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Molecular characterization and in-silico analysis of complete coding sequence of bubaline mLYS

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ABSTRACT

The role of immune-relevant genes involved in disease biology as well as innate and adaptive immunity is at the centre stage while exploring the genetic basis of disease occurrence. The macrophage expressed lysozyme gene product contributes to innate immunity by cleaving the ß- 1,4 linkage of bacterial peptidoglycan in phagocytosis. Higher specific activity observed in bubaline milk/ serum lysozymes over their bovine counterparts is expected to contribute higher resistances to diseases in general and mastitis in particular. To explore the reasons for higher activity in terms of sequence variations, macrophage expressed lysozyme cDNA was synthesized, cloned and sequenced. The 593 bp mRNA sequence revealed a 444 bp (27nt to 470nt) ORF bearing usual start codon ATG and end codon TAA with GC content of 46 % coding for precursor polypeptide of 147 amino acids. The comparative sequence analysis of cattle and buffalo revealed the difference at 10 places leading to 3 non-synonymous (5^{th}, 116^{th} and 142^{th}) substitutions which did not affect the predicted 3D structure. The *mLYS* polypeptide had signal sequence (1^{st} to18^{th} residue) and the mature peptide had two lysozyme catalytic sites, three Ca^{++} binding sites as well as eleven catalytic clefts prominent among the conserved domains. Secondly, bubaline *mLYS* was phylogenetically closer to the abomasum type than mammary gland type. The high similarity in the coding sequences and predicted structure suggested that bubaline lysozyme gene if hyper-expressed either in native or recombinant form in bovine udder may result in better udder health.

Key words*:* Buffalo, cDNA sequencing, In-silico analysis, Macrophage lysozyme

Among various diseases affecting dairy enterprises, mastitis causes heavy economic loss worldwide (Gill *et al*. 1990, Maga 2005) by affecting quality, safety and quantity of milk. The host susceptibility is influenced by various genetic (i.e. physical and physiological) as well as environmental factors and heritability of reduced susceptibility (to mastitis) is very low i. e. $h^2 < 0.1$ (Simianer *et al*. 1991). The mammary gland is lined with innate sentinels such as PMN (polymorphonuclear) leucocytes and macrophages who routinely defend invading bacteria like Gram +ve *Staphylococcus aureus* through phagocytosis. In other words, phagocytosis is only little option left with mammary gland as it is anatomically restricted to adaptive immune arm such as antibody complement system. Therefore the genes involved in phagocytosis are in focus to understand the mechanism, search of putative markers as well as for therapeutic modulation. Among the antimicrobial peptides found in the body as components of immune system, the bacteriolytic lysozyme gene is noteworthy whose product degrades the peptidoglycan (PGN), an unique and essential component of all type of bacterial cell wall, but not found in eukaryotes (Dziarski and Gupta 2005). In ruminants, mainly two types of lysozymes are reported; stomach specific c- type (Chicken) and g- type (Goose) expressed in cells of myeloid lineage (*mLYS*), specifically in macrophages and granulocytes (Steinhoff *et al.* 1994) and product found in non-digestive tissues and secretions. Ruminants (except buffalo) are generally deficient in lysozyme activity with a prominent exception of abomasums of cattle (Henke *et al*. 1996). Understanding the mechanism of lower activity in terms of DNA sequence variations is inevitable pre-requisite to modulate the activity by genetic manipulation. Buffalo milk lysozyme, a 16kDa basic protein had 10 times more specific activity than its bovine counterpart as one of the contributors of higher resistance of buffaloes to mastitis (White *et al*. 1988, Priyadarsini and Kansal 2002). The higher serum lysozyme activity in Indian buffalo *(bubalus bubalis)* than that of *bos indicus* (Sahoo *et al*. 2010) was also reported. A single gene codes for both milk as well as immune-relevant

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lysozyme (mLYS) in cattle and human beings (Peters *et al.* 1989). But the consistency of this relationship in bubaline species (although the activity is relatively higher) is not known. As the macrophage expressed immuno-relevant lysozyme gene sequence in bubaline species was yet to be reported, an attempt was made to characterize it and to explore its intra and/or inter species phylogenetic relationship.

