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Bovine CD14 Gene Polymorphism and its Association with Milk Yield, Milk Constituents and Somatic Cell Count in Holstein Friesian Crossbred and Gir Cows

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ABSTRACT

The present study was undertaken to explore polymorphism of the CD14 gene and its association with milk yield, fat, protein, SNF % and somatic cell count (SCC) (lakhs/ml) in Gir and Holstein Friesian (HF) crossbred cattle. A total of 80 cows comprising 40 each of Gir and HF crossbreds were included in the study. Genomic DNA isolation, quantification, standardization of PCR protocol and restriction digestion of PCR product using enzyme *Hinf*I were done as per standard methodologies. Data on the lactation yield were collected from the history sheets and records maintained at the farms. Fat, Protein and SNF % were determined by milk analyzer. Somatic Cell Count in the milk was performed using microscope. The RFLP pattern yielded three genotypic variants viz., CC, CD and DD with their respective frequencies of 0.425, 0.400 and 0.175 in Gir and 0.200, 0.425 and 0.375 in Crossbreds. The effect of breed on lactation yield was significant ($P < 0.01$); while the effect of the CD14 genotype was non-significant. For Fat% the effects of breed ($P < 0.01$) and genotype ($P < 0.05$) were significant; Mean milk protein% and SNF% were significantly higher for cows possessing CD14 genotype CC in comparison to the cows of genotype CD and DD. For SCC, effect of breed ($P < 0.05$) and genotype ($P < 0.01$) were both significant. It could be concluded from the present study that the DD genotype was the favoured genotype for milk yield, CC for milk fat % Protein %, SNF% and SCC.

HIGHLIGHTS:

- Gir has a comparatively higher frequency of allele C while HF crossbreds has a comparatively higher frequency of allele D for CD14 gene.
- DD genotype has higher milk yield while CC genotype excelled for milk fat % Protein %, SNF%.
- CC genotype of the CD 14 gene is less susceptible for mastitis.
- CD14 genetic variants may be used as a genetic aid to improve dairy traits.

Keywords: Cattle, CD14, Fat %, Lactation yield, PCR-RFLP, Somatic cell count

Mastitis mostly impacts milk production and costs dairy producers' money worldwide and in India. Bovine mastitis is one of the main illnesses impacting dairy animals' capacity to produce milk, which ultimately has an effect on both the national economy and farmer economics (Sharma *et al.*, 2012). The condition has significant public health significance in addition to its economic significance since contaminated milk may act as a vehicle for the spread of certain infections to the people (Bhat *et*

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al., 2016). This production disease, which causes losses in milk quality and quantity, losses from rejected milk, early culling, treatment expenses, and additional labour costs, is regarded as a significant disease of dairy cows (Hogeveen *et al.,* 2011). The estimated economic loss due to mastitis in India was about INR 7165.51 crore per annum (Sinha *et al.,* 2014). The somatic cell count (SCC) has been used by the dairy industry as a measure of intramammary health because SCC is positively correlated with mastitis (Shook., 1989). There are a number of candidate genes for mastitis resistance traits. Cluster of Differentiation-14 (CD14) gene is one of the excellent candidates for this trait in cattle (Ogorevc *et al.,* 2008 & 2009). The protein encoded by this gene is a component of the innate immune system which is the first line of defence against microbial infection (Janeway *et al.,* 2002). Its pattern recognition receptor binds mainly with lipopolysaccharide, lipoteichoic acid, arachidonic acid and thus releases various cytokines which act for the body's defence. CD14 gene has been mapped on chromosome 7 (BTA 7) of cattle and spans over 2630 nucleotides comprising of two exons and one intron. The total coding sequence is 1122 bp long which encodes 373 amino acids (Ikeda *et al.,* 1997). There are studies in cattle that showed the association of CD14 gene polymorphism with several animal diseases e.g., mastitis (Kumar *et al.,* 2014, Selvan *et al.,* 2014b) and milk, fat and protein yield (Beecher *et al.,* 2010). Five single-nucleotide polymorphism (SNP) markers have been reported in Taurine cattle (lbeagha *et al.,* 2008). 25 SNPs with 17 amino acid changes were recently reported in crossbred cattle (*Bos indicus* × *Bos Taurus*) (Pal *et al.,* 2011). The lack of knowledge about the CD14 gene polymorphism with milk yield, fat, protein, solid-not-fat percentage and somatic cell count in indigenous and crossbred cattle prompted the current study to investigate if this gene may be employed as a marker gene for selection.

