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Research Article

CXCR1 Gene SNP Variability that Affects Mastitis Resistance in Holstein Cows in Türkiye

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Abstract

Genotyping 16 SNPs of C-X-C motif chemokine receptor 1 (CXCR1) gene region which affects host resistance against mastitis disease was carried out in Holstein cows raised in Türkiye. In this study, the frequency of the undesirable C allele in the CXCR1 c.771C>G polymorphic region, associated with an incomplete response, was found to be high. Additionally, the genotypes c.1016AA and c.1016GG, which contribute to mastitis resistance, were observed at low frequencies. Several SNP loci in the CXCR1 gene, including c.606G>A, c.678G>A, c.1104G>A, c.1119+6C, c.1119+7A, and c.1119+10, significantly deviated from Hardy-Weinberg equilibrium (HWE) (P<0.0001), indicating violations of HWE assumptions such as random mating and absence of selection. The deviations at c.606G>A, c.678G>A, and c.1104G>A suggest strong selection pressures, likely due to artificial selection in Holstein cattle. These variants are synonymous mutations that do not alter the amino acid sequence but may influence protein synthesis through effects on mRNA stability, splicing, or translation efficiency. Furthermore, the absence of heterozygotes at loci c.1119+6C, c.1119+7A, and c.1119+10, which are located on untranslated regions (UTRs), potentially affecting gene expression by regulating mRNA stability, localization, or translation initiation, points to genetic drift or population substructure. These findings are important for understanding genetic variability and can inform marker-assisted selection programs to enhance breeding strategies while preserving genetic diversity for traits like disease resistance and milk production.

Keywords: Cattle, PCR, SNP, Mastitis, Resistance

Introduction

In recent years, to increase profitability and meet the growing demand for milk, enhancing daily milk yield has become essential, alongside intensive genetic selection and improved nutrition and management conditions. Achieving this high yield requires the cows to possess a healthy and well-developed mammary structure. However, mammary health is consistently threatened by pathogens attempting to enter the mammary gland through the teat canal. Intramammary infections cause inflammation known as mastitis. The disease can manifest with or without symptoms. The terms clinical mastitis and subclinical mastitis are used for observable symptoms and unobservable symptoms, respectively. Clinical mastitis is characterized by clotting in the milk and swelling, redness, or an overall increase in body temperature in the affected udders [1]. Subclinical mastitis can be identified by the increased somatic cell concentration in the milk obtained from the affected quarter. Both clinical and subclinical mastitis lