Molecular characterization of Toll Like Receptor 4 *(TLR-4)* gene in Jaffrabadi buffalo (*Bubalus bubalis)*

G.S. Sonawane[1], V.B. Dongre[2*], A.R. Ahlawat[3] and S.R. Badhe[4]

[1]Livestock Farm Complex, Krantisinh Nana Patil College of Veterinary Science, Shirwal Dist- Satara [2]Livestock Farm Complex, College of Veterinary & Animal Sciences, Udgir Dist- Latur, MAFSU, Nagpur; [3]Department of Animal genetics & Breeding, College of Veterinary Science & Animal Husbandry,Anand, Kamdhenu University, Gandhinagar; [4]Department of Livestock Products Technology, Nagpur Veterinary College, MAFSU, Nagpur
*Corresponding Author e-mail: vilasdongre@mafsu.ac.in

Journal of Livestock Science (ISSN online 2277-6214) 16: 121-126
Received on 24/10/24; Accepted on 4/1/25; Published on 15/1/25
doi. 10.33259/JLivestSci.2025.121-126

Abstract

The present study was conducted on 60 unrelated Jaffrabadi buffaloes from Cattle Breeding Farm (CBF), College of Veterinary Science & Animal Husbandry, Junagadh to explore genetic polymorphism on Toll like receptor 4 (TLR-4) which is a candidate gene mastitis resistance. Total nine primers were used for amplification. The gene amplified and the PCR products were 180 bp, 280 bp, 410 bp, 420 bp, 478 bp, 440 bp, 406 bp, 410 bp and 286 bp for primers 1 to 9, respectively. Amplicon were subjected to PCR-RFLP analysis using *Alu I*restriction enzyme. PCR-RFLP analysis with *AluI* restriction enzyme of each primer produced different patterns, resulting in their identification as homozygotes AA, BB and heterozygote AB genotypes. *Alu I* revealed polymorphism at primers 3, 4, 5, 7 and 9 with AA, AB and BB genotypes. Multiple alignment revealed a total of 6 mutations. Out of these six mutations, three were non-synonymous resulting in change in threonine to methionine, arginine to valine and threonine to glutamine. The contiguous TLR-4 nucleotide sequence was subjected to basic local alignment search tool (BLAST) at NCBI database to know the sequence homology with the corresponding regions of other species. Therefore, further study on large number of samples is required to explore possibility of genetic polymorphism and its association with mastitis resistance in Jaffrabadi buffaloes.

Key Word: Jaffrabadi buffaloes; PCR-RFLP; Toll like receptor-4

Introduction

The milk production of India is 236.35 million tonnes in 2023-24 and ranks first in the world. Out of the total milk production of the country, buffalo contribute 56% milk (NDDB, 2024). Jaffrabadi is major milk production breed in Saurashtra region of Gujarat. The Jaffrabadi buffalo is a notable breed originating from the Gir forest region of Gujarat, India. The breeding tract includes Amreli, Bhavnagar, Jamnagar, Junagadh, Porbandar and Rajkot districts of Gujarat state. It is one of the heaviest and most productive buffalo breeds in the country, known for its high milk yield. The average milk yield is around 2000-2500 liters per lactation with 7.7. fat %, making it one of the top milk-producing buffalo breeds (Kathiaravan et al, 2007). Jaffrabadi has been characterized for various genes at the molecular level. Mastitis is a disease having most deleterious economic impact on dairy industry in both developed and developing countries (Rasouli et al 2017; Danilov et al 2019). Toll like receptor 4 (TLR-4) is an important candidate gene, which affects the host disease resistance. It is associated with the mastitis resistance and is not characterized in Jaffrabadi breed. *Gene* possesses *3* exons and 2 introns. Entire coding sequence of TLR-4 gene consist of 2526 bp. In cattle and buffalo this gene has been revealed polymorphism suggesting its establishment as a molecular marker to understand basis of variation in mastitis resistance between animals which indeed will be beneficial for marker assisted selection in buffaloes. Therefore, the aim of this work was to characterize TLR4 gene and explore genetic polymorphism in Jaffrabadi buffaloes.

