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Association of *prolactin, interleukin-8* genes polymorphism and the incidence of some diseases with milk production traits in Holstein-Friesian Cows

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ABSTRACT

The present study was conducted to identify the association between *prolactin and interleukin-8* gene polymorphism and their effects on hormonal concentration, milk yield, and composition traits in Holstein-Friesian cows. Sixty-four Holstein-Friesian milking cows from Gammasa dairy farm close to Mansoura city were categorized into three groups based on milk yield (G1, n= 20 high, G2, n= 24 medium and G3, n= 20 low-producing animals). Serum, blood, and milk samples were collected from the three groups to measure hormone concentration, sequence analysis, and milk components (fat, protein, lactose, and ash). Two novel single nucleotide polymorphisms (SNPs) known as C54G and G75A were detected within the exon three of the prolactin gene. Although high-producing animals with CG prolactin genotype were more frequent in the population (0.62) and significantly showed high serum prolactin hormone concentration, the impact of these SNPs on milk yield traits was statistically non-significant. Moreover, the prolactin C54G showed a significant effect on milk components. Furthermore, a significant association existed between high-producing animals and a higher incidence of mastitis. The results indicated that the population is in Hardy–Weinberg equilibrium, suggesting no change in the distribution of alleles among consequent generations. The CG SNP of the prolactin gene may be a helpful marker for assisted selection programs to improve Holstein-Friesian cattle health and milk composition traits.

1. INTRODUCTION

It is a challenge to find genes that influence polygenic features that characterize milk or meat production. Several putative candidate genes, however, have been identified. They can be investigated as quantitative trait loci (QTLs) if chosen according to a known association between biochemical or physiological processes and production traits (Oprzadek et al., 2003). Animals should be periodically selected based on their genotype, impacting their productivity and national economy and increasing their performance. There is a great interest in adopting productive traits (e.g., milk and meat production) based on molecular DNA markers technology for more effective and reasonable simple animal selection. (El-Magd et al., 2014). Also, breeders are advised to consider the environmental factors in the management program because they significantly impact farm profitability (Shalan et al., 2022). Lactogenic genes are candidate genes that are linked to milk traits. In this group of genes, the prolactin (PRL) gene was included (El-Magd et al., 2015). Prolactin is a crucial hormone in regulating mammary gland processes, mainly galactopoiesis (Lacasse et al., 2016). Although the mechanism governing PRL secretion is not well understood, basal PRL concentration and the volume of PRL released during milking decrease as lactation progresses (Koprowski and Tucker, 1973; Miller et al., 2000; Bernier-Dodier et al., 2011). Additionally, as the period between milking or manual stimulations shortens, cows' milking induced PRL release decreases (Lacasse and Ollier, 2014). Linkage QTL analysis for milk production traits appears to be a candidate for using the bovine prolactin gene (Thuy et al., 2018).

The Prolactin gene, which is found on chromosome 23 (Barendse et al., 1997), has five exons and four introns that cover a 10-kilobase (kb) genomic tract and is encoded for a mature protein of 199 amino acids (Wallis, 1974; Cao et al., 2002). According to Lewin et al. (1992), the bovine PRL gene's silent AG (adenosine-guanine) transition mutation at the codon for amino acid 103 in exons 3 produced a polymorphism site for the RsaI restrictions enzyme. Prolactin concentration and SCC in milk from bovines infected with chronic mastitis are positively correlated; prolactin activates nuclear factor κB (NF-κB) in bovine mammary epithelial cells, resulting in an inflammatory response, implying a function for prolactin in the etiology of chronic mastitis (Boutet et al., 2007).

The bovine IL8 gene has been identified as a QTL for traits related to milk production, and it has been located on Bos Taurus autosome 6 (BTA6) (Ridhowi et al., 2018). The innate immune response significantly influences the development of bacterial infections. The efficiency of innate immunity depends on the expression of several genes, some related to neutrophil activity. The CXCR1 gene, which codes for the interleukin 8 (IL-8) receptor, is found on the surface of neutrophils and has a high affinity for the pro-inflammatory IL-8. Consequently, the bovine CXCR1 gene

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can potentially be used as a mastitis marker in dairy cattle (Pawlik et al., 2015).

