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Tejas C Shende
Department of Animal Genetics and Breeding, College of Veterinary Science, Shirwal, Maharashtra, India

Vipul
Department of Animal Genetics and Breeding, College of Veterinary Science, Shirwal, Maharashtra, India

Dr. Abhijit K Barate
Department of Veterinary Biochemistry, KNP College of Veterinary Science, Shirwal, Maharashtra, India

Aakash Doiphode
Department of Animal Genetics and Breeding, College of Veterinary Science, Shirwal, Maharashtra, India

Corresponding Author:
Dr. Abhijit K Barate
Department of Veterinary Biochemistry, KNP College of Veterinary Science, Shirwal, Maharashtra, India

Sequence characterization of partial TLR4 gene in Indian crsossbred cattle

Tejas C Shende, Vipul, Dr. Abhijit K Barate and Aakash Doiphode

Abstract
In this study, partial exon 3 of TLR4 gene (T4CRBR2) of Indian crossbred cattle was characterized using PCR and bioinformatics tools. Analysis revealed that T4CRBR2 of Indian crossbred cattle had 100% identity with *Bubalus bubalis*, *Bos taurus* and Sahiwal breed sequences (both at nucleotide and amino acid level). At nucleotide level, high identity of T4CRBR2 (more than 99%) was also seen with Vechur cattle, *Bos indicus x Bos taurus* crossbreed cattle, Tharparkar cattle, bison and gayal sequences. At amino acid level 100% identity of T4CRBR2 was also seen with Vechur cattle, *Bos indicus x Bos taurus* crossbreed cattle, Tharparkar cattle, bison and gayal sequences. Nilgai T4CRBR2 had lowest identity compared to T4CRBR2 sequences of Indian crossbred cattle. In phylogenetic tree analysis T4CRBR2 of Indian crossbred cattle, *Bubalus bubalis*, *Bos taurus* and Sahiwal fall in one group, both at nucleotide and amino acid level.

Keywords: Tlr4, t4crbr2, crossbred, bioinformatics, India

Introduction
Toll-like receptors (TLRs) are multigene family of pattern recognition receptors (PRRs) that are members of TLR-interleukin 1 super family. They are important censors against microbial infection capable of stimulating both the innate and adaptive immune response [1]. Presently at least 13 members of family have been reported in mammals out of which only 10 receptors (TLR1 – TLR10) are expressed in bovine species [2, 3]. Amongst the different TLRs, TLR4 is essential for initiating the innate response against lipopolysaccharide (LPS) of Gram-negative bacteria [4-6]. It also recognizes bacteria (*Mycobacterium tuberculosis*) [7], fungi (*Aspergillus fumigatus*, *Cryptococcus neoformans*, *Candida albicans*) [8-10] parasites (*Setaria digitate*) [8] and host inflammagens such as heat-shock protein (Hsp60), fibrinogen, fibronectin, and hyaluronic acid [11, 12]. TLR4 is also involved in regulation of neutrophil lifespan [13], migration of polymorphonuclear leukocytes [14] production of cytokines [15] and intramammary innate immune response against gram-negative organisms [9].
The TLR4 gene of bovines contains 3739 nucleotides. Open reading frame (ORF) of TLR4 has 2526 nucleotides (contains three exons encoding for 841 amino acids) whereas remaining 1213 nucleotides are in untranslated region (UTR) (470 nucleotides 5′ UTR and 743 nucleotides in 3′ UTR, respectively) [16]. TLR4 ORF has been reported to contain several single nucleotide polymorphisms (SNP) [6, 17]. Previous studies have reported the association between TLR4 polymorphism and susceptibility to brucellosis [18], susceptibility to Paratuberculosis [19], mastitis resistance [12, 20-22], somatic cell score in Canadian Holsteins [23], somatic cell score in Chinese Holsteins, Sanhe and Chinese Simmental cattle [17]. Sequence characterization studies are important as they may reveals non-synonymous SNPs (nsSNPs) in genes. The nsSNPs could result in altered structure and/or function of corresponding protein due to change in proteins catalytic or ligand binding site, its inappropriate folding, inaccurate intracellular transport, decreased stability or loss of function of that gene product [24]
In this study, DNA sequence of partial exon 3 of TLR4 gene (T4CRBR2) of Indian crossbred cattle was characterized and analyzed using bioinformatics tools.