Materials and Method

A total of 60 buffaloes from field and from Cattle Breeding Farm (CBF), College of Veterinary Science & Animal Husbandry, Junagadh were selected for blood sample collection. The latitude of the farm is 21.515471, and the longitude is 70.456444. About10 ml of blood collected from each buffaloes in sterile vacutainer tube containing 15% of 0.12 ml EDTA solution. The samples were transported to the laboratory in an icebox and stored at 4^0C till further processing for DNA isolation. DNA was isolated from 10 ml of blood by phenol-chloroform method, as described by Sambrook et al. (1989) with few modifications. The laboratory work and PCR was carried out at Department of Animal genetics & Breeding, College of Veterinary Sciences & Animal Husbandry, JAU, Junagadh. Quality of DNA was checked by electrophoresis by loading 2 ul DNA on 0.8% agarose in horizontal mini electrophoresis unit using 1xTBE as running buffer at 30-40 volts for about one and a half hours. After electrophoresis, the gel was stained with Ethidium Bromide solution (0.5 µg/ml). The gel was photographed by Gel Documentation System. Quality and quantity of DNA was estimated by spectrophotometer method. DNA (2 ul) was dissolved in 98 ul of double distilled water and loaded into a 100 ul cuvette. Optical Density (O.D.) was determined at wavelengths 260 nm and 280 nm in spectrophotometer against distilled water as blank sample. Quantity of DNA was calculated using the following formula:

Quantity of DNA in ug/ml $= O.D._{260}\ 50_{\ X}$ Dilution Factor

The ratio between OD260 and OD280 was calculated. The sample possessing a ratio of less than 1.7 and more than 2.0 was subjected to proteinase K digestion and DNA extracted with phenol chloroform isoamyl alcohol. The present study was conducted on bovine TLR-4 gene by using PCR-RFLP technique and a total of nine primers (one each for exon 1 and 2 and 7 for exon 3) were designed to cover entire coding sequence of TLR-4 gene using Primer 3 plus software. PCR conditions were standardized. After PCR amplification, the PCR product was checked on 1.5% agarose to verify the amplification of target region.

Figure 1: Adult Jaffrabadi buffalo

Results and Discussions

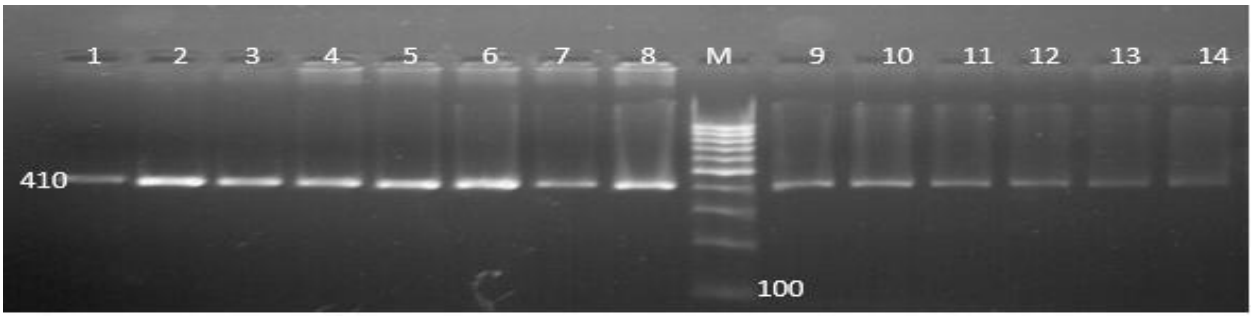
The PCR products were 180 bp, 280 bp, 410 bp, 420 bp, 478 bp, 440 bp, 406 bp, 410 bp and 286 bp for primers 1 to 9 respectively (Fig. 2 & 3). The regions amplified with amplicon sizes (Table-1).

PCR-RFLP Analysis

Amplicon were subjected to PCR-RFLP analysis using *Alu I*restriction enzyme. PCR-RFLP analysis with *AluI* restriction enzyme of each primer produced different patterns, resulting in their identification as homozygotes AA, BB and heterozygote AB genotypes (Fig 4). *Alu I* revealed polymorphism at primers 3, 4, 5, 7 and 9 with AA, AB and BB genotypes (Table-2).

