Associations between polymorphisms of LAP3 and SIRT1 genes with clinical mastitis and milk production traits in Sahiwal and Karan Fries dairy cattle

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Abstract
Background: Bovine mastitis continues to remain as the most challenging disease in dairy cattle, as a result improvement of selection methods has great economic relevance while a deeper understanding of the genetic mechanisms regulating milk production traits and mastitis is of general scientific interest.

Objectives: This study aimed to evaluate the association of SNPs of the LAP3 and SIRT1 genes with estimated breeding values (EBVs) of milk production traits and clinical mastitis in dairy cattle of Indian origin.

Methods: DNA samples from 263 animals (Sahiwal and Karan Fries cattle) were genotyped by PCR-RFLP to assess their pattern of genetic variation. EBVs of milk production traits and phenotypic records of incidences of clinical mastitis were used for association analysis.

Results: A total of 9 SNPs were identified, including three (rs110932626: A>G, rs716493845: C>T and rs43702363: C>T) in intron 12, four (g.24904G>C, rs110839532: G>T, rs43702361: T>C and rs41255599: C>T) in exon 13 and within 3’UTR of LAP3 gene and two (rs110250233: G>A and rs42140046: C>G) in the promoter region of SIRT1 gene. Eight of these identified SNPs were chosen for subsequent genotyping and association analyses. Association analysis revealed that SNP rs41255599: C>T was significantly associated with lactation milk yield, 305-day milk yield, 305-day fat yield, 305-day solid not fat yield, lactation length and incidence of clinical mastitis ($p < 0.05$) in Sahiwal cattle. For Karan Fries cattle, two SNPs including rs110932626: A>G and rs43702363: C>T showed significant association with 305-day milk yield.

Conclusion: Overall, these findings provide evidence for association of the LAP3 gene with milk production traits and clinical mastitis in dairy cattle, indicating the potential role of LAP3 variants in these traits.
1 | INTRODUCTION

Within India’s economy, the dairy industry is regarded as one of the most important and dynamic agrifood sectors, and it serves as the primary source of income for dairy farmers. The cattle population in the country is 192.49 million (37.28%) comprising of about 142.11 million indigenous and 50.42 million crossbred cattle, as per the 20th Livestock census (BAHS, 2019). Of this, indigenous and crossbred cattle contribute 9.63% and 27.68% to the total milk output (BAHS, 2019), respectively. Sahiwal is one of the most important milk breeds of cattle famous for higher milk production, remarkable power of endurance for hot climate of subtropics, comparatively resistant to diseases and low maintenance cost (Poonam et al., 2019). However, long-term selection to increase milk yield increases the incidence of mastitis in dairy cows (Negussie et al., 2008; Oltenacu and Broom, 2010). Whereas Karan Fries is a synthetic breed evolved by crossing Holstein Friesian (75%) and Tharparkar cattle in 1980 at ICAR-National Dairy Research Institute, Karnal, India. This breed has good production potential but is more prone to mastitis as compared to their Indian counterpart, while the prevalence of clinical mastitis in crossbred cows ranges between 5% and 37% (Bangar et al., 2016). The negative effects of mastitis are provoked by a combination of animal characteristics (age, lactation stage, etc.), genetic (breed, inbreeding, etc.) and environmental factors (season, management, etc.) (Oget et al., 2019). Therefore, the high prevalence of the incidence of clinical mastitis in Karan Fries cattle is partly due to the genetic influence of its Holstein Friesian ancestors added to the intensive selection for milk production traits. However, Fang et al. (2017) claim that different breeds could share similarities in genetic architecture underlying mastitis resistance and milk production traits.

Mastitis resistance and milk production are complex traits of economic importance in the dairy sector and are associated with intramammary infection (Fang et al., 2017). In the dairy industry, mastitis is one of the costliest diseases owing to its consequences of reduced milk production and quality and the need for the treatment and replacement of animals (Aitken et al., 2011). Since mastitis is difficult to record objectively and heritability is low, genetic selection to improve mastitis by traditional selection is not very effective (Sutera et al., 2021) and efforts to improve the genetic immune resistance have met with little success. To overcome this challenge, marker-assisted selection (MAS) approach for specific quantitative trait loci (QTL) or known genetic variants may be an alternative (Weigel & Shook, 2018). Traits of economic importance in animals are polygenic, that is, there are several QTLs that explain the phenotypic trait variation (Meuwissen et al., 2016). These QTLs indicate the genetic architecture of the traits, helping to find candidate genes. The candidate gene approach, which selects useful markers associated with milk production and health traits can be utilised in the breeding program through MAS, for instance, when the trait’s heritability is low.

Association of variants of candidate genes with economically important traits has been found to be significant in various studies; few examples that include well-characterised functional candidate genes (GHR, PAEP, CSN2, DGAT1, GPAT4 and FASN) are truly causative for milk production and composition traits in the French dairy cattle breeds (Tribout et al., 2020). Recent studies have revealed that numerous candidate genes, APP, FOXL2, SSFA2, OTUD3, ADORA2A, TXNRD2 and NDUFS6 (Szyda et al., 2019), and GC and NPFFR2 (Sahana et al., 2014) genes have been reported to be associated with clinical mastitis in dairy cattle. Moreover, the candidate genes MHC, IL, TLR, LTF, CARD15, FEZL, CD14, C4A and MBL1 found to be associated with resistance against mastitis (Ogorevc et al., 2009). Overall, identification of genetic variants that could be used in breeding programs to improve mastitis resistance and milk production traits in dairy cattle is of paramount importance given their future utility.

