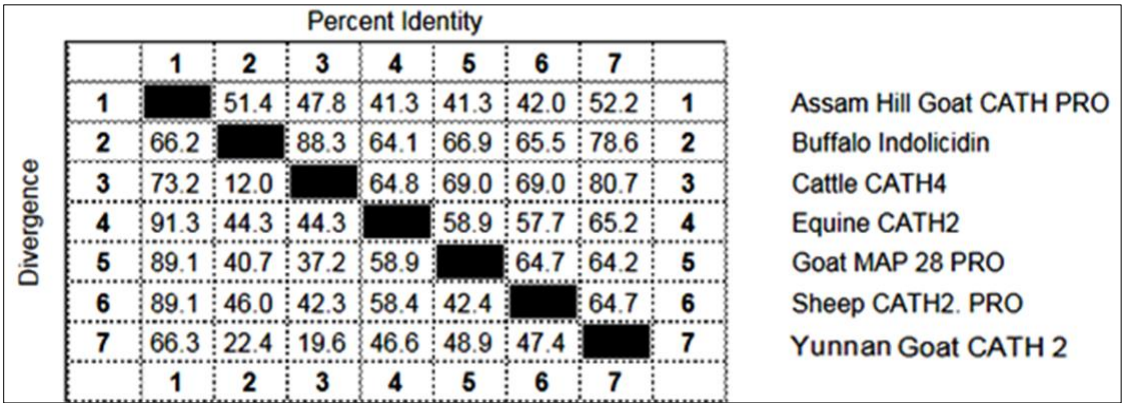


Fig 4: Percent identity and divergence of Assam hill goat cathelicidin at amino acid level with cathelicidin of different species

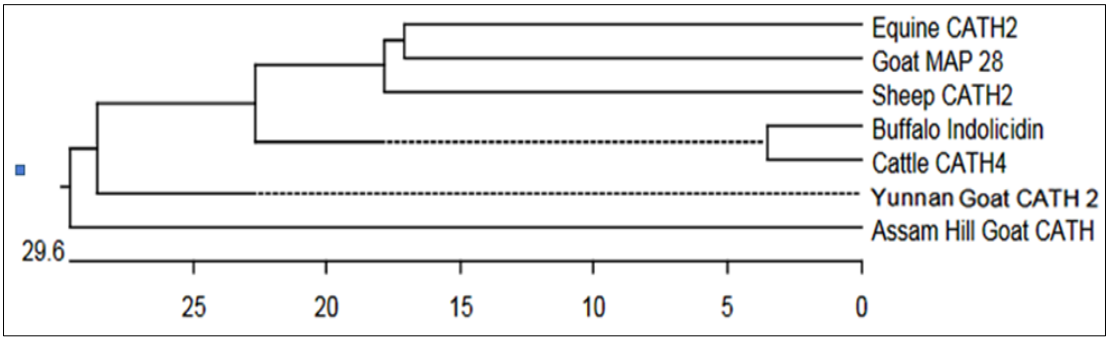


Fig 5: Phylogenetic tree of Assam hill goat cathelicidin gene with cathelicidin gene of different other species

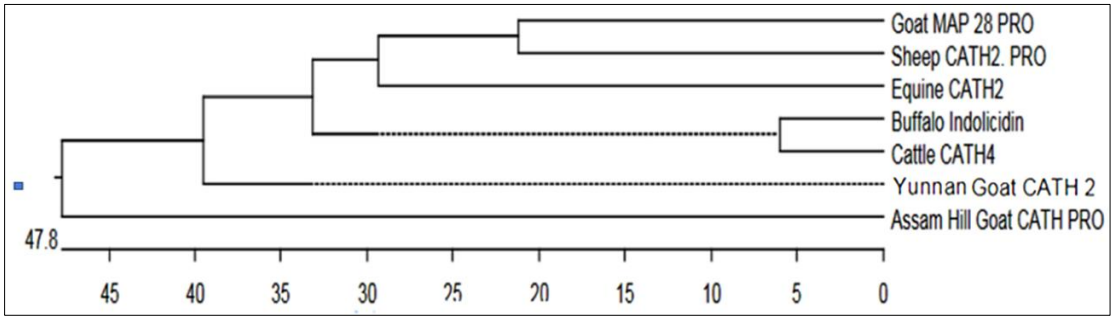


Fig 6: Phylogenetic tree of Assam hill goat cathelicidin with cathelicidin of different other species at amino acid level (deduced)

4. Discussion

Multicellular organisms have several different families of antimicrobial peptides that act as host defense effector molecules stimulating innate immune response against a broad array of microbes including bacteria, virus, fungi and parasites. The present investigation was undertaken with an aim to characterize the cathelicidin gene in Assam hill goat. The nucleotide sequence of Assam hill goat cathelicidin showed highly conserved nucleotides at region 5' and a limited identity in 3' region with other published sequences, which is a common feature of the cathelicidin gene [6, 7]. The deduced pre-propeptides of Assam hill goat cathelicidin has conserved N-terminal and the diverse heterogeneous C-terminal is in agreement with other cathelicidin congeners [8, 9]. Alanine at position 29 is conserved in most of the congeners and it might be the probable site of elastase mediated proteolytic cleavage to separate the signal sequence from the prosequence. The signal sequence of Assam hill goat cathelicidin comprised of 1-29 hydrophobic stretch of amino acid residues, which corroborated with other congeners [10, 11]. Valine at position 126 is common to almost all pre-pro-peptides including that of Assam hill goat indicating the common processing sites to yield the mature carboxyl terminal peptides [7, 12]. The prosequence of Assam hill goat cathelicidin comprised of 101 amino acid residues from 30 to 130, which are highly identical to the cathelin motif, an inhibitor of thiol proteases [13]. Assam hill goat cathelicidin had 11 amino acid residues in the mature peptide from 127-137. The glycine is the last residue in most of the congeners as well as in the Assam hill goat cathelicidin and it might probably act as amide donor in post translation amidation of the C-terminus end [12, 14]. The C-terminal amidation is essential for its optimum antimicrobial activity which increases the lipopolysaccharide binding ability and enhances the outer membrane permeabilization [15, 16]. The mature peptide of Assam hill goat cathelicidin has 2 glycine, 2 arginine and 1residue each of lysine, alanine, serine, ileisoleusine, leucine, phenylalanine and proline. The positively charged arginine and lysine are essential to initiate interaction with negatively charged outer microbial surface [15, 17]. Proline is an important amino acid to enhance the microbicidal activity by forming flexible helical kink and more ordered structure, which increases membrane permeability [18]. Besides, proline also provides some protection against nonspecific proteolytic degradation of proteases secreted by microorganisms [19]. Peptides rich in arginine and proline are shown to have strong microbicidal activity against gram-positive and gram-negative bacteria [20], fungi [21, 16] and viruses [22]. Phylogenetic tree of Assam hill goat cathelicidin with other cathelicidin sequences revealed a closer relationship of Yunnan goat CATH2 with Assam hill goat cathelicidin which suggested that it might have diverged from the same ancestral gene. From the present study it can be concluded that Assam hill goat bone-marrow myeloid cells express a very potent and unique antimicrobial peptide. The mature region of the peptide may be used for designing of new peptide analogue in future for synthesis of novel antimicrobial agent.

5. Conclusion

From the present study it can be concluded that Assam hill goat bone-marrow myeloid cells express a very potent and unique antimicrobial peptide. The mature region of the peptide may be used for designing of new peptide analogue in future for synthesis of novel antimicrobial agent.

6. References

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