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Polymorphism study of *TLR4* gene in crossbred cattle of Kerala

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Abstract

The present study was conducted to identify single nucleotide variations in exon 3 region of the bovine Toll-Like Receptor gene (TLR4) associated with somatic cell score (SCS) in crossbred cattle of Kerala using polymerase chain reaction-single stranded conformational polymorphisms (PCR-SSCP) technique. Blood samples were collected from 200 lactating crossbred cattle in different farms of Kerala Veterinary and Animal Sciences University (KVASU) and field centres of ICAR- FPT scheme, KVASU. Genomic DNA was isolated and polymorphisms were identified by SSCP analysis. The 231 bp fragment of exon 3 in TLR4 gene was found to polymorphic and two genotypes viz. CC and CD were obtained with frequencies 77 per cent and 23 per cent respectively. The frequencies of C and D allele in crossbred were found to be 0.88 and 0.12. On sequencing, C to T transition had led to one non-synonymous change at 2021th position of ORF. Cattle with CC genotype were associated with significantly lower SCS ($p \leq 0.05$) as compared to CD genotype. The association between TLR4 polymorphism and SCS described in this study suggests the possible role of SNP during mastitis infection. Thus, could be employed as a useful marker for genetic selection to promote mastitis resistance in dairy cattle.

Keywords: *TLR4, PCR-SSCP, somatic cell score, crossbred cattle*

Mastitis, the inflammation of mammary gland, usually involves invasion of udder by harmful pathogens resulting in decreased yield, compositional alterations and elevated somatic cells count (SCC) in the milk (Alhussien and Dang, 2018). This is a dairy disease of economic

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importance as its management includes milk discarding, medications, veterinary care etc. (DeGraves and Fetrow, 1993). As per the Newsletter of National Dairy Research Institute (NDRI), Karnal, India (2019) had suffered huge economic loss due to mastitis which amounted to INR 7165.51 crores per annum. In Kerala, screening of apparent healthy crossbred dairy cows show that there was a high prevalence of sub clinical mastitis cases (54 per cent) recorded in their study (Amritha *et al*., 2021). In comparison, research in Jammu and Kashmir found that there was much higher (81.48 per cent) prevalence of sub clinical mastitis in crossbred dairy cows (Ali *et al*., 2021). Somatic cell score monitoring is critical as it provides information about the status of the animal and milk quality, which is often accompanied with the rise of somatic cells in the milk and hence, SCS is considered as useful indicator of udder health (Vallimont *et al*., 2009). Somatic cells score has high genetic correlation with SCC and multitude of studies reported that cattle with low SCS are more resistant to mastitis infections (Koeck *et al*., 2014).

The innate immune system act as the first line of defence against microbial pathogens, and genes that codes for immune factors that detect and eliminate pathogens are significant potential markers during mastitis infection. One such example is Toll-Like receptor 4 (*TLR4*) gene that codes for a *TLR4* protein which can recognise lipopolysaccharides, a component of the outer membrane found in most of the Gram-negative bacteria (Akira and Takeda, 2004). Following *TLR4* activation, gene expression is characterised by the synthesis of significant mediators of innate immunity (Wu *et al*., 2015). Bovine *TLR4* is highly variable, with 36 single nucleotide polymorphisms (SNP) observed among 14 breeds of cattle (Deb *et al*., 2013). As *TLR4* has important role in detection of pathogens and eliciting subsequent immune response during mastitis infection, it has been investigated as a candidate gene with the goal of increasing mastitis resistance in dairy cattle. Several reports of genetic variants in the bovine *TLR4* have been made in different breeds of cattle, which have been linked to SCS and mastitis (Sharma *et al*., 2006; Wang *et al*., 2007). In India, Girish *et al*. (2016) studied association of *TLR4* gene with SCC in Indigenous and crossbred cattle of Tamil Nadu. Likewise, Gupta *et al*. (2015) conducted similar association studies of *TLR4* with SCS in Kankrej and Triple cross cattle. However, there have been no previous studies on the association of *TLR4* with SCS in crossbred cattle or any other breeds of cattle in Kerala. Crossbred cattle of Kerala were developed by crossing local indigenous breeds with exotic breeds mainly Holstein Friesian (exotic inheritance limited to 50–62.5%).