The bovine leucine amino peptidase three (LAP3) gene, which is located on chromosome 6, is made up of 13 exons that span a 25-kilobase genomic stretch and encodes a 519 amino-acid mature protein. In mammals, the LAP3 gene contributes to the processing of bioactive peptides (oxytocin, vasopressin, enkephalins) and is involved in vesicle trafficking to the plasma membrane for MHC-I antigen presentation (Kloetzel and Ossendorp, 2004; Matsui et al., 2006). Previous studies revealed that the LAP3 candidate gene is located within QTL region on bovine chromosome 6 with large effect on milk production traits (Cohen-Zinder et al., 2005; Khatkar et al., 2004; Ogorevc et al., 2009; Olsen et al., 2005; Sheely et al., 2009). On the other hand, it has been shown that polymorphisms in LAP3 gene variants are associated with milk production traits and somatic cell score in dairy cattle (Ju et al., 2012; Zheng et al., 2011) and birth weight in sheep (Lai, 2016).

The bovine silent information regulator one (SIRT1) gene, which is found on chromosome 28, contains 9 exons and is highly expressed in kidney and adipose tissue (Ghinishozumi et al., 2011). SIRT1 plays a role in physiological functions in liver, muscle, pancreas, testis, ovary and adipose tissues to regulate cell proliferation, cell survival and apoptosis. In addition, it has been discovered that calorie restriction in mammalian cells activates silent information regulator gene, which minimises stress-induced apoptosis, thus increasing the life span of the cell (Cohen et al., 2004). In mammals, the SIRT1 gene has also evolved to modify the activity of a growing number of transcription factors, including p53, NF-κB and PGC-1α, suggesting that SIRT1 functions in a wide range of cellular responses to stress, inflammation and nutrients. Previous polymorphism studies have reported several significant SNPs in SIRT1 gene for body size traits in the Nanyang cattle (Li et al., 2013), intramuscular fat content in Chinese Qinchan cattle (Gui et al., 2019) and carcass traits in Luxi cattle (Liu et al., 2017). Recently, Selvaggi et al. (2019) reported the significant effect of g.274C>G SNP locus
for milk production and reproductive performance traits in Agerolese cattle breed.

Based on the evidence presented above, it is believed that the LAP3 and SIRT1 genes could be an important candidate gene for marker-assisted selective breeding of resistance to mastitis and milk production traits in dairy cattle. However, to date, no studies have reported the genetic association of SNPs variation in bovine LAP3 and SIRT1 genes with clinical mastitis and milk production traits in Sahiwal and Karan Fries dairy cattle. Therefore, this study aimed to explore the genetic polymorphism in bovine LAP3 and SIRT1 genes and analyse their association with clinical mastitis and estimated breeding values (EBVs) of milk production traits in dairy cattle.

2 | MATERIALS AND METHODS

2.1 | Animals and phenotypic data collection

Milk recording data were collected from 935 Sahiwal cows (1979–2019) and 1426 Karan Fries cows (1988–2019) with all parities maintained at ICAR-National Dairy Research Institute (ICAR-NDRI), India. The animals were maintained under the loose housing system and were machine milked three times a day (4 a.m., 12 noon and 4 p.m.) with the recording of milk yield at every milking. The estimated breeding values for lactation milk yield (LMY), 305-day milk yield (305dMY), 305-day fat yield (305dFY), 305-day solid not fat yield (305dSNFY) and lactation length (LL), were estimated by repeatability animal model using BLUPF90 (Misztal et al., 2015). Phenotypic data pertinent to the incidences of clinical mastitis (CM) were recorded for Sahiwal and Karan Fries cattle from the treatment register of Animal Health Complex, NDRI, Karnal. The criteria for judging and identification of clinical mastitis was done by the farm veterinarian based on symptoms of abnormalities of udder or teat such as swelling, heat, pain, redness and hardness and/or change in colour/consistency and presence of flakes/shreds in milk. Depending on the incidence of clinical mastitis, animals were grouped as not affected/healthy (0 = no mastitis) with no episode of clinical mastitis and mastitis affected (1 = clinical mastitis) with more than one episode of clinical mastitis during the lactations considered.

2.2 | DNA isolation and PCR amplification

Genomic DNAs were isolated from whole blood samples of 263 lactating dairy cattle (Sahiwal = 125 and Karan Fries = 138) by phenol-chloroform extraction method following standard procedures (Sambrook & Russell, 2001). The quantity and quality of extracted DNAs were measured by NANODROP 2000 Spectrophotometer (Thermo Scientific, DE, USA). Primers used to amplify the target regions of bovine LAP3 (Accession number: ENSBTAG00000005989) and SIRT1 (Accession number: ENSBTAG00000014023) genes were designed using Primer3Plus (v.0.4.0) online software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) according to Bos taurus sequence provided in Ensemble genomic browser (http://www.ensembl.org). The sequences of PCR primers used for amplification, target regions, expected product size and annealing temperature are presented in Table 1. Other primers with no polymorphism detected in their amplification regions are not listed in this table. The PCR reaction were carried out in a total volume of 25 µl on a Thermo-Cycler (Bio-Rad T100) containing 50 ng genomic DNA: 2.0 µl and 0.5 µl of each primer, 13.0 µl of 2× PCR Master Mix and 9.0 µl of nuclease free water. The PCR reaction cycling protocol was performed with an initial denaturation at 95°C for 3 min, followed by 34 cycles of 94°C for 30 s, respective annealing temperature for 30 s and 72°C for 40 s, with a final extension step at 72°C for 8 min. A 1.5% agarose gel electrophoresis stained with ethidium bromide was used to evaluate the PCR results (Supplementary Figures S1 and S2).

