# TLR-4 GENE POLYMORPHISM IN INDIAN MURRAH BUFFALOES (*Bubalus bubalis*)

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## ABSTRACT

TLR-4 is an important candidate gene, which affects the host disease resistance. Its role in pathogen recognition and subsequent initiation of the inflammatory and immune responses makes it a suitable candidate gene for enhancing disease resistance in dairy animals. TLR-4 gene also provides an ideal model to study the consequences of genetic variation and their relation to the function of receptor and their susceptibility to diseases. TLR-4 gene mainly recognizes the conserved lipopolysaccharide and lipoteichoic acid patterns of Gram-negative and Gram-positive bacteria, respectively. The present study was undertaken with the objectives of sequence characterization and studying the genetic variation in exon 3 of Toll-like receptor 4 (TLR-4) gene in Murrah buffalo by PCR-RFLP. Exon 3 (2265 bp) of TLR-4 gene was amplified by PCR using oligonucleotide primers designed by Primer 3 plus software and subsequently, RFLP study was carried out to identify genotypes of the animals with *Bsp1286I* restriction enzyme. It exhibited AA, AB and BB genotypes in exon 3 for primer 3.1, 3.5, 3.6 and 3.7. Amplicons were sequenced and compared with published *Bubalus bubalis* (EU386358) sequence and variation at four nucleotide positions were occurs and out of which one non synonymous SNP result into change of amino acid (Threonine to Glutamine).

**Keywords**: *Bubalus bubalis*, buffaloes, TLR4 gene, Murrah buffaloes, PCR-RFLP, *Bsp*1286I

## INTRODUCTION

India ranks first in world for buffalo and has 57% of world's buffalo population. Total number of buffaloes in the country is 108.7 million numbers (Nineteenth Livestock Census, 2012). There are increasing in momentum on buffalo genetic improvement program in India particularly focused on milk production traits, institutionalizing organized evaluation and selection system, and increasing use of artificial reproductive technologies (ARTs). It is an acknowledged fact that the buffalo is a better converter of coarse feeds into fat-rich milk even under harsh agro-climatic situations. The buffalo contributes about 54% of the total milk produced in India and Murrah buffalo is backbone of milk industry of India. The Murrah buffalo, which is described as the "Asian tractor",

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is in fact triple purpose animal-for milk, meat and work. Murrah buffalo is the finest genetic material of milk producing buffalo in the world. This breed has beaten the best dairy cows of the world in performance.

Selection of animals solely based on their milk yield increasing the incidence of disease. A managemental, chemotherapeutic and prophylactic measure reduces the incidence of mastitis and other infectious diseases but not up to desired level. So, enhancing the host genetic resistance is another approach to reduce the incidence of infectious diseases. The use of polymorphic markers in breeding programmes could make selection more accurate and efficient.

Toll-like receptors (TLR) are a multigene family of pattern recognition receptors that are members of the TLR-interleukin 1 superfamily. Currently, at least 13 members of the TLR family have been identified in mammals; genes encoding 10 of these receptors have been mapped to the bovine genome (McGuire *et al*., 2005). Toll-like receptors (TLRs) are membrane proteins mainly expressed in macrophages and dendritic cells (De Schepper *et al*., 2008). Toll like receptor-4 (TLR-4) gene is an important pattern recognition receptor that recognizes endotoxins associated with gram negative bacterial infections (Takeda *et al*., 2003). They are the first line of defense against pathogens by sensing their presence through the recognition of specific molecular signatures associated with the invading pathogen, which are collectively known as pathogen-associated molecular patterns (PAMPs) (Lu *et al*., 2008). Its role in pathogen recognition and subsequent initiation of the inflammatory and immune responses and highly polymorphic nature in the bovine species, make it a suitable candidate gene for use in MAS for enhancing disease resistance in dairy animals (White *et al*., 2003).

The bovine TLR4 gene has been mapped to the distal end of BTA8 (White *et al*., 2003). TLR-4 gene consisting of 3 exons and 2 introns. Bovine TLR4 has three exons, exon 1 includes coding base pairs 1-95, exon 2 consists of base pairs 96-260 and exon 3 comprises base pairs 261-2526. The whole genomic length is estimated to be approximately 11 kb, of which the first intron comprises about 5 kb and the second, 3 kb. Bovine TLR-4 protein consists of 841 amino acids (Wang *et al*., 2007).

