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Genetic polymorphism in bubaline mLYS (Exon-IV) and its effect on serum lysozyme activity and somatic cell count

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Mastitis, one of the most important diseases of dairy industry, severely affects quality, safety and quantity of milk. The phagocytic activity is considered as the major active defense against this disease (Reiter and Bramley 1975, Paape *et al.* 1979). In phagocytosis, lysozyme contributes to innate immunity by cleaving the ß- 1, 4 linkage of bacterial peptidoglycan and serum lysozyme activity reflects the homeostatic expression of the reticulo-endothelial system (Lie 1980). India possesses a vast reservoir of buffalo genetic resources, which besides efficient conversion of course fodder to valued milk, confer high innate resistance to wide range of tropical diseases (White *et al.* 1988). Buffalo milk lysozyme, a 16kDa basic protein had 10 times more specific activity than bovine milk lysozyme, which might be one of the causes of higher resistance of buffaloes to mastitis (White *et al.* 1988, Priyadarsini and Kansal 2002). Sahoo *et al*. (2010) reported higher serum lysozyme activity in Indian buffalo. While the infection protective function of milk/serum proteins was established beyond doubt (Callewaert and Michiels 2010), role of lysozyme gene had been suggested as a candidate gene for improvement of mastitis resistance (Sayfert *et al.* 1996). However, for practical utility it would require demonstration of a variation either in quality or concentration correlated with genetic polymorphisms for trait improvement. The present investigation was undertaken in Indian buffalo breeds to identify SSCP (Single strand conformation polymorphism) patterns, characterize them by sequencing and to study the effect of different genotypes as well as nongenetic factors on serum lysozyme activity and somatic cell count (SCC).

Random blood samples from 280 buffaloes comprising 4 breeds, viz. Murrah (135), Bhadawari (44), Mehsana (50) and Surti (51) maintained at 6 different livestock farms (IVRI, Izatnagar ; CCSHAU**,** CIRB, Hisar; Govt. Livestock Farm, Etawah; AAU, Anand and Navsari Agricultural University**,** Navsari) across India, were collected for polymorphism study. Peripheral blood (5 ml) was collected from the animals with and without 2.7% EDTA for DNA isolation and serum separation. Genomic DNA was isolated by the phenol-chloroform-isoamyl alcohol method (Sambrook and Russell 2001). A 230 bp fragment spanning from intron-III to exon-IV was amplified with primers (F: 5'- AAT ACT TGG ATC TGT CTG T -3' and R: 5'- AAA ATG GGT TGA AGT AAA -3') designed on the basis of bovine lysozyme gene (BTU25810). The PCR conditions involved were denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 42°C for 45 sec and extension at 72°C for 1 min and one final 10 min elongation cycle at 72°C. The buffaloes were screened for polymorphism using SSCP technique (Orita *et al.* 1989). For visualization of bands highly sensitive silver staining of gels was carried out (Bassam *et al.* 1991). The gene and genotype frequencies were estimated and representative alleles were extracted, cloned with pGEMT Easy vector and transformed into the *Escherichia coli* DH5α strain. Sequencing was performed by the Sanger's di-deoxy chain termination sequencing method and the obtained sequences were submitted to Gen bank (GQ 995483; GQ 995484 and GQ 995485) as allele A, B and C. The serum lysozyme activity was estimated using 'lysoplate' assay method (Lie 1980) while SCC of the collected milk samples was done as per Schalm *et al.* (1971). The effects of various SSCP variants, season as well as sex on the lysozyme activity and SCC were studied with SAS version 9.3. The least square means were compared among various factors affecting the concentration of serum lysozyme as well as somatic cell count using PROC GLM procedures using the model $Y_{ijkl} = \mu + X_i + Y_j + Z_k + e_{ijkl}$

Where, Y_{ijk}, k^{th} observation of i^{th} genotype recorded in j^{th} season with k^{th} sex; e_{ijkl}, random error.