MATERIALS AND METHODS

Animals, blood collection and RNA extraction: Blood samples (5 ml) of 5 Murrah buffaloes maintained at the institute farm of IVRI were collected in DEPC treated sterile vials. The white blood cells were separated through the density gradient centrifugation using Histopaque. The buffy coat was washed with phosphate buffer saline (PBS) twice. The total RNA was isolated using acid guanidinium thiocyanate- phenol- chloroform extraction method (Sambook and Russel 2001). The quality and concentration of RNA was judged by spectrophotometrics as well as using 2.2 M formaldehyde denatured agarose gel electrophoresis. The separation of mRNA from total RNA was done using Oligotex mRNA spin columns following the standard protocol supplied with the kit and stored at –70°C for further use.

cDNA synthesis and amplification with gene specific primers: The basic gene structure among the vertebrate lysozyme is remarkably constant. As the bubaline lysozyme sequence was not available in public database, the primers were designed targeting the 5' and 3' flanking region of bovine immune-relevant lysozyme mRNA (NM_180999) taking care not to exclude any nucleotide from the reading frame. The primer sequences used for amplification of cDNA were 5'-TCT GGA CAT TTG ACT TCT C -3' (Forward) and 5'-CAT GCT CCT GCT TCT GT- 3' (Reverse). RT-PCR was carried out for cDNA synthesis with specific primers using a kit in one step RT-PCR using the protocol supplied with the kit with a control reaction bearing all the components except the template RNA. The standardized programme used was 50°C for 30 min (reverse transcription), 95°C for 16 min (RT-inactivation), 94°C for 1 min (PCR-denaturation), 47°C for 1 min (PCR-annealing), 72°C for 1 min (PCR-extension), 72°C for 10 min (PCR-final extension). The programme was set for 30 cycles for PCR amplification. The amplified product was checked in 1.2% agarose gel with standard DNA molecular weight marker.

Cloning and sequencing of cDNA: The amplified cDNA was run in 0.8% low EEO agarose gel and the bands of interest were cut by scalpel and extracted from gel by elute

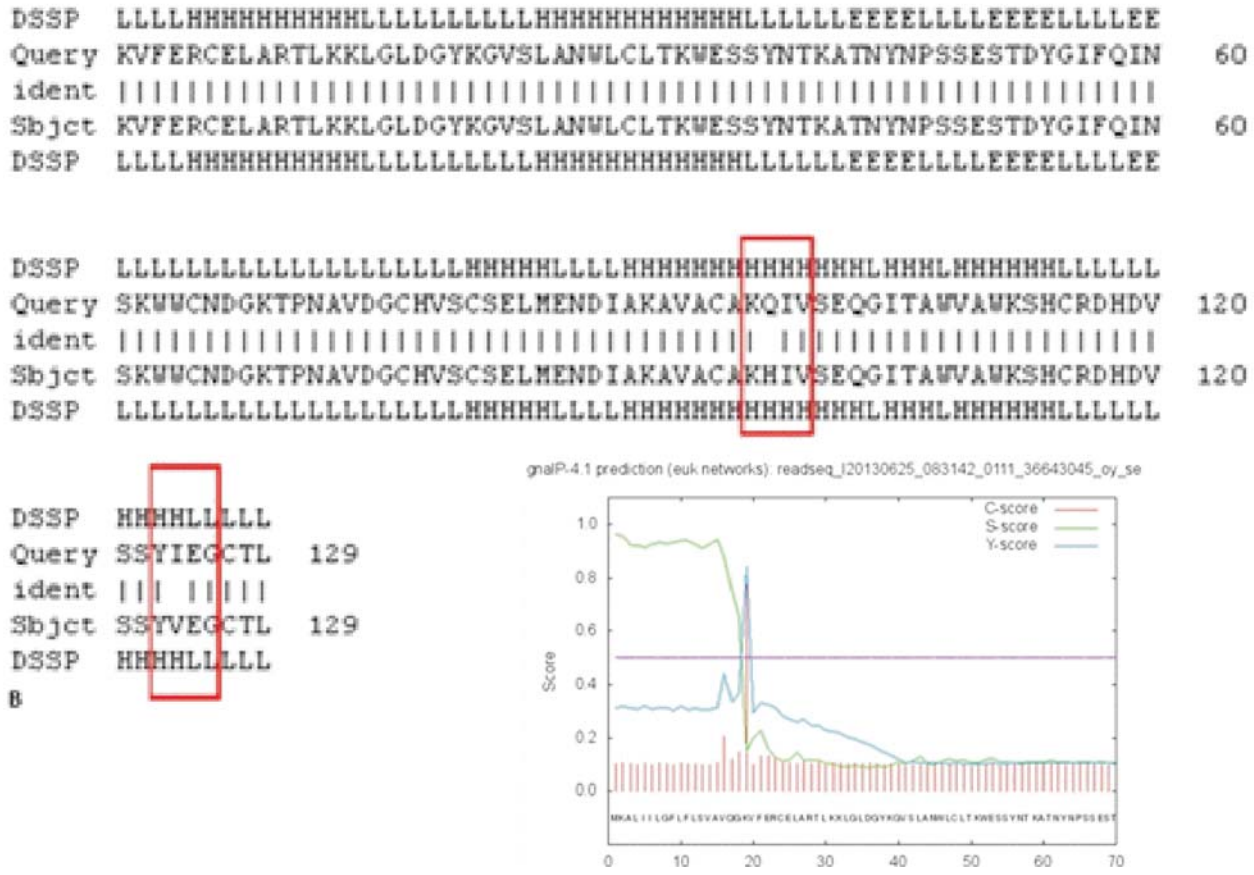


Fig. 1A. Prediction of the signal peptide was using the Signal P 3.0 Server. Out of 147 amino acid precursor molecules, 1st to18th is signal peptide and from 19th to 147th is mature chain. B. Comparison of predicted 3D structures using online server Dali pair wise comparison shows the differences 116th (Q→H) and 142th (I→V) position did not affect 3D structure.

Table 1. In silico sequence analysis of bubaline and bovine macrophage expressed lysozyme

Positions	*BB*	*BT*	*Type of change*	*Amino acid change*	$P_{deleterious}$ *	subPSEC score*	MSA position*	P_{wt} *	$P_{substituted}$*	NIC*	DDG free energy change value †
13th	A	G	Transition	Ile/Val Part of Signal sequence–0.98							
42th	T	C	Transition	Synonymous substitution (42th, 87 th, 132th, 270th, 336th)							
87 th	G	T	Transversion								
132th	G	A	Transition								
270th	C	T	Transition								
336th	C	G	Transversion								
348th	G	T	Transversion	Gln/His	0.101	–0.818	95	0.058	0.061	2.194	–0.05
351th	C	T	Transition	Synonymous substitution (351th, 375th)							
375th	A	C	Transversion								
424th	A	G	Transition	Ile/Val	0.129	–1.092	121	0.268	0.41	2.12	–0.53

BB, *Bubalus bubalis*; BT, *Bos taurus*; Positions, Nucleotide distance from start of ORF; DDG <0, decrease stability; DDG>0, increase stability; * Panther; †i-mutant 2.0.

gel extraction kit. The concentration was checked by electrophoresis, ligated with pGEMT Easy and subsequently transformed in to the *Escherichia coli* DH5α strain. After overnight culture, white colonies were screened for the presence of insert by performing colony PCR and Plasmid PCR with the same set of primers used for amplification. The positive clones with the insert of serum lysozyme gene were selected from the master plate and sequencing was performed by the Sanger's di-deoxy chain termination sequencing method in genetic analyzer.

In-silico analysis and sequence comparison: The nucleotide sequence was confirmed using non-redundant (nr) database at the NCBI with blastn algorithm (www.ncbi.nlm.nih.gov/BLAST) and submitted to Gen bank (EF535848). The ORF was predicted, translated and signal peptide was predicted using the SignalP 3.0 Server (http://www.cbs.dtu.dk/ services/SignalP/). To study the functional motifs, the conserved domain was searched by Conserved Domain Search Service (CD Search) of NCBI. The 3D structures of bubaline as well as bovine immuno-relevant lysozyme were predicted using SWISS MODEL (http://swissmodel.expasy.org/) in automated mode. These models had been compared using online server Dali pair wise comparison (http:// ekhidna. biocenter. helsinki. fi/ dalilite/) for the structural differences. In silico functional analysis of non-synonymous mutations was obtained using PANTHER as well as I-Mutant 2.0 online programme. In addition to the bubaline macrophage expressed lysozyme gene sequenced in the present study, coding sequences of the several mammalian species available in public database were also included for comparison as well as phylogenetic analysis using MEGA 6.0.

