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Study of polymorphism of toll like receptor-4 gene in crossbred cattle and its association with somatic cell count

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India has total livestock population of 535.78 million which shows an upsurge of 4.6% over Livestock census 2012 (Department of Animal Husbandry and Dairying, 20th Livestock Census, 2019). The female cattle population was 145.12 million which increased by 10% in 2019, as compared to previous census (2012). The exotic/crossbred population in 2019 was 25.67 million. The indigenous cattle population and the crossbred cattle population has raised by 10% and 26.9% in 2019 as compared to previous census (DAH&D, 20th Livestock census). The economic losses due to subclinical form of mastitis were in the range of ₹21,677/- to ₹88,340/- for one lactation period depending on the condition of the animal (Rathod *et al*. 2017). Mastitis is under polygenic control as there are many genes that control the traits such as Major Histocompatibility Complex (MHC), Lactoferrin, Lysozyme, Toll-Like Receptor (TLR), CD14, NLR (Nod-Like Receptor) as reported by Gulhane and Sangwan (2012). The maximum of somatic cells are leukocytes (white blood cells), which are present in increasing numbers in milk generally as an immune response to a mastitis-causing pathogen. Considering the above fact, the studies on the role of TLR4 in pathogen recognition and consecutive initiation of the inflammatory and immune responses and also on differential expression of the gene during mastitis are essential. TLR4 has been suggested as a strong candidate for increasing subclinical mastitis resistance in breeding programs. Therefore, present investigation was undertaken to evaluate polymorphism in exon 3 of TLR4 gene and its association with SCC in crossbred cows.

The present study comprised of 60 unrelated HF × Gir crossbred cows (40 affected and 20 normal) from Bombay Gowrakshak Mandali, Betegaon Farm, Betegaon. Approximately 5 ml of venous blood samples were collected from 40 unrelated and affected cows between 3rd to 5th lactation in sterile vacutainer tubes containing 0.5 M EDTA as anticoagulant. The confirmation of these cows for subclinical mastitis was done with California Mastitis Test (CMT). The blood samples were also collected from 20 normal cows and transported to the Genetic Investigation Laboratory (GIL), Department of Animal Genetics and Breeding, Mumbai Veterinary College, Parel, Mumbai, maintaining the cold chain. The samples were stored at 4°C until DNA extraction. Further, milk samples of the same cows were collected for detection of somatic cell count in a sterile vial for analysis. The milk somatic cell count was measured using a De Laval Cell counter at Department of Veterinary Physiology, Mumbai Veterinary College. Using disposable sterile cassettes, the De Laval Cell Counter evaluates the somatic cell count of the milk sample from the affected cow and non-affected cow. The somatic cell count readings were converted into somatic cell score by the formula (Rupp *et al.* 1999):

$$SCS = \log2 [SCC/100] + 3$$

Hillerton (1999) proposed that somatic cell count of individual milk samples and bulk milk samples above 2 × 10^5 cells/ ml and 4 × 10^5 cells/ml, respectively suggests mastitis. The genomic DNA was isolated from the blood samples using traditional Phenol: Chloroform: Isoamyl alcohol (P:C:I) method. Purity and integrity of the isolated genomic DNA samples were checked by nanodrop and also by running DNA with loading dye on 0.8% agarose gel at 90 V for 60 min.

The set of primer sequences described by Sentitula *et al*. (2012) for locus TLR31 of TLR4 gene for exon 3 were utilized to amplify the DNA sample using polymerase chain reaction in mixture of 25 µl. The master mix was prepared for each sample by adding 10.5 µl Nuclease free water, PCR master mix 12.5 µl, Forward primer F: 5'-CATTTTGGTTTCCTATTCAGCA-3' (0.5 µl), Reverse primer R:5'-GATCCAAGTGCTCCAGGTTG-3' (0.5 µl), Template DNA 1.0 µl. The PCR products (10 µl) were

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digested using *Hae*III restriction enzyme in the final reaction volume of 20 µl and analyzed on 2.5% of agarose gel.