This investigation aims to find polymorphisms in the prolactin and interleukin 8 genes in Holstein-Friesian cows. Furthermore, analyzes the association of these polymorphisms with milk production and composition traits.

2. MATERIAL AND METHODS

2.1. Ethical Approval:

Before conducting the present study, we received the ethical approval with a license number BUFVTM: 01-08-21 from the Animal Care and Welfare Committee, Egypt, Faculty of Veterinary Medicine Benha University. The current study was conducted at the Central Laboratory and Laboratory of Animal Wealth Development, Faculty of Veterinary Medicine, Benha University.

2.2. Animals:

Two hundred and twenty-five productive data records of sixty-four Holstein-Friesian cows, from 1st to 7th lactations, at 2-8 years old, were collected from Gammasa private farms for agricultural investment between 2018 and 2020. Moreover, records were collected for further analysis of the diseases [mastitis (671 and 349 records) and lameness (451 and 238 records)] that affect 305-day milk yield and total milk yield, respectively.

2.3. Herd management:

All year long, animals were maintained in open sheds, provided by a cool spraying system in the summer. They were fed one of three total mixed rations (T.M.R.): 22.50, 20.58, and 19.50 kg of dry matter for high, medium, and low-producing cows, respectively, based on the dry matter intake according to NRC (2001). Cows are milked three times daily and breeding with artificial insemination.

2.4. Sample collection:

Sixty-four blood, serum, and milk samples were collected from three groups (Group 1 n= 20 high, Group 2 n= 24 medium and Group 3 n= 20 low-producing animals). Blood was collected from the jugular veins of animals in EDTA vacutainer tubes kept in the ice box and stored at -20 °C till further processing for DNA isolation. The genomic DNA was then extracted from the whole blood using the TIANam Genomic DNA Kit. Then, the quantification of DNA was done using SpectroStar Nanodrop (BMG LAB TECH). Serum samples were obtained by collecting blood samples from the jugular veins of animals on plain tubes that were kept in the ice box and stored at four °C overnight. Serum was separated by centrifugation at 3000 rpm for 10 min and held at -20 °C till further processing. Serum samples were then analyzed using the electrochemiluminescence immunoassay "ECLIA", which is intended for use on Elecsys and Cobas e immunoassay analyzers (prolactin II reagent kits, Roche diagnostics) for measuring prolactin hormone concentration. Also, morning milk samples were collected in sterile falcon tubes kept in the ice box and stored at -20 °C till further analysis. Then, they were analyzed using a Lactoscan device (Narodnibuditeli str., Nova Zagora, 8900, Bulgaria, Milkotronic Ltd 4) for measuring milk components (fat, protein, lactose, and ash).

Genotyping

PCR reactions were conducted in a total volume of 25 µl including 50 ng of genomic DNA, 12.5 µl of Taq green mastermix and 0.20 mM primers. The PRL gene's 156 bp fragment was amplified using (5'- CGAGTCCTTATGAGCTTGATTCTT-3') forward and (5'- GCCTTCCAGAAGTCGTTTGTTTTC-3') reverse primers (Mahajan *et al.*, 2012) and 260bp fragment of Interleukin-*8* gene forward (5'-CAGATGACTCAGATGTGCTCTCA-3') and reverse (5'-CAGGAAAAGCTGCCAAGAGA-3') primer (Accession No. AY627308.1). The PCR procedures involved an initial denaturation of 2 minutes at 95 °C, then 35 cycles, each comprising 30 sec. at 95 °C, 1 minute at an annealing temperature of 56 °C for *prolactin* and 63 °C for 30 sec. for *interleukin-8* gene and 1-minute extension at 72 °C and a final extension for five minutes at 72 °C. Then, according to Kryndushkin *et al.* (2003), agarose gel electrophoresis was done. Ten samples for each gene (prolactin (156 bp), based on milk production (high and low-producing animals) and interleukin eight genes (260 bp), based on health conditions (normal and diseased animals)) were sequenced using forward primer in 3500 genetic analyzers (Applied Biosystems®). Geneious 4.8.4 software (http://www.geneious.com/web/geneious/home) was applied for sequence analysis. The MEGA 6 (Kumar *et al.*, 2008) and Bioedit 7.0.5.3 (Hall, 1999) software were employed for sequence alignment and polymorphism detection.