RESULTS AND DISCUSSION

Lysozyme c with an important role in host defense has been extensively studied as a model in molecular biology, enzymology, protein chemistry and crystallography (Irwin *et al*. 2011). It is widespread in nature and its protein and gene sequences have been characterized from numerous diverse vertebrate species (Callewaert and Michiels 2010, Sahoo *et al*. 2012). In the ruminants other than buffaloes, the lysozyme activity was reported to be less in other organs with a prominent exception of abomasum of cattle. The bubaline lysozyme activity at the same time was several times more than its bovine counterpart. In bovines the neutrophil granulocytes expressed as well as mammary gland expressed lysozyme genes are proposed to be derived from a different gene other than bovine stomach lysozyme gene (Steinhoff *et al.* 1994).

In-silico sequence analysis of bubaline lysozyme cDNA: Sequencing of the selected clones confirmed the 593 bp insert as lysozyme mRNA by using NCBI BLAST. The analysis revealed an ORF was of 444 bp (from nt 27 to 470) bearing usual start (ATG) and stop (TAA) codon with GC content of 46 %. The translated product revealed that lysozyme gene encoded a precursor polypeptide of 147 amino acids with a molecular weight of 16.32 kD.

Previous reports also revealed molecular weight of

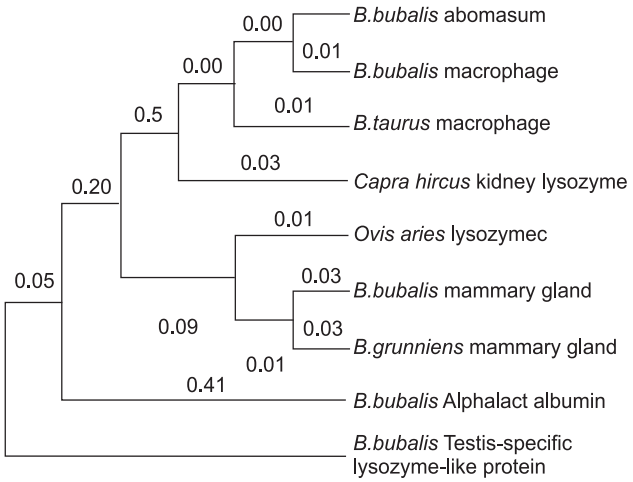


Fig. 2. Phylogenetic tree constructed using the coding sequence similarity.

buffalo milk lysozyme to be about 16 kD (Priyadarshini and Kansal 2002). However, the molecular weight of bovine lysozyme was reported as 18 kD, which is higher than that of buffalo (Henke *et al.* 1996). The number of strongly acidic residues (15 D and E) were equal with number of strongly basic (15 K and 4 R) ones in the matured peptide leading to neutral isoelectric point 7.095. The amino acid sequence when subjected to BLAST revealed 3 types of conserved domains i.e. lysozyme catalytic site, Ca^{++} ion binding site and catalytic cleft. The functionally active lysozyme catalytic sites are responsible for hydrolysis of 1, 4 ß linkage between N-acetyl D-glucosamine and N-acetyl muramic acid in the bacterial peptidoglycan ultimately leading to antibacterial activity. Two catalytic sites were present at residue 34 and 51.

Also there were 3 Ca^{++} ion binding sites at residue 84th, 89th and 90th position of the peptide which were characteristic features of a-lactalbumin and a few other lysozymes. There were presence of 11 catalytic clefts which bind Glc NAC + Mur NAC hexa-saccharide to the active sites at residue position 33rd, 34th, 36th, 43rd, 56th, 58th, 61st, 97th, 100th, 106th and 107th. These conserved domains regulate the activity of the enzyme.

Difference from bovine counterpart: From the alignment with bovine immune-relevant lysozyme gene (NM180999), 10 nucleotides differences were found which (Table 1) led to 3 amino acid differences (non-synonymous) whereas, others were synonymous mutations. Out of 147 amino acids in bubaline immune-relevant lysozyme gene precursor molecule, 1st to18th residue is signal peptide (Fig. 1a) and from 19th to 147th is mature chain. The comparison revealed differences in 5th (I→V), 116th (Q→H) and 142th (I→V) positions out of which one found within signal peptide and other 2 were present in mature chain**.** The amino acids in signal peptides were same in cattle and buffalo except at one place i.e. 5th (I→V). Looking to the nature of the gene and the role it plays in immune system the number of changes in amino acids is justified. While the mature peptide did not show any predicted structural differences the change in signal sequence may explain the change in activity. Also previous reports have shown that manipulation of signal peptide might affect the enzyme activity (Tsuchiya *et al.* 2007) in human beings. The mature chain secondary 3D structure had been predicted and compared which revealed 98 % similarity in amino acid sequence and identical 3D structure with a z score of 29.5.

