

Characterization of a novel antimicrobial peptide gene from the reproductive tract of indigenous cows (*Bos indicus*) of Asom

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ABSTRACT

Antimicrobial peptides are innate immune defense peptides protecting against infection. Defensins and cathelicidins are the two major antimicrobial peptides in eukaryotes. In the present study, female reproductive tract was collected from apparently healthy local cows (*Bos indicus*) of Asom after slaughter. cDNA was synthesized from the extracted RNA by reverse transcription and amplified the Lingual Antimicrobial Peptide (LAP) gene (227 bp) using specific primers. The purified product was sequenced and sequence were aligned Nucleotide sequence was BLAST with twelve published sequences and analyzed using DNA Star software. At nucleotide level, *Bos indicus* LAP of reproductive tract showed the highest similarity of 97.4% with *Bos taurus* LAP of tongue followed by 92.8% with buffalo EBD. We identified the highest similarity (93.8%) of *Bos indicus* LAP of reproductive tract with *Bos taurus* LAP of tongue followed by buffalo EBD (86.2%). The phylogenetic analyses at nucleotide and amino acid level showed that *Bos indicus* LAP of reproductive tract and *Bos taurus* LAP of tongue are closely evolutionarily which implied that they might have diverged from ancestral gene. We conclude that female reproductive-tract epithelium of local cows of Asom express a potent AMP similar to that of *Bos taurus* LAP of tongue.

Keywords: *Bos indicus*, cDNA, Female reproductive tract, Lingual antimicrobial peptide, RNA

Antimicrobial peptides are important constituents of the innate immune defense of all living organisms including mammals, amphibians, insects and plants. The innate immune system is the first line of defense against pathogenic insult, which is followed by acquired immune responses (Fearon *et al.* 1996, Medzhitov *et al.* 2000). In meat industry, 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 resistant microbes (Gorbach 2001). Antibiotic resistance has been posing increasingly serious concern to the public, health specialist and animal food producers. Therefore, newer strategies are required for synthesis of novel antimicrobial agents to deal with threat of bacterial resistance (Ravi *et al.* 2011). Antimicrobial peptides represent ancient host defense molecules and act as key elements in non-specific immunity (Ganz and Lehrer, 1989). Their wide spread distribution throughout the animal and plant kingdoms suggest that antimicrobial peptides serve a fundamental role in the successful evolution of complex multicellular organisms (Bals. 2000). Antimicrobial peptides are candidates as broad-spectrum alternatives to conventional antibiotics (Ganz and Lehrer 1994). Antimicrobial peptides have been

classified in several families on the basis of their structural features, functional properties and expression patterns (Boman *et al.* 1995). Mammalian defensins and cathelicidins are two broad classes of natural antimicrobial peptides constituting a large family of endogenous peptide antibiotics with broad spectrum activity against various bacteria, fungi and viruses. Expression of human cathelicidin namely hCAP-18 and LL-37, has been reported in the reproductive tract (Malm *et al.* 2000) and skin epithelial cell (Markus *et al.* 2012), respectively. Several β -defensin such as, human β -defensin-4 from testis (Garica *et al.* 2001), cryptidin from mouse sertoli cells (Grandjean *et al.* 1997), Bin1b from rat epididymis (Li *et al.* 2001) have been isolated. Lingual antimicrobial peptide (LAP) gene from buffalo tongue has been sequenced and characterized (Kalita and Kumar 2009). Synthesis of partial and complete sequences of natural analogue of buffalo LAP and functional study revealed its potency against both gram positive and negative bacteria (Kalita *et al.* 2009). However to date, antimicrobial peptide gene from reproductive tract of indigenous cattle of Asom has not been characterized. Keeping this in view, the present study was carried out to characterize the lingual antimicrobial peptide gene of reproductive tract from indigenous cattle (*Bos indicus*) of Asom as the LAP expressed by the reproductive tract is hypothetically supposed to be more potent due to its physiological function and anatomical location which may

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provide a template for synthesis of novel peptide antibiotic in future.