MATERIALS AND METHODS

DNA Extraction

Blood samples were collected from a total of 40 Holstein Friesian crossbred and 40 Gir cows maintained at Shri Ahilyamata Jeev Dayamandal Trust Gaushala, Indore and Livestock farm, College of Veterinary Science and A.H., Mhow. The extraction of genomic DNA from collected blood samples was done by the standard method (John *et al.,* 1991) with minor modifications. The required solutions and reagents were prepared in the laboratory using molecular (Sigma) grade chemicals. The concentration and purity of DNA were checked by UV – spectrophotometer. Optical density (OD) values at 260 nm and 280 nm were measured using UV-spectrophotometer. The samples with higher concentrations of DNA were diluted to 30 ng/µl using nuclease-free water (Sigma). Three microlitres of DNA were used as a template for the PCR reaction. The primers used for the amplification of the CD14 gene were selected on the basis of the previous reports (Ibeagha - Awemu *et al.,* 2008) The primers were synthesized by Eurofins Genomics India Pvt. Ltd., Bangalore. Amplification was performed in a thermal cycler (Applied Biosystems) programmed for 34 cycles with an initial denaturation at 94°C for 2 minutes, denaturation at 94°C for 40 seconds, annealing at 60°C for 40 seconds and extension at 72°C for 50 seconds with a final elongation at 72°C for 10 minutes. An 832bp fragment of CD14 gene was amplified and digested with the *Hinf*I restriction enzyme. Subsequently, digested PCR products were electrophoresed on 3% agarose gel containing 1 % ethidium bromide at a constant voltage of 80 V for 90-120 minutes using 0.5 x TBE buffer. On completion of electrophoresis, the gel was visualized under a UV transilluminator (UVITECH, Cambridge, U.K.) to reveal different restriction patterns and allele types obtained.

Collection and Estimation of data on milk yield, composition and Somatic cell count

Lactation yields of all the 40 Holstein Friesian crossbred and 40 Gir cows included in the study were collected from the history sheets and records maintained at the respective farms. For estimation of milk constituents and SCC milk samples (50 ml) were collected from each cow. Fat %, protein % and solid-not-fat% were estimated in fresh milk by Lactoscan (Unitech) milk analyzer. Data on lactation yield and milk fat %, milk protein %, SNF% and somatic cell count were classified according to breed and CD 14 genotype.

The SCC count in the milk was performed to assess the degree of infection in the udder. SCC above 2, 50, 000 cells/ml of milk is generally considered as positive for subclinical mastitis (NMC, 2012). The somatic cell count

in milk was done under the microscope as per the method of Schalm *et al.* (1971) which was slightly modified.

Estimation of gene and genotype frequencies

Gene and genotype frequencies for the CD14 gene under study were estimated using standard formulae (Falconer, 1996) Microsoft Windows-based freeware for population genetic analysis. The chi-square test (Snedecor and Cochran, 1994) was used to test the populations for Hardy-Weinberg equilibrium at the locus under study.

Association of CD14 gene polymorphism with milk yield, fat%, protein% and somatic cell count

To study the effect of various polymorphic variants of the CD14 gene on lactation yield, fat %, protein % and SCC the data were subjected to analysis of variance by the SPSS software package using the following general linear model.

$$Y_{ijk} = \mu + B_i + G_j + e_{ijk}$$

Where,

Y_{ijk} is the observed value of trait; μ is the population mean; B_i is the effect of breed (i = 1, 2); G_j is the effect of genotype (j = 1, 2, 3); e_{ijk} is the random error.

