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PCR-SSCP of Serum Lysozyme Gene (Exon-III) in Riverine Buffalo and Its Association with Lysozyme Activity and Somatic Cell Count

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ABSTRACT : Serum lysozyme gene is one of the important genes influencing the immune system as its product can cause lysis of bacterial cell wall by cleaving the peptidoglycan layer. The present investigation on the serum lysozyme gene of Indian riverine buffalo was undertaken with the objectives to identify and characterize single nucleotide polymorphic patterns by PCR-SSCP method as well as to study the effect of different genotypes on serum lysozyme activity and somatic cell count. A total of 280 animals comprising four different famous bubaline breeds (Murrah, Mehsana, Surti and Bhadawari), spread over six different farms across the country were used for this study. A 276 bp (partial intron 2, complete exon 3 and partial intron 3) fragment of lysozyme gene was screened for polymorphism using the SSCP technique. Four genotypes namely AA, AB, BC and AC were observed, out of which BC genotype was found to be the most frequent. Among these three alleles, C allele (0.38) was most prevalent in these populations. Various SSCP allelic variants were cloned for sequencing and sequences were submitted to NCBI Genbank. From the alignment of the nucleotide sequences of various allelic variants, it was found that there were differences in 12 positions among the alleles, out of which maximum variation (at 8 places) was found in the intronic region. The allele A was closer to allele-C than allele-B. Allele B was phylogenetically equidistant from both of the other alleles. Mean lysozyme activity determined in serum samples of different animals of Murrah buffalo was 27.35±2.42 μg per ml of serum, whereas the mean somatic cell count was $1.25\pm0.13\times10^5$ cells per ml of milk. The SSCP pattern-wise effects of various genotypes on lysozyme activity and SCC were analyzed. Although the mean values were apparently different in various genotypes, these differences were statistically non-significant. It can be concluded that the riverine buffaloes are sufficiently polymorphic with respect to serum lysozyme gene. The absence of AA genotype in Bhadawari breed of buffalo can be considered as a marker for breed characterization. The difference of four nucleotides in exon-3 indicates high selection pressure on the gene. (**Key Words :** Polymorphism, Lysozyme Gene, SSCP, Buffalo, Association, SCC, Sequencing)

INTRODUCTION

India possesses a vast diversified reservoir of buffalo genetic resources in terms of the best dairy breeds. These animals are not only capable of utilizing course fodder as milk machines but also able to confer higher innate resistance to wide range of tropical diseases e.g. mastitis (White et al., 1988; Priyadarsini and Kansal, 2002). The predominant mastitis causing organism are Gram +ve cocci, in general and *Staph. aureus* in particular. The role of antibody complement system in providing immunity in case of udder infections is in-conspicuous as its action is restricted mainly to Gram -ve bacteria. The phagocytic activity of polymorphonuclear (PMN) leucocytes and to some extent macrophages is considered as the major active defense against udder infections (Schalm et al., 1966; Reiter and Bramley, 1975; Paape et al., 1979). Experimental findings have revealed the lysozyme level as an index of macrophage functional status (Di Luzio, 1979). The serum lysozyme activity reflects the homeostatic expression of the

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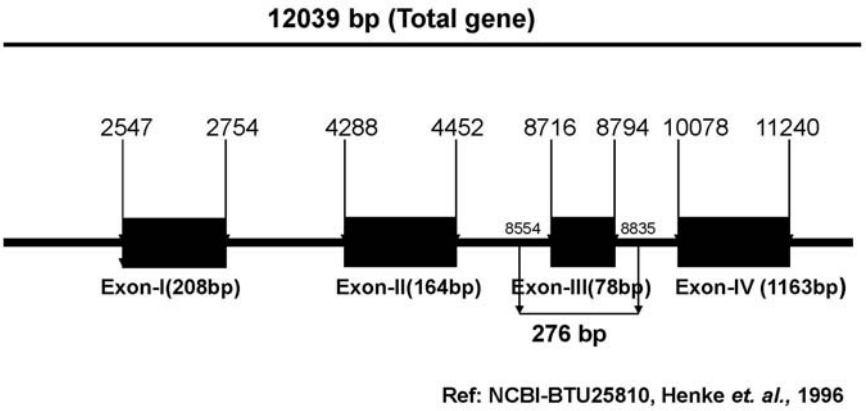

Figure 1. Amplified fragment of serum lysozyme gene.

reticulo-endothelial system (RES), which is one of the most fundamental defense mechanism against infection (Lie, 1980). Buffalo milk lysozyme, a 16 kDa basic protein found in buffalo milk has 10 times more specific activity than its bovine counterpart. This may be one of the causes of higher resistance of buffaloes to mastitis (White et al., 1988; Priyadarsini and Kansal, 2002). While the infection protective function of milk/serum proteins has been established beyond doubt (Jolles and Jolles, 1984), the role of lysozyme gene has been suggested as a candidate gene for improvement of mastitis resistance (Sayfert et al., 1996). This would require demonstration of a variation either in quality or quantity (concentration), which may be correlated with trait for improvement and genetic polymorphisms responsible for that variation can be exploited.

