

LACK OF ASSOCIATION OF LACTOFERRIN GENE POLYMORPHISM WITH MASTITIS IN MURRAH BUFFALOES

RICHA KHIRBAT, M. L. SANGWAN*, JITENDER YADAV, RAVINDER SINGH,
VIRENDER and VIJAY KUMAR

Department of Animal Biotechnology, College of Veterinary Sciences
Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar-125 004

ABSTRACT

The present investigation was undertaken to study the genetic polymorphism in lactoferrin gene by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and single strand conformational polymorphism (PCR-SSCP) technique and its association with mastitis in Murrah buffaloes. Genomic DNA was isolated by phenol chloroform isoamyl alcohol method from blood of 40 randomly selected Murrah buffaloes (including 20 mastitic animals). The PCR was standardized to amplify partial promoter region (370bp) and exon 15 (261bp) of lactoferrin gene. When 261bp PCR products of exon 15 of lactoferrin gene were digested with Taqα I and Nru I restriction endonucleases, it detected single genotype viz. xx, i.e. monomorphism. When SSCP analysis of 370 bp PCR products of promoter region of lactoferrin gene was done, it revealed 5 SNPs between healthy and mastitis animals. On comparison with reference sequence *Bubalus bubalis* (EF650854.1) and Seyfert sequence, some important SNPs at positions +35, -19 and -156 were observed. The T-C transition (+35) was observed in the 5' UTR of exon1, 4 bp upstream of the start codon which could play a crucial role in the regulation of gene expression. No association of lactoferrin gene with mastitis was found. In phylogenetic tree, the lactoferrin gene (exon 15 and promoter region) of *B. bubalis* were found more closely related to *Bos taurus* than to *Capra hircus*.

Key words: PCR-SSCP, PCR-RFLP, lactoferrin, buffalo

Lactoferrin is an iron binding glycoprotein found in variety of mammalian biological fluids such as tears, saliva and notably in milk (Sorenson and Sorenson, 1939) and belongs to serum transferrin gene family. Lactoferrin concentration in milk samples from cows with clinical mastitis has been reported to be significantly higher than that of normal cows (Kawai *et al.*, 1999). Its direct bactericidal action occurs due to its strong iron binding property which deprives the growing microbes of their demand for ferric ions. It also has a bacteriostatic action which is mediated by two antimicrobial peptides of an N-terminal part of amino acid chain of this protein, called lactoferricin and lactoferrampin, released from native protein by pepsin-mediated digestion (Kraan *et al.*, 2004; Orsi, 2004; Exposito and Recio, 2006). Its well-documented anti-bacterial activity makes it a candidate marker for genetic selection towards reduced susceptibility in dairy animals. The lactoferrin gene was mapped to bovine chromosome 22 (BTA 22), contains 17 exons and spreads on about 34.5 kbp (Schwerin *et al.*, 1994).

Mastitis continues to be an economically

important infectious disease of bovine throughout the world. In India, annual losses due to mastitis in cows and buffaloes has been calculated to the tune of Rs.7165.51 crores (Bansal and Gupta, 2009). Of the various candidate genes for mastitis resistance, lactoferrin has been reported to be a important candidate gene in cattle (Ogorevc *et al.*, 2009). However, information regarding the polymorphisms within the bubaline lactoferrin gene is very scanty. In the present study, polymorphism within exon 15 and fragment of promoter region of bubaline lactoferrin gene was studied through PCR-SSCP / PCR-RFLP as well as its association with mastitis.

MATERIALS AND METHODS

DNA Samples: Blood samples of 40 unrelated Murrah buffaloes (*B. bubalis*) (including 20 mastitic animals) were collected from Government Livestock Farm, Hisar, and Animal Farm and Veterinary Clinics, LLRUVAS, Hisar. Genomic DNA was extracted from whole blood following standard phenol-chloroform extraction method (Sambrook and Russell, 2001).