The main objectives of the present study were to identify the variations at nucleotide level in exon 3 of *TLR4* in the crossbred cattle of Kerala using PCR-SSCP technique and to further analyse their associations with SCS.

Materials and methods

Animals and sample collection for DNA isolation

Around 6 mL of blood samples were collected from each 200 lactating crossbred cattle using EDTA vials from University Livestock Farm, CVAS Mannuthy; Cattle Breeding Farm Thumburmuzhy; Livestock Research Station Thiruvazhamkunnu, and field centres of ICAR-FPT scheme, KVASU, Kerala (Institutional Biosafety ethic committee permission number 17-22). All the cattle were screened for *TLR4* polymorphisms. Genomic DNA was isolated from the collected blood using the standard phenol: chloroform method. The concentration, purity and quality of DNA were checked by NanoDrop spectrophotometer (Thermo Scientific, USA). The purity of DNA was verified by measuring absorbance at 260 nm and 280 nm. A 260/280 ratio of approximately 1.8 was generally accepted as “pure” for DNA (Sambrook and Russel, 2001).

PCR amplification

Primers (forward 5′-TCAGTGTGTCGGTGGTCACT-3′ and reverse 5′- CCCTGTAGTGAAGGCAGAGC-3′) were designed using Primer3 software using the published information available in GenBank (accession no: NC_037335.1 *Bos taurus* -whole genome shotgun sequence) for the

amplification of exon 3 region of *TLR4* and were custom synthesised (Sigma-Aldrich). The PCR reactions were carried out in 20 µL volume using 50 ηg of genomic DNA, 1×PCR Buffer, 1.2mM $MgCl_2$, 10 mM dNTP, 10 pM each of forward and reverse primers, and 2.5U/mL of JumpStart AccuTaq LA DNA Polymerase with proofreading 5 Activity (Sigma–Aldrich) in a thermal cycler (Bio-Rad – My Cycler). PCR cycling conditions were as follows: one cycle at 94ºC for 5 min, followed by 34 cycles (30 sec at 94ºC, 30 sec at 62.8ºC and 30 sec at 72ºC), followed by 1 cycle at 72ºC for 3 min, stopped at 4ºC. Amplified PCR products were loaded into the wells of 2 per cent agarose gel with a standard 50 bp DNA ladder (GenerRuler, MBI Fermentas, Germany) as a marker to check the size of the fragment. Electrophoresis was carried out at the rate 4 Volts/cm in 1X TBE buffer. Gels were stained with ethidium bromide and visualised under UV light and documented in a gel documentation system (Bio-Rad, USA).

Genotyping

Genotyping of the samples was done by Single Stand Confirmation Polymorphism (SSCP). The amplified fragments were mixed with SSCP loading buffer in the ratio of 1:2 (10 µL sample with 20 µL dye), denatured at 95°C for 10 min and immediately snap chilled in ice. The products were run in 12% polyacrylamide gel at 4°C for 3 h 45 min at 120 V/cm. Polyacrylamide gel was prepared with composition of 30% acrylamide/bis-acrylamide (29:1)–8 mL, 10% ammonium per sulphate–100 µL, TEMED–20 µL, 10X TBE–1.88 mL and distilled water-10 mL. Gels were stained with silver nitrate as per the procedure described by Byun *et al*. (2009) and SSCP fragments were visualised directly. Individual genotypes were defined according to band patterns. Representative PCR products, which showed different banding patterns in SSCP, were sequenced using Sanger's dideoxy chain termination method to confirm nucleotide variations and aligned with other sequences in GenBank employing BLASTn from NCBI.