2.3 | SNPs identification and genotyping

Representative samples of PCR products were purified and sequenced on an automated ABI3730XL DNA sequencer (ABI, Foster city,
RESULTS AND DISCUSSION

Statistical analysis

Identification and genotyping of SNPs

This study revealed a total of nine SNPs, including three (rs110932626: A>G, rs716493845: C>T and rs43702363: C>T) in the intron 12, four (g.24904G > C, rs110839532: G>T, rs43702361: T>C and rs41255599: C>T) in exon 13 and within 3’UTR of LAP3 gene and two (rs110250233: G>A and rs42140046: C>G) in the promoter region of SIRT1 gene of studied population (Figure 1). Further, 6 SNPs in LAP3 gene (rs110932626: A>G, rs716493845: C>T, rs43702363: C>T, g.24904G > C, rs110839532: G>T and rs41255599: C>T) and the 2 SNPs in SIRT1 gene (rs110250233: G>A and rs42140046: C>G) were finally individually genotyped while using PCR-RFLP method for 125 Sahiwal and 138 Karan Fries cows. Pictorial representation for genotypes is given in Figures 1 and 2. Identified SNPs, restriction enzymes used and sizes of fragments are presented in Table 2. The SNP at rs110932626: A>G locus of 560 bp PCR product in intron 12 of LAP3 gene was digested with TaqI restriction enzyme (Supplementary Figure S3a). Three genotypes were identified in Karan Fries cattle, the genotype AA defined by the presence of four restriction bands of 228, 169, 134 and 29 bp, genotype AG represents five restriction fragment bands of 303, 228, 169, 134 and 29 bp and genotype GG represents three bands of 303, 228 and 29 bp, respectively (Table 2). Restriction digestion using HindIII for LAP3 gene at the SNP rs716493845: C>T locus revealed polymorphic profile of three genotypes with lengths of 358 and 202 bp for genotype CC, 560, 358 and 202 bp for genotype CT, and 560 bp for genotype TT in Sahiwal cattle population (Supplementary Figure S3b). However, this SNP locus found to be nearly monomorphic (CC genotype) in the tested Karan Fries cattle population. Likewise, the SNP rs43702363: C>T in LAP3 gene was also digested with NmuCI restriction enzyme (Table 2) and resulted in fragment length of 560 bp for CC genotype, 560, 407, 128 and 25 bp for CT genotype and 407, 128, and 25 bp for TT genotype in Karan Fries cattle population (Supplementary Figure S3c) and proven to be monomorphic (TT genotype) in the tested Sahiwal cattle population. Notably, the SNP at position g.24904G>C was novel and digested with PstI restriction enzyme and revealed two genotypes (Supplementary Figure S3d) with fragment length of 500, 42 and 18 bp for genotype GG and 560, 500, 42 and 18 bp for genotype GC (Table 2) in Sahiwal cattle population. The genotype CC was not observed in the sampled population; the probably reason may be because the experimental population size was insufficient to capture full genetic variation (Gui et al., 2015). This SNP tended to have monomorphic pattern in Karan Fries cattle population. Moreover, digestion of the PCR product of the LAP3 gene for the mutation site rs110839532: G>T with TaqI restriction enzyme (Table 2) revealed 449 and 57 bp fragments for genotype GG; 506, 449 and 57 bp fragments for genotype GT and 506 bp fragments for genotype TT in Karan Fries cattle population (Supplementary Figure S3e). The other SNP (rs41255599: C>T) locus in exon 13 within 3’UTR held no suitable endonuclease restriction site; it was genotyped by employing Artificially Created Restriction Site (ACRS-PCR) containing

2.4 Statistical analysis

The population genetics statistical analyses, genotype frequency, allelic frequency, Hardy-Weinberg equilibrium (HWE) $\chi^2$ test and polymorphism information content (PIC) for the identified SNPs, were performed using PopGen2 software (Yeh et al., 1997). The EBVs of 263 dairy cows (Sahiwal = 125 and Karan Fries = 138) were used as a phenotype to test the association of LAP3 and SIRT1 genes SNPs with EBVs of milk production traits. Thus, the effect of genotypes on the EBVs of milk production traits was analysed using the general linear model (GLM) procedure of SAS version 9.2 software (SAS, 2008), with the following model. Moreover, the Tukey–Kramer multiple comparison test was used to test significance of differences between groups. Significant level was set to be $p < 0.05$.

$$Y_{ij} = \mu + G_i + e_{ij},$$

where $Y_{ij}$ is estimated breeding value of $j$th animal of $i$th genotype, $\mu$ is overall mean, $G_i$ is the effect of $i$th genotypes and $e_{ij}$ is the residual error NID (0, $\sigma^2_e$).