RESULTS AND DISCUSSION

Frequencies of genotypes and alleles at CD14 locus

Three genotypic variants viz., CC, CD and DD were observed in the animals screened in both the breed groups (Fig. 1&2). The pattern with three bands of size 377, 272, and 183 bp was designated as CC, the pattern with four bands of size 377, 225,183 and 47 bp was designated as DD and the pattern showing all the five bands (377, 272, 225, 183 and 47 bp) was designated as CD. The same three genotypes (restriction patterns) have also been reported by Kumar *et al.* (2014) in Sahiwal and Selvan *et al.* (2014b) in Karan Fries breeds of cattle.

In Gir breed, out of 40 cows under study, 17 were of genotype CC, 16 were of genotype CD and 7 were of genotype DD (Table 1). Thus, the frequency of three genotypes in the Gir breed was found to be 0.425, 0.400 and 0.175, respectively for CC, CD and DD genotypes. Out of 40 HF crossbreds included in the study, 8 belonged to genotype CC, 17 belonged to genotype CD and 15 belonged to genotype DD giving genotypic frequencies of 0.200, 0.425 and 0.375, respectively for CC, CD and DD genotypes (Table 1).

Table 1: Frequency of genotypes and alleles at CD 14 locus

Genotype/Allele	Frequency	
	Gir (40)	HF crosses (40)
CC	0.425 (17)	0.200 (8)
CD	0.400 (16)	0.425 (17)
DD	0.175 (07)	0.375 (15)
C	0.6250	0.4125
D	0.3750	0.5875

The frequencies of alleles C and D were 0.6250 and 0.3750, respectively in Gir and 0.4125 and 0.5875 in HF crossbred cows (Table 1). The allelic frequency in the Gir breed in the present study is close to that reported by Kumar *et al.,* (2014) in Sahiwal cows (0.655 for allele C and 0.345 for D). However, the frequency of three genotypes in their study (0.54 for CC, 0.23 for CD and 0.23 for DD) differed from the present results. Comparatively higher frequency of allele D and lowest genotypic frequency of CC in Holstein crosses as observed in this study has also been reported by Selvan *et al.,* (2014b) in Karan Fries cows. Thus, it appears that Indian breeds have a comparatively higher frequency of allele C and exotic breed (Holstein Friesian) has a comparatively higher frequency of allele D.

Association of CD14 gene polymorphic variants with lactation yield, milk constituents and somatic cell count

Lactation Yield

The results of the least squares analysis of variance for lactation yield have been presented in Table 2. It was revealed that the effect of breed on lactation yield was significant (P <0.01); while the effect of the CD14 genotype was non-significant. The least-square means for lactation yield have been presented in Table 3. The mean lactation yield for HF crosses (1719.71±88.91 kg) was significantly higher as compared to the mean lactation

