Indian Journal of Animal Sciences **90** (4): 588–591, April 2020/Article
https://doi.org/10.56093/ijans.v90i4.104206

Association of polymorphic variant of exons 6 and 11 of lactoferrin gene with mastitis in Murrah buffalo

KRISHANENDER DINESH*, ARCHANA VERMA, I D GUPTA and S K DASH

ICAR-National Dairy Research Institute, Karnal, Haryana 132 001 India

Received: 25 July 2019; Accepted: 29 October 2019

ABSTRACT

Lactoferrin is one of the important candidate genes for mastitis resistance in dairy animals. The gene is located on *Bos taurus* autosome (BTA) 22 and consists of 17 exons spanning over 34.5 kb of genomic DNA. The present study was undertaken to identify allelic variant in exons 6 and 11 of lactoferrin gene and to analyze association with incidence of clinical mastitis in Murrah buffalo. The amplification of exons 6 and 11 of lactoferrin gene yielded 301 and 131 bp amplicon size. Comparison of nucleotide sequence of exonic region of lactoferrin gene with *Bos taurus* (NCBI accession number AC_000179.1) revealed 6 mutations; among them 3 were in coding DNA sequence and remaining 3 were in flanking intronic region. All these mutations were found in exon 6 and synonymous in nature without affecting the sequence of amino acid. PCR-restriction fragment length polymorphism (RFLP) analysis of 301 bp amplicon using *Fok*I restriction enzyme exhibited polymorphic pattern with two genotypes (AA and AB) with respective frequency of 0.625 and 0.375. The frequencies of two alleles, A and B were estimated as 0.81 and 0.19 respectively. RE *Hpy*188I and *Hinf*I used for digestion of exon 11 had exhibited monomorphic pattern. The chi-square (χ^2) analysis revealed significant association between incidence of clinical mastitis and genetic variant of exon 6 and animals with AA genotype were found to be less susceptible to mastitis. The findings indicate potential scope for incorporation of lactoferrin gene in selection and breeding of Murrah buffaloes for improved genetic resistance to mastitis.

Keywords: Lactoferrin gene, Murrah buffalo, PCR-RFLP, SNP

Mastitis is the most common, costly and devastating disease in dairy animals resulting in reduced milk yield, poor milk quality and lactation persistency as well as early culling contributing to huge economic losses. In India, about 1–10% and 5–20% 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 a polygenic trait with very low heritability. Earlier mastitis was considered purely as a managemental disease, but at present many candidate genes for mastitis have been identified. Lactoferrin is one of the important candidate genes for mastitis resistance. 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 *Bos taurus* autosome (BTA) 22 and spans 34.5 kb consisting of 17 exons and 16 introns (Seyfert and Kuhn 1994). It is a potent activator and regulator of various immunological functions such as granulopoiesis, cytokine production, antibody synthesis *in vitro*, natural killer cell cytotoxicity, lymphocyte proliferation, complement activation and production of interleukins (Sanchez *et al*. 1992, Kimber *et al*. 2002).

*Corresponding author email: krishanender25@gmail.com

Analysis of genetic polymorphism in lactoferrin gene and its relationship with udder infections have practical significance in dairy animals. The information may be utilized for marker assisted selection so as to exploit the genetic resistance of lactating animals to the maximum extent to prevent occurrence of mastitis. Identification of lactoferrin variants as a genetic marker associated with mastitis susceptibility or resistance in buffalo would allow producers to decrease costs associated with mastitis by improving herd health through animal selection. The polymorphism in lactoferrin gene and its association with mastitis has been described in *Bos taurus* (Li *et al*. 2004) but very little information is available with respect to exonic region of lactoferrin gene in Murrah buffalo except for its promoter and 5′ flanking regions (Kathiravan *et al.* 2009, Kathiravan *et al.* 2010). Hence, the present study was undertaken with the objective to identify polymorphism through PCR-RFLP in exons 6 and 11 of lactoferrin gene and identify association with mastitis in Murrah buffaloes.