Association of genotypes with mastitis affected and non-affected animals was calculated using the chi-square ($\chi^2$) procedure of SAS Version 9.2 software. Further logistic regression model was applied to analyse the effect of calving period, calving season, parity and genotypes on the incidence of clinical mastitis by the maximum likelihood method of the logistic procedure of SAS Version 9.2 software. Further logistic regression model was applied to analyse the effect of calving period, calving season, parity and genotypes on the incidence of clinical mastitis by the maximum likelihood method of the logistic procedure of SAS Version 9.2 software. Further logistic regression model was applied to analyse the effect of calving period, calving season, parity and genotypes on the incidence of clinical mastitis by the maximum likelihood method of the logistic procedure of SAS Version 9.2 software. Further logistic regression model was applied to analyse the effect of calving period, calving season, parity and genotypes on the incidence of clinical mastitis by the maximum likelihood method of the logistic procedure of SAS Version 9.2 software. Further logistic regression model was applied to analyse the effect of calving period, calving season, parity and genotypes on the incidence of clinical mastitis by the maximum likelihood method of the logistic procedure of SAS Version 9.2 software. Further logistic regression model was applied to analyse the effect of calving period, calving season, parity and genotypes on the incidence of clinical mastitis by the maximum likelihood method of the logistic procedure of SAS Version 9.2 software. Further logistic regression model was applied to analyse the effect of calving period, calving season, parity and genotypes on the incidence of clinical mastitis by the maximum likelihood method of the logistic procedure of SAS Version 9.2 software. Further logistic regression model was applied to analyse the effect of calving period, calving season, parity and genotypes on the incidence of clinical mastitis by the maximum likelihood method of the logistic procedure of SAS Version 9.2 software. Further logistic regression model was applied to analyse the effect of calving period, calving season, parity and genotypes on the incidence of clinical mastitis by the maximum likelihood method of the logistic procedure of SAS Version 9.2 software. Further logistic regression model was applied to analyse the effect of calving period, calving season, parity and genotypes on the incidence of clinical mastitis by the maximum likelihood method of the logistic procedure of SAS Version 9.2 software.
a nucleotide mismatch, which enabled the use of restriction enzymes for discriminating nucleotide mismatches (Zhao et al., 2003). Thus, the newly created restriction site, adopted from Zheng et al. (2011), was digested with HhaI and produced three genotypes with three distinct patterns in Sahiwal cattle population. These genotypes were CC (122 and 20 bp), TC (142, 122 and 20 bp) and TT (142 bp), respectively (Table 2).

Regardless of SIRT1 gene, the SNP at the nucleotide position g-382G > A (rs110250233: G > A) in the promoter region held no suitable endonuclease restriction site; it was genotyped by employing Created
FIGURE 2  Schematic representation of bovine SIRT1 gene structure showing promoter in green bars, exons 1 to 8 in dark blue bars and introns in grey intervals, the position of identified SNPs and results of sequencing chromatogram of the polymorphic loci in the Karan Fries cattle population.

TABLE 2  Identified SNPs, location, restriction enzymes used for genotyping and size of fragment pattern in LAP3 and SIRT1 genes for Sahiwal and Karan Fries cattle breed

| Gene | SNP ID   | Location | Nucleotide substitution | Breed | RE     | Sizes of fragment pattern (bp) |
|------|----------|----------|-------------------------|-------|--------|-------------------------------|
| LAP3 | rs110932626 | Intron 12 | G/A                     | KF    | Tasi   | 560/303+ 228+ 169+ 29         |
|      | rs716493845 |          | C/T                     | SW    | HindII | 560/358+ 202                   |
|      | rs43702363  |          | C/T                     | KF    | NmuCl  | 560/407+128+25                |
|      | g.24904G>C  | Exon 13  | G/C                     | SW    | PstI   | 560/500+42+18                 |
|      | rs110839532 |          | G/T                     | KF    | TaqI   | 506/449+57                    |
|      | rs41255599  |          | C>T                     | SW    | HhaI   | 142/122+20                    |
| SIRT1| rs110250233 | Promoter | G>A                     | KF    | VspI   | 166/139+27                    |
|      | rs42140046  |          | C>G                     | KF    | Smal   | 273/235+38                    |

KF, Karan Fries; SW, Sahiwal; RE, restriction enzyme.

g.24904G>C rs110839532: G>T rs43702361: T>C rs41255599: C>T.
### TABLE 3  The genotype and allelic frequencies and genetic diversity parameters of LAP3 and SIRT1 genes in Sahiwal and Karan Fries cattle

| Breed | SNP | Genotypic frequency | Allelic frequency | HWE ($\chi^2$) | PIC | He | Ho | ne* |
|-------|-----|---------------------|-------------------|----------------|-----|-----|-----|-----|
| SW    | rs716493845: C>T | CC        | CT        | TT        | C     | T     | 0.527 | 0.362 | 0.477 | 0.51 | 1.69 |
|       | g.24904G>C   | GG        | GC        | CC        | G     | C     | 16.238*** | 0.316 | 0.39 | 0.53 | 1.64 |
|       | rs41255599: C>T | CC        | CT        | TT        | C     | T     | 16.087*** | 0.355 | 0.46 | 0.296 | 1.85 |
| KF   | rs110932626: A>G | AA        | AG        | GG        | A     | G     | 5.67* | 0.311 | 0.387 | 0.464 | 1.627 |
|       | rs43702363: C>T | CC        | CT        | TT        | C     | T     | 3.89* | 0.305 | 0.376 | 0.446 | 1.60 |
|       | rs110839532: G>T | GG        | GT        | TT        | G     | T     | 2.84 | 0.276 | 0.338 | 0.386 | 1.50 |
|       | rs110250233: G>A | GG        | GA        | AA        | G     | A     | 0.903 | 0.136 | 0.143 | 0.154 | 1.166 |
|       | rs42140046: C>G | CC        | CG        | GG        | C     | G     | 3.46 | 0.374 | 0.499 | 0.421 | 1.99 |

*Significant at $p < 0.05$.
***Significant at $p < 0.001$.