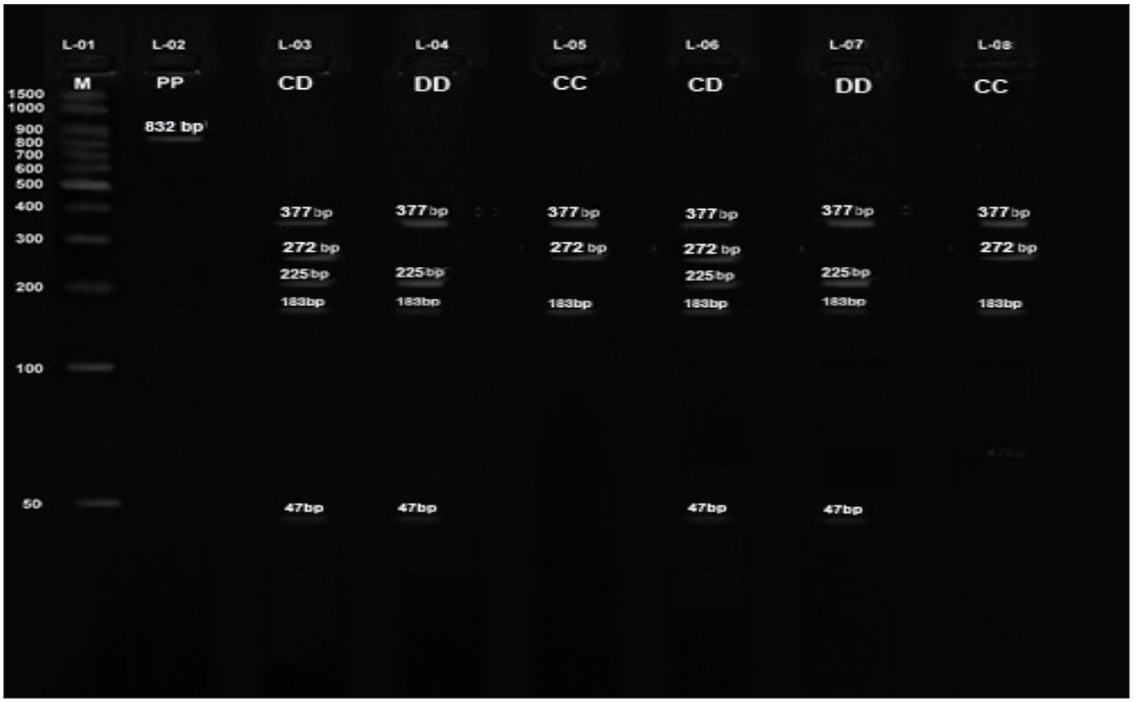

Fig. 1: PCR- RFLP patterns of CD 14 gene digested with *Hinf I* in Gir cows

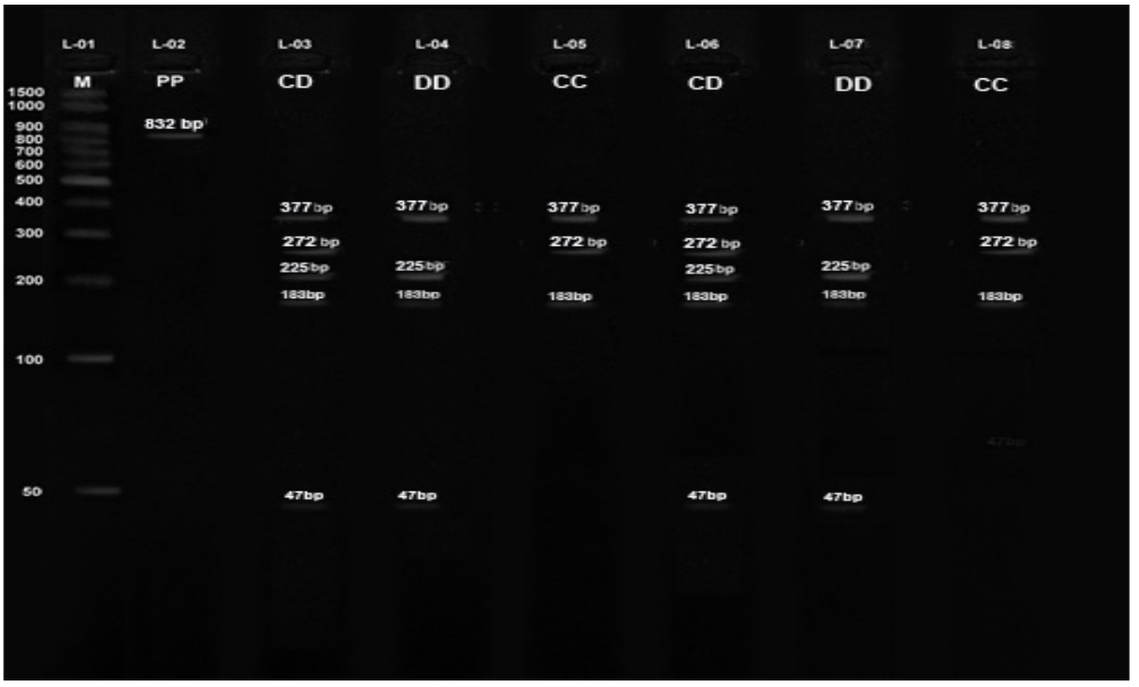

Fig. 2: PCR- RFLP patterns of CD 14 gene digested with *Hinf I* in HF Crossbred cows

yield of the Gir breed (1024.19±74.83 kg). This is quite obviously expected as there are inherent differences in milk producing ability of the two breed groups and crossbreds are definitely superior for this trait over Indian breeds.