Estimation of SCS

SCS is a log conversion of conversion of somatic cell count (SCC). It was calculated by the formula given by (Wiggans *et al*., 1987).

$$SCS = \log [SCC/(100\text{-}000)+3]$$

Association analysis between the genotypes and SCS was done using using Fixed Linear Model by Harvey Software (Harvey,1990).

$$Y_{ijklm} = \mu + S_i + P_j + L_k + C_l + G_m + e_{ijklmn}$$

Where,

Y_{ijklmn}=SCS of the n^{th} crossbred cattle; µ= population mean; S_i, effect i^{th} season of calving, (i= 4 seasons: winter, summer, southwest monsoon and post monsoon); P_j, effect of j^{th} year of calving, (j= 3 years: from 2018-2020); L_k=effect of k^{th} stage of lactation; (k=3 stages: early, mid and late lactation); C_l = effect of Centres (l= 4 Centres); G_m = effect of the m^{th} genotype (CC, CD); e_{ijklmn}= random residual error associated with each observation which is normally and independently distributed with mean zero and unit variance.

Results and discussion

Polymerase chain reaction- single stranded conformational polymorphisms (PCR-SSCP) was performed to detect the SNPs in exon 3 of *TLR4* gene. In the present investigation, 231 bp fragment of *TLR4* gene was amplified using the specific set of primers designed (Fig.1). The SSCP analysis for exon 3 *TLR4* showed two different band patterns of two bands (CC) and four bands (CD) as shown in Fig.2. Upon sequencing PCR products from each type of bands, revealed the polymorphism of *TLR4* exon 3 was induced by C-T transition single nucleotide polymorphism (SNP) located at 117^{th} position of 231 bp (Fig.3 and 4). The observed SNP was located at c.2021 bp position of ORF. On further analysis the SNP identified (c.2021 C>T) was found to be non-synonymous single nucleotide polymorphism. The codon changed from ACC to ATC resulted in an amino acid substitution of an amino acid, Threonine (neutral, polar, amino acid) to Isoleucine (neutral, non-polar, hydrophobic amino acid). Similarly, several studies by Sharma *et al*. (2006), Noori *et al*. (2013) and Carvajal *et al*. (2013), Sharma *et al*. (2015) also

reported transition C→T SNP at position 2,021 bp in exon 3 of *TLR4* in Holstein Friesian cattle.

The genotypic and allelic frequencies of two loci were calculated by the method as suggested by Falconer and Mackay (1996). In the present study, allele C (0.88) was predominant than D (0.12) allele. The value of CC (0.77) genotypic had higher frequency than CD (0.23) genotype. CC diplotype frequency and the frequency of C haplotypes were found to be predominant in studied population. In the present study homozygote, DD diplotype was not observed in the screened population and the 'C' haplotype was almost fixed in the population. Chi square analysis showed no significant differences between cattle with respect to *TLR4* locus. It revealed that the screened population is under Hardy Weinberg equilibrium.

In the association study, there was significant lower (<0.05) SCS observed for cattle with genotype CC (4.48±0.06), compared

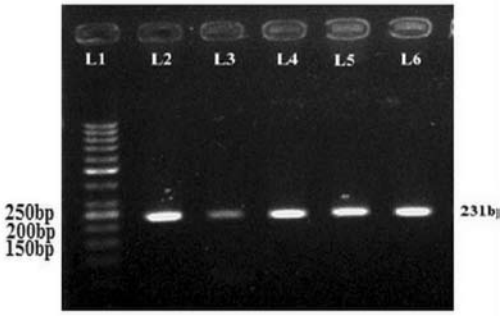

Fig. 1. PCR amplification of exon 3 of *TLR4* (231 bp fragment)