Hence considering the above potential of this gene, the present investigation was undertaken on serum lysozyme gene of Indian riverine buffalo to identify single nucleotide polymorphic patterns by Single Strand Conformation Polymorphism (SSCP) method, characterize them by sequencing and subsequently study the effect of different genotypes on serum lysozyme activity and somatic cell count.

MATERIALS AND METHODS

Animals

A total of 280 riverine buffaloes (*Bubalus bubalis*), comprising of four well known milch breeds viz Murrah (135), Bhadawari (44), Mehsana (50) and Surti (51) maintained at six different livestock farms were used in this study. The Murrah samples were collected from the farms of Indian Veterinary Research Institute, Izatnagar, Bareilly, Utter Pradesh; C. C. S. H. A. U. and Central Institute for Research in Buffaloes, Hisar, Haryana. Bhadawari samples were collected from Govt. Livestock Farm, Etawah, Utter Pradesh whereas, Mehsana and Surti samples were collected from Anand Agricultural University and Navsari Agricultural University in the state of Gujrat, respectively in the present investigation for search of SNPs. Samples were collected randomly from these farms. For the association studies 80 lactating and 120 animals of Murrah buffalo were used for milk somatic cell count and serum lysozyme activity, respectively.

Collection of blood samples

Approximately 5 ml of blood was collected from the jugular vein of each animal in a sterile polypropylene vial containing 2.7% EDTA (0.25 ml/5 ml of blood). After thorough mixing, the vials were kept in icebox containing ice and gel cool packs and were transported to the laboratory and subsequently kept in deep freeze at -20°C till the isolation of DNA. Equal amount of blood was collected from Murrah buffaloes without adding anticoagulant for the separation of serum which was to be used in the evaluation of serum lysozyme activity.

Isolation of genomic DNA and serum

Genomic DNA was isolated from the leucocytes of blood samples by the method as described by Sambrook and Russell (2001) using Proteinase-K and Phenol. DNA was isolated from fresh, refrigerated (4°C for 1-2 days) and frozen (-20°C) blood samples. Although, all the samples yielded sufficient amount of DNA, it was found that yield from fresh samples was appreciably higher than the frozen samples. The isolated DNA samples were stored at -20°C for further analysis. The serum was extracted after clotting the blood and then stored at -70°C till further study.

PCR-Amplification of lysozyme gene

The immuno-relevant lysozyme genes (*mlys*) of most ruminants bear almost similar structure and have evolutionary relationships among them. Here the bovine *mlys* gene sequence (Figure 1) had been used as reference

Table 1. Primer sequences used to amplify lysozyme gene

Primers	Sequence	Length	Fragment size
P_1	5'- CAA, ATG, GGA, TGG, AAT, GAA -3'	18 bp	276 bp
P_2	5'- CAA, TAA, AAC, TGA, AAG, GAA, AAA -3'	21 bp	

P_1 and P_2 are forward and reverse primers, respectively.

sequence (BTU25810) as buffalo lysozyme gene structure was not reported. A fragment of length 276 bp spanning over a part of intron II (Figure 1) to intron III was amplified using the forward (P_1) and reverse (P_2) primers (Table 1) designed with the help of Lasergene Software (DNASTAR), by using standard PCR protocols. The 25 µl PCR reaction mixture was prepared using dNTPs of 100 µm (each), Primers of 20 pm (each), $MgCl_2$ 1.5 mM and 1 µl of Taq DNA polymerase of 1 unit/µl concentration (MBI Fermentas). Finally 80-100 ng of good quality genomic DNA was added and set for PCR amplification. The PCR programme used was 95°C for 5 min (initial denaturation), 95°C for 1 min (denaturation), 43°C for 45 sec (annealing), 72°C for 1 min (extension), 72°C for 10 min (final extension). Denaturation, annealing and extension steps were repeated for 30 cycles. The PCR was performed in an Eppendroff thermal cycler which resulted in quality amplified product.