The PCR products were genotyped using single-stranded conformational polymorphism (SSCP) modified after (Abo-Gamil, 2021). Each sample of 5 µl of PCR products was combined with five µl denaturing buffer containing 98 % formamide, 0.09% bromophenol blue, and 0.09% xylene cyanole. After being denatured at 98 °C for five minutes, each sample underwent a quick cooling on ice for 10 minutes. *The prolactin* gene was separated on a 12% polyacrylamide gel (Acrylamide 20% (39 gm): Bis acrylamide (1 gm) for 3 hours at 80V, while the *interleukin* gene was separated for 6 hours at 60V. Finally, gel ethidium bromide staining (0.5 g/ml in 1x TBE) was used to identify the DNA fragments. The SSCP genotypes were identified by differential migration due to fragment size.

2.5. Statistical analysis:

2.5.1. Gene and genotypic frequencies of prolactin locus SNP were calculated by direct counting, according to the following (Falconer and Mackay, 1997)*:*

Gene frequency P (C) =

$$\frac{(Number\ of\ homozygous\ individuals\ for\ C) + \frac{1}{2}(Number\ of\ heterozygous\ individuals\ for\ C)}{Total\ number\ of\ individuals\ in\ the\ sample}$$

Gene frequency Q (G) =

$$\frac{(Number\ of\ homozygous\ individuals\ for\ G) + \frac{1}{2}(Number\ of\ heterozygous\ individuals\ for\ G)}{Total\ number\ of\ individuals\ in\ the\ sample}$$

Genotypic frequency P^2 (CC) =

$$\frac{Number\ of\ individuals\ of\ particular\ genotype\ CC)}{Total\ number\ of\ individuals\ in\ the\ population}$$

Genotypic frequency 2pq (CG) =

$$\frac{Number\ of\ individuals\ of\ particular\ genotype\ CG)}{Total\ number\ of\ individuals\ in\ the\ population}$$

Genotypic frequency Q^2 (GG) =

$$\frac{Number\ of\ individuals\ of\ particular\ genotype\ GG)}{Total\ number\ of\ individuals\ in\ the\ population}$$

2.5.2. The Hardy–Weinberg (H–W) equilibrium was evaluated using the Chi-square test according to Court (2012) for determining whether the observed genotypic frequencies are in consistence with H-W equilibrium.

2.5.3. Association among genotypes and some diseases (mastitis and lameness)

General linear model (GLM) of the Minitab statistical program (version 15) (SAS, 2001) was applied to the milk yield traits as follows:

Model 1:

For analysis of the association among prolactin genotypes with *prolactin* hormone concentration, days in milk (DIM), days open (DO), total milk yield (TMY) and 305-day milk yield (305-DMY), and milk composition.

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

Where

Y_{ij} is the milk yield trait of the j^{th} cow,

μ is the overall mean,

G_i: The effect of the genotype (i= 1, 2, and 3: where 1=CC genotype; 2=CG genotype; 3=GG genotype),

e_{ij} is random error.

Model 2:

For analysis the association of the incidence of diseases (mastitis and lameness) with 305-DMYand TMY

$Y_{ijk} = \mu + L_i + M_j + e_{ijk}$.

Where

Y_{ijk} is the measured value (i.e., 305-DMY),

μ is the overall mean,

L_i is the effect of lameness (i= 1, 2, and 3),

M_j is the effect of mastitis; (i= 1, 2, and 3),

e_{ijk} is the random error.

3. RESULTS

3.1. Amplification of PCR for prolactin and interleukin-8 gene

The DNA fragment of 156 bp of *prolactin* and 260 bp of *interleukin-8* was obtained after PCR amplification (Figure1).