MATERIALS AND METHODS

Isolation of total cellular RNA

Female reproductive tract from approximately 5 years of local cows (*Bos indicus*) were collected immediately after slaughter in phosphate buffered saline (pH 7.4) under cold and aseptic condition from local slaughter house of Guwahati under Kamrup district for extraction of RNA from the epithelial layer of uterus. Total RNA was isolated using RNeasy Mini Kit (Qiagen). The integrity of RNA was checked by 1% agarose gel electrophoresis and quantity was estimated by spectrophotometric method using nanodrop spectrophotometer. The purity of RNA was judged on the basis of optical density (OD) ratio at 260:280 nm.

Polymerase chain reaction (PCR)

The cDNA synthesis was carried out using Revert Aid™ First Strand cDNA Synthesis Kit (Thermoscientific) from the extracted total RNA. Specific published primers (Kalita *et al.* 2009): forward 52 CGGCACCGACAGC ATGAG32 and reverse 52 GCCACGTCTTCGCC TTCT32, were used for amplification of the cDNA. To confirm the size of the amplified PCR product, agarose gel electrophoresis was performed using 1.5% agarose with ethidium bromide staining. After confirmation of the size of the PCR product, the product was purified by gel purification kit (Qiagen) and sequenced at the DNA Sequencing Facility, Department of Biochemistry, South Campus, University of Delhi.

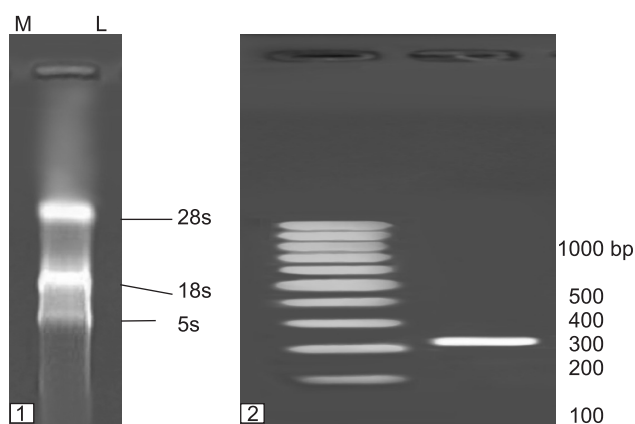
Analysis of nucleotide sequence

The sequences were aligned with other published sequences to determine homology. Sequence analysis was performed using DNA Star (USA) sequence analysis software.

RESULTS AND DISCUSSION

The concentration of total RNA extracted from epithelium of female reproductive tract was 164.67 ng/μl. The Optical Density (OD) ratio (260:280) of extracted RNA was 1.99 and upon 1% agarose gel electrophoresis yielded two high intensity ribosomal RNA bands of 28S and 18S and a faint band of 5S RNA (Fig. 1). The PCR product at optimum annealing temperature (53°C) yielded a specific product of 227 bp upon 1.5% agarose gel electrophoresis (Fig. 2).

The Open Reading Frame (ORF) of *Bos indicus* LAP of reproductive tract was comprised of 195 bases (Fig.3) from the total size of 227 bp. The translated region had 49A (25.13%), 52G (26.67%), 45T (23.08%) and 49C (25.13%). The nucleotide sequences identified in this study aligned with twelve other published sequences of β-defensin of different species. The cDNA of *Bos indicus* reproductive



Figs 1–2. 1. Agarose gel electrophoresis of RNA of female reproductive tract epithelia of local cattle (*Bos indicus*) of Asom. 2. Agarose gel electrophoresis of PCR product of Lingual Antimicrobial Peptide gene of female reproductive tract of local cows (*Bos indicus*) of Asom M, 100 bp DNA ladder; L, PCR product (227 bp).

tract LAP and other aligned sequences showed 68 conserved bases. Among ruminants, 137 nucleotides were found to be conserved. Reproductive tract LAP cDNA sequence of *Bos indicus* had highest similarity of 97.4% with *Bos taurus* LAP of tongue followed by 92.8% with buffalo EBD and 90.8% with reindeer BD1 (Fig. 4).

The ORF of *Bos indicus* reproductive tract LAP contained 64 amino acid (Fig.3). The predicted molecular mass of the translated precursor sequence was about 6.99 kDa. Six cysteine residues at C31, C38, C43, C53, C60 and C61 of *Bos indicus* LAP were conserved in all β-defensin molecules. M1, R2, L7, L15, G²⁰, Q49, I50, G51, T52 and K⁵⁹ were found to be conserved with other β-defensin. In ruminants, conserved positions were 1–4, 6–8, 10, 12, 14–17, 19–24, 30, 31, 34, 36, 38, 42–43, 49–53 and 57–61. Glutamine, histidine and proline amino acids of *Bos indicus* reproductive LAP were substituted respectively by histidine, leucine and arginine at 5, 54 and 62 position in *Bos taurus* LAP of tongue. The percent divergence and similarity at amino acid level showed highest similarity (93.8%) between *Bos indicus* reproductive LAP and *Bos taurus* LAP of tongue followed by 86.2% with buffalo EBD (Fig.5).