The mean somatic cell score in normal cows ranged between 2.86 to 2.99 $\times 10^5$ cells/ml, whereas for affected animal the range of mean for somatic cell score was 3.45 to 5.72 $\times 10^5$ cells/ml. Bytyqi *et al.* (2010) reported less than 1×10^5 cells/ml somatic cell count in healthy cows which increased above 1×10^6 cells/ml during infections. Somatic cell count is extensively used as indirect selection tool for selecting the animals for mastitis resistance as the genetic correlation is found to be high in SCC and milk yield in first lactation (Koivula *et al.* 2005). Nanodrop Spectrophotometer was used to check the purity as well as the concentration of the extracted DNA. The DNA concentrations ranged from 85 ng/µl to 1640 ng/µl. The samples with OD ratio less than 1.8 and more than 2.0 were purified and/or re-extracted, so as to obtain desired OD ratio 1.8 to 2.0. The quality of extracted DNA was also checked by agarose gel electrophoresis (0.8%) to ensure good quality. All the extracted samples revealed the single compact and bright band of DNA (343 bp) indicating good quality of DNA for further analysis

The gradient PCR was used to standardize the annealing temperature during the PCR reaction. It was observed that single compact DNA fragment of TLR4 gene was obtained at 56.5°C annealing temperature. Panigrahi *et al.* (2021) and Noori *et al.* (2013) reported the annealing temperature of 61.5°C and 59°C, respectively. The agarose gel electrophoresis of PCR products revealed single DNA fragment of 343 bp (Fig.1) in all studied samples. Kumar *et al.* (2017) and Sentitula *et al.* (2012) also reported the 343 bp exon 3 amplicon of TLR4 gene in Rathi and Sahiwal cattle, respectively.

The PCR products (343 bp) were digested with *HaeIII* restriction enzyme and analyzed on 2.5% of agarose gel. The *HaeIII* PCR-RFLP analysis revealed polymorphisms with two genotypes as 'BB' and 'AB' in mastitis population, while, only 'BB' genotype in normal cows. The 'BB' genotype was represented by three fragments of 49 bp, 98 bp and 196 bp, while four fragments of 49 bp, 98 bp,

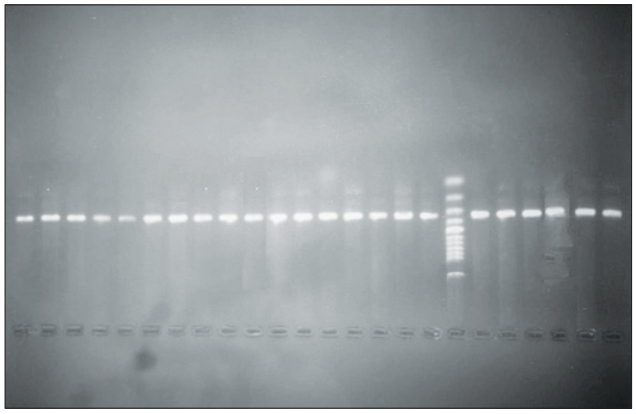

Fig. 1. Agarose gel (1.8%) electrophoresis of exon 3 (343 bp) of TLR4 using 100 bp ladder. Lane no. 1 to 23 (PCR amplification of exon 3).

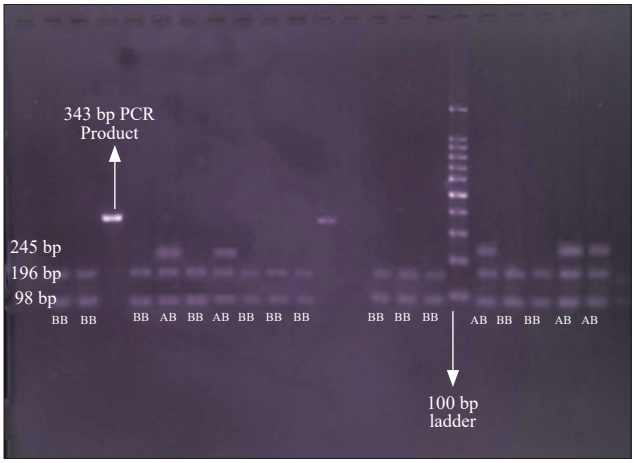

Fig. 2. PCR-RFLP of exon 3 of TLR4 using *HaeIII* restriction enzyme.