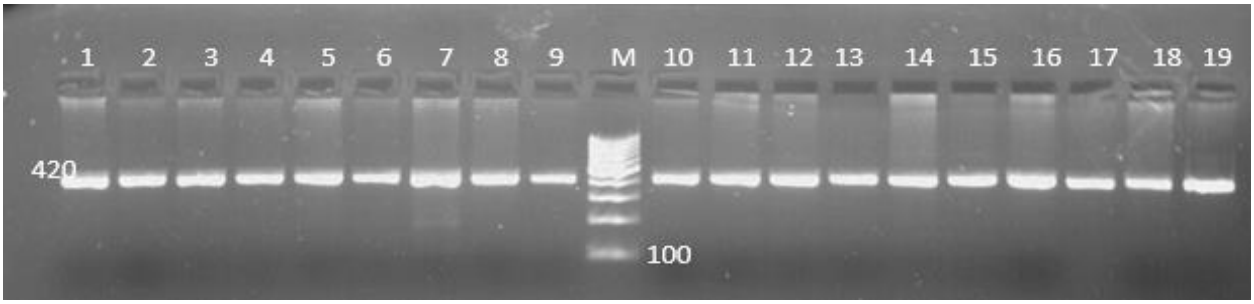
Table 1: PCR amplified product of TLR4 gene in Jaffrabadi buffalo

Exon	Primer No.	Annealing Temperature (ºC)	Amplified Region	Amplicon Size (bp)
Exon 1	1	54	881-1045	180
Exon 2	2	48	1168-1467	280
Exon 3	3.1	54	1571-1980	410
	3.2	54	1974-2384	420
	3.3	54	2365-2842	478
	3.4	54	2664-3091	440
	3.5	54	3071-3476	406
	3.6	54	3433-3841	410
	3.7	53	3659-3930	286



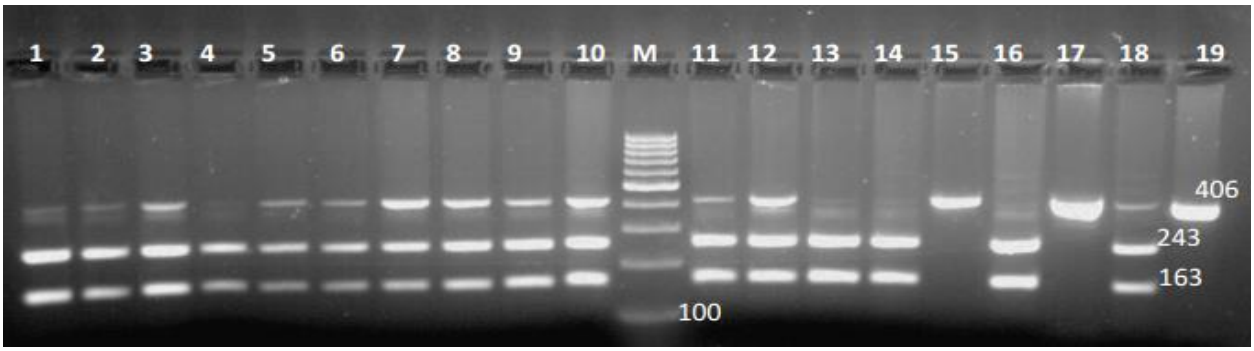
Lane 1 to 14: PCR product (410 bp); Lane M: 100 bp molecular marker

Fig.2 Resolution of primer 3.1 (Exon 3) amplicon of TLR-4 gene in Jaffarabadi buffaloes



Lane 1 to 19: PCR product (420 bp); Lane M : 100 bp molecular marker

Fig 3 Resolution of primer3.2 (Exon 3) amplicon of TLR-4 gene in Jaffarabadi buffaloes



Lane 1-3,5-12,18:AB Genotype (406, 243,163 bp), Lane 4,13,14,16: BB Genotype (243,163 bp), Lane 15,17 : AA Genotype (406 bp), Lane 19: PCR Product (406 bp)' Lane M: 100 bp DNA Ladder

Fig.4. PCR-RFLP in TLR-4 gene using *AluI* in Jaffarabadi buffaloes

Table 2: Amplicon Size and PCR-RFLP bands using *AluI*inTLR4 gene of Jaffrabadi buffalo

