Lactoferrin gene polymorphism of exons 8 and 13 in Murrah buffalo

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Abstract: Lactoferrin is one of the important candidate genes for mastitis resistance in dairy animals. The gene is located on chromosome BBU21 and consists of 17 exons spanning over 32.95 kb of genomic DNA. The present study was undertaken to identify allelic variants in exon 8 and 13 of lactoferrin gene and their association with incidence of clinical mastitis in Murrah buffalo. A total of 200 lactating Murrah buffaloes, grouped as mastitis affected and non-affected, were included in the study. Genomic DNA was isolated from the whole blood sample of each animal. Two primer sets were used to amplify exon 8 and 13 of lactoferrin gene, which yielded respective amplicons of 216 bp and 211 bp. The polymerase chain reaction-restriction fragment length polymorphism analysis of lactoferrin gene revealed monomorphic pattern in exon 8 and polymorphic pattern in exon 13. *Hpy 188I*-RFLP for exon 13 exhibited polymorphism with three genotypes: AA, AB and BB with respective frequencies of 0.20, 0.58 and 0.22 whereas, frequencies for A and B alleles were estimated as 0.49 and 0.51. Comparison of nucleotide sequence of exonic region of lactoferrin gene in Murrah buffalo with that of *Bos taurus* cattle revealed a total of 5 mutations out of which 2 were transition and 3 were transversion. The SNPs in exon 8 were found to be non synonymous and revealed two amino acid changes in exon 8 of Murrah buffalo as compared to *Bos taurus* cattle. Chi-square (χ^2) analysis indicated non significant association between genetic variants of exon 13 and incidence of clinical mastitis.

Keywords: Lactoferrin, Murrah buffalo, PCR-RFLP, Single nucleotide polymorphism

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Introduction

Among various health disorders in dairy animals, mastitis is one of the most expensive and devastating diseases in dairy animals including buffaloes. Mastitis is the inflammation of the mammary glands caused predominantly by entry through the teat by certain bacteria especially *Streptococcus*, *Staphylococcus* and *Escherichia* etc. It causes reduced milk yield, poor milk quality and lactation persistency and early culling contributing to huge economic losses to dairy farmers. In India, about 1 to 10% and 5 to 20% of buffaloes are affected with clinical and subclinical mastitis respectively every year (Joshi and Gokhale, 2006). Selective breeding of buffaloes for increased resistance to mastitis is difficult as it is polygenic trait with very low heritability. Earlier mastitis was considered purely a managerial disease, but at present several candidate genes (lactoferrin, BoLA-DRB-3, CARD15, interleukins, FEZL, CD14, etc.) have been identified for mastitis resistance. Lactoferrin is an important candidate gene having relation with the innate immunity and is considered to be a promising candidate gene in selection for mastitis resistance (Seyfert et al. 1996). It is a minor whey non-heme iron binding protein with molecular weight of 80 kDa containing a single polypeptide chain of 708 amino acids. The gene is located on chromosome BBU21 and consists of 17 exons spanning over 32.95 kb of genomic DNA. It is a potent activator and regulator of various immunological functions such as granulopoiesis, in vitro antibody synthesis, natural killer cell cytotoxicity, lymphocyte proliferation, complement activation and production of interleukins (Sanchez et al. 1992); (Kimber et al. 2002). Analysis of genetic polymorphism in lactoferrin gene and its relationship with udder infections has practical significance in marker-assisted selection (MAS) in dairy animals to maximally exploit their genetic potential for milk yield. Identification of lactoferrin variants as a genetic marker associated with mastitis resistance in buffalo would allow producers to decrease costs associated with mastitis by improving herd health through genetic selection. The polymorphism in lactoferrin gene and its association with mastitis has been described in *Bos taurus* (Li et al. 2004), but little information is available for exons 8 and 13 of lactoferrin gene in Murrah buffalo except for its promoter and 5' flanking regions (Kathiravan et al. 2010). Murrah breed of buffaloes is categorized among the best dairy breeds of riverine buffaloes, but its true

yield potential and economic contribution are hampered by increasing prevalence of mammary infections. Hence, the present study was undertaken with the objectives to identify polymorphism in exons 8 and 13 of lactoferrin gene through PCR-RFLP and its association with incidence of clinical mastitis in Murrah buffalo.