196 bp and 245 bp indicated 'AB' genotype. The fragment of 49 bp was not visible on agarose gel (Fig. 2). Kumar *et al*. (2017) reported the similar pattern of 'BB' and 'AB' genotype in TLR4 (exon 3) gene of Rathi cattle. Sentitula *et al.* (2012) reported two genotypes 'BB' (98 bp and 196 bp) and 'AB' (98, 196, 245, 294 and 343 bp) in exon 3 of Sahiwal cattle and Murrah buffalo. Valentina *et al.* (2020) reported 13 polymorphisms for sequenced region of TLR 4 gene in water buffaloes most of which were in coding region.

The gene and genotypic frequencies of normal and affected animals were estimated separately and details are shown in Table 1. In case of normal animals, the monomorphic pattern was observed with all the 20 animals having 'BB' genotype. The genotypic frequency of 'BB' genotype and the gene frequency of 'B' allele were found to be one in this population. However, in mastitis affected animals, out of 40 animals, 31 animals were of 'BB' genotype while, remaining 9 animals were of 'AB' genotype. In mastitic population, the observed genotypic frequencies of 'BB' and 'AB' genotype were 0.775 and 0.225, respectively. While, in same population, the observed allelic frequencies of 'B' and 'A' were 0.887 and 0.112, respectively.

The mean and SE of the somatic cell score for genotype 'AB' was found to be 3.66±0.03 (N=9) whereas the mean of 'BB' genotype was 3.59±0.13 (N=51), respectively. The statistical analysis with two mean 't' test revealed that there was no significant difference between the somatic cell count of 'BB' and 'AB' genotype. It indicated that there was no association between the genotypes of exon 3 of TLR4 gene with somatic cell count in HF×Gir crossbred cattle.

The PCR product of 343 bp was successfully amplified for exon 3 of TLR4 gene in HF×Gir cows. The PCR-RFLP of exon 3 with *HaeIII* enzyme revealed two genotypes 'BB' and 'AB' with band pattern 49 bp, 98 bp and 196 bp and 49 bp, 98 bp, 96 bp and 245 bp, respectively. Non-significant association between mean somatic cell scores and genotypes 'BB' and 'AB' in exon 3 of TLR4 gene was observed, hence it was concluded that TLR4 gene

Table 1. Total number of animals with gene and genotypic frequencies

Normal/ Mastitis	No. of animals	Genotype	Genotypic frequency	Gene frequency	
				B	A
Normal	20	'BB'	1	1	0
Mastitis	31	'BB'	0.775	0.887	0.112
	09	'AB'	0.225		

polymorphism in the studied crossbred cow population was not associated with mastitis resistance.

SUMMARY

The present study was undertaken to study the TLR4 gene polymorphism in exon 3 and its association with somatic cell count in HF×Gir cows using PCR-RFLP. Sixty crossbred cows (40 affected and 20 normal) between 3rd to 5th lactation were selected from Bombay Gowrakshak Mandali, Betegaon, Maharashtra. The confirmation of mastitis was done with the help of California Mastitis Test (CMT) and the blood samples of 40 affected animals were collected aseptically along with 20 normal animals. The mean of somatic cell score in normal cows ranged between 2.86 to 2.99 × 10^5 cells/ml, whereas for affected animal the range of somatic cell score was 3.45 to 5.72 × 10^5 cells/ml. The genomic DNA was isolated and exon 3 of TLR4 gene was successfully amplified by PCR using a specific primer. The PCR product was digested with *HaeIII* restriction enzyme. The PCR-RFLP of exon 3 with *HaeIII* enzyme revealed two genotypes 'BB' and 'AB' with band pattern 49 bp, 98 bp & 196 bp (BB) and 49 bp, 98 bp, 196 bp & 245 bp (AB), respectively. The mean of somatic cell score estimated for animals with genotypes 'BB' and 'AB' was 3.99±0.13 and 3.66±0.03, respectively. The two mean 't' test analysis showed non-significant difference between somatic cell count with genotypes 'BB' and 'AB'. It indicated that there was no association between the genotypes of exon 3 of TLR4 gene with somatic cell count in crossbred cows under present study.

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