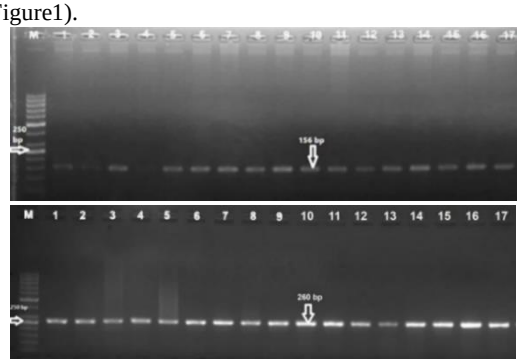

Figure 1 Ethidium bromide-stained agarose gel showing 156-bp PCR product of prolactin gene (upper raw, lanes 1-17) and 260-bp PCR product of interleukin -8 gene (lower raw, lanes 1-17). M: 50-bp ladder.

3.2. Sequence and GenBank alignment of prolactin gene:

Using sequence analysis, two SNPs known as C54G and G75A were identified within the bovine *prolactin* gene exon 3. For C54G SNP, three genotypes were discovered and given the names CC, CG, and GG, respectively (Figure 2a, b, and c).

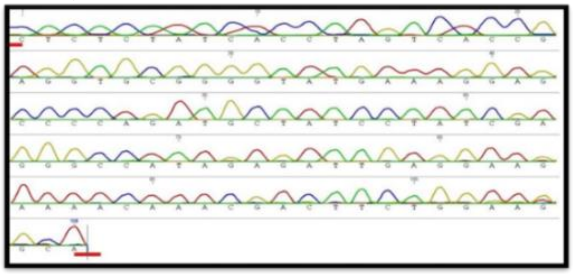

Figure 2a Sequence of prolactin gene (156bp) in Holstein-Friesian cows.

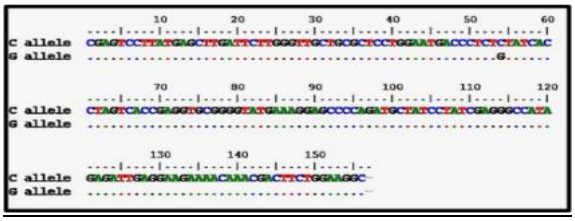

Figure 2b Sequence alignment of PRL in Holstein-Friesian cows shows one novel SNP (C54G) at exon 3.

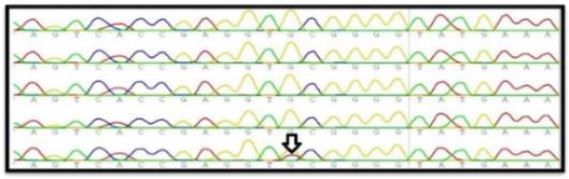

Figure 2c Sequence alignment of *PRL* in Holstein-Friesian cows shows one novel SNP (G75A) at exon 3.

3.3. Sequence and GeneBank alignment of interleukin-8 gene

Sequence analysis and Genebank alignment of the 260 bp DNA fragment of *interleukin-8* gene (Figure 3)

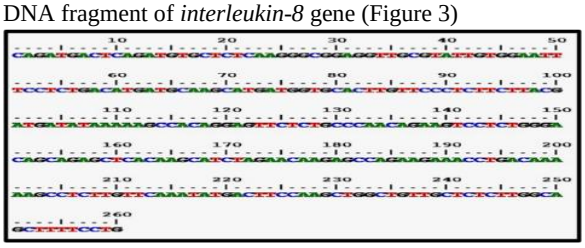

Figure 3 Sequence of *interleukin-8* gene (260 bp) in Holstein-Friesian cows.

3.4. Identification of Prolactin SNPs and their correlation with milk production.

Genotyping of *Prolactin* locus was identified using PCR-SSCP Single strand conformational polymorphism (SSCP) method; four unique SSCP patterns were detected in *prolactin* gene (Figure 4a and 4b). The low-production animals have four bands of SSCP pattern (CC genotype), five or six bands pattern (GG genotype), while high-production animals have only three bands (CG genotype) patterns. Indeed, medium-production animals have either three bands (CG genotype) patterns or four bands of SSCP pattern (CC genotype).