SW, Sahiwal; KF, Karan Fries; $\chi^2$, chi-square; PIC, polymorphic information content; He, expected heterozygosity; Ho, observed heterozygosity; ne, Effective number of alleles.

Restriction Site PCR (CRS-PCR), following the protocols adopted from Li et al. (2013), which enabled the use of restriction enzymes for discriminating sequence variations. The third base C was replaced by A at the 3’ end of forward primer, which created a new VspI restriction site (ATGAA). Thereupon, digestion of the PCR product of 273 bp size harbouring SNP at rs110250233: G>A mutation in the promoter of SIRT1 gene with the enzyme VspI revealed two genotypes GG (166 bp) and GA (166, 139 and 27 bp) in Karan Fries cattle population (Table 2), but this SNP detected to be monomorphic in the studied Sahiwal cattle population (Supplementary Figure S4a). These results differ from those presented by Li et al. (2013), where three genotypes (GG, GA and AA) in this SNP region (rs110250233: G>A) in five cattle breeds of China was reported. Moreover, SNP locus of rs42140046: C>G in the promoter region of SIRT1 gene was digested with the enzyme Smal and revealed monomorphic patterns in Sahiwal cattle population and polymorphic patterns of CC (273 bp), CG (273, 235 and 38 bp) and GG (235 and 38 bp) genotypes (Supplementary Figure S4b) in Karan Fries cattle population (Table 2), respectively. This agreed with the previous findings (Gui et al., 2019; Li et al., 2013; Selvaggi et al., 2019), who reported the three genotypes in this SNP region in indigenous cattle breeds of China and Agerolese cattle breed in Italy, respectively.

### 3.2 Genotype and allelic frequencies

The genotype and allelic frequencies, expected heterozygosity (He), observed heterozygosity (Ho), effective population size (ne) and its corresponding chi-squared test ($\chi^2$) to determine whether the population was in Hardy-Weinberg equilibrium (HWE) or not are presented in Table 3. The level of significance used in the test was 0.05; thus, values above 0.05 indicate that the population was in HWE. Notably, two SNPs (g.24904G>C and rs41255599:C>T) in Sahiwal ($p < 0.01$) and two SNPs (rs110932626: A>G and rs43702363: C>T) in Karan Fries cattle population ($p < 0.05$) are the individual frequencies deviated from HWE. The possible explanation for this could be as follows: (1) Sahiwal and Karan Fries are the well-established Zebu and cross-bred cattle breeds known to have good genetic potential to produce considerably large quantities of milk in India, selection pressure for the increase in milk production infer the loss of non-favourable alleles and (2) the effect of small population size used for the study cannot be neglected. The genetic indices of gene homozygosity (Ho), gene heterozygosity (He), polymorphic information content (PIC) and $\chi^2$ values are effective to assess the genetic diversity from different loci of candidate genes. According to the results of the present study, the PIC and He values of the identified SNPs in LAP3 gene exhibited that the genetic diversity of Sahiwal and Karan Fries cattle was in medium polymorphism level (0.25<PIC<0.50). Moreover, the studied population possess medium to high effective allele number (ranging from 1.50 to 1.85). This indicates that these SNPs have a potential for selection. With respect to SIRT1 gene, Karan Fries cattle population had a low genetic diversity (PIC<0.250) at rs110250233: G>A locus, while they possessed a moderate genetic diversity (0.25<PIC<0.500) at rs42140046: C>G locus. Li et al. (2013) reported similar results in the Nanyang, Jiaxian and Qinchuan cattle populations in China.
addition, Gui et al. (2019) reported moderate genetic diversity for the SNP rs42140046: C>G locus in Chinese indigenous cattle. The low genetic diversity in Karan Fries cattle breed of this SNP reflected selective breeding, where this herd is being selected for several generations for better lactation performance.

The results of the present study revealed that the genotypic frequency of GG and AG genotypes at locus rs110932626: A>G were 0.51 and 0.46 in Karan Fries cattle population, whereas the frequency of AA genotype was essentially rare and close to zero (0.03). In total there were relatively few cows that were homozygous for the A variant, though the G variant and the GG genotype were more frequent among the Karan Fries cows (Table 3). Even though, Zheng et al. (2011) reported the three genotypes in this SNP locus in Chinese cattle breeds, by comparison, the occurrence of the three genotypes differed for different Chinese cattle breeds. In terms of allelic frequency, they reported that G allele was more frequent, which supports our finding. It was noted that CT genotype was the most frequent amongst SNP rs716493845: C>T locus genotypes (0.51), with CC and TT being less frequent (0.36 and 0.13) in Sahiwal cows. Though C allele was the most abundant in Sahiwal cattle population under the study (Table 3). Within rs43702363: C>T locus of LAP3 gene, through digesting with NmuCI restriction enzyme resolved into three polymorphic patterns of CC, CT and TT genotypes. Accordingly, the CC genotype occurred at a very low frequency (0.03) than CT (0.45) and TT (0.52), respectively (Table 3). For SNP rs42140046: G>T locus, the genotype frequencies of GG and GC in Sahiwal cattle population were 0.47 and 0.53, respectively. However, CC genotype was not prevalent in the studied population. The G allele frequency was significantly higher compared to its counterpart C allele in the studied population (Table 3). In the same way, at rs110839532: G>T locus, the genotypic frequency of TT (0.59) was also the maximum, while that of GG genotype occurred at a very low frequency (0.02), which was essentially close to zero in Karan Fries cattle population. Whereas the frequency of GT genotype was 0.39 (Table 3). Similarly, at locus rs41255599: C>T, the frequency of TT genotype (0.49) was higher than CT (0.30) and CC (0.21) genotypes. At this locus, the T allele frequency was maximal.

