Polymorphism in TLR4 gene and its association with mastitis in Murrah buffaloes

A B Gulhane and M L Sangwan*
Department of Animal Biotechnology, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar 125 004, India

Received 7 February 2011; revised 23 July 2011; accepted 7 September 2011

Genomic DNA was isolated from blood samples, collected from 60 unrelated Murrah buffaloes (Bubalus bubalis) including 30 animals suffering/suffered from mastitis. Exon 3 of TLR4 gene was amplified from genomic DNA, which gave a PCR product of 386 bp. The digestion of 386 bp PCR product of TLR4 gene with StyI restriction enzyme detected two genotypes, viz., aa and ab with frequencies 0.57 and 0.43, respectively and alleles a and b were observed with frequencies 0.78 and 0.22, respectively. There was significant (P ≤ 0.05) difference in the genotypic frequencies of these two genotypes in healthy and mastitis animals. The frequency of aa genotype was significantly higher (P ≤ 0.05) in healthy animals, which indicates the association of aa genotype with resistance to mastitis. In phylogenetic analysis, TLR gene of B. bubalis was found more closely related to goat and sheep than taurine and zebu cattle.

Keywords: Mastitis, Murrah buffalo, PCR-RFLP, TLR4

Mastitis continues to be an economically important infectious disease of bovine throughout the world. The estimated annual losses due to clinical and subclinical mastitis in India are ₹ 1683.83 crores and ₹ 4369.32 crores, respectively1. Many countries have recently updated their breeding programme to include mastitis resistance2 as a trait for selection of animals. Mastitis is caused by many factors, which include microorganisms, genetic factors and poor management practices. Many genes are found to be associated with mastitis, such as, major histocompatibility complex (MHC), β-defensin, lactoferrin, lysozyme, toll like receptors (TLR), etc.

TLRs play an important role in the recognition of components of pathogens and subsequent activation of the innate immune response, which leads to development of adaptive immune responses3-5. TLR4 mainly recognizes the molecular patterns presented by lipopolysaccharides (LPS) that is a component of the ectoblast of Gram-negative bacterium, and induces the over expression of inflammatory factors IL-1, IL-6 and IL-10, which participate in innate immune responses and then confer disease resistance6. A previous study showed increased expression of TLR4 during mastitis infection in the bovine mammary gland7.

Analysis of proteins extracted from fat globule membranes of bovine milk has identified several proteins, including CD14, TLR2 and TLR48. These reports indicate that the mammary gland may have direct role in detecting an infection and that TLR4 proteins are involved in pathogen recognition in the mammary gland. Therefore, this gene may be a potential candidate for use in marker-assisted selection to enhance mastitis resistance in dairy animals.

TLR4 gene is 4256 bp long consisting of 3 exons and 2 introns in buffalo and is located on chromosome number 8 (NCBI Acc. No. EU386358) and encodes 841 amino acids. Among these regions, the putative co-receptor (a receptor that suppose to exit but have not direct evidence) binding region is a very important region for pathogen recognition and it is encoded by exon 3 of TLR4 gene9.

In the present study, 386 bp segment of TLR4 (exon 3) gene from co-receptor binding region in Murrah buffalo was amplified by polymerase chain reaction (PCR) and genetic polymorphism of population was detected by digesting the fragment with restriction endonuclease StyI.

For the present study, blood samples were collected upto 3rd lactation from 60 unrelated Murrah buffaloes (Bubalus bubalis) from Government Livestock Farm, Hisar; Central Institute for Research on Buffaloes, Hisar; Buffalo Research Centre, College of Animal Sciences and Veterinary Clinics, CCS Haryana Agricultural University (CCCHAU), Hisar, India. Among 60 animals, 30 were suffering/suffered from mastitis and rest were healthy. Genomic DNA was extracted from blood samples as described elsewhere10. The quality and quantity of genomic DNA was determined as per the standard

*Author for correspondence:
Email: sangwanml@yahoo.co.uk
Genomic DNA was evaluated on 0.7% (w/v) agarose gel electrophoresis and all samples showed single intact distinct band near the wells. Primers (forward primer: 5’ AGACAGCATTTCACTCCCTC 3’ and reverse primer 5’ ACCACCGACACACTGATGAT 3’) described elsewhere were used to amplify exon 3 of TLR4 gene. PCR reactions were carried out in 25 µL reaction mixture containing, 3.5 µL of 10× Taq DNA polymerase buffer (New England Biolabs), 0.5 µL dNTPs (MBI fermentas), 0.25 µL (40 ng) each primer, 0.5 µL (2.5 U) Taq DNA polymerase (New England Biolabs) and 1 µL of genomic DNA for each sample. The 25 µL reaction mixture was kept for amplification in programmed thermocycler (BioRad). After initial denaturation at 95°C for 5 min, 35 cycles were given, each comprising of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. After final extension, PCR products were resolved in 1.8% agarose gel. Exon 3 of TLR4 gene was amplified from genomic DNA of Murrah buffalo to obtain a 386 bp PCR product which was resolved on 1.8% agarose gel (Fig. 1).