Primer No.	Restriction Fragments size (bp) of Genotypes		
	AA	AB	BB
1	--	---	121, 59
2	No Cutting Site		
3	410, 300, 238 and 100	300,238,100 and 72	238,100 and 72
4	420, 372, 293 and143	420, 372, 293, 143 and 69	293, 143 and 69
5	478,350,272 and 169	478,350,272,169 and 74	272,169 and 74
6	----	440, 337 and 94	337, 94 and 83
7	406	406, 243, and 163	243 and 163
8	---	---	292, 200 and 100
9	280,244,186 and 116	244,186 and 116	186 and 116

Table 3: Genotypic and Allelic Frequencies of *TLR4 gene for AluI* in Jaffrabadi buffalo

Primer*	Genotypic Frequency			Allelic Frequency	
	AA	AB	BB	A	B
1	0.000(00)	0.00(00)	1.000(102)	0.000	1.000
3	0.059 (06)	0.686(70)	0.254(26)	0.402	0.598
4	0.529(54)	0.176(18)	0.294(30)	0.617	0.383
5	0.088(09)	0.294(30)	0.618(63)	0.235	0.765
6	0.000 (00)	0.196 (20)	0.804(82)	0.098	0.902
7	0.088(09)	0.618(63)	0.294(30)	0.397	0.603
8	0.000 (00)	0.000	1.000(102)	0.000	1.000
9	0.186(19)	0.667(68)	0.147(15)	0.520	0.480

Number of animals exhibiting a particular genotype have been indicated in parenthesis

Allelic frequencies for A and B alleles for these primers were: 0.399 and 0.601; 0.617 and 0.383; 0.236 and 0.764; 0.396 and 0.604; 0.520 and 0.480 respectively. Primer 6 exhibited only 2 genotypes AB and BB with allelic frequencies of A and B as 0.098 and 0.902 respectively. Primer 1 and 8 revealed only BB genotype, whereas, Primers 2 did not show any cutting site for *AluI* in exon 2 (Table-3).

In accordance, Roldan-Montes *et al.*, (2020) reported total of 13 polymorphisms were identified for the sequenced regions of the *TLR4*, most of which are in the coding region. The association with the somatic cell score was highly significant ($p < 0.001$) for all identified polymorphisms of *TLR4* gene in Water Buffaloes. Sonawane *et al.*, (2018) reported that 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 in Murrah buffalo. 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 *HaeIII* RE and by TaqI 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 StyI 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*

Table 4: Primers for the study of TLR-4 gene in Jaffarabadi improvement

Exon (s)	Primer No.	Primer Sequence 5'- 3'	Amplicon Size (bp)
Exon 1	Primer-1	Forward 5'-CACAGAGCCACTTCTGGTCA-3' Reverse 5'- TTTTCAGAAGCAAGGCCAAG-3'	180
Exon 2	Primer-2	Forward 5'- ACCTGAGCTTTAACTACCT-3' Reverse 5'-AATATTTCTGCTGAATAGGA-3'	280
Exon 3	Primer-3	Forward 5'-CTGGGCTCTCAAGTTTACGG-3' Reverse 5'-AACCAGCCGGTTGATTTTTA-3'	410
	Primer-4	Forward 5'-GGCTGGTTTTGGGAGAATTT-3' Reverse 5'-TGTGAGAACAGCAACCCTTG-3'	420
	Primer-5	Forward 5'-CAAGGGTTGCTGTTCTCACA-3' Reverse 5'-GAGCGAGTGGAGTGGTTCAT-3'	478
	Primer-6	Forward 5'-TGCTCCCTGACATCTTCACA -3' Reverse 5'-TCTGACAAGTGGCATTCCTG-3'	440
	Primer-7	Forward 5'-TCAGGAATGCCACTTGTCAG-3' Reverse 5'-CAGGTCTGGGCAATCTCATA-3'	406
	Primer-8	Forward 5'-CCAGAGCCGATGGTGTATCT-3' Reverse 5'-CACTGAATCACCGGGCTTT-3'	410
	Primer-9	Forward 5'-GGTAAACCCACGAGTCCAGA-3' Reverse5'-CCCCCGGGAAGTTCTATATT-3'	286

Sequence Alignment and Homology across Species

The coding DNA sequence of bubaline TLR-4 gene compared with sequence of *Bubalus bubalis* with NCBI Accession Number EU386358. Multiple alignment revealed a total of 6 mutations. Out of these six mutations, three were non-synonymous resulting in change in threonine to methionine, arginine to valine and threonine to glutamine. Lactoferrin has been identified as a candidate gene for mastitis resistance in different dairy breeds (Huang et al., 2010; Pawlik et al., 2009; Zielak-Steciwko et al., 2014). The contiguous TLR-4 nucleotide sequence was subjected to basic local alignment search tool (BLAST) at NCBI database to know the sequence homology with the corresponding regions of other species. The sequence homology in TLR-4 gene for Jaffarabadi buffalo with the corresponding regions of other species revealed 97%, 97%, 99%, 98% and 80% homology with Bos indicus, *Bos taurus*, *Ovis aries*, *Capra hircus* and *Homo sapiens*, respectively.