3.5. Genotyping of interleukin-8 locus was identified using PCR-SSCP Single strand conformational polymorphism (SSCP) method.

PCR-SSCP patterns of *interleukin-8* gene in Holstein-Friesians cows showed one SSCP three bands pattern (Figure 4c).

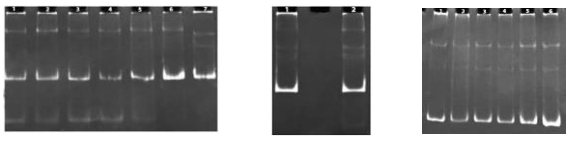

Figure (a) **Figure (b)** **Figure (c)**

Figure 4 PCR-SSCP patterns of *prolactin* gene in Holstein-Friesians cows showed two SSCP patterns, lanes (1-6) are three bands pattern (CG genotype), lane (7) are four bands pattern (CC genotype) (Figure a); (Figure b) showed two SSCP patterns, lane (1) is five bands pattern (GG genotype), lane (2) is six bands pattern (GG genotype). Figure (C) PCR-SSCP patterns of *interleukin-8* gene in Holstein-Friesians cows showed one SSCP pattern, lanes (1-6) are three bands pattern.

3.6. Genotypic frequencies of prolactin gene:

Four SSCP conformation patterns representing 3 genotypes of *prolactin* gene were detected among the three studied cattle groups; the CG genotype showed the maximum frequency (0.62 %) while the GG genotype had the minimal frequency (0.08%), as shown in Table (1).

Table 1 Genotype distribution and allele frequencies at C54G SNP of cattle Prolactin gene and the estimated Chi-Square (χ^2), HWE = Hardy-Weinberg equilibrium.

SNP	Genotype frequencies			Allele frequencies		χ^2 (HWE)	P value
C54G	CC	CG	GG	C	G	0.09	0.76
	0.30 (19)	0.62 (40)	0.08(5)	0.61	0.39		

χ2(HWE): is the test value for Hardy-Weinberg equilibrium. $P > 0.05$ means that observed genotype frequencies are consistent with Hardy-Weinberg equilibrium. The figures in parenthesis represent the number of observations.

3.7. Effect of prolactin genotype on prolactin hormone and milk production traits.

Findings of the current investigation revealed that the *prolactin* genotype had highly significant effect ($P \leq 0.01$) on prolactin hormone while it had a non-significant effect on other milk production traits as shown in Table 2.

Table 2 Least-square means (±SE) of *prolactin* genotype affecting prolactin hormone, milk production traits, and days open.

Traits / Diseases	Number of records	Genotype		
		CC	CG	GG
Hormone (n mol)	20	0.07^{b} ±0.02	0.43^{a}±0.55	0.04^{bc} ±0.04
Days in milk (days)	225	308 ±15.62	309±12.44	317±32.06
Days open (days)	225	142 ±11.64	152 ±9.82	145±23.89
Total milk yield (kg)	162	10804±421	11647±348	10773±873
305 days milk yield (Kg)	222	11775±859	12288±671	10371±1729

Least squares appearing in various letters within the same raw differ significantly ($p \leq 0.05$).

3.8. Effect of prolactin genotype on milk components.

Results shows significant higher milk fat for the individuals carrying CG genotype while the individuals carrying CC genotype were the highest for milk protein, lactose, and ash content (Table 3).

Table 3 Least-square means (±SE) of prolactin genotype affecting milk components.