PCR-SSCP Analysis: Published primers (Kathiravan

*Corresponding author: manoharsangwan@gmail.com

et al., 2009) were used to amplify the fragment of promoter region of the lactoferrin gene. The primer sequence was: 5'GGGCTGCGGACAAGTGGGAAAGAA3' (F) and 5'GACAGCAGGGCGGGGACGAAGAG3' (R) which generated a product of 370bp. The reaction mix consisted to 100 ng of genomic DNA, 0.25 µL of each primer (50pmol), 0.5 µL of 10 mM of dNTP mix, 3.5 µL of 10× buffer with 1.5 mM MgCl₂ and 2.5 U of Taq DNA polymerase (0.5 µL of 5U/ µl) (Fermentas Pvt. Ltd) in a final reaction volume of 25 µl. Amplification was performed using programmable thermal cycler (Bio-Rad i-cycler) with an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 sec., annealing at 62°C for 30 sec. and extension at 72°C for 1 min, with final extension at 72°C for 10 min.

The PCR products were visualized on a 1.8% ethidium bromide stained agarose gel. The 370 bp PCR products of 5' regulatory region were then resolved by SSCP analysis after optimization of non-denaturing PAGE concentration and other running conditions. Each PCR product was diluted in denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, 20 mM EDTA), heated at 95°C for 5 min, snap chilled on ice and resolved on 8% polyacrylamide gel. The electrophoresis was carried out in a Protean II xi vertical electrophoresis unit (BioRad, USA) using 1×TBE buffer. Gels were silver-stained (Sambrook and Russell, 2001) and photographed for scoring. The phylogenetic tree was also constructed using Phylogeny.fr server to show relatedness between the species with respect to fragment of promoter region of lactoferrin gene.

PCR-RFLP Analysis: The primers were designed to amplify exon 15 of the bubaline lactoferrin gene based on the published bovine sequence (Seyfert *et al.*, 1994; GenBank accession number L19991). Sequence of the self-designed primers were: 5'CGTGGCTGATGCAGCCTTCTC3' (F) and 5'GCCCCCTGAAGGTAAGCACAGGC3' (R) with an expected product size of 261 bp. The PCR was carried out in a reaction volume of 25 µL containing 100 ng of genomic DNA, 0.25 µL of each primer (50pmol), 0.5 µL of 10 mM of dNTP mix, 3.5 µL of 10× buffer with 1.5 mM MgCl₂ and 2.5 U of Taq

DNA polymerase (0.5 µl of 5U/ µl). Amplification was performed in a thermal cycler with an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation 94°C for 45 sec, annealing at 60°C for 30 sec and extension at 72°C for 45 sec, with a step of final extension at 72°C for 10 min. The PCR products were visualized through a 1.8% ethidium bromide stained agarose gel. The 261 bp PCR products of exon 15 were then digested with restriction enzymes (RE) *Taq*I and *Nru* I. The reaction mixture was prepared by adding 1.8 µl nuclease free water (NFW), 2 µl of 10X buffer and 1 µl of RE, 15 µl of PCR product was added in each tube. The 19.8 µl reaction mix was kept for digestion in an incubator at 37°C overnight. After the digestion, heat inactivation of the enzymes was done at 80°C (*Taq*I and *Nru* I) for 20 min. The restriction fragments were resolved by electrophoresis on 3% agarose gel (Sigma, USA) in 1X TAE buffer. After staining with ethidium bromide, the fragments were visualised. The phylogenetic tree was also constructed using Phylogeny.fr server to show relatedness between the species with respect to exon 15 of lactoferrin gene.

Sequence Analysis of SSCP and RFLP Variants: SSCP variants from fragments of 5' regulatory region and RFLP variants of exon 15 of lactoferrin gene were purified by QIAquick gel extraction kit (QIAGEN). Purified PCR products were sequenced in both directions using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) on an automated Genetic Analyzer ABI 3100 (Applied Biosystems, USA). Sequence data were analyzed using Bioedit programme. Multiple sequence alignments were performed with Clustal W (<http://align.genome.jp>) method of the Bioedit software.