Materials and Methods

The experimental animals for the present study were taken from dairy herd of Murrah buffalo maintained at National Dairy Research Institute, Karnal, India. A total of 200 animals were sampled to identify polymorphism in exons 8 and 13 of lactoferrin gene. The animals were classified into affected and non-affected groups on the basis of past history of incidences of clinical mastitis from the treatment records of the herd. Among all, 50% of animals were mastitis-affected, while the remaining 50% were non-affected. Under sterile conditions, 10 ml of venous blood was collected from the jugular vein of buffalo in a 15 ml vacutainer tube containing 0.5 ml of 0.5M EDTA solutions, as an anticoagulant. Phenol-chloroform extraction method, as described by Sambrook and Russell (2001) with minor modifications was used for DNA isolation. The lactoferrin gene primers both forward (P1) and reverse (P2) for coding region of exons 8 and 13 were taken from published literature of (Li et al. 2004) and (Kathiravan et al. 2010) respectively (details of oligonucleotide sequence, annealing temperature and amplicon size are presented in Table 1). PCR amplification was carried out in programmed thermal cycler comprising final reaction volume of 25 µl containing 3 µl (100 ng) genomic DNA, 12.5 µl 2X PCR Master Mix (Fermentas), 0.5 µl of each primers and 8.5 µl nuclease free water. Amplification was performed using initial denaturation at 95°C for 2.5 minutes followed by 35 cycles of 94°C for 30s, respective annealing temperature for 30s and 72°C for 1 minute, with a final extension for 5 minutes at 72°C. PCR products were used for sequencing of lactoferrin gene through sequencing service provided by M/s. SciGenom Labs Pvt. Ltd. Basic Local Alignment Search Tool (BLAST) analysis was performed to find out sequence identity of lactoferrin gene of Murrah buffalo with other species. For determining the single nucleotide polymorphism (SNPs) in exons 8 and 13 of lactoferrin gene in Murrah buffalo, the available sequence in the NCBI for *Bos taurus* (Accession number-000179.1) was compared and aligned with the edited sequences of Murrah buffalo using ClustalW software.

PCR-RFLP condition

The preliminary selection of restriction enzymes to be used was done using NEB cutter V2.0 by submitting sequences obtained after amplification of experimental samples. PCR amplification of exons 8 and 13 revealed amplicon size of 216 and 211 bp by agarose gel electrophoresis. Amplified PCR products of exons 8 and 13 were digested using restriction enzymes *HpaII*, *HaeIII* and *Hpy188I*, *HinfI* respectively. For restriction enzyme digestion,

the reaction mixture comprised of a final volume of 20 µl containing 0.3 µl (10 U/µl) RE, 2.0 µl buffer, 7.70 µl milli Q water and 10 µl PCR product. Incubation was done for 10-12 hours at 37°C for all enzymes. Restriction enzyme digested products were separated by 2.5-3% agarose gel electrophoresis and visualized with ethidium bromide staining (@ 2µl/100 ml of gel) under UV light with a Gel Doc system (BioRad).

Statistical analysis

Chi-square statistic was used to analyze differences among genotypes and to assess significant association for susceptibility or resistance to mastitis. The chi square statistic (χ^2) was calculated by the formula $\chi^2 = \sum (\text{Observed} - \text{Expected})^2 / \text{Expected}$ (Snedecor and Cochran, 1994). The observed genotype frequencies based on RFLP patterns were tested for Hardy-Weinberg Equilibrium (HWE) by chi-square test. The observed and expected frequencies were analyzed for goodness of fit at probability $p > 0.05$. The calculated chi-square value was compared with table p value to ascertain the significance of association between genotype frequency and mastitis incidence.

Results and Discussion

In the present study polymorphism was detected in exon 13 of lactoferrin gene through PCR-RFLP. Exons 8 and 13 of lactoferrin gene were amplified successfully which yielded amplicon size of 216 and 211 bp. The PCR-RFLP was performed on the amplified fragment of exons 8 and 13. Restriction enzymes (REs) *HaeIII* and *HpaII* were used for digestion of exon 8 having single cutting site. PCR-RFLP analysis of exon 8 revealed monomorphic pattern (AA) with fragment size of 122 and 94 bp using *HaeIII* restriction enzyme. Similarly *HpaII* restriction enzyme had single cutting site, producing two fragments of 125 and 91 bp (BB) that exhibited monomorphic pattern. (Fig.1 and 2).