SSCP analysis revealed 4 genotypes (Table 1) viz. AA, AB, AC, and BC and 3 alleles (A, B and C). The AA genotype was most frequent in Surti (0.61) and Bhadawari

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Table 1. Gene and genotype frequency of lysozyme polymorphism in riverine buffalo

Genotypes	Genotype frequency				
	Murrah	Mehsana	Surti	Bhadawari	Pooled
AA	0.02	0.14	0.61	0.53	0.32
AB	0.52	0.48	0.39	0.36	0.44
BC	0.16	0.14	—	—	0.08
AC	0.30	0.24	—	0.11	0.16
Alleles	Allelic frequency				
A	0.43	0.50	0.80	0.71	0.61
B	0.34	0.31	0.20	0.18	0.26
C	0.23	0.19	—	0.11	0.13

Table 2. Factors affecting lysozyme activity and somatic cell count in Murrah buffalo

Factors	*LA*	Factors	SCC
Overall mean	27.35±2.42 (120)	Overall mean	1.25±0.13 (80)
Genotypes			
AB	28.02±6.07 (62)	AB	1.30±0.22 (45)
BC	27.57±8.48 (19)	BC	1.30±0.42 (12)
AC	25.08±3.84 (36)	AC	1.10±0.18 (21)
AA	27.85±12.7 (03)	AA	1.51±1.25 (02)
Seasons			
Monsoon	26.08±1.51 (32)	Monsoon	1.09±0.85 (19)
Summer	28.14±1.33 (49)	Summer	1.30±0.70 (36)
Winter	27.76±1.37 (39)	Winter	1.29±1.0 (25)
Sex			
Female	27.46±1.10 (80)		
Male	27.21±1.51 (40)		

LA, Lysozyme activity in g/ml of serum; SCC, somatic cell count in 10^5 number/ml of milk; none of the values differed significantly (P<0.05) along the columns.

(0.53) breeds whereas, AB genotype was frequent in Murrah (0.52) and Mehsana (0.48) breeds of buffalo, respectively. AB genotype had highest genotypic frequency (0.44), followed by AA (0.32), AC (0.16) and BC (0.08) in the pooled data. The A allele was most prevalent (0.61) in these populations. The genotypes BC and AC were absent in Surti buffaloes. The results indicated that natural selection may be favouring the AA and AB genotypes in general and A allele in particular. The serum lysozyme activity was highest in AB genotype (28.02±6.07) with an overall mean 27.35±2.42 µg/ml (Table 2).

This level is an index of macrophage function and reflects the status of RE (reticulo endothelial) system in the body. These values are quite higher in comparison to cattle i.e. 3.16µg/ml and 2.26 µg/ml in Rathi and Tharparkar breeds (Sharma 2002). The SCC in buffalo milk of IVRI farm was highest for AA genotype (1.51×10^5) with an