For RFLP analysis, 15 µL of the amplified PCR products were digested with 10 U of StyI restriction enzyme (site 5’ C*CWWGG/3’ GGWWC*C) in a final volume of 20 µL at 37°C overnight. The restriction fragments were resolved by electrophoresis with 3% agarose gel (Sigma, USA). The fragments were visualised by gel documentation system.

Cloning of PCR products of TLR4 gene were performed using the pJET1.2/blunt cloning vector supplied with Clone JET™ PCR cloning kit (Fermentas). The JM107 strain of Escherichia coli was used as a host system for transformation with recombinant plasmid vector. Clones were screened by touch PCR using TLR4 gene specific primers to check the presence of insert. Recombinant plasmid DNA was isolated by alkaline lysis method and resolved on 0.7% agarose gel. It was sequenced using automated DNA sequencer in the Department of Animal Biotechnology, College of Veterinary Sciences, CCSHAU, Hisar. The sequences were analyzed using NCBI BLASTn. ClustalW2 program was used for multiple sequence alignment of TLR4 gene sequences of sheep, goat, taurine and zebu cattle and phylogenetic tree was also constructed using ClustalW2 and Jalview programs. The gene frequencies were tested by student’s t test.

The digestion of 386 bp PCR products with StyI resulted in two different genotypes, viz., aa and ab (Fig. 2). The number of healthy animals with aa and ab genotypes were 21 and 9, while the mastitis cases with aa and ab genotypes were 13 and 17, respectively. The overall frequencies of aa and ab genotypes were 0.57 and 0.43; while their frequencies in mastitis cases were 0.43 and 0.57, respectively (Table 1). However, the differences in the genotypic frequencies of the two genotypes in healthy and mastitis animals were found significant (P<0.05). The genotype aa was found more frequently (0.70) in the group of healthy animals and, thus, it may be associated with resistance to mastitis. The overall frequencies of alleles a and b were 0.78 and 0.22, respectively and it may be due to natural selection.

### Table 1—TLR4 genotype frequencies in healthy and mastitis animals

<table>
<thead>
<tr>
<th>Category of animals</th>
<th>Genotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aa</td>
</tr>
<tr>
<td>Healthy</td>
<td>0.70 (21)</td>
</tr>
<tr>
<td>Mastitis</td>
<td>0.43 (13)</td>
</tr>
<tr>
<td>Total</td>
<td>0.57 (34)</td>
</tr>
</tbody>
</table>

*Figure in parenthesis is the number of animals*
favouring the fixation of allele \( a \). On the other hand, the frequencies of \( a \) allele in healthy and mastitis animals were 0.70 and 0.43, respectively.

So far, there has been no report available on PCR-RFLP of TLR4 gene with \( S_{ly} \) restriction endonuclease in buffalo. \( HinfI \) and \( DraI \) restriction endonucleases did not show any restriction site in partial exon III of TLR4 gene, while \( AluI \) restriction endonuclease exhibited two fragments of 112 bp and 282 bp in all Murrah buffaloes under study\(^9\). Two alleles \( a \) and \( b \) were identified by using \( AluI \) restriction endonuclease in Chinese Holstein, Sahi cattle and Chinese Simmental cattle\(^9\). In the present study, it was found that allele \( a \) showed lower somatic cell score than that of allele \( b \) and might play an important role in mastitis resistance in bovine as allele \( a \) was found more frequently in healthy animals.

Amplified PCR products of two different alleles \( a \) and \( b \) were cloned into pJET 1.2 blunt end cloning vector. The transformants having 386 bp TLR4 gene were streaked on Luria broth-ampicillin agar plates, which yielded 20 to 70 colonies in each plate after 17 h of incubation at 37°C. Colonies were subjected to touch PCR using gene specific primer pair for confirmation of insert in the vector. Plasmids were isolated from recombinant clones by alkaline lysis method.

Nucleotide sequencing of alleles \( a \) and \( b \) showed that allele \( a \) can be distinguished from allele \( b \) because of substitution of cytosine by guanine (C→G) at 217 nucleotide position in allele \( b \), which causes substitution of amino acid arginine by threonine. ClustalW2 program was used for multiple sequence alignment of buffalo TLR4 gene sequence with other species DNA sequences retrieved from NCBI database. The accession numbers of the sequences are: EU386358.1 (\( B. \) bubalis), DQ839567.1 (\( Bos \) taurus), DQ922636.1 (\( Ovies \) aries), EU386357.1 (\( B. \) indicus) and DQ922635.1 (\( Capra \) hircus).

The phylogenetic tree was also constructed using ClustalW2 program to see the relatedness among the species with respect to TLR4 gene fragment (Fig. 3). In the phylogenetic tree, allele \( a \) and \( b \) of \( B. \) bubalis were found more closely related to goat (\( C. \) hircus) and sheep (\( O. \) aries) than taurine cattle (\( B. \) taurus) and zebu (\( B. \) indicus). Eventually, present study helps in establishing a possible association between TLR4 gene and mastitis in Murrah buffaloes. However, these findings need to be explored in a large population size.

Fig. 3—Phylogenetic tree of TLR4 gene depicting the evolutionary relationship among different species

References