MATERIALS AND METHODS

The present study was conducted on Murrah buffaloes maintained at cattle yard of ICAR-National Dairy Research Institute, Karnal, India. Blood samples of animals (200) were selected to find out polymorphism in exons 6 and 11

Table 1. Oligonucleotide sequence, annealing temperature and amplicon size of exons 6 and 11 of lactoferrin gene in Murrah buffalo

Primer		Sequence (5′-3′)	Annealing Temp.	Amplicon size (bp)
Exon 6	F	AGGAGCCCACAGAAGACAA	54°C	301
	R	TGTGCTCTACAGAGAGCTTTGA		
Exon 11	F	TGACATTTTGCTTCTCTTTATTCT		
	R	TTCCCAACCTCTCCTCCTC	57°C	131

of lactoferrin gene through PCR-RFLP and to analyze association with incidence of clinical mastitis in Murrah buffalo. Among them, 50% animals had suffered from mastitis. Under sterile conditions, 10 ml of venous blood was collected from the jugular vein of buffalo in a 15 mL polypropylene centrifuge tube containing 0.5 mL of 0.5 M EDTA solutions, as an anticoagulant. Phenol-chloroform extraction method, as described by Sambrook and Russel (1989) with minor modifications was used for DNA isolation. The lactoferrin gene primers both forward (P1) and reverse (P2) for coding region of exon 6 was designed using published NCBI sequence of *Bos taurus* cattle (Accession number 000179.1) through Primer 3.0 software and for exon 11 primers were taken from published literature (Kathiravan *et al*. 2009). The 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 2× 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 min followed by 35 cycles of 94°C for 30 sec, respective annealing temperature for 30 sec and 72°C for 1 min, with a final extension for 5 min at 72°C. PCR amplified products were sent to M/S. SciGenom Labs Pvt. Ltd. (Kochi, India) for purification and sequencing in both directions. To find out sequence of lactoferrin gene in Murrah buffalo, the raw sequence data which were obtained was edited to reduce overlapping sequences by using BioEdit software of DNASTAR. For determining the single nucleotide polymorphism (SNPs) in exons 6 and 11 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 *Bos taurus* reference sequence (NCBI Accession No. 000179.1). PCR amplification of exons 6 and 11 revealed amplicon size of 301 and 131 bp by agarose gel electrophoresis. For PCR-RFLP analysis, amplified PCR products of exon 6 was digested with *Fok*I restriction enzyme and restriction enzymes *Hpy*188I and *Hinf*I were used for digestion of exon 11. The digestion was done in a final volume of 20 µL containing 0.3 µL (10 U/mL) RE, 2.0 µL buffer, 7.70 µL milli Q water and 10 µL PCR product. Incubation temperature for all the enzymes was 37°C for 10–12 h. RE digested products were separated by 2.5–3% agarose gel electrophoresis and visualized with ethidium bromide staining (@ 2 µL/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, if any for susceptibility or resistance to mastitis. The chi square statistic (χ^2) was calculated by the formula $\chi^2=\Sigma$ (Observed−Expected)^2/Expected (Snedecor and Cochran 1994). The observed genotype frequencies based on RFLP patterns were tested for Hardy-Weinberg Equilibrium by chi-square test. The observed and expected frequencies were analyzed for goodness of fit at probability $P\leq0.05$. The calculated chi-square value was compared to the table p value at desired (1) degrees of freedom to ascertain the significance of association between genotype frequency and mastitis incidence.

RESULTS AND DISCUSSION

The PCR-RFLP analysis revealed genetic polymorphism in exon 6 of lactoferrin gene. Primers for exons 6 and 11 of lactoferrin gene were amplified successfully which yielded amplicon size of 301 and 131 bp. The PCR RFLP was performed on amplified fragment of exons 6 and 11. Restriction enzyme *Fok*I was used for digestion of exon 6 have single cutting site. PCR-RFLP analysis of exon 6 with *Fok*I exhibited polymorphic pattern with two genotypes: AA and AB with respective frequencies of 0.625 and 0.375. Genotype BB was not detected. The frequencies for A and B alleles were estimated as 0.81 and 0.19 respectively. The genotype AA was characterized by single fragment of 86 bp while AB genotype was observed by the presence of two restriction fragments of 215 and 86 bp (Supplementary Fig. 1). The results of present study are consistent with earlier report of Srubarova and Dvorak (2010) who observed two genotypes AA and AB in lactoferrin gene with respective frequencies of 57.14% and 42.86% and genotype BB was not detected. Similarly, 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. Rodriguez *et al*. (2013) also reported polymorphism in intron 6 of lactoferrin gene using PCR-RFLP technique. However, Raja (2007) reported monomorphic pattern in exons 6 through PCR- SSCP analysis in Sahiwal cattle. The RE digestion for exon 11 was carried out using *Hpy*188I and *Hinf*I. Monomorphic pattern with fragment sizes of 103, 28 bp and 102, 29 bp could be seen using *Hpy*188I and *Hinf*I restriction enzyme