Conclusions

Total six mutations occurs and three were non-synonymous resulting in change in threonine to methionine, arginine to valine and threonine to glutamine. Analysis of the coding region of TLR-4 gene using *Alu I* PCR-RFLP does not indicate a definite genotypic and allelic trend with respect to the primers in Jaffrabadi buffaloes; but allele B is more frequent than allele A.

References

1) Danilov M.S., Valitova N.V., Vorobyov A.L., Asangaliev E.A., Kalachev A.A. 2019. Needles of Abies Sibirica in the Treatment of Subclinical Mastitis in Cows. Journal of Livestock Science 10:9-18. doi. 10.33259/JLivestSci.2019.9-18
2) Gulhane, A.B. and Sangwan, Manohar. (2012). Polymorphism in TLR4 gene and its association with mastitis in Murrah buffaloes. Indian Journal of Biotechnology. 11. 330-332.
3) Huang J., Wang H., Wang C., Li J., Li Q., Hou M., Zhong J. Single nucleotide polymorphisms, haplotypes and combined genotypes of lactoferrin gene and their associations with mastitis in Chinese Holstein cattle. Mol. Biol. Rep. 2010;37:477–483. doi: 10.1007/s11033-009-9669-1.
4) Kathiaravan P., Mishra B.P., Sadana D.K., Katariya, R.S. and Singh, G. 2007. Buffalo Genetic Resources of India: Jaffarabadi. Monograph, National Bureau of Animal Genetic Resources, Karnal
5) Pawlik A., Sender G., Korwin-Kossakowska A. Bovine lactoferrin gene polymorphism and expression in relation to mastitis resistance-a review. Anim. Sci. Pap. Rep. 2009;27:263–271.
6) Roldan-Montes V, Cardoso DF, Hurtado-Lugo NA, do Nascimento AV, Santos DJA, Scalez DCB, de Freitas AC, Herrera AC, Albuquerque LG, de Camargo GMF, Tonhati H. 2020. Polymorphisms in TLR4 Gene Associated with Somatic Cell Score in Water Buffaloes (*Bubalus bubalis*). *Front Veterinary S*cience, 5;7:568249. doi: 10.3389/fvets.2020.568249. PMID: 33251259; PMCID: PMC7676892.
7) Rasouli A., Mosaferi S. 2017. Accuracy rate of mastitis on-farm test (udder check) in comparison to somatic cell count (S.C.C) in dairy herd. Journal of Livestock Science 8: 191-195.
8) Sambrook J., Fritsch E. F. and Maniatis T. 1989 Molecular cloning: a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

9) Sentitula, R. Kumar and B.R. Yadav. 2012. Molecular analysis of TLR4 gene and its association with intra-mammary infections in Sahiwal cattle and Murrah buffaloes. *Indian Journal of Biotechnology*, 11(3): 267-273
10) Sonawane G.S., Archana Verma, Gupta I.D. and Indrasen Chauhan. 2018. TLR-4 gene polymorphism in Indian Murrah buffaloes (*Bubalus bubalis*). *Buffalo bulletin*, Vol.37 No.1: 01-08
11) Wakchaure, R.S., I.D. Gupta, A. Verma, D. Kumar, R.S. Kumar and N.P. Soumya. 2012. Genetic polymorphism in TLR4 gene and its association with incidences of mastitis in Sahiwal cattle. *Wayamba Journal of Animal Science*, 307-311.
12) Zielak-Steciwko A.E., Pecka E., Kęsek M., Kuczaj M., Szulc T. Changes in the proportion of proteins fractions depending on lactoferrin polymorphism gene and the somatic cells count in the milk of Polish Holstein-Frisian and Polish Red-White cattle. Vet. Ir Zootech. 2014;66:83–89

.