The results of the analysis of variance (Table 2) revealed that the effect of CD14 genotype was not significant on lactation yield. However, cows of genotype DD exhibited superiority (Table 3) producing higher mean lactation yield (1504.98±101.06 kg) by 326.69 kg than the cows of genotype CC (1178.29±81.16 kg). Genotype CD (1432.58±78.42 kg) also exhibited superiority over CC genotype (1178.29±81.16 kg) for lactation yield. However, these differences were statistically non-significant. Reports on the association of polymorphism at this region of the CD 14 gene and milk yield do not appear to be readily available. However, the present finding showing no significant association of CD14 gene polymorphism with lactation yield is in agreement with the finding of Gupta *et al.,* (2018). They have also reported that there was no association between polymorphic variants at a different exon straddling region of the CD 14 gene and 305-day milk yield in crossbred cows. However, Beecher *et al.* (2010) on the basis of single nucleotide polymorphism (SNP) study reported that the CD14-1908 A allele was associated with increased (P < 0.1) milk yield and G allele of CD14-1908 was associated with decrease in milk production.

Milk fat per cent

The results of the least squares analysis of variance for milk fat per cent have been presented in Table 2. It is evident from this table that the effects of breed (p<0.01) and genotype (P < 0.05) were significant. The least-square means for milk fat per cent have been presented in Table 3. The mean milk fat % of Gir cows (4.17±0.08) was significantly higher than that recorded for crossbred cows (3.19±0.07). This is as per expectation as Indian breeds of cows produce milk with higher butter fat content as compared to exotic and crossbred cows. The cows with genotype CC (3.96±0.08) had significantly higher milk fat % as compared to the cows of genotype DD (3.40±0.09). The difference between genotypes CC and CD and between genotypes CD and DD was not significant for milk fat per cent (Table 3). The reports on the association of polymorphism at the CD 14 gene region under study and milk fat % in dairy cattle do not appear to be readily available. Beecher *et al.,* (2010) have reported a significant association of SNPs at CD 14 gene locus and milk fat, where the G allele of CD14-1908 was associated with lower milk fat and CD14-1908 A allele was found to be associated with increased (P < 0.05) milk fat.

Table 2: Least squares analysis of variance for lactation yield, milk fat % and protein % and somatic cell count (Mean Square values)

Source of variation	Degree of freedom	Lactation yield	Fat%	Milk protein%	SNF%	SCC (Lakhs/ml)
Breed	1	27862835.25**	14.90**	0.152^{NS}	0.37^{NS}	1.78*
Genotype	2	2804813.71^{NS}	4.15*	0.179*	0.54*	2.20**
Error	76	947572.20	0.845	0.053	0.17	0.43

** Significant (P < 0.05): ** Significant (P < 0.01); NS- Non significant.*

Table 3: Least squares Mean ± SE for lactation yield, milk fat % and protein % and somatic cell count

Effect	No. of observations (N)	Lactation Yield Mean ± SE (kg)	Fat Mean ± SE (%)	Milk protein Mean ± SE (%)	SNF Mean ± SE (%)	SCC Mean ± SE (Lakhs/ml)
Breed						
Gir	40	1024.19 ± 74.83^{b}	4.17 ± 0.08^{a}	2.99±0.03	8.59 ± 0.08	1.67 ± 0.15^{b}
HF crossbreds	40	1719.71 ± 88.91^{a}	3.19 ± 0.07^{b}	2.93±0.04	8.49 ± 0.07	2.09 ± 0.19^{a}
Genotype						
CC	25	1178.29 ± 81.16	3.96 ± 0.08^{a}	3.09 ± 0.04^{a}	8.71 ± 0.10^{a}	1.42 ± 0.13^{b}
CD	33	1432.58 ± 78.42	3.68 ± 0.06^{ab}	2.93 ± 0.03^{b}	8.48 ± 0.09^{b}	1.98 ± 0.15^{a}
DD	22	1504.98±101.06	3.40 ± 0.09^{b}	2.86 ± 0.05^{b}	8.43 ± 0.12^{b}	2.24 ± 0.18^{a}

a, b : Values with a common alphabet as superscript do not differ significantly within a column.