RESULTS AND DISCUSSION

PCR-SSCP Analysis: PCR-SSCP was used to determine the SNPs in the lactoferrin gene promoter region. SSCP analysis of the 370 bp fragment of the 5' regulatory region revealed a total of two distinct patterns across mastitic and healthy animals. When resolution of single stranded PCR product of promoter region (370 bp) was done in non-denaturing 8% PAGE,

two SSCP variants A and B were observed with overall frequencies of 0.95 and 0.05, respectively. In all the 20 healthy animals, single SSCP variant i.e. A was detected. While in 18 mastitic animals, SSCP variant A and in remaining 2 animals, SSCP variant B was detected (Figs. 1 and 2). In all the healthy animals, SSCP variant B was absent. These two SSCP variants further revealed 5 single nucleotide polymorphic sites (SNPs) between healthy and mastitic animals. The SNPs were : G, G, G, T and T in mastitic animals and C, C, A, C and C in healthy animals at nucleotide positions -155, -156, -112, -19 and +35, respectively. Comparison of SSCP variants with reference sequence of the riverine buffalo (EF650854.1) from India revealed five SNPs and two insertions (Table 1).

Kathiravan *et al.* (2009) also reported SNPs in promoter region of lactoferrin gene in different breeds of Indian buffaloes. However, there are no reports of SNPs in promoter region of lactoferrin among healthy and mastitic buffaloes. SNPs were observed at positions -19 and +35 which were consistent with those reported by Kathiravan *et al.* (2009) and while SNP at position -156 was consistent with those reported by Li *et al.* (2004) in comparison with the Seyfert *et al.*

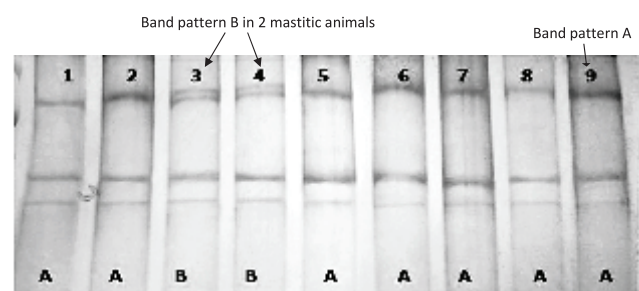


Fig 1. Resolution of fragment of promoter region of lactoferrin gene by 8% non denaturing PAGE showing SSCP variants. Lanes: 1-9 = Samples of mastitic animals; Lanes 1, 2, 5, 6, 7, 8 and 9 = Pattern A; Lanes 3 and 4 = Pattern B

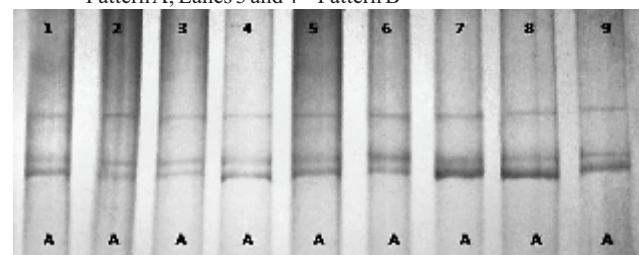


Fig 2. Resolution of fragment of promoter region of lactoferrin gene by 8% non denaturing PAGE showing SSCP variants. Lanes 1-9 = Samples of healthy animals; Lanes 1-9 = Pattern A

Table 1 Position of SNP's in comparison with reference sequence			
Position of nucleotide	Mastitic sample	Healthy sample	<i>Bubalus Bubalis</i> (Ref) EF650854.1
-155	G	C	C
-156	G	C	C
-112	G	A	G
-19	T	C	T
+35	T	C	T
-241 (b/w -241 & -240 of ref.)*	T	T	-
-245 (b/w -244 & -243 of ref.)*	C	C	-

(1994) sequence (Table 2). The T–C transition (+35) was observed in the 5' UTR of exon1, 4 bp upstream of the start codon which could play a crucial role in the regulation of gene expression. The BLAST results of the consensus 5' flanking region of bubaline lactoferrin gene revealed 99% homology with *B. bubalis*, 94% with *Bos taurus*, 94% with *Bos grunniens*, 94% with *Bos indicus* and 92% with *Capra hircus*. In phylogenetic tree, fragment of 5' flanking region lactoferrin gene of *B. bubalis* was found more closer to *B. taurus* than to *C. hircus*. In the phylogenetic analysis of promoter region of the lactoferrin gene, *Sus scrofa* was the farthest species.