The LAP is a member of the β-defensin family and was first isolated from the inflamed bovine tongue epithelium (Schonwetter *et al.* 1995). Subsequent investigation found wide spread LAP expression in infected bovine intestinal and respiratory tissues (Stolzenberg *et al.* 1997). RT-PCR of the isolated total RNA gave a specific product 227 bp which matched with *Bos taurus* LAP (Schonwetter *et al.* 1995) and differed by 5 nucleotides at 15, 162, 185, 186 and 187 position. Variations of 13, 19, 20, 25 and 27 nucleotides were also recorded with buffalo EBD (Das *et al.* 2005), buffalo LAP of tongue (Kalita *et al.* 2009), goat LAP of tongue (Sharma *et al.* 2010) of tongue, cattle TAP of trachea (Diamond *et al.* 1991) and cattle EBD of intestine (Tarver *et al.* 1998) respectively. The nucleotide sequence of *Bos indicus* reproductive tract LAP showed highest

	atg	agg	ctc	cat	cag	ctg	ctc	ctt	gcg	ctc	ctc	ttc	ctg	gtc	ctg	
	M	R	L	H	Q	L	L	L	A	L	L	F	L	V	L	15
46	tct	gct	ggg	tca	gga	ttt	act	caa	gga	gta	aga	aat	tct	caa	agc	
	S	A	G	S	G	↓	F	↑	T	Q	G	V	R	N	S	30
91	tgc	cgt	agg	aat	aaa	ggc	atc	tgt	gtg	ccg	atc	agg	tgc	cct	gga	
	C	R	R	N	K	G	I	C	V	P	I	R	C	P	G	45
136	agc	atg	aga	cag	att	ggc	acc	tgt	cac	gga	gcc	caa	gta	aaa	tgc	
	S	M	R	Q	I	G	T	C	H	G	A	Q	V	K	C	60
181	tgc	cca	agg	aag	taa											
	C	P	R	K	Stop	64										

Fig. 3. Nucleotide and predicted amino acid sequence of lingual antimicrobial peptide gene of reproductive tract of local cows (*Bos indicus*) of Asom.

Percent Similarity															
	1	2	3	4	5	6	7	8	9	10	11	12	13		
1		97.4	89.7	86.2	92.8	84.6	87.2	86.7	88.7	90.8	68.7	55.4	52.3	1	Cattle LAP (local).SEQ
2	2.6		92.8	87.2	95.9	87.7	87.7	87.7	88.7	92.3	69.2	56.4	51.8	2	Cattle LAP.seq
3	10.4	7.6		87.7	96.4	86.7	90.3	88.2	88.2	89.7	69.2	55.9	53.8	3	Buffalo LAP.seq
4	14.3	12.6	12.4		90.8	84.6	85.1	99.5	94.4	90.3	66.7	55.9	50.3	4	Goat LAP.seq
5	7.0	4.2	3.7	9.5		89.2	88.2	91.3	90.8	93.3	67.7	54.9	53.8	5	Buffalo EBD.seq
6	15.4	12.3	12.9	15.6	10.5		82.1	85.6	84.1	82.1	64.6	54.4	51.8	6	Cattle EBD.seq
7	14.1	12.3	8.7	15.5	11.1	17.3		85.6	86.2	86.7	67.7	54.9	56.9	7	Cattle TAP.seq
8	13.7	11.9	11.8	0.5	8.9	14.9	14.8		94.9	90.8	67.2	56.4	50.3	8	Goat BD1.seq
9	12.4	11.8	11.1	5.4	8.8	16.2	14.8	4.8		90.8	68.7	56.9	52.3	9	Goat BD2.seq
10	9.3	7.6	11.0	10.6	7.0	16.1	14.1	9.9	9.9		65.6	56.4	51.8	10	Reindeer BD1.seq
11	32.0	31.3	30.6	33.8	32.9	36.3	32.1	32.9	31.2	34.7		59.0	52.3	11	Swine BD1.seq
12	53.0	51.3	52.1	54.7	54.7	53.8	51.1	53.4	50.8	50.3	46.2		52.8	12	Equine BD1.seq
13	58.6	59.0	56.7	59.7	56.6	57.8	49.8	60.1	57.5	60.1	54.4	52.4		13	Human BD4.seq
	1	2	3	4	5	6	7	8	9	10	11	12	13		