Materials and Methods
Blood sample was collected aseptically from Holstein Friesian crossbred bull maintained at KNP College of Vet. Sci. Shirwal, India. 5 ml of blood was collected and used for genomic DNA extraction using method as described previously [25].

T4CRBR2 was amplified with PCR using specific primers reported previously [17]. PCRwas carried out in a final volume of 50 µl reaction mixture containing 50 ng of template DNA, 1X PCR assay buffer, 1.5 mM of Mg^{2+}, 200 µM of dNTPs, 1 µM of each primer and 1U of Taq DNA polymerase. Amplification was carried out in Thermal cycler (Eppendorf, USA). PCR condition were: initial denaturation at 94º C for 5 minutes; followed by 94º C for 40sec, 62º C for 30sec, 72º C for 25 sec, and a final extension of 72º C for 5min. PCR products were purified and quantified according to manufacturer's instructions (QIAquick PCR Purification Kit; Qiagen Inc). Purified PCR products were submitted to geneOmbio technologies pvt ltd. Sequence analysis was done by comparing T4CRBR2 amplicon sequence to published sequences available at National Center for Biotechnology Information (NCBI, USA) using DNAstar software (USA).

Results and Discussion

The PCR amplification of T4CRBR2 of Shirwal HF crossbred cattle is shown in Fig. 1. This result is in agreement with previous report [17]. PCR amplification of 382 bp was checked using 1.5% agarose gel electrophoresis. T4CRBR2 nucleotide sequence of Shirwal HF crossbred cattle showed 100% identity with *Bubalus bubalis*, *Bos taurus* and sahiwal cattle sequences (Fig 2a). Further, high homology of HF crossbred sequence was also seen with Vechur (99.7%), *Bos indicus x Bos taurus* (99.7%), Tharparkar (99.5%), Bison (99.5%), Domestic Yak (99.5%) and Gayal sequences (99.2%). Lowest identity of T4CRBR2 of HF crossbred was seen with Nilgai sequence (95.5%). Nucleotide homology of full length TLR4 gene of Chinese Simmental cattle has been studied previously, wherein sheep, pig, human, and mouse were reported to have identities of 97, 84, 81, and 73% with cattle sequence, respectively [16]. Likewise full length TLR4 nucleotide sequence of Vrindavani cattle was reported to have Sahiwal (99.9%) and Bos taurus (99.9%) followed by Tharparkar (99.7%), mithun (99.5%), yak (99.4%), buffalo (99.2%), nilgai (97.3%), sheep (96.8%), goat (96.1%), cat (80.7%), and horse (80.1%) identity [26]. Further nucleotide sequence of partial TLR4 (T4CRBR1) of Vrindavani crossbred cattle was reported to have 99.7, 97.9, 96.2, 95.7% homology to *Bos Taurus*, *Bubalus bubalis*, sheep and goat sequences, respectively [22]. At amino acid level T4CRBR2 of HF crossbred showed 100% identity with *Bos isssndicus x Bos taurus*, Bison, Sahiwal, Tharparkar, Vechur, Gayal, *Bos taurus* and buffalo sequences (Fig 2b). Next high identity of T4CRBR2 amino acid sequence was seen with Domestic Yak (99.2%). Similar to nucleotide findings, lowest identity of HF crossbred T4CRBR2 amino acid sequence was seen with Nilgai sequence (93.7%). In 2009, homology identity of full TLR4 between cattle with from sheep, porcine, human, and murine was reported be 96, 81, 75, and 66%, respectively [16]. Phylogenetic tree at nucleotide level (Fig 3a) revealed that T4CRBR2 of HF crossbred, *Bubalus bubalis*, *Bos taurus* and Sahiwal fall in one group. Vechur, *Bos indicus x Bos taurus* and Tharparkar nucleotide sequences are less distant from T4CRBR2 of HF crossbred and fall in separate group. Sheep, goat and Swamp type water buffalo sequences fall in separate and more distant group. In phylogeny based on nucleotides Nilgai sequence was highest distant compared to T4CRBR2 of HF crossbred. Similar phylogeny findings were reported by Panigrahi *et al.* [22] where in partial TLR4 (T4CRBR1) of Vrindavani crossbred cattle and sequences of *Bubalus bubalis*, *Bos Taurus* fall in close groups. Sheep and goat nucleotide sequences were more distant compared to Vrindavani crossbred cattle [22]. Our phylogeny findings are also in agreement with Mishra *et al.* [26], wherein Indian cattle, *Bubalus bubalis*, *Bos Taurus* are in close group whereas sheep and goat in distant group.