Genotype	Number of observed genotypes	L.S.M ± S.E.			
		Fat%	Protein %	Lactose%	Ash%
CC	19	5.08^{b}±0.26	2.51^{a}±0.02	6.76^{a}±0.07	3.55^{a}±0.04
CG	40	6.69^{a}±0.21	2.37^{b}±0.02	6.36^{b}±0.06	3.33^{b}±0.03
GG	5	6.67^{a}±0.55	2.29^{bc}±0.05	6.16^{bc}±0.15	3.21^{bc}±0.08

Least squares appearances in various letters within the same categorization differ significantly ($p \leq 0.05$)

3.9. Association of some diseases (mastitis and lameness) with 305-day milk yield (305-DMY) and total milk yield (TMY).

Results showed that animals that produced more milk were more significantly ($P \leq 0.05$) affected with mastitis, while the animals that produce less milk were less affected with mastitis. However, there was a non-significant ($P \geq 0.05$) effect of mastitis on 305-DMY. Moreover, the effect of lameness on both 305-DMY and TMY was non-significant ($P \geq 0.05$) (Table 4).

Table 4 Least-square means (±SE) of some diseases (mastitis and lameness) affecting 305-day milk yield (305-DMY) and total milk yield (TMY).

Classification	305-DMY (Kg)		TMY (Kg)	
	N	L.S.M ± S. E	N	L.S.M ± S. E
Mastitis (incidence)				
1	363	11868.09± 270.89	187	11885.72^{b} ± 206.93
2	157	11266.49± 411.90	79	11421.89^{bc}± 318.38
≥3	151	11637.15± 419.71	83	12820.60^{a} ± 310.16
Total	671		349	
Lameness (incidence)				
1	294	11701.08± 129.41	157	12044.81± 248.05
2	107	11541.49± 214.49	57	12656.66± 411.68
≥3	50	11253.40± 313.78	24	11480.41± 634.44
Total	451		238	

Least squares appearing in various letters within the same categorization differ significantly ($p \leq 0.05$).N: the number of records.
305-DMY: 305-day milk yield, TMY: total milk yield.

4. DISCUSSION

Prolactin, one of the most important pituitary hormones, controls vital physiological processes, from the development of the mammary gland to the initiation and maintenance of lactation. The coding regions of the bovine prolactin gene were shown to have polymorphisms associated with economically significant features (He et al., 2006).

In the current study two SNPs within the bovine prolactin gene at exon three at 54 (C/G) and 75 (G/A) positions were discovered. Regarding the C54G SNP, the animals carrying the heterozygous CG genotypes showed higher frequencies (0.62), followed by the CC genotype (0.30), while the homozygous GG animals showed the lowest frequency (0.08). Moreover, the non-significant (P<0.05) Chi-square value for these genotypes in Holstein cattle indicated that the population was in H-W equilibrium, suggesting no change in the distribution of alleles among consequent generations. Similarly, Kumari et al. (2008) reported greater frequencies for genotype AB in Gyr (0.49), Red Sindhi (0.62), Jersey (0.65) and Kankrej (0.62) cattle. This is consistent with Ünal et al. (2015), who pointed out that the AB heterozygous genotype was the most prevalent across all breeds.

It is comparable to the information gathered by other researchers in various parts of India using different sample sizes and breeds, e.g., Patel and Chauhan (2017), who

observed a greater frequency of the AB genotype (0.77) of the PRL gene in Gir cattle. This outcome disagreed with Dybus (2005), who reported that A and B showed two alleles with the highest frequencies: 0.88 for A and 0.12 for B. The heterozygosity degree was 0.196. Consequently, the presence of the Hardy-Weinberg equilibrium is rejected as the null hypothesis (X2, $P < 0.05$). The different genotypic frequencies found in further studies, linked to polymorphism of the prolactin gene, can be due to differed breeds and the low sample sizes ($n < 50$), which made the genotypes not to be efficiently expressed (Brym et al., 2005).

In this study, the prolactin genotype had a highly significant effect ($P\leq 0.01$) on prolactin hormone; CG had the highest hormone concentration (0.43), while GG had the lowest hormone concentration (0.04). While it had a non-significant effect on other milk production traits, as shown in Table 2. Alfonso et al. (2012) findings showed that the genotype AA had the best mean value for the prolactin gene's impact on milk production (305-DMY), with 3252 kg/l, followed by the AB and BB with 2790 and 2604 kg/l, respectively. Thuy et al. (2018) reported that cows with the PP genotype produced more milk (20.41±4.45 kg) than those carrying PC (19.11±3.83 kg) and CC (19.18± 4.98 kg) genotypes. However, Mahajan et al. (2012) indicated that genotypes had no significant impact on lactation length and that the BB genotype had the longest lactation (329.14 days), which attributed this non-significant effect to a small sample size and higher error variance.