Fig. 4. Percent divergence and percent similarity of reproductive tract lingual antimicrobial peptide gene of local cows (*Bos indicus*) of Asom at nucleotide level with different β - defensins.

similarity of 97.4% with *Bos taurus* LAP of tongue (Schonwetter *et al.* 1995) followed by 92.8% with buffalo EBD (Tarver *et al.* 1998) and 90.8% with reindeer BD (Stolzenberg *et al.* 1997). The mature peptide of *Bos indicus* reproductive tract LAP is comprised of 42 amino acids from 23–64. The length of mature peptide of buffalo LAP (Kalita *et al.* 2009), buffalo EBD (Das *et al.* 2005), *Bos taurus* LAP (Schonwetter *et al.* 1995), cattle TAP (Diamond *et al.* 1991) and cattle EBD (Tarver *et al.* 1998) was also of 42 amino acids from 23–64. Glutamine, histidine and proline of *Bos indicus* reproductive LAP was substituted respectively by histidine, leucine and arginine at 5, 54 and 62 position in *Bos taurus* LAP. Presence of 10 basic, 8

hydrophobic amino acids and 3 proline in the mature peptide might make the *Bos indicus* reproductive tract LAP, a highly potent endogenous antimicrobial peptide as compared to other β -defensins as the indigenous cows are more resistance to different diseases as compared to other animals (Bishop and Woolliams 2014). The cationic nature of different defensin peptides help to pass the anionic phospholipids rich bacterial membranes and acts by disrupting the physical integrity of the bilayer (Robert *et al.* 2006). Histidine which has the high cationic charge density enables the β -defensins to bind and insert into the cellular membrane, leading to the killing of the microorganism by forming multiple membrane pores (Yang *et al.* 2011). Proline, present in the

Percent Similarity													
	1	2	3	4	5	6	7	8	9	10	11	12	13
1		93.8	81.5	69.2	86.2	72.3	80.0	69.2	73.8	80.0	53.8	41.5	35.4
2	4.8		86.2	72.3	90.8	75.4	83.1	72.3	75.4	84.6	53.8	44.6	35.4
3	19.6	13.7		75.4	92.3	76.9	84.6	75.4	80.0	81.5	55.4	44.6	41.5
4	37.8	32.8	28.2		78.5	69.2	69.2	98.5	86.2	73.8	50.8	44.6	36.9
5	13.7	8.3	6.5	23.7		78.5	83.1	78.5	80.0	86.2	52.3	44.6	38.5
6	32.8	28.2	25.9	37.8	23.7		72.3	69.2	70.8	67.7	46.2	43.1	40.0
7	21.6	17.6	15.6	37.8	17.6	32.8		69.2	73.8	78.5	53.8	46.2	43.1
8	37.8	32.8	28.2	0.0	23.7	37.8	37.8		86.2	73.8	50.8	44.6	36.9
9	30.4	28.2	21.6	13.7	21.6	35.2	30.4	13.7		75.4	52.3	46.2	38.5
10	21.6	15.6	19.6	30.4	13.7	40.4	23.7	30.4	28.2		50.8	46.2	36.9
11	68.2	68.2	64.6	75.8	71.9	88.6	68.2	75.8	71.9	75.8		50.8	46.2
12	103.6	93.3	93.3	93.3	93.3	98.3	88.6	93.3	88.6	88.6	75.8		46.2
13	128.3	128.3	103.6	121.4	115.1	109.1	98.3	121.4	115.1	121.4	88.6	88.6	
	1	2	3	4	5	6	7	8	9	10	11	12	13

Cattle Lap (local).PRO
 Cattle LAP.PRO
 Buffalo LAP.PRO
 Goat LAP.PRO
 Buffalo EBD.PRO
 Cattle EBD.PRO
 Cattle TAP.PRO
 Goat BD1.PRO
 Goat BD2.PRO
 Reindeer BD1.PRO
 Swine BD1.PRO
 Equine BD-1.PRO
 Human BD4.PRO

Fig. 5. Percent divergence and percent similarity of reproductive tract lingual antimicrobial peptide gene of local cows (*Bos indicus*) of Asom at amino acid level with different β - defensin.