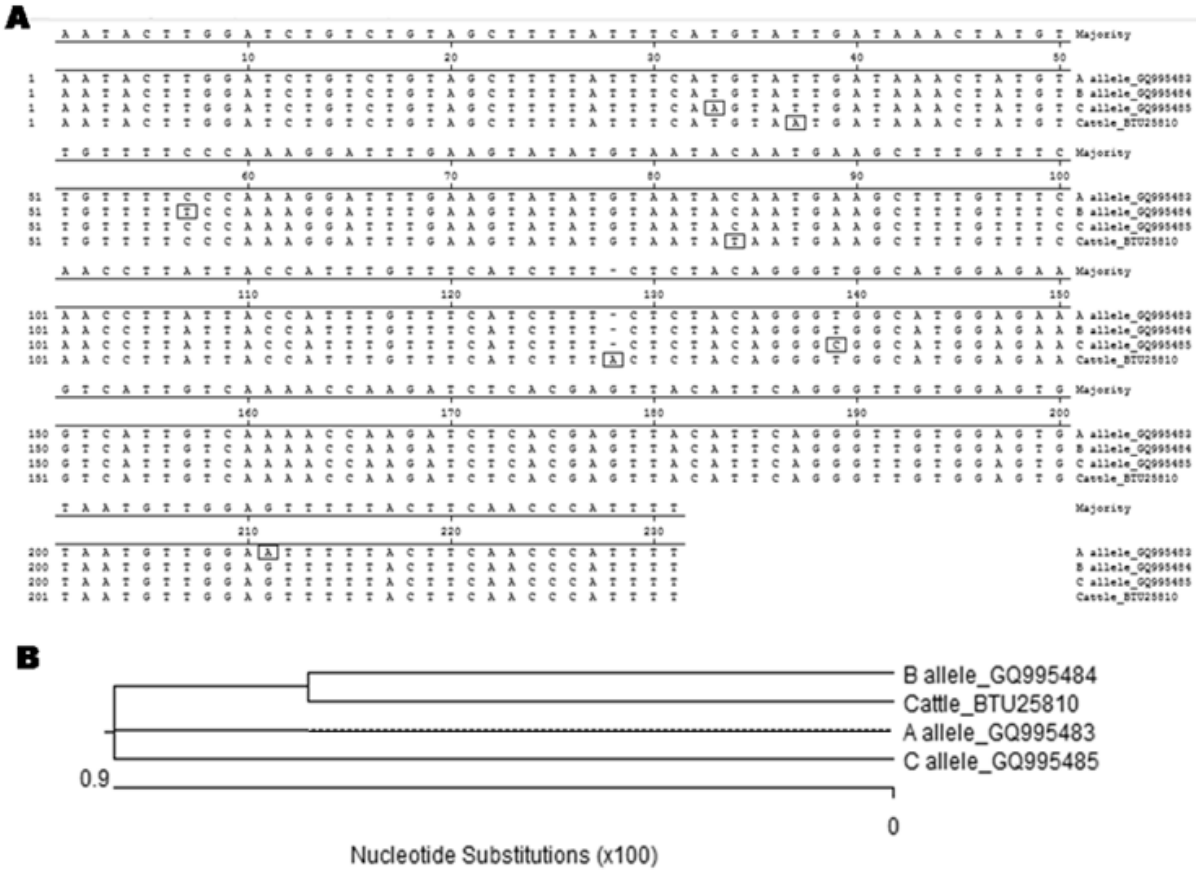

Fig.1.A. Nucleotide sequence comparison among alleles and with bovine counterpart, B. Phylogenetic tree based on the sequence analysis of alleles and cattle.

overall mean of 1.25× 10^5 cells/ml of milk (Table 2). Although the mean values of lysozyme activity and SCC were apparently different across various genotypes seasons and sex, differences were statistically nonsignificant (P=0.92 and P=0.83, respectively). This may be due to the fact that the gene fragment contributes to a smaller part of total variation present. Alternatively the study of bubaline lysozyme promoter or other regulatory region can give better insight to explain the differences. Chen *et al*. (2013) reported SNP Lys c.118T>G correlated significantly with SCS (Somatic cell score) and 305 day milk yield in Chinese Holstein. Salehin *et al*. (2009) also reported polymorphic lysozyme gene associated with total milk production and highest yield during first lactation in Indian crossbred cattle.

Four nucleotide changes were observed among 3 (i.e. A, B and C) alleles/haplotypes (Fig.1A) on sequence comparison. A haplotype differed from B and C by 2 and 3 nucleotide substitutions, out of which one was present in intronic region in both the cases. B haplotype differs from C by 3 bases out of which 2 were present in the intronic region. A differs from others with a transition (A>G) at 211th position whereas, B and C differ from others at 57th and 139th positions, respectively. The corresponding sequence of cattle revealed an insertion (A) at 128th position which can be utilized as a species specific marker. Among all the alleles, A and B are closer with a homology of 99.1%. The phylogenetic tree (Fig. 1B) revealed that A and C formed a cluster and were closer to each other than the cattle and B allele. It might be due to the fact that the gene fragment under study mostly covers the exonic part. In conclusion, the lysozyme gene was sufficiently polymorphic in Indian buffalo and although some temporal differences were found in lysozyme activity and SCC across genotypes in Murrah buffalo, no statistically significant association was observed.

SUMMARY

Animals (280) consisting of 4 different buffalo breeds (Murrah, Mehsana, Surti and Bhadawari) spread over 6 different farms across the country were used for this study. A 230 bp fragment spanning from intron-III to exon-IV was screened for SSCP which revealed frequent occurrence of AB genotype and A allele. Although, different values of lysozyme activity and somatic cell count were observed in Murrah buffalo across the genotypes and seasons, no statistically significant association was observed. Nucleotide analysis revealed A allele differed from B and C allele by 2 and 3 nucleotide substitutions, respectively, out of which one was present in intronic region in both cases. The polymorphism identification and characterization may provide a baseline tool for future studies to further delineate the role of this gene as a putative candidate gene for selection against mastitis.

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