PCR- SSCP analysis of gene fragment

Single nucleotide polymorphisms had been screened in the fragment under study using SSCP technique (Orita et al., 1989). The PCR products were resolved on 12% polyacrylamide gel. For optimization of SSCP assay, different concentrations of Acrylamide: Bis-crylamide i.e 100:1, 50:1, and 29:1 as well as presence or absence of glycerol in gel solution was tested to get sharp and dark bands with optimum resolution. Finally 40 ml of PAGE solution was prepared by adding 9.6 ml of Acrylamide:Bis-crylamide (50:1), 2 ml of Glycerol, 28.3 ml of 1X TBE, 100 µl of 10% Ammonium persulfate, and 20.0 µl of TEMED.

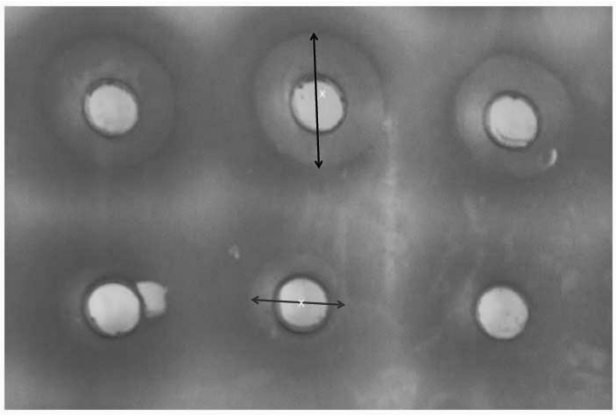

Figure 2. Serum lysozyme activity of Murrah buffalo.

After thorough mixing, the freshly prepared PAGE gel mix was poured into the space between plates and spacer. Then the comb was inserted immediately with care so as to leave no air bubble inside the gel. Then the gel was allowed to polymerize at room temperature for 1 h and was given a pre-run at 200 V for 30 min, in a vertical gel electrophoresis system. About 3 µl of PCR product was taken in a 0.2 ml PCR tube and 17 µl denaturing formamide dye (formamide, 95%; Xylene cyanol, 0.025%; Bromophenol blue, 0.025%; 0.5 M EDTA, 4%) was added and mixed properly. PCR product and formamide dye mix were denatured at 95°C for 5 min (by keeping on 95°C hot water) and snap chilled on ice for 15 minutes. The product was loaded in gel carefully. The electrophoresis was performed at 4°C temperature at 14 mA constant current for 12 h. After running, the gels were silver stained.

Silver staining

For visualization of bands, silver staining of gels was carried out as per the method described by Basam et al. (1991) with appropriate modifications made to suit this fragment in our laboratory condition. The gel was agitated in 200 ml of 10% glacial acetic acid slowly for 45 minutes or until the tracking dye was no longer visible. After rinsing the gel for 3 times in double distilled water, 200 ml of 0.2% silver nitrate solution was added to the tray and agitated slowly for 30 minutes followed by brief rinsing in distilled water for 30 seconds. Subsequently, the gel was developed in 200 ml of 3% sodium carbonate with 450 µl of formaldehyde solution (37%). The reaction was stopped by adding 200 ml of 10% glacial acetic acid. The gel was visualized and documented in gel documentation system.

Gene and genotype frequency

The genotypes were detected from the SSCP patterns of each sample in the gels. The frequency of serum lysozyme gene and genotypes were estimated by standard procedure (Falconer and Mackey, 1996).

$$\text{Genotype frequency} = \frac{\text{Total No. of individuals of a particular genotype}}{\text{Total No. of individuals of all genotypes}}$$

and

$$\text{Gene frequency} = \frac{D + 1/2\ H}{N}$$

Where,
D = No. of homozygotes
H = No. of heterozygotes
N = Total no. of individual