Table 2. Mastitis affected and not affected animals with respect to exon 6 of lactoferrin gene in Murrah buffalo

Exon-RE combination	Genotype	Mastitic		Non-mastitic		Total
		No.	(%)	No.	(%)	
Exon 6-*FoKI*	AA	53	42.4	72	57.6	125
	AB	47	62.7	28	37.3	75

respectively (Supplementary Figs 2 and 3). Since fragment size of 28 bp and 29 bp is very small hence it could not be visible in agarose gel electrophoresis. Kathiravan *et al*. (2009) performed PCR-SSCP analysis for bubaline lactoferrin gene and revealed monomorphic patterns in exons 2, 11 and 14. Similarly, Bukhari *et al*. (2015) observed monomorphism in promoter region of lactoferrin gene using *Taq* 1 restriction enzyme in Jersey crossbred cattle. Singh *et al*. (2016) also reported monomorphic pattern in exons 2, 3, 14 and their flanking intronic regions of lactoferrin gene in Deoni cattle by PCR-SSCP. The results of association study done by chi-square analysis indicated that in exon 6, animals with AA genotype were less susceptible to mastitis than those with AB genotype (Table 2). Since calculated χ^2 (7.69) value is greater than tabulated χ^2 (3.84) value at 1 degrees of freedom at P<0.05%, hence, AA and AB genotypes of Murrah buffaloes differed significantly for mastitis incidence (P≤0.05). The observed genotypic frequencies were tested for Hardy-Weinberg Equilibrium (HWE) and a significant (9.174) χ^2 value indicated deviation from equilibrium in gene and genotypic frequency. There is evidence that resistance to infectious diseases have genetic basis. These findings support the observations of Zhao *et al*. (2009), Rahmani (2011), Sharifzadesh and Doosti (2011) and Dinesh *et al*. (2015) who reported that cows with A/A genotype were resistant to mastitis infection. However, on the contrary, Sender *et al*. (2006) reported that BB genotype animals exhibited the lowest somatic cell count and more resistant to mastitis than heterozygotes (AB).

All these finding suggest that lactoferrin gene can be used as a genetic marker and allele A of this gene is suitable marker for resistance to mastitis infection in dairy cows. 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 6 mutations; among them 3 were in coding DNA sequence and remaining 3 were in flanking intronic region. Out of which, 3 were transition and 1 was transversion mutation. Two SNPs that showed remarkable changes from the reference sequence were insertion of G at 10204 position and deletion of C at 10215 position in intron 6 of Murrah buffalo. All the SNP changes were found to be synonymous in nature. Kathiravan *et al.* (2010) reported 4 synonymous mutations in exon 6 and flanking intronic region.

BLAST results were used to check the percent homology of the sequenced exonic region of lactoferrin gene with that of other species. The sequence homology of exon 6 was 100% with *Bubalus bubalis*, 96% with *Bos taurus and Bos indicus,* 98% with *Capra hircus* and 94% with *Ovis aries*. Similarly, the percent homology of exon 11 was 97% with *Bubalus bubalis* and 100% with *Bos taurus, Capra hircus* and *Ovis aries.* BLAST results revealed that exonic region of lactoferrin gene in Murrah buffalo was 94 to 100% identical with several species. This is consistent with the earlier finding on exonic region of the bubaline lactoferrin gene by Kathiravan *et al*. (2009, 2010). A similar homology (65–100%) in a gene sequence among different mammalian species was reported by Teng (2002).

In conclusion, the PCR-RFLP analysis of exon 6 of lactoferrin gene in Murrah buffalo exhibited polymorphic pattern and monomorphic pattern in exon 11. Chi-square analysis revealed significant association of exon 6 with incidence of clinical mastitis in Murrah buffalo. It was observed that AA and AB genotypes differ significantly for mastitis incidence and animals with AA genotypes were less susceptible to mastitis as compared to AB genotype. The identified genetic variant (AA genotype) may be validated in a larger herd and used as potential marker in the breeding programs for selection of buffaloes less susceptible to mastitis. The findings have potential scope in buffalo breeding programmes for genetic selection of Murrah buffaloes for higher resistance against clinical mastitis.