The RE digestion for exon 13 was carried out by using *Hpy 188I* and *HinfI*. Restriction enzyme *Hpy 188I* for 211bp amplicon revealed polymorphic pattern with three genotypes : AA (211 bp), AB (211, 164, and 47 bp), and BB (164 and 47 bp) with respective frequencies of 0.20, 0.58 and 0.22 (Fig. 3). Allelic frequencies of A and B genotype were estimated as 0.49 and 0.51, respectively. Genotypic frequency of AB heterozygote was more than the homozygote animals. However, restriction enzyme *HinfI* revealed monomorphic pattern with band size of 130, 81 bp (CC) in exon 13. Kathiravan et al. (2009) performed PCR-SSCP analysis for bubaline lactoferrin gene and revealed monomorphic patterns in exons 2, 11 and 14. In another study Kathiravan et al. (2010) identified polymorphisms in exons 6, 7, 13 and their flanking intronic regions in the bubaline lactoferrin gene by PCR-SSCP analysis and revealed two SSCP variants with the frequencies of 0.92 and 0.08 in exon 13 of lactoferrin gene. Similarly Khatibi et al. (2013) reported four SSCP patterns with frequencies of 0.341, 0.259, 0.118 and 0.282 for A, B, E and F alleles respectively in

Table 1 Oligonucleotide Sequence, annealing temperature and amplicon size of Exons 8 and 13 of lactoferrin gene in Murrah Buffalo

Primer	Sequence (5'-3')	Annealing Temp.	Amplicon size (bp)
Exon 8	F- CTCTACCACTGACATCATAAT R-CACCTTCCCTGAGGTTCTTC	54.0°C	216
Exon13	F-AGAGCTGGCTCCCCATGTTTCTT R-AGGGCCCTGTCCTGATGAAGC	58.5°C	211

Fig 1. PCR-RFLP of Exon 8 of Lactoferrin Gene Using *Hae*III Restriction Enzyme

Lane 1-12 : (AA) 2 Bands of 122, 94 bp
 Lane M : Marker (50 bp)
 Lane P : PCR Product (216 bp)

Fig 2. PCR-RFLP of Exon 8 of Lactoferrin Gene Using *Hpa*II Restriction Enzyme

Lane 1-19 : (BB) 2 Bands of 125, 91 bp
 Lane M : Marker (50 bp)

lactoferrin gene in 85 Iranian buffaloes. Wojdak-Maksymiec et al. (2013) studied lactoferrin polymorphism in 588 Holstein cows and reported two genotypes AA and AB with respective frequencies of 0.568 and 0.432. However, Bukhari et al. (2015) observed monomorphic pattern in promoter region of lactoferrin gene using *Taq* 1 restriction enzyme in Jersey crossbred cattle. However, Dinesh et al. (2015) reported polymorphism in exon 7 and 12 of lactoferrin gene in Murrah buffalo. Singh et al. (2016)

reported monomorphic pattern in exons 2, 3, 14 and their flanking intronic regions of lactoferrin gene in Deoni cattle by PCR-SSCP.

Association analysis was performed by chi-square test. For exon 13 with *Hpy*188I RE, calculated χ^2 (2.10) value is less than tabulated χ^2 (5.99) value at 2 degrees of freedom and 5 % level of significance. Hence, AA, AB and BB genotypes do not differ significantly with respect to mastitis incidence. The observed genotypic frequencies were tested for Hardy-Weinberg

Fig 3. PCR-RFLP of Exon 13 of Lactoferrin Gene Using *Hpy188I* Restriction Enzyme

Lane 15, 16 : AA (211 bp)
Lane 4, 7, 10-14 : AB (211, 164, 47 bp)
Lane 1-3, 5, 6, 9 : BB (164, 47 bp)
Lane M : Marker (50 bp)