Another SNP, targeting rs42140046: C>G locus in the promoter region of SIRT1 gene, resulted polymorphic patterns of CC, CG and GG genotypes. The CG genotype (0.42) was somewhat more frequent compared with CC (0.32) and GG (0.26) genotypes in Karan Fries cattle population (Table 3). These results resemble with the previous studies (Li et al., 2013; Liu et al., 2017; Selvaggi et al., 2019) for different cattle breeds.

### 3.3 Association of SNPs with estimated breeding values of milk production traits

The association analysis results between SNPs genotypes of bovine LAP3 and SIRT1 genes and EBVs of milk production traits are presented in Table 4. The present study revealed that the SNP rs41255599: C>T remained significantly associated with EBVs of all the studied milk production traits LMY, 305dMY, 305dFY, 305dSNFY and LL (p value = 0.0425–0.0001) in Sahiwal cows, respectively. Interestingly, the CT genotype of SNP rs41255599: C>T locus possessed the highest EBVs for all milk production traits as compared to CC and TT genotype, indicating that allele T was associated with superior genetic merit for milk production traits (Table 4) of the studied population. However, the remaining two SNPs did not show significant effect on the EBVs of the studied traits (p values > 0.05) of Sahiwal cows. In the other hand, the SNPs rs110932626: A>G and rs43702363: C>T located within the same gene had significant effect on EBV of 305dMY (p = 0.0302) in Karan Fries cows, and cows with GG and TT led to much better performance than AG and CT genotypes (Table 4), respectively. Zheng et al. (2011) studied five SNPs in the introns 12 and exon 13 of the LAP3 gene, and SNP g.25415T>C was found to be significantly associated with protein percentage. Furthermore, Ju et al. (2012) reported two novel SNPs in the promoter region of the bovine PEPS (LAP3) gene other than the SNP locus identified in this study, and they found that the combined analysis of these SNPs, g. -534>T and g. -2545>G>A, was notably significantly associated with fat percentage and SCS traits in Chinese Holstein cattle. In light of all these, it is believed that milk production traits of the studied breeds were influenced by QTL effects in chromosome 6 of LAP3 gene. This confers well with several studies; for example Ogorevc et al. (2009) and Khatak et al. (2004) illustrated the presence of one or more QTL for milk production traits on BTA6. A QTL containing six milk production candidate genes including the LAP3 gene was mapped to a 420-kb region on bovine chromosome 6 (Olsen et al., 2005). Bongiorni et al. (2012) detected the strongest signal on chromosome 6 between 37.8 and 38.7 Mb, where LAP3 gene, involved in oxytocin hydrolysies, is located in this region. Another evidence claims that LAP3 gene, close to QTL, plays an important role for milk performance traits (Cohen-Zinder et al., 2005; Sheely et al., 2009). Such data clearly demonstrated that the LAP3 gene could be an important candidate gene for milk production traits in dairy cattle. In a nutshell, this study underlines the importance of LAP3 gene for better lactation performance in dairy cattle.

Regarding association between SNP genotypes of SIRT1 gene and EBV of milk production traits, this study did not find the suggestive significant effect (p < 0.05) of SNPs rs110250233: G>A and rs42140046: C>G on any of the studied traits in Karan Fries cattle population (Table 4). The non-significant relationship of this SNP could be owing to the small size of the studied population, which had smaller number of genotyped cows for the association analysis. On the contrary, the SNP rs42140046: C>G polymorphism in bovine SIRT1 gene was reported to be associated with milk production (Selvaggi et al., 2019), intramuscular fat content (Gui et al., 2019) and growth traits (Li et al., 2013) in different cattle breeds, respectively. Experiment to directly determine the allele-specific effects of the c.-274C>G (rs42140046: C>G) polymorphism on native promoter activity affirms that the binding sites for the cell cycle-dependent element (generated in the presence of C allele) were abolished in the presence of the G allele, which indicated that the c.-274C>G polymorphism might affect the binding affinity of the surrounding sequences and further diminish the activity of SIRT1 promoter, which may reduce SIRT1’s function as a deacetylase and then results in increased PPARc transcriptional activity and fat storage.
### Table 4: Association of different genotypes of SNPs in LAP3 and SIRT1 genes with EBV of milk production traits for Sahiwal and Karan Fries cattle