PCR-RFLP analysis: The digestion of 261 bp PCR product of exon 15 with *TaqI* resulted in similar restriction pattern between healthy and mastitic animals. It detected single genotype (viz. xx.) in all the 40 samples. Digestion with *NruI* also revealed single genotype viz. ss. in all the 40 samples. There seems to be no report available in literature on PCR-RFLP of lactoferrin gene exon 15 with these REs. No polymorphism was found with these REs within this locus. The BLAST analysis revealed 99% homology of buffalo sequence with *B. bubalis*, 97% with *B. taurus*, 97% with *B. grunniens*, 96% with *B. indicus*, 96% with *Ovis aries* and 96% with *C. hircus*. In phylogenetic analysis, lactoferrin gene of *B. bubalis* was found more closely related to *Bos taurus* than to *C.*

Table 2 Comparison with Seyfert <i>et al.</i> (1994) <i>Bos taurus</i> sequence				
Position of nucleotide	Mastitic sample	Healthy sample	<i>Bubalus bubalis</i> (Ref) EF650854.1	Seyfert <i>et al.</i> (1994)
-112	G	A	G	G
-19	T	C	T	C
+35	T	C	T	C
-156	G	C	C	G

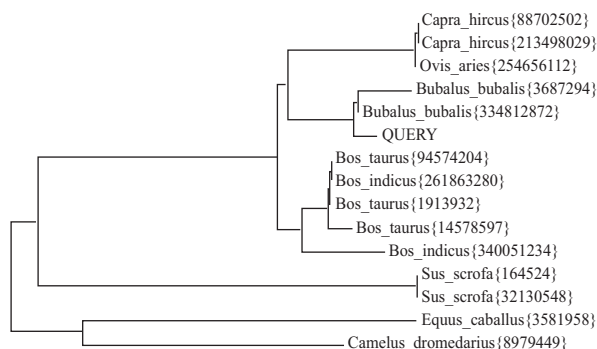


Fig 3. Phylogenetic tree of lactoferrin gene exon 15 depicting the evolutionary relationship among different species

hircus. In the phylogenetic analysis of exon 15 of the lactoferrin gene, *Equus caballus* was the farthest species (Figs. 3 and 4).

In this study, there were 5 SNPs in the promoter region of lactoferrin gene among healthy and mastitic animals, but there was no significant association of lactoferrin gene promoter region with mastitis. In the exon 15 of the lactoferrin gene no polymorphism was found using *TaqI* and *NruI* REs, thus, there was no association of lactoferrin gene exon 15 with mastitis. Kathiravan *et al.* (2009) investigated polymorphism in the 5' flanking region of bubaline (*B. bubalis*) lactoferrin gene in five different Indian buffalo breeds (Murrah, Jaffarabadi, Marathwada, Toda and Pandharpuri) using PCR-SSCP and revealed two SNPs at 57 and -61 positions in the putative estrogen response element and one SNP at -636 positions in putative recognition sequence for IRF-1. Li *et al.* (2004) from China investigated polymorphism of bovine lactoferrin gene in 100 Holstein dairy cattle using PCR-SSCP and its association with mastitis. In our study no significant

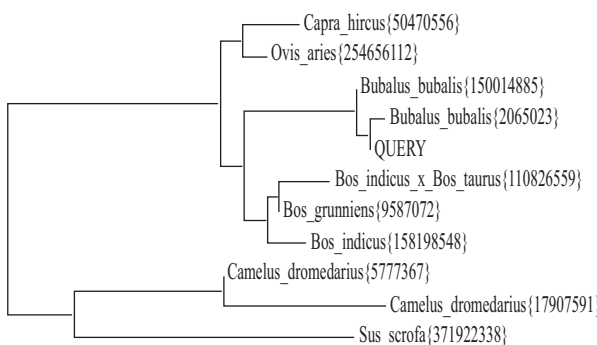


Fig 4. Phylogenetic tree of lactoferrin gene promoter region depicting the evolutionary relationship among different species

association was observed between lactoferrin gene and mastitis resistance, hence the observations need to be confirmed on large sample size.

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