Cloning and characterization of allelic patterns

The electrophoretic bands of various allelic patterns were eluted from the gel by Gel Extraction kit (Qiagen, Germany), following the kit protocol. The concentration was checked by running 2 μl eluted product in 1.2% agarose gel along with standard DNA molecular weight marker. Cloning was carried out in pGEMT Easy vector system (Qiagen, Germany) as per the protocol provided along with the kit. The ligation reaction was set at 4°C over night in 0.2 ml PCR tube, adding 2X rapid ligation buffer (having 1 unit T_4 DNA Ligase), pGEMT Easy vector (50 ng/ml) and purified PCR product. The competent cells were prepared using overnight grown culture of *E. coli* DH5α. For transformation, 10 μl ligation mixture was diluted to 200 μl TCM (1X), to which, 200 μl of the competent cells were added with gentle mixing and subsequently kept on ice (0°C) for one hour. Heat shock was given to this mixture at 45°C for two min and then it was rapidly chilled on ice for 10 min. To this Ependroff tube, 600 μl of SOC medium was added and incubated at 37°C for one hour in the incubator shaker. The transformed cells were spread on LB agar plate containing Ampicillin (100 μg/ ml) and Kanamycin (25 μg/ ml). Appropriate positive and negative controls were processed simultaneously. Plates were incubated over night at 37°C and later stored at 4°C. Recombinant clones were identified by the screening of blue/white colonies. Positive clones were also confirmed by colony PCR and Plasmid PCR using the same pair of primers. DNA sequencing was performed from all the genotypes by the Sanger's dideoxy chain termination sequencing method in Automatic ABI Prism DNA sequencer. These sequences were compared with available sequences of NCBI Gen Bank using Lasergene Software (DNASTAR) and submitted to Gen bank (Acc No GQ497214, GQ497215 and GQ497216).

Estimation of serum lysozyme level

Serum lysozyme level was determined using 'lysoplate' assay method (Lie, 1980) with appropriate modifications. Lypolised *Micrococcus lysodeikticus* was used as substrate in this assay. The standard curve was prepared with solutions of known lysozyme concentrations (Sigma, USA) to determine the lysozyme level in serum of individual sample. The concentrations (after $\log_2$ transformation) of the known standards were regressed on the diameter of the lysed zones around them. The slope of the curve and intercept were determined. The lysozyme concentration of the unknown sera samples were determined by following regression equation.

$$Y = bx+c$$

Where,
Y = Concentration of the unknown sample
b = Slope of regression equation
c = Intercept of regression equation
x = Diameter of lysed zone around unknown sample

Somatic cell count

Somatic cell count (SCC) of the collected milk samples was estimated by the method of Schalm et al. (1971) using a stage micro meter. In clean grease free slide, an area of 1 cm^2 square was marked. The milk samples were mixed thoroughly by shaking and then 10 μl (0.01 ml) sample was added on this 1 cm^2 field on the glass slide. It was uniformly smeared with a fine glass rod and allowed to air dry in room temperature. One drop of Newman's Lambert stain was applied and kept for 5-10 minutes for staining. The slide was washed in running tap water for few seconds before air-drying and observed under oil immersion lens (100×) in compound microscope. Counting of somatic cells was done on at least 30 fields from each slide using stage micro meter. The total number of cells in the milk were estimated by multiplying total number of cells recorded to the working factor of microscope and was expressed per ml of milk sample.

Statistical analysis

The difference of the lysozyme activity and somatic cell count among various SSCP genotypes were analyzed using standard statistical tools (Snedecor and Cochran, 1994). The pattern wise effects of various genotypes on lysozyme activity and somatic cell count were analyzed using SPSS 10.0 package.

RESULTS AND DISCUSSION

DNA polymorphism of serum lysozyme gene

SSCP analysis of 276 bp fragment of serum lysozyme gene revealed various patterns depending on their single strand conformation. Each animal had shown a specific conformation pattern (genotype). The SSCP analysis revealed a total of 4 patterns viz AA, AB, AC, BC in this fragment with three alleles i.e A, B and C. The observed

Table 2. Gene and genotype frequency of various breeds of buffalo

Genotypes	Genotype frequency				
	Murrah	Mehsana	Surti	Bhadawari	Pooled
AA	0.03	0.04	0.27	-	0.09
AB	0.22	0.14	0.25	0.12	0.18
BC	0.35	0.30	0.22	0.68	0.39
AC	0.40	0.52	0.26	0.20	0.34
Alleles	Allelic frequency				
A	0.34	0.37	0.53	0.11	0.34
B	0.28	0.22	0.23	0.40	0.28
C	0.38	0.41	0.24	0.49	0.38

gene and genotype frequencies are presented in Table 2. Among these genotypes, the absence of AA genotype in Bhadawari buffalo can be considered as a marker for breed identification and characterization. The frequencies of AC genotype were highest in Murrah (0.40) and Mehsana (0.52), where as AA and BC genotypes were predominant in Surti (0.27) and Bhadawari (0.68) breeds of buffalo (Table 2), respectively. There were three alleles found in these populations, out of which C allele was predominant (Gene frequency 0.38). This indicates the natural selection may be favouring the BC and AC genotypes in general and C allele in particular. These findings could not be compared as no SSCP reports are available in the literature for this particular fragment.