Phylogenetic tree at amino acid level (Fig 3b) revealed that T4CRBR2 of HF crossbred, Domestic Yak, *Bos indicus x Bos taurus*, Bison, Sahiwal, Tharparkar, Vechur, Gayal, *Bubalus bubalis* and *Bos taurus* fall in one group. Swamp type water buffalo sequence falls in separate distant group. Goat and sheep amino acid sequences form separate distance group. Again at amino acid level Nilgai sequence shows highest distance compared to T4CRBR2 of HF crossbred.

Findings of this study will provide foundation for future studies on TLR4 polymorphisms in crossbred cattle and their resistance against disease conditions.

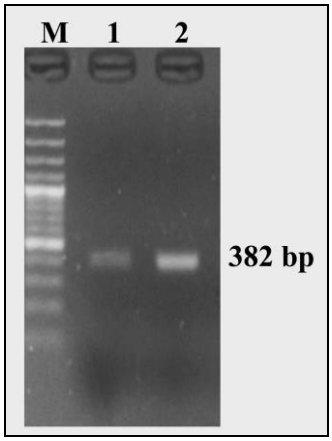

Fig 1: 1.5% agarose gel electrophoresis of T4CRBR2 of Shirwal HF crossbred cattle

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		100.0	100.0	100.0	99.7	99.7	99.5	99.5	99.5	99.2	97.9	97.6	97.6	95.5	1	HF crossbreed Shirwal.seq
2	0.0		100.0	100.0	99.7	99.7	99.5	99.5	99.5	99.2	97.9	97.6	97.6	95.5	2	Bubalus bubalis KM058708.seq
3	0.0	0.0		100.0	99.7	99.7	99.5	99.5	99.5	99.2	97.9	97.6	97.6	95.5	3	Bos taurus DQ839567.seq
4	0.0	0.0	0.0		99.7	99.7	99.5	99.5	99.5	99.2	97.9	97.6	97.6	95.5	4	Sahiwal Bos indicus JX276459.seq
5	0.3	0.3	0.3	0.3		100.0	99.7	99.2	99.2	99.0	97.6	97.4	97.4	95.3	5	Vechur cattle Bos indicus Vechur KX138
6	0.3	0.3	0.3	0.3	0.0		99.7	99.2	99.2	99.0	97.6	97.4	97.4	95.3	6	Bos indicus x Bos taurus KM114870.seq
7	0.5	0.5	0.5	0.5	0.3	0.3		99.0	99.0	99.2	97.4	97.1	97.1	95.0	7	Tharparkar Bos indicus KM102982.seq
8	0.5	0.5	0.5	0.5	0.8	0.8	1.1		99.5	99.2	98.4	98.2	98.2	96.1	8	Bison bison AH015104.seq
9	0.5	0.5	0.5	0.5	0.8	0.8	1.1	0.5		99.2	97.9	97.6	97.6	95.5	9	Domestic Yak Bos grunniens KF878306
10	0.8	0.8	0.8	0.8	1.1	1.1	0.8	0.8	0.8		97.6	97.4	97.4	95.3	10	Gayal Bos frontalis KF905590.seq
11	2.1	2.1	2.1	2.1	2.4	2.4	2.7	1.6	2.1	2.4		99.7	99.7	97.1	11	Swamp type water buffalo Bubalus carab
12	2.4	2.4	2.4	2.4	2.7	2.7	3.0	1.9	2.4	2.7	0.3		100.0	96.9	12	Goat Capra hircus HQ263215.seq
13	2.4	2.4	2.4	2.4	2.7	2.7	3.0	1.9	2.4	2.7	0.3	0.0		96.9	13	Sheep Ovis areis DQ922636.seq
14	4.6	4.6	4.6	4.6	4.9	4.9	5.2	4.0	4.6	4.9	2.9	3.2	3.2		14	Nilgai Boselaphus tragocamelus DQ286
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		