Milk protein per cent

Table 2 shows the results of the analysis of variance for milk protein %. This milk constituent was significantly ($p<0.05$) influenced by the CD14 genotype, while the effect of the breed was non-significant. Mean milk protein % was significantly higher for cows possessing CD 14 genotype CC (3.09 ± 0.04) in comparison to the cows of genotype CD (2.93±0.03) and DD (2.86±0.05) as presented in Table 3. Survey of relevant literature revealed that the parallel report on the association of PCR-RFLP in this region of CD 14 gene and milk protein per cent in cattle does not appear to be readily available. Hence, results could not be compared. However, Beecher *et al.* (2010) have studied the association of SNPs in CD 14 gene and milk protein and have reported that the CD14-1908 A allele was associated with increased ($p<0.05$) milk protein yield and the G allele of CD14-1908 was associated with lower milk protein yield.

Solid-not-fat per cent

The results of the least squares analysis of variance for solid-not-fat (SNF) % in the milk have been presented in Table 2. The effect of breed was non-significant while the effect of the CD 14 genotype was significant ($p<0.05$) on milk SNF %. The least-square means for milk SNF% have been presented in Table 3. Mean milk SNF % was significantly higher for cows possessing genotype CC (8.71±0.10 %) as compared to the cows having genotype CD (8.48±0.09 %) and DD (8.43±0.12 %). The results could not be compared as the parallel study on the association of CD 14 gene polymorphism and milk SNF % in cattle does not appear to be readily available.

Somatic cell count (lakhs/ml)

Table 2 depicts the results of the least squares analysis of variance for somatic cell count (SCC) in the milk. The effect of breed ($p<0.05$) and genotype ($p<0.01$) were both significant on milk SCC. The least square means for milk SCC have been presented in Table 3.The mean SCC of crossbred cows (2.09±0.19 lakhs/ml) was significantly higher as compared to Gir cows (1.67± 0.15 lakhs/ml). There were significant differences in mean milk SCC for different CD 14 genotypes. The mean SCC for genotype CC was the lowest (1.42±0.13 lakhs/ml) and it was significantly different from the mean SCC of genotypes CD (1.98±0.15 lakhs/ml) and DD (2.24±0.18 lakhs/ml). The difference between the mean SCC of genotypes CD and DD was not significant. These findings are corroboratory to the findings of Kumar *et al.* (2014) in Sahiwal and Selvan *et al.* (2014b) in Karan Fries cows. These workers have reported the CC genotype at the same region of the CD 14 gene to be significantly less susceptible for mastitis. But in Deoni cows, Shivashanker *et al.* (2018) have reported a significantly lower incidence of mastitis among cows of the CD genotype as compared to CC and DD genotypes obtained by PCR-RFLP of the same region of the CD 14 gene. This discrepancy may be due to the small sample size, and varied managemental and environmental conditions prevailing over different farms/locations. Significant association of polymorphic variants at promoter and other regions of the CD 14 gene with mastitis incidence and somatic cell score has also been reported by Selvan *et al.* (2014a) in Karan Fries and Gupta *et al.,* (2018) in crossbred cows. Further, significant association of SNPs of CD14 gene with morbidity of mastitis and somatic cell count has also been reported by Jun *et al.* (2017) in Chinese Holstein cows and by Pal *et al.* (2019) in buffaloes.

CONCLUSION

Thus, the present study confirms the associations of polymorphisms at the CD 14 gene locus and SCC. Based on the findings, CD14 may be a key gene for mastitis, and its polymorphism regions may be further investigated as potential genetic indicators of mastitis resistance or susceptibility.

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