Serum lysozyme activity and somatic cell count in Murrah buffalo

Serum lysozyme is widely known for its immuno protective action. The genotype wise mean serum lysozyme activity (μg/ml) is presented in Table 3. The study revealed that AA and AC genotypes had highest (32.97±9.16) and lowest (25.36±4.03) lysozyme activity with a mean of all genotypes as 27.35±2.42 μg/ml of serum. This level is an index of macrophage function and reflects the status of RE (Reticulo endothelial) system in the body. These values are higher in comparison to cattle i.e. 3.16 μg/ml and 2.26 μg/ml in Rathi and Tharparkar breeds (Sharma, 2002).

The genotype wise mean somatic cell count in Murrah buffalo milk is presented in the Table 3. The study revealed that animals of AC genotype had highest i.e. (1.16±0.15)× 10^5 and those of AA genotype had lowest i.e. (0.92±0.72)× 10^5 somatic cell count with overall mean of 1.25±0.13×10^5 cells per ml of milk (Table 3). Although the pattern wise mean values are apparently different in various genotypes in both the cases (lysozyme activity and somatic cell count), they were found to be statistically non-significant.

Nucleotide sequence analysis

Various alleles of 276 bp fragment of serum lysozyme gene were sequenced by Sanger's dideoxy chain termination sequencing method in Automatic ABI Prism DNA sequencer. The sequences obtained were subjected to NCBI BLAST. After comparing with other available sequences of cattle (as no information available in buffalo), the amplified fragment was confirmed to comprise of partial intron 2 spanning over exon 3 upto partial intron 3. These sequences were submitted to NCBI Gen bank data with Acc No GQ497214, GQ497215 and GQ497216 for A, B and C alleles, respectively. All the three sequences as well as the corresponding sequence of cattle were aligned using MEGALIGN program of DNASTAR software (Figure 3). The allele wise nucleotide differences for 276 bp fragment of serum lysozyme gene are presented in Table 4. From the alignment of the allelic variants, it was found that there are differences in 12 positions among the alleles, out of which maximum variations (at 8 places) were found in intronic region in contrast to 4 variations in exonic region. A allele differed from B

Table 3. Lysozyme activity and somatic cell count of different genotypic groups in Murrah buffalo

Mean lysozyme activity		Mean somatic cell count	
Genotypes	Concentration in μg/ml of serum	Genotypes	Somatic cell count in 10^5 number/ml of milk
AB (23)	27.43±5.52^{NS}	AB (14)	1.42±0.16^{NS}
BC (44)	28.61±3.85^{NS}	BC (30)	1.30±0.36^{NS}
AC (50)	25.36±4.03^{NS}	AC (34)	1.16±0.15^{NS}
AA (3)	32.97±9.16^{NS}	AA (2)	0.92±0.72^{NS}
Total (120)	27.35±2.42	Total (80)	1.25±0.13

Figures in the parenthesis indicate number of individuals under study

Values with superscript 'NS' along the column don't differ significantly (p≤0.05).