Although there were changes in amino acids in 2 places, no change in the predicted 3D structure was observed (Fig. 1b). The effects of these changes were estimated using Panther as well as I-Mutant 2.0 server (Table 1). No structural difference with respect to the original two amino acids (H116Q and V142I) was observed which suggested that the structure might not have contributed to the differential functional activity of bovine and bubaline lysozymes.

Higher similarity in the coding sequences as well as predicted structure suggested that this higher specific activity type bubaline lysozyme gene can be used in native or recombinant form would elicit least immunological cross reactivity in bovine udder in place of human recombinant lysozyme.

Lysozymes comparison from other body parts, ruminants and homologous proteins: The alignment of nucleotide sequences (Table 2) revealed that the bubaline nucleotide composition was more than 97, 95, 95 and 82% similar to its bovine, ovine, caprine and yak counterparts, respectively

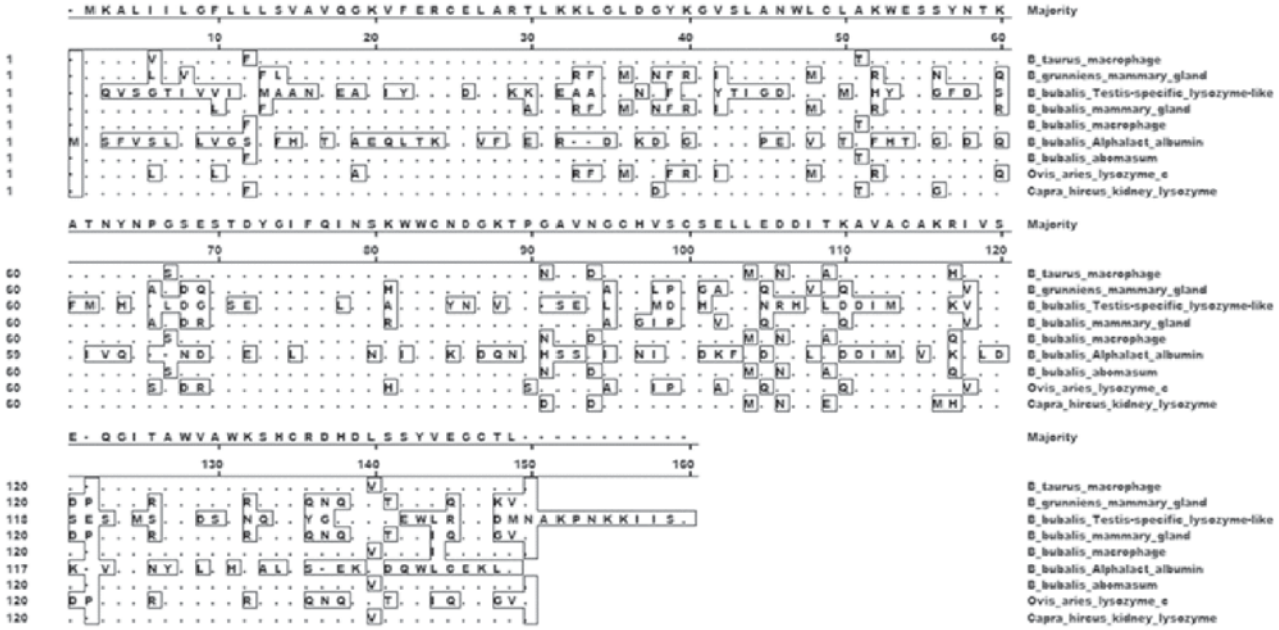


Fig. 3. Multiple alignment of derived amino acid sequences of bubaline mLYS with that of lysozymes expressed in other body parts, of other ruminants as well as homologus proteins.

with higher similarity of cattle and buffalo mLYS sequences over that of sheep and goat sequences. Similarly, phylogenetic analysis (Fig.2) of cDNA sequences revealed that mLYS and abomasum lysozyme formed one cluster whereas, mammary gland expressed one formed another cluster. An insertion of 3 bases (TCC) was prominent, in case of mammary gland expressed lysozyme and was not present in other two types which can be used as a diagnostic marker. Similarly an insertion of Pro^{121} was the characteristic difference found in mammary gland expressed one. The per cent similarity and divergence among the lysozymes expressed in other tissues (Table 3) revealed that the bubaline gene was more closely related to their abomasum counterpart (97.3%, 98.6%) than the mammary gland expressed one (82.7%, 68.9%).