| Breed | Gene  | Loci          | Genotype | LMY      | 305dMY   | 305dFY   | 305dSNF | LL      |
|-------|-------|---------------|----------|----------|----------|----------|----------|---------|
| SW    | LAP3  | rs716493845: C>T | CC (44)  | 42.7     | 42.6     | -0.21    | 0.19     | 2.69    |
|       |       |               | CT (63)  | 16.02    | 18.74    | -0.40    | -0.04    | 3.20    |
|       |       |               | TT (16)  | -24.76   | -13.73   | -0.37    | 0.001    | 1.27    |
|       |       |               | p        | 0.4733   | 0.5221   | 0.8892   | 0.9429   | 0.9388  |
|       |       |               | g.24904G>C | GG (57)  | 49.28    | 50.21    | -0.08    | 0.48     | 2.05    |
|       |       |               | GC (68)  | -7.58    | -2.73    | -0.54    | -0.33    | 3.24    |
|       |       |               | p        | 0.0980   | 0.0886   | 0.1873   | 0.1918   | 0.7300  |
|       |       | rs41255599: C>T | CC (26)  | 19.79    | b        | 21.64    | b        | -0.41b   | 2.33b   |
|       |       |               | CT (36)  | 147.46   | a        | 137.82   | a        | 1.02a    | 2.24a   |
|       |       |               | TT (61)  | -54.54   | b        | -43.85   | b        | -1.07b   | -1.18b  |
|       |       |               | p        | 0.0001   | 0.0001   | 0.0001   | 0.0001   | 0.0425  |
| KF    | LAP3  | rs110932626: A>G | AG (66)  | 143.98   | 105.33   | 0.49     | -0.11    | 2.07    |
|       |       |               | GG (72)  | 274.15   | 206.75   | -0.004   | -0.92    | 3.16    |
|       |       |               | p        | 0.0803   | 0.0302   | 0.3963   | 0.4431   | 0.4860  |
|       |       | rs43702363: C>T | CT (64)  | 143.33   | 93.10    | -0.11    | -0.91    | 2.13    |
|       |       |               | TT (74)  | 275.26   | 206.91   | 0.33     | -0.37    | 3.50    |
|       |       |               | p        | 0.1281   | 0.0332   | 0.5121   | 0.6526   | 0.4533  |
|       |       | rs110839532: C>G | TT (54)  | 176.56   | 123.70   | 0.36     | -0.05    | 2.83    |
|       |       |               | TT (84)  | 234.62   | 180.46   | 0.15     | -0.59    | 2.52    |
|       |       |               | p        | 0.4481   | 0.2387   | 0.7311   | 0.9007   | 0.8458  |
|       | SIRT1 | rs110250233: G>A | GG (114) | 223.30   | 158.14   | 0.11     | -0.69    | 2.65    |
|       |       |               | GA (21)  | 181.19   | 285.15   | 0.92     | 0.66     | 3.09    |
|       |       |               | p        | 0.6858   | 0.6799   | 0.3196   | 0.3625   | 0.8408  |
|       |       | rs42140046: C>G | CC (45)  | 211.10   | 144.13   | -0.40    | -1.25    | 3.04    |
|       |       |               | CG (58)  | 239.66   | 175.75   | 0.46     | -0.28    | 2.86    |
|       |       |               | GG (35)  | 166.92   | 147.39   | 0.68     | -0.03    | 1.76    |
|       |       |               | p        | 0.7416   | 0.8182   | 0.2996   | 0.6345   | 0.8072  |

Note: Least square means within a column with no common superscript differ significantly (p < 0.05). SW, Sahiwal; KF, Karan Fries.

Further study indicated that transcriptional activity of SIRT1 was likely to be modified by different cis-acting elements binding to the promoter region because of the presence of c.-274C>G SNP (Gui et al., 2019).

### 3.4 Association of SNPs with incidence of clinical mastitis

The association analysis results between SNPs genotypes and clinical mastitis are shown in Tables 5 and 6. Association study was done using the chi-square ($\chi^2$) analysis as well as logistic regression approach. The probability was modelled to the event of incidence of clinical mastitis. In the present study, we identified rs41255599: C>T locus in LAP3 gene significantly contributed to the incidence of clinical mastitis at p < 0.05 in Sahiwal cattle (Table 5). Among the genotypes, referring to rs41255599: C>T locus, Sahiwal cows expressing genotype CC were less susceptible to the incidence of clinical mastitis in comparison to cows with genotype CT ($p < 0.01$): 68.00% versus 31.03%, respectively (Table 5). Sahiwal cows tended to have high incidence of clinical mastitis when the CT genotype was expressed as compared with the CC and TT genotype; the odds ratio of CT against TT for the incidence of clinical mastitis was found to be high, that is, 3.59 with confidence interval 1.25-10.3 (Table 6). This was confirmed in the $\chi^2$ values given in Table 4, where the frequency of CT genotype for mastitis affected Sahiwal cows was extremely higher than the frequency of TT genotype (68.97% vs. 33.90%). Regrettably, a variant in the SNP locus rs41255599: C>T LAP3 gene causes enhanced estimated breeding values of milk production traits in heterozygote (Table 4), while less incidence of clinical mastitis in homozygotes (CC and TT) in Sahiwal cows (Table 6). In the same way, variants that cause a heterozygote disadvantage for bovine tuberculosis have been described by Tsairidou et al. (2018), who...
TABLE 5  Chi-square values for mastitis affected and not affected animals with respect to genetic variants of LAP3 and SIRT1 genes in Sahiwal and Karan Fries cattle