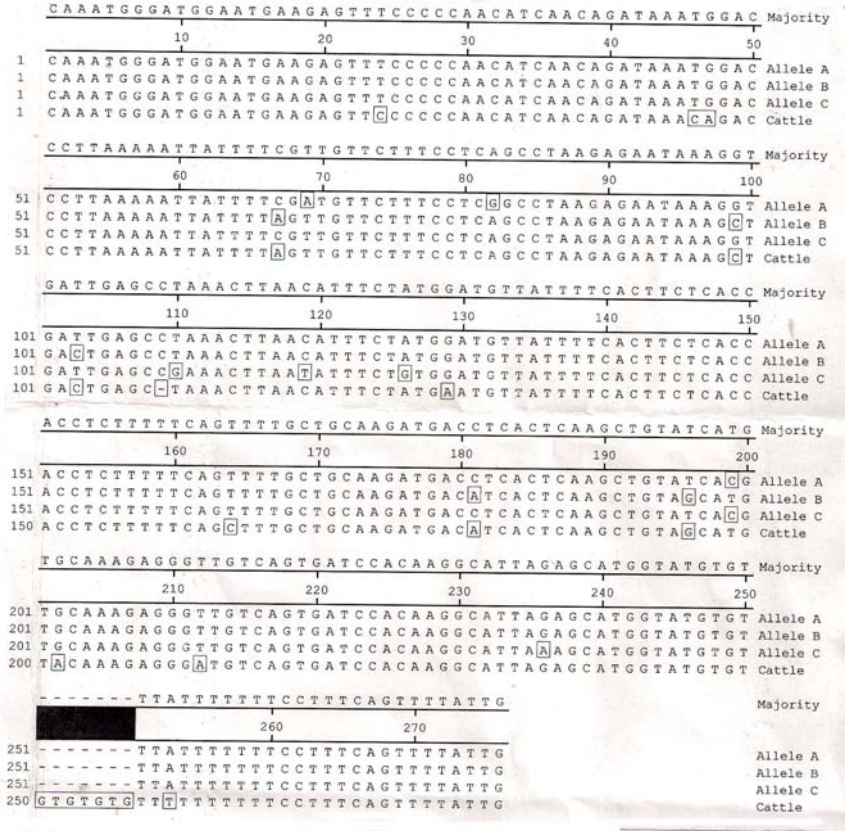

Figure 3. Nucleotide sequence comparison among alleles and with bovine counterpart.

and C allele by 8 and 6 nucleotide substitutions respectively, out of which 5 are present in intronic region in both cases. B allele differed from C by 10 bases out of which 7 were present in the intronic region. From the sequence analysis it was observed that two mutations A→T (69th) and G→A (82 th) differentiated A allele from others. There are 6 and 4 mutations observed to differentiate allele B and C alleles, respectively.

Percentage similarity between alleles

Percentage similarity study was carried out between nucleotide sequences of different alleles and the corresponding fragment of cattle lysozyme gene (Acc No-BTU25810) by using DNASTAR software and presented in Table 5. The allele A was closer to allele-C (97.8%) than allele-B (97.1%). Allele B was phylogenetically equidistant from both other alleles (Figure 4). The cattle sequence was more close to B allele (97.1%) than A and

Table 4. Allele-wise nucleotide differences

Alleles	Nucleotide positions											
	67th	69th	82nd	99th	103rd	110th	119th	126th	181th	196th	199th	236th
A	C	A	G	G	T	T	C	A	C	T	C	G
B	A	T	A	C	C	T	C	A	A	G	T	G
C	C	T	A	G	T	G	T	G	C	T	C	A

Nucleotide positions corresponds to 1 to 276 positions along the amplified product length from 5' to 3'.

Table 5. Percentage identity and divergence among alleles

Percent divergence	Percent identity			
	Allele-A	Allele-B	Allele-C	Cattle
Allele-A	-	97.1	97.8	94.2
Allele-B	2.9	-	96.4	97.1
Allele-C	2.2	3.7	-	93.5
Cattle	6.0	3.0	6.9	-

All values in the table are expressed in percentage.
Above diagonal values represent percent identity and off-diagonal values represent percent divergences.

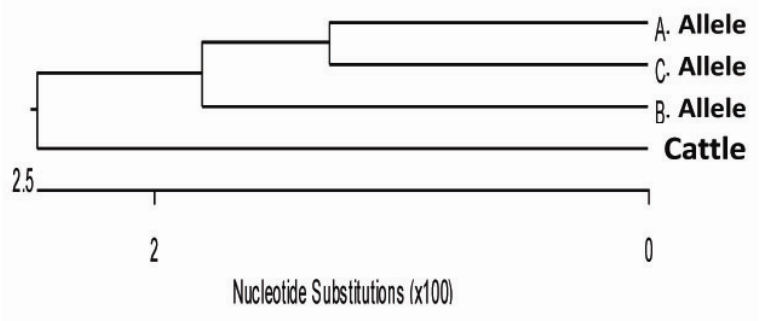

Figure 4. Phylogenetic tree based on the sequence analysis of alleles and cattle.

C alleles of buffalo.

CONCLUSION

Lysozyme gene was found to be sufficiently polymorphic in various breeds of Indian riverine buffalo. The gene and genotype frequency of lysozyme gene are different in different breeds of buffaloes available at different locations of India. The presence and absence of particular genotype can be utilized as a marker for breed identification. No significant effect of genotypes was observed on milk somatic cell count and serum lysozyme activity, though there were some apparent differences among the mean values. This may be due to the fact that the 276 bp fragment of lysozyme gene might contribute to a smaller part of total variation present. The change of amino acid in protein/nucleotide sequence in coding region may influence secondary structure of protein ultimately leading to change in biological activity of protein through protein substrate interaction.

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