Although, the economic importance of buffaloes has always been known, yet very little work has been carried out to exploit the genetic potentials of this animal. Though studies have been carried out on characterization in cattle, similar studies in buffaloes are scarce. Information on TLR-4 gene polymorphism in Murrah buffalo is very scanty; hence, the present study was undertaken to reveal the Polymerized Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) pattern of the TLR-4 locus in the Murrah buffalo.

## MATERIALS AND METHODS

### Buffalo population, sampling, DNA extraction and quality checking

To analyze the status of TLR-4 / *Bsp*1286I polymorphism, blood samples (10 ml) were collected randomly from 102 unrelated lactating animals from the herd of Murrah buffaloes well maintained at cattle yard of National Dairy Research Institute (NDRI), Karnal, Haryana state. DNA was extracted using standard protocol by the phenol: chloroform extraction procedure (Sambrook *et al*., 2001). Quality and quantity of DNA was estimated by spectrophotometer method. Quantity of DNA was calculated using the following formula:

Quantity of DNA in µg/ml = $O.D._{260}$ x 50 x Dilution Factor

The overall yield of DNA in Murrah buffalo ranged from 350 to 510 µg with a mean of 414.64±4.87 µg/ml and the overall purity of DNA ($OD_{260/280}$) ranged from 1.70 to 1.90 with a mean of 1.80±0.01. The results are in agreement with earlier reported yields of DNA ranges 300 to 500 µg per 10 ml of blood of Murrah buffaloes (Mitra, 1994).

**Molecular genotyping**

The primer pairs for TLR-4 gene were designed by using bioinformatics tools, Primer 3 plus software on the basis of published *Bubalus bubalis* (EU386358) sequence.

PCR was carried out in a final reaction volume of 25 µl. Amplification cycling conditions involved initial denaturation 94°C for 10 minutes, followed by 35 cycles at 94°C for 1 minute, annealing temperature varied for each primer (range 53°C to 55°C) for 45 seconds and 72°C for 1 minute with a final extension at 72°C for 10 minutes. After confirmation of amplification of target region, PCR-RFLP analysis, by *Bsp*1286I restriction enzyme (RE) had been carried out. PCR amplified product 10 µl of each primer was digested with 5 units of the *Bsp*1286I (5'…GD*GCH'C…3'; D* = A or G or T) in a 20 µl total reaction and kept for incubation at 37°C for 4 h in incubator.

The RE digested products were separated by electrophoresis on a 2.5% agarose gel in 1X Tris-borate-EDTA (TBE) at 100 volts for 30, 60, and 90 minutes till complete separation and visualization of all fragments of RE digested gene fragments, DNA ladder and PCR marker.

**Sequence data analysis**

Sequence data were analyzed mostly by DNASTAR software. Sequence data was analyzed by using Chromas (Ver. 1.45, http: //www. technelysium. com.au/chromas.html). Sequence data from variants of different regions were subjected to multiple alignments (DNASTAR, Clustal W) for identifying the SNPs. Seven contigs were re-assembled with overlaps to form the contiguous sequence of bubaline TLR-4 gene and compare with base sequence (*Bubalus bubalis* EU386358).

**Database search**

The database search of sequences for a possible match to the DNA sequence of TLR-4 gene was conducted using the Basic Local Alignment Search (BLAST) algorithm available at the National Center for Biotechnology Information (NCBI, Bethesda, MD) database to know the sequence homology with the corresponding regions of other species.

**Statistical analysis**

The restriction fragments were resolved in 2.5% agarose gel and visualized in gel documentation system. The band patterns were scored to assign specific genotype and the genotypic and gene frequencies were calculated for each genotype and the alleles thereof. The frequencies of gene and genotypes were estimated for the identified locus as per the method suggested by Falconer and Mackay (1998).