ACKNOWLEDGEMENTS

The authors are grateful to the Director, NDRI, Karnal and Head, Animal Genetics and Breeding Division, NDRI for providing necessary research facilities.

REFERENCES

Bukhari Saba, Das A K, Kumar N, Raghuwanshi P, Taggar R K, Chakraborty D, Vohra V and Gupta P. 2015. Genetic polymorphism of promoter region of lactoferrin gene and its association with mastitis resistance in Jersey crossbred cattle. *Indian Journal of Animal Research* **49**(2): 165–67.

Dinesh K, Verma A, Gupta I D, Thakur Y P, Verma N and Arya A. 2015. Identification of polymorphism in exons 7 and 12 of Lactoferrin gene and its association with incidence of clinical mastitis in Murrah buffalo. *Tropical Animal Health Production.* DOI 10.1007/s 11250-015-0765-z.

Joshi S and Gokhale S. 2006. Status of mastitis as an emerging disease in improved and periurban dairy farms in India. *Annals of the New York Academy of Sciences* **1081**(1): 74–83.

Kathiravan P, Kataria R S, Mishra B P, Dubey P K, Selvakumar M and Tyagi N. 2009. Seven novel single nucleotide polymorphisms identified within river buffalo (*Bubalus bubalis*) lactoferrin gene. *Tropical Animal Health Production* **42**: 1021–26.

Kathiravan P, Kataria R S, Mishra B P, Tyagi N and Selvakumar M. 2010. Sequence characterization and polymorphism detection in exons 6, 7 and 13 of the Bubaline lactoferrin gene. *Buffalo Bulletin* **29**(3).

Kimber I, Cumberbatch M, Dearman D R, Headon D R, Bhushan M and Griffiths C E M. 2002. Lactoferrin: influences on Langerhans cells, epidermal cytokines, and cutaneus inflammation. *Biochemistry and Cell Biology* **80**: 103–07.

Li G H, Zhang Y, Sun D X and Li N. 2004. Study on the

polymorphism of bovine lactoferrin gene and its relationship with mastitis. *Animal Biotechnology* **15**: 67–76.

Rahmani S F. 2011. 'Studies on bovine lactoferrin gene and its association with mastitis in cattle.'Ph.D. Thesis, Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka.

Raja K N. 2007. Characterization of bovine lactoferrin gene and its polymorphism in Sahiwal cattle. PhD Thesis, NDRI, Karnal.

Rodriguez Nancy C, Lopez Albeiro H and Julia Echeverri Z. 2013. Population genetic structure of bovine lactoferrin gene in Holstein breed of Antioquia Department. *Revista MVZ Córdoba* **18**(1): 3355–61.

Sambrook J and Russell D W. 1989. 3rd edition. Cold Spring Harbor Laboratory Press, New York 6.1–6.62.

Sanchez L, Calvo M and Brock J H. 1992. Biological role of lactoferrin. *Archives of Disease in Childhood* **67**: 657–61.

Sender G, Korwin-Kossakowska A, Galal K and Prusak B. 2006. Ocena wp³ywu polimorfizmu wybranych genów na wystêpowanie mastitis u krów. *In Polish*. *Medycyna Weterynaryjna* **62**: 563–65.

Seyfert H M and Kuhn C. 1994. Characterization of a first bovine lactoferrin gene variant based on an EcoRI polymorphism. *Animal Genetics* **25**: 54.

Sharifzadesh A and Doosti A. 2011. Study of lactoferrin gene polymorphism in Iranian Holstein cattle using PCR-RFLP technique. *Global Veterinaria* **6**(6): 530–36.

Singh AP, Ramesha K P, Isloor S, Divya P, Arya A and Mir M A. 2016. Sequence characterization and polymorphism detection in lactoferrin gene of Deoni (*Bos indicus*) cattle. *Indian Journal of Animal Research* **50**(4): 455–59.

Snedecor G W and Cochran W G. 1994. *Statistical Methods*. 8th ed. State University Press, USA.

Srubarova P and Dovrak J. 2010. Association between Lactoferrin gene polymorphism and bovine mammary gland inflammation.

Teng C T. 2002. Lactoferrin gene expression and regulation: an overview. *Biochemistry and Cell Biology* **80**: 7–16.

Zhao C, He G, Wan Y and Zhang Z. 2009. Polymorphism of lactoferrin gene with PCR-RFLP and its association with subclinical mastitis in dairy cows. *Modern Applied Sciences* **3**: 144–46.