| Breed | Gene | SNPs | Genotype | Non-affected animals (No (%)) | Affected animals (No (%)) | Total | χ² value |
|-------|------|------|----------|-------------------------------|----------------------------|-------|---------|
| SW    | LAP3 | rs716493845: C>T | CC       | 27 (61.36)                    | 17 (38.64)                 | 44    | 0.489   |
|       |      | g.24904 G>C   | CT       | 33 (51.56)                    | 31 (48.44)                 | 64    |         |
|       |      |               | TT       | 7 (46.67)                     | 8 (53.33)                  | 15    |         |
|       |      |               |          | 28 (49.12)                    | 29 (50.88)                 | 57    | 0.268   |
|       |      | rs41255599: C>T | CC       | 17 (68.00)                    | 8 (32.00)                  | 25    | 11.22** |
|       |      |               | CT       | 9 (31.03)                     | 20 (68.97)                 | 29    |         |
|       |      |               | TT       | 39 (66.10)                    | 20 (33.90)                 | 59    |         |
| KF    | LAP3 | rs110932626: A>G | AG       | 40 (67.80)                    | 19 (32.20)                 | 59    | 0.02    |
|       |      | rs43702363: C>T | GG       | 46 (66.70)                    | 21 (33.30)                 | 69    |         |
|       |      | rs110839532: G>T | CT       | 28 (59.60)                    | 19 (40.40)                 | 47    | 0.56    |
|       |      |               | TT       | 38 (66.66)                    | 19 (33.20)                 | 57    |         |
|       |      | SIRT1         | GG       | 72 (64.86)                    | 39 (35.14)                 | 111   | 0.56    |
|       |      | rs110250233: G>A | GA       | 15 (71.43)                    | 6 (28.57)                  | 21    |         |
|       |      | rs42140046: C>G | GG       | 29 (67.44)                    | 14 (32.55)                 | 43    | 0.32    |
|       |      |               | CC       | 38 (66.66)                    | 19 (33.33)                 | 57    |         |
|       |      |               | GG       | 21 (61.76)                    | 13 (38.23)                 | 34    |         |

**p < 0.01.
SW, Sahiwal; KF, Karan Fries.

TABLE 6  Effect of genetic variants of LAP3 gene on the incidence of clinical mastitis in Sahiwal cattle

| Effect  | Genotype | Estimate ± SE | Wald chi square | Odds ratio | 95% CI     |
|---------|----------|---------------|-----------------|------------|------------|
| rs41255599: C>T | CC       | −0.23 ± 0.56  | 0.16            | 0.797      | 0.26–2.41  |
|         | CT       | 1.28 ± 0.53   | 5.65            | 3.593*     | 1.25–10.31 |
|         | TT       | -             | -               | -          | -          |

*p < 0.05.
CI, confidence interval; SE, standard error.

reported that cattle heterozygous for the locus on BTA6 was linked to increased susceptibility to bovine tuberculosis in comparison with homozygotes in cattle, that is, a heterozygote disadvantage. Nevertheless, when considering why LAP3 gene highly associated with both the traits, LAP3 involved in glucose transport, defence, MHC I antigen presentation, hormone control such as oxytocin and vasopressin catabolism, protein maturation, protein inactivation and protein digestion and other physiological functions (Kloetzel & Ossendorp, 2004; Matsui et al., 2006). In that way, LAPs and LAP-related aminopeptidases provide a surprising diversity of functions and impacts on cellular and physiological activities. However, it is noted that our results are based upon a relatively small cattle population and it needs to expand the population size to further verify the effects of this specific SNP on the incidence of clinical mastitis in Sahiwal cattle.

Association of the SNP locus (rs41255599: C>T) with the incidence of clinical mastitis in Karan Fries cattle was non-significant (p > 0.05). Significant differences were not detected in either of the remaining SNP genotypes, neither in LAP3 nor in SIRT1 genes, and percentage observation essentially remains the same for the incidence of clinical mastitis in Sahiwal and Karan Fries cattle population included in this study (Table 5). Few studies have been conducted on the association of LAP3 genes with mastitis incidence in dairy cattle. Even though reports are available, but few polymorphism studies attempt to crystalize the association of candidate genes with somatic cell score of dairy animals. In fact, somatic cell count (SCC) in milk reflects the health state of cows; their level is an important indicator for cattle breeders. In cows producing milk with a somatic cell count of less than 200,000 cells/ml indicate a healthy mammary quarter; however, a greater SCC is an
influence or bias the content of the paper.

4 | CONCLUSIONS

In conclusion, the results presented herein demonstrated that the SNP variants (rs110932626:A>G and rs43702363:C>T) in LAP3 gene had significant effects on EBV of 305-day milk yield in Karan Fries cattle. Further, we identified another SNP variant (rs41255599: C>T) in the LAP3 gene associated with clinical mastitis and EBVs of milk production traits in Sahiwal cattle. This study revealed better lactation performance in heterozygous genotype of rs41255599: C>T locus, while less resistant to clinical mastitis in Sahiwal cattle and could not be used as an aid to select for simultaneous improvement of animals. However, further studies in a larger population size are warranted to explore the role of LAP3 variants on milk yield traits and clinical mastitis in dairy cattle.

AUTHOR CONTRIBUTIONS

Destaw Worku: Conceptualisation; data curation; formal analysis; investigation; methodology; software; supervision; visualisation; writing – original draft; writing – review & editing. G.R. Gowane: Conceptualisation; data curation; methodology; supervision; visualization; writing-review & editing. Anupama Mukherjee: Resources; writing – review & editing. Rani Alex: Writing – review & editing. Pooja Joshi: Writing – review & editing. Archana Verma: Conceptualisation; funding acquisition; investigation; supervision; validation; writing – review & editing.

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CONFLICT OF INTEREST

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

DATA AVAILABILITY STATEMENT

The data sets supporting the conclusions of this article are included within the article and its additional files.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.924.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental procedures were approved by the Institutional Animal Ethics Committee of ICAR-National Dairy Research Institute, Karnal, India and were performed according to guidelines and rules framed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Reg. No. 1705/GO/ac/13/CPCSEA).

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