## RESULTS AND DISCUSSION

The amplicons of exon 3 in TLR-4 gene obtained by optimization of PCR conditions and were resolved in 1.5% agarose gel and visualized in gel documentation system (Table 1 and Figure 1). PCR-RFLP analysis of each PCR product was

carried out using *Bsp* 1286I RE for all samples. The restriction fragments were resolved in 2.5% agarose gel and visualized in gel documentation system.

The restriction fragment patterns generated by using *Bsp* 1286I RE were identified as AA, AB and BB genotypes. The band pattern size (Table 2 and Figure 2), genotypic and allelic frequencies of these primers have been presented (Table 3). Amplicons 3.2, 3.3 and 3.4 of exon-3 of TLR-4 gene did not have any cutting site with *Bsp*1286I.

For Amplicon 3.1 three genotypes AA, AB and BB with their respective frequencies of 0.274, 0.206 and 0.520. The allelic frequencies of A and B alleles were 0.377 and 0.623 respectively. Amplicon 3.5 exhibited AA, AB and BB genotypes

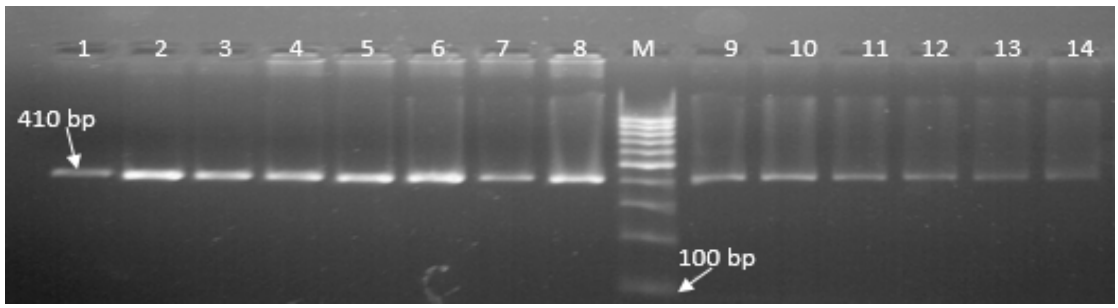


Figure 1. Resolution of primer 3.1 (exon3) of TLR-4 gene in Murrah buffalo.
Lane 1 to 14 : PCR product (410 bp).
Lane M : 100 bp molecular marker (ladder).

Table 1. Amplicons of Exon-3 in TLR-4 gene with designed specific primers.

| Primer | Primer sequence ( 5'-3') | | Region amplified | Product size | Annealing temp. (°c) |
|---|---|---|---|---|---|
| 3.1 | F | 5'-CTGGGCTCTCAAGTTTACGG-3' | 1571-1980 | 410 | 54 |
| | R | 5'-AACCAGCCGGTTGATTTTTA-3' | | | |
| 3.2 | F | 5'-GGCTGGTTTTGGGAGAATTT-3' | 1974-2384 | 420 | 55 |
| | R | 5'-TGTGAGAACAGCAACCCTTG-3' | | | |
| 3.3 | F | 5'-CAAGGGTTGCTGTTCTCACA-3' | 2365-2842 | 478 | 54 |
| | R | 5'-GAGCGAGTGGAGTGGTTCAT-3' | | | |
| 3.4 | F | 5'-TGCTCCCTGACATCTTCACA -3' | 2664-3091 | 440 | 54 |
| | R | 5'-TCTGACAAGTGGCATTCCTG-3' | | | |
| 3.5 | F | 5'-TCAGGAATGCCACTTGTCAG-3' | 3071-3476 | 406 | 55 |
| | R | 5'-CAGGTCTGGGCAATCTCATA-3' | | | |
| 3.6 | F | 5'-CCAGAGCCGATGGTGTATCT-3' | 3433-3841 | 410 | 54 |
| | R | 5'-CACTGAATCACCGGGCTTT-3' | | | |
| 3.7 | F | 5'-GGTAAACCCACGAGTCCAGA-3' | 3659-3930 | 286 | 53 |
| | R | 5'-CCCCCGGGAAGTTCTATATT-3' | | | |

Table 2. PCR-RFLP bands using *Bsp* 1286I *RE* for TLR4 gene in Murrah buffaloes.