The current study detected that the effect of the prolactin genotype on some milk components (fat, protein, lactose, and ash) was highly significant ($P\leq 0.01$). The C54G SNP was associated with significantly higher milk fat percent for the individuals carrying the CG (6.69 %) genotype, followed by the GG (6.67%) genotype. The CC genotype shows the lowest percentage (5.08 %). The individuals carrying the CC genotype had the highest milk protein, lactose, and ash content, followed by the CG genotype, while the GG genotype showed the lowest percentage. He et al. (2006) discovered three SNPs in the promoter, which were (A767C), (G485T) and (C247A) and one SNP (C427T) in intron1 of the prolactin gene; findings revealed that (G485T) SNP was associated significantly ($P\leq 0.05$) with milk yield, fat yield, protein yield, and protein percentage. Cows with the genotype BB produced considerably more milk, fat, and protein yield than cows with the genotype AA. In contrast, cows with the genotype AA produced the most significant protein percentage, suggesting that this SNP could be a potential marker affecting milk yield and composition traits. Also, Karuthadurai et al. (2021) discovered a SNP (G55A) to confer an increased test-day milk yield of roughly 321.5 g, a fat yield of about 13.9 g, and an increased SNF yield of 19.4g. Contrary to current results, in the PRL gene promoter region, a unique T72C SNP was found, although statistical analysis revealed no correlation between this SNP and various milk parameters in Egyptian dairy water buffaloes, including milk yield, fat, protein, lactose, and solid percentages, this may be due to that analysis of one SNP may be of no impact on the phenotype of polygenic traits like milk traits (El-Magd et al., 2015). In accordance with Bangar et al. (2021), who reported a negative effect (mean difference = − 0.51) of the AA genotype on fat%. Also, Gilmanov et al. (2021) showed that the fat mass fraction content indicator in milk was 3.77 % to 3.88 % (AA and AB genotypes, respectively). While in disagreement with that, the analysis of variance revealed a significant ($P< 0.05$) influence of the AA genotype on fat % compared to the AB genotype. However, these genotypes did not affect lactose %, protein %, or SNF %, which could be attributed to the small sample size and increased error variance (Dudule et al., 2020).

Results showed that animals that produced more milk (12820.60 kg) were more significantly ($P\leq 0.05$) affected with mastitis (three times), while the animals that had less milk (11421.89 kg) were less affected with mastitis (two times). However, mastitis had a non-significant ($P\geq 0.05$) effect on 305-DMY. Moreover, the effect of lameness on both 305-DMY and TMY was non-significant ($P\geq 0.05$). Similar to our results regarding milk yield, Abd-El Hamed and Kamel (2020) recorded that mastitis was more common in high-producing cows than in low-producing cows. Moreover, Sinha et al. (2021) recorded the maximum incidence of clinical mastitis in high-producing cows. Nevertheless, the effect of 305-DMY on the incidence of clinical mastitis was non-significant (Abo-Gamil et al., 2021).

The interleukin-8 gene is vital in innate and acquired immunity by activating neutrophils (Mitchell et al., 2003). However, in the current study, we couldn't detect any polymorphisms related to this gene that may affect these animals. In disagreement with the current results, researchers detected a single nucleotide polymorphism (IL8-T2862C polymorphism), which showed a significant association with SCC ($p<0.05$) (Stojkovic et al., 2017) and has an impact on the milk production traits this may be due to the CT cows have more milk-secreting cells and so increased milk yield (Ridhowi et al., 2018).

5. CONCLUSIONS

It could be concluded that identifying the polymorphism of prolactin in this cattle population may make the process of selecting breeding animals more effective, while the interleukin-8 gene showed no impression on this population.

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