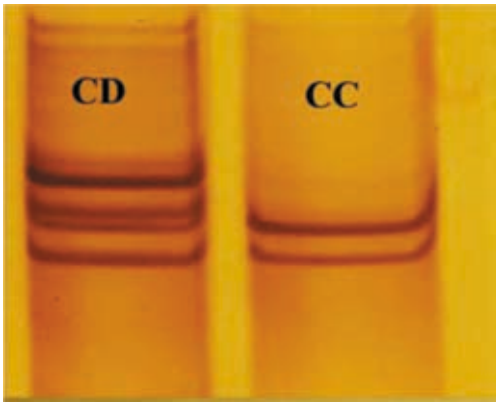

Fig. 2. SSCP pattern of 231 bp fragment of *TLR4* exon 3 (with CC and CD genotypes)

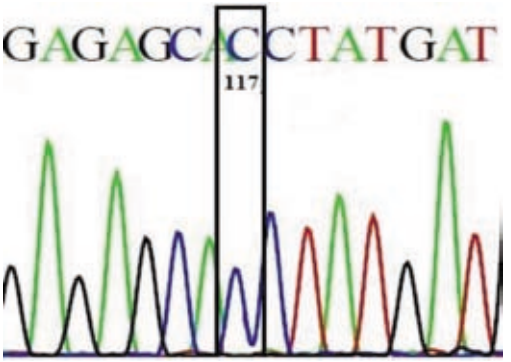

Fig.3. Allele sequencies of CC of 231 bp fragment of *TLR4* (exon 3)

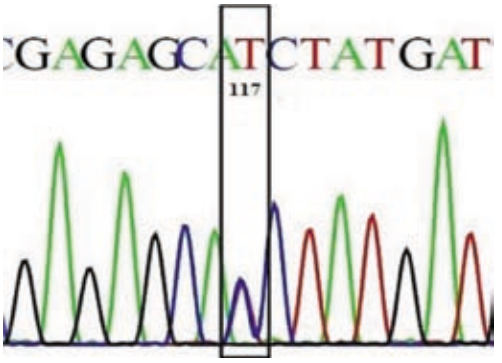

Fig.4. Allele sequencies of CD of 231 bp fragment of *TLR4* (exon 3)

to the heterozygous genotype CD (4.82±0.09). Similar result was reported by Sharma *et al*. (2006) in Holstein cattle, the genotypes CC and CT had significant association with SCS and lactation persistency. The genotype CC had significant lower SCS (2.98 ± 0.02) and higher than CT genotype (3.08 ± 0.04). Noori *et al*. (2013) reported three genotypes AA, AB and BB in exon 3 of *TLR4* in Holstein dairy cows of Iran. They found that genotype BB had a lower SCS than genotypes AB or AA. Sharma et al. (2015) reported that the SNP (c.2021C>T) with a combined genotype of CC and CT was related with susceptibility during *Mycobacterium sp.* infection. On the contrary to the present study, association study by Beecher *et al*. (2010) and Miseikiene *et al*. (2020) determined that there was no association in *TLR4* genotypes with SCS in Holstein-Friesian cattle.

In the present study, the association analysis with major non-genetic factors showed that SCS was significantly (<0.05) influenced by

season of calving and centre. Crossbred cattle calved during winter season have lower SCS than other seasons. Similar result was reported by Sewalem *et al.* (2006) in Holstein Friesian cattle where the SCS level was lowest during the during winter season. In the present study among the four centres crossbred cattle from Cattle breeding farm, Thumburmuzhy were found to be having lower SCS than cattle form other centres.

Conclusion

The results of the present study showed a significant association between polymorphisms in 231 bp fragment of exon 3 *TLR4* with somatic cell score in crossbred cattle. Therefore, this SNP may be employed as a genetic marker to promote mastitis resistance in selection programmes for this trait in crossbred cattle of Kerala. To corroborate the findings, more research into the various polymorphisms in different locations of the *TLR4* related with SCS in other breeds and different populations of cattle is needed.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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