Fig 2a: Sequence distance of HF crossbred cattle T4CRBR2 at nucleotide level

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.2	96.8	96.0	96.0	93.7	1	HF crossbreed Shirwal.pro
2	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.2	96.8	96.0	96.0	93.7	2	Bos indicus x Bos taurus AIS73003.pro
3	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	99.2	96.8	96.0	96.0	93.7	3	Bison bison AAZ38830.pro
4	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	99.2	96.8	96.0	96.0	93.7	4	Sahiwal AGG22586.pro
5	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	99.2	96.8	96.0	96.0	93.7	5	Tharparkar AIS93171.pro
6	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	99.2	96.8	96.0	96.0	93.7	6	Vechur cattle APQ40215.pro
7	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	99.2	96.8	96.0	96.0	93.7	7	Gayal AIA59646.pro
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	99.2	96.8	96.0	96.0	93.7	8	Bos taurus ABH09760.pro
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		99.2	96.8	96.0	96.0	93.7	9	Bubalus bubalis AIQ82906.pro
10	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8		96.0	95.2	95.2	92.9	10	Domestic Yak AIA59623.pro
11	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	4.1		99.2	99.2	96.8	11	Swamp type water buffalo.pro
12	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.9	0.8		100.0	96.0	12	Goat ADZ13672.pro
13	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.9	0.8	0.0		96.0	13	Sheep ABI96901.pro
14	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6	7.5	3.2	4.1	4.1		14	Nilgai ABB97024.pro
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		

Fig 2b: Sequence distance of HF crossbred cattle T4CRBR2 at amino acid level

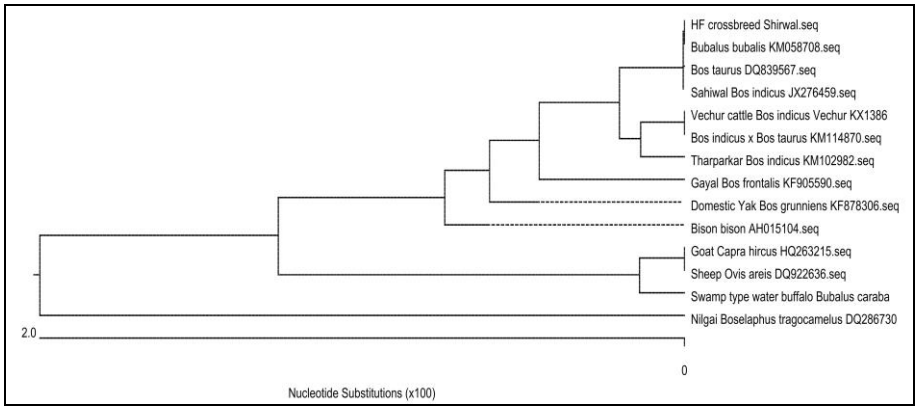

Fig 3a: Phylogenetic tree of HF crossbred cattle T4CRBR2 at nucleotide level

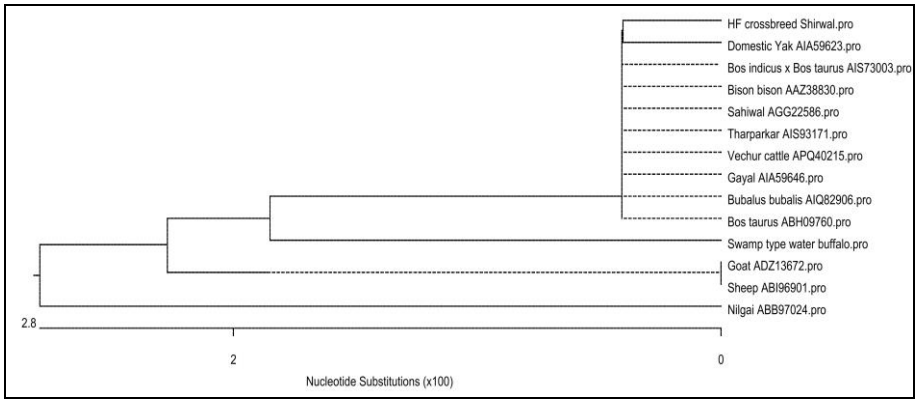

Fig 3b: Phylogenetic tree of HF crossbred cattle T4CRBR2 at amino acid level

Conclusion

Analysis of T4CRBR2 of HF crossbred cattle revealed that it is 100% identical with *Bubalus bubalis*, *Bos taurus* and sahiwal sequences, both at nucleotide and amino acid level. Furthermore, T4CRBR2 of HF crossbred, *Bubalus bubalis*, *Bos taurus* and Sahiwal fall in one group. Similarities of T4CRBR2 of HF crossbred cattle sequences with Indian cattle Vechur and Tharparkar were also observed.

Application of research: Results of this study are useful with regards to molecular markers for selection of animals.

Research category: Veterinary Science

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Conflict of Interest: The authors declare that there are no conflicts of interest.

Ethical committee approval number: Not applicable

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