Fig 4. PCR-RFLP of Exon 13 of Lactoferrin Gene Using *HinfI* Restriction Enzyme

Lane 1-18 : (CC) 2 Bands of 130, 81 bp
Lane M : Marker (50 bp)
Lane P : PCR Product (211 bp)

equilibrium and found that gene and genotypic frequency of exon 13 was in equilibrium, showing a non-significant association between genotype of exons 13. The results of present study are consistent with earlier reports of Kaminski et al. (2006) who reported non significant association between polymorphic variant in promoter region of lactoferrin gene and somatic cell count. Zhao et al. (2009) observed genetic polymorphism in promoter region of lactoferrin gene by PCR-RFLP using *HinfI* and found that frequency of AA genotype was higher in healthy dairy cows, while BB genotype was found in dairy cows affected with sub-clinical mastitis. Huang et al. (2010) also reported non significant association between identified SNPs and somatic cell count. Similarly Dinesh et al. (2015) reported non significant association between genetic variant of exon 12 of lactoferrin gene and mastitis, however they observed significant association between genetic variant of exon 7 of lactoferrin gene and mastitis. Nanaei et al. (2016) did not find significant association between intron 6 of lactoferrin gene and 305 days milk yield, protein percentage, pregnancy length and milk days. In another study Dinesh et al. (2020) observed significant association between incidence of

clinical mastitis and genetic variant of exon 6 and they reported that animals with AA genotype were found to be less susceptible to mastitis.

Comparison of nucleotide sequences of exonic regions of the lactoferrin gene with that of *Bos taurus* cattle by ClustalW multiple alignments revealed a total of five mutations. Two transitions and one transversion were observed in exon 8 of the Murrah buffalo lactoferrin gene as compared to cattle, while one transition and one transversion were found in exon 13 of lactoferrin gene in Murrah buffalo. The coding sequences were translated into amino acid sequence by using ExPASy translate tools and the resulting amino acid sequence was aligned with corresponding sequence of *Bos taurus* by ClustalW. Conceptualized translation of nucleotide sequence of exon 8 revealed two amino acid changes in Murrah buffalo as compared to that of *Bos taurus* cattle. Amino acid changes observed in exon 8 were arginine to glycine and threonine to alanine at 303 and 346 position respectively. However, in exon 13 both the mutations were found to be synonymous in nature without

affecting the sequence of amino acid. Thus only the nucleotide changes in exon 8 were found to be non synonymous in nature affecting the sequence of amino acid. In a similar study, Li et al. (2004) found polymorphisms in exons 4, 8, 9, 11, and 15 and in intron 4 in Holstein-Friesian cattle. A mutation occurring in exon 4 led to the amino acid substitution (isoleucine to valine), while other mutations were silent. O'Halloran et al. (2009) identified 47 polymorphisms in lactoferrin coding sequences. Out of these, 18 SNPs were synonymous causing no change in amino acid sequence, while 27 SNPs were associated with amino acid changes. The result of present study are in agreement with the earlier report of Kathiravan et al. (2010) who observed two point mutation in exon 13 of lactoferrin gene as compared to cattle.

BLAST results were used to check the percent homology of Murrah buffalo lactoferrin gene with that of other species. The sequence homology of exon 8 was 99% with *Bubalus bubalis*, 98% with *Bos taurus*, 98% with *Bos indicus*, 92% with *Capra hircus* and 92% with *Ovis aries*. Similarly, the percent homology of exon 13 was 100% with *Bubalus bubalis*, 98% with *Bos taurus*, 97% with *Capra hircus* and 96% with *Ovis aries*. BLAST results revealed that exonic region of lactoferrin gene in Murrah buffalo was 92 to 100% identical with several species. This is consistent with the finding on exonic region of the bubaline lactoferrin gene as reported by Kathiravan et al. (2010). A similar homology (65-100%) in a gene sequence among different mammalian species was reported by Teng, 2002.

Conclusions

PCR-RFLP analysis in exon 13 of lactoferrin gene in Murrah buffalo using *Hpy*188I restriction enzymes revealed polymorphism with three genotypes (AA, AB and BB), whereas exon 8 exhibited monomorphic pattern. Genotype AB occurred with higher frequency as compared to AA and BB. Multiple sequence alignment of Murrah buffalo with that of cattle showed five SNPs. SNP found in exon 8 were found to be non synonymous. BLAST results revealed that exonic region of lactoferrin gene in Murrah buffalo was 92 to 100% identical with other species.

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