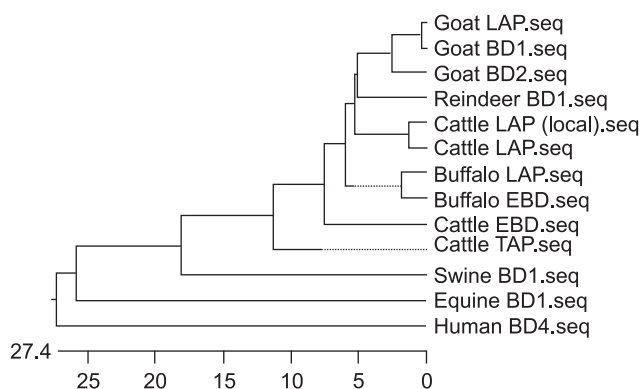


Fig. 6. Phylogenetic tree of lingual antimicrobial peptide gene of reproductive tract of local cows (*Bos indicus*) with other β - defensin at nucleotide level.

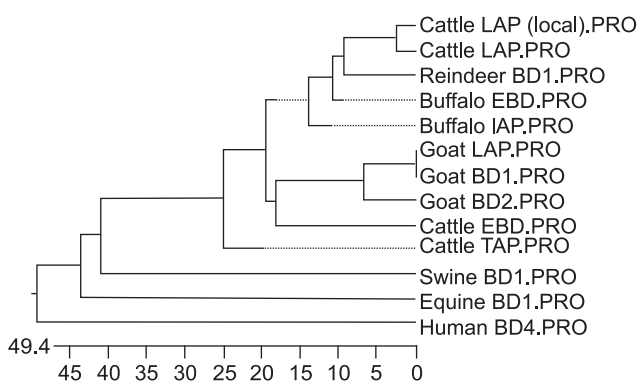


Fig. 7. Phylogenetic tree of lingual antimicrobial peptide gene of reproductive tract of local cows (*Bos indicus*) with other β - defensin at amino acid level.

mature peptide enhances the microbicidal activity by forming flexible helical link which increases membrane permeability and allows the hydrophobic residues to reside in the concave helical region (Suh *et al.* 1999, Park *et al.* 2002). Moreover proline rich peptides can enter the cells

without membrane lysis and inhibit the activity of specific molecular targets essential to bacterial growth, thereby causing cell death (Gennaro *et al.* 2002). *Bos indicus* reproductive tract LAP shared highest similarity of 93.8% with *Bos taurus* LAP followed by 86.2% with buffalo EBD at amino acid level.

The phylogenetic trees of reproductive tract LAP gene of *Bos indicus* with β -defensin of different species were generated by DNA Star software both at nucleotide (Fig.6) and predicted amino acid level (Fig.7). Phylogenetic tree revealed that *Bos indicus* LAP of reproductive tract and *Bos taurus* LAP of tongue is more close to each other at nucleotide and deduced amino acid level among all the ruminant β -defensin as they lie in the close proximity to each other. Beside ruminants, other species like swine, horse and human β -defensin were distantly related to *Bos indicus* LAP of reproductive tract as they exist separate clad in the phylogenetic tree. In the phylogenetic tree both at nucleotide and amino acid level, the β -defensin of different ruminant species formed single cluster and it implied that they might have diverged from same ancestral gene (Kalita *et al.* 2009 and Joseph *et al.* 2011). It can be concluded that female reproductive tract epithelium of indigenous cows of Asom expressed a potent antimicrobial peptide similar to *Bos taurus* LAP of tongue and the mature LAP of local cows of Asom had 6 arginine, 3 lysine 1 histidine and 3 proline residues. Arginine, lysine and histidine residues are responsible for disruption of microbial membrane due to its cationic nature and proline due to its special structure can form flexible helical kink which increases the permeability of the microbial membrane to LAP and thus the LAP of *Bos indicus* expressed by reproductive tract provide a template for synthesis of novel peptide antibiotic in future.

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