| Primer no. | Restriction fragments sizes (bp) of genotypes | | |
|---|---|---|---|
| | **AA** | **AB** | **BB** |
| 3.1 | 410 | 410, 236 and 174 | 236 and 174 |
| 3.2 - 3.4 | No cutting site | | |
| 3.5 | 406, 234 and 176 | 406, 234, 176, 174 and 100 | 176, 174 and 100 |
| 3.6 | 410 and 310 | 310, 200, 142 and 100 | 200, 142 and 100 |
| 3.7 | 286 and 186 | 286, 186 and 100 | 186 and 100 |

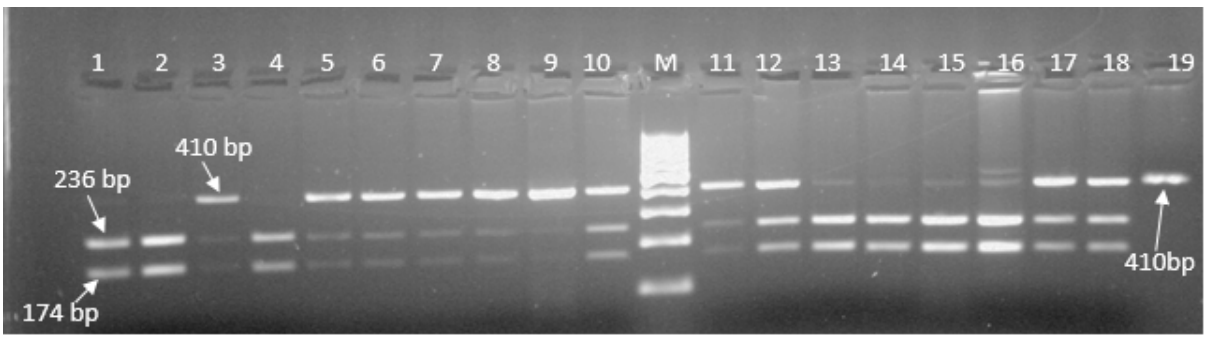


Figure 2. Resolution of PCR-RFLP of primer 3.1 (exon 3) of TLR-4 gene in Murrah buffalo using *Bsp* 1286I RE.

Lane 3, 5-9, 11 : AA Genotype (410 bp)
Lane 1, 2, 4, 13-16 : BB Genotype (236, 174 bp)
Lane 10,12,17,18 : AB Genotype (410, 236, 174 bp)
Lane 19 : PCR Product (410 bp)
Lane M : 100 bp DNA Ladder

Table 3. Genotypic and gene frequencies of TLR4/*Bsp* 1286I in Murrah buffaloes.

| Primer | Genotypic frequency | | | Allelic frequency | |
|---|---|---|---|---|---|
| | **AA** | **AB** | **BB** | **A** | **B** |
| 3.1 | 0.274 (28)* | 0.206 (21) | 0.520 (53) | 0.377 | 0.623 |
| 3.5 | 0.579 (59) | 0.029 (03) | 0.392 (40) | 0.594 | 0.406 |
| 3.6 | 0.676 (69) | 0.275 (28) | 0.049 (05) | 0.814 | 0.186 |
| 3.7 | 0.098 (10) | 0.774 (79) | 0.128 (13) | 0.485 | 0.515 |

*Number of animals have been indicated in parenthesis for that genotype.

with frequencies as 0.579, 0.029 and 0.392 and allelic frequencies of A was 0.594 and B was 0.406 respectively. Amplicon 3.6 exhibited AA, AB and BB genotypes with frequencies as 0.676, 0.275 and 0.049 and allelic frequencies was 0.814 and 0.186 for A and B alleles respectively. Amplicon 3.7 also exhibited AA, AB, and BB genotypes with frequencies of 0.098, 0.774 and 0.128 respectively and frequencies of 0.485 and 0.515 for A and B alleles respectively. It was reported by Sentitula *et al.* (2012) in Murrah buffalo three genotypes AA, AB and BB with BB genotypes frequency higher than other two genotypes by *Hae*III RE and by *Taq*I RE two genotypes *AB and BB* in TLR-4 gene. Gulhane and Sangwan (2012) also reported two genotypes aa and ab in exon 3 of TLR-4 gene in Murrah buffalo with *Sty*I RE. Wakchaure *et al.* (2012) reported three genotypes CC, CD and DD in exon 3 region of TLR4 gene of Sahiwal cattle and CC has higher frequency than other two genotypes by *Hinf* I RE.