On comparison with homologous proteins this revealed higher similarity of lysozyme like protein from testis than the α-lact albumin. The per cent similarity and divergence among them shows the similar picture (Table 4). It is reported that the same gene codes for both milk as well as immuno-relevant lysozyme (*mlys*) in cattle (Steinhoff *et al.* 1994). However, we observed the bubaline mLYS was found to be phylogenetically closer to abomasum expressed lysozyme than its mammary gland counterpart which can provide further insight regarding the lysozyme evolution. Genetic changes in lysozyme gene family might be associated with the evolution of the ruminant lifestyle. Irwin (2004) reported more than 4 different lysozymes in cows in non-stomach tissues with a probable function as antibacterial defense enzymes, in addition to genes expressed in the stomach. Recombination, through concerted evolution, might have allowed some lysozymes to acquire the ability to survive in occasional acidic environments. The comparison with homologous proteins reveals that the mLYS was phylogenetically closer to testis specific lysozyme like protein than that of alpha-lacto albumin (Table 4).

Alpha-lactalbumin, a modifier protein for galactosyl transferase is believed to share a common ancestor with lysozyme. They share about 40% identical amino acids in their sequence with conserved disulphide bridges and a similar gene organization along with common secondary and tertiary structures. However, in contrast to the widespread occurrence of lysozyme in different body fluids, α-lactalbumins are limited to mammalian milk and colostrums which also do not have catalytic activity. This indicates that other than duplication of lysozyme genes which express in different body parts, there are several proteins structurally similar to lysozyme whose functions are important for different body physiology or immuno-protective mechanism. Furthermore, looking to the catalytic nature of lysozyme as well as its effective antimicrobial properties, it can be speculated that this enzyme would find an important place even in therapeutic use either alone or in combination.

In conclusion, the macrophage expressed bubaline

Table 2. Comparison of bubaline cDNA and derived amino acid with other ruminants

	Per cent divergence										
	Per cent identity (nucleotide)					Per cent identity (amino acid)					
	BB	BT	OA	CH	BG	BB	BT	OA	CH	BG	
BB	***	97.8	95.7	95.1	82.7	***	97.3	93.9	*93.9*	*66.2*	*BB*
BT	2.3	***	96.0	95.5	83.4	2.1	***	94.6	*94.6*	*66.9*	*BT*
OA	4.4	4.2	***	95.5	81.3	5.6	4.9	***	92.6	66.2	*OA*
CH	5.1	4.7	4.6	***	83.1	5.6	4.9	7.1	***	66.9	*CH*
BG	*19.9*	19.0	21.7	19.3	***	43.5	42.4	43.5	42.4	***	*BG*

BB, *Bubalus bubalis*; BT, *Bos taurus*; OA,*Ovis aries*; CH,*Capra hircus*; BG, *Bos grunniens*.The above diagonal values are per cent identity where as below diagonal values are per cent divergence.

Table 3. Comparison of cDNA and derived amino acid of mLYS with their counterparts expressed in other body parts

Per cent divergence							
Percent identity (nucleotide)				Percent identity (amino acid)			
	MP	*AB*	*MG*	*MP*	*AB*	*MG*	
MP	***	97.3	82.7	***	98.6	*68.9*	*MP*
AB	2.8	***	82.2	0.7	***	*68.2*	*AB*
MG	20.0	21.0	***	38.9	40.1	***	*MG*

MP, macrophage; AB, abomasums; MG, mammary gland. The above diagonal values are per cent identity where as below diagonal values are per cent divergence.

Table 4. Comparison of bubaline mLYS with other homologues proteins

	Per cent identity (nucleotide)			
	MPL	*LLP*	*α-LA*	
Percent divergence				
MPL	***	37.4	34.7	*MPL*
LLP	75.2	***	33.6	*LLP*
α-LA	68.0	78.8	***	*α-LA*

MPL, macrophage expressed lysozyme; LLP, lysozyme like protein; a-LA, a-lactalbumin. The above diagonal values are per cent identity where as below diagonal values are per cent divergence.

lysozyme cDNA was characterized which revealed 10 nucleotide changes with respect to its bovine counterpart of which 3 were non-synonymous and did not affect the predicted 3D structure. The mLYS was found to be phylogenetically closer to the abomasum type than mammary gland type. Considering the striking similarity found in the cDNA sequence and higher activity, the bubaline immune-relevant lysozyme may be considered to be hyper expressed either in recombinant or natural form in the bovine udder may result better udder health.

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