The allelic distribution in present study does not clearly indicate any notable deviation but overall B allele was higher than A. In Murrah buffalo for TLR-4 gene, B allele was more prevalent than A allele reported by Sentitula *et al.* (2012). It was reported in exon 3 of TLR-4 gene in Brazilian Holsteins with frequencies of 53.5% for allele C and 46.5% for allele T (de Mesquita *et al*., 2012).

**Sequence alignment and homology across species**

The contiguous TLR-4 nucleotide sequence was subjected to BLAST at NCBI database and revealed 97%, 97%, 99%, 98% and 80% homology with *Bos indicus, Bos taurus, Ovis aries, Capra hircus and Homo sapiens* respectively (Table 4).

**SNP identification**

The partial CDS (Coding DNA Sequence) of Murrah TLR-4 gene (exon3) were conceptually translated and compared with published *Bubalus bubalis* (EU386358) sequence. Variation at four nucleotide sequences at positions 1650, 2075, 3642 and 3643 has been found and one non synonymous SNP result into change of amino acid (Threonine to Glutamine) (Table 5 and Figure 3).

Gulhane and Sangwan (2012) reported 1 SNPs at 217 nucleotide position in exon 3 of TLR-4 gene in Murrah buffalo with amino acid change in arginine by threonine. In another study on TLR4 gene in mixed population of cattle, 32 SNPs were found among the 40 individuals and 28 SNPs of these were coding SNPs (White *et al*., 2003). In Holstein cattle, Sharma *et al.* (2006) have reported 3 SNPs with 1 in promoter region (P-226) and 2

Table 4. BLAST analysis of TLR-4 nucleotide sequence of Murrah buffalo.

| Accession No. | Species | Identity |
|---|---|---|
| DQ839567.1 | *Bos taurus* | 97% |
| EU386357.1 | *Bos indicus* | 97% |
| DQ922636.1 | *Ovis aries* | 99% |
| AY753179.1 | *Sus scrofa* | 89% |
| AB445638.1 | *Homo sapiens* | 80% |

Table 5. SNPs identified in coding region of TLR-4 gene (Murrah buffaloes).

| Coding region | Position | Base change | Amino acid substitution* |
|---|---|---|---|
| Exon-3 | 1650 | A>C | - |
| | 2075 | T>C | - |
| | 3642 | A>C | - |
| | 3643 | C>A | T>Q |

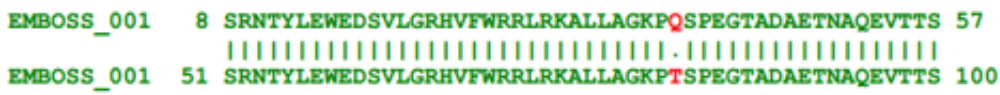


Figure 3. Amino acid substitution: Threonine (T) to Glutamine (Q) in TLR-4 gene (Exon-3) in Murrah buffalo.

in exon 3 (1656 and 2021). Wang *et al.* (2007) identified 1 SNP at nucleotide 1397 in exon 3 of TLR-4 gene. Wang *et al*. (2008) have reported in Chinese mixed population of cattle that 31 SNPs scattered through 5 flanking region to exon 3 of TLR-4 gene with 12 of which were novel SNPs and sixteen of these SNPs were in coding regions (cSNP); but five of these SNPs induced amino acid substitution. Wakchaure *et al.* (2012) reported six SNPs in exon 3 region of TLR4 gene of Sahiwal cattle and out of which one non synonymous SNP result into change of amino acid (Valine to Isoleucine).

One genomic nucleotide sequence of exon 3 of Murrah TLR-4 gene was submitted to Genbank of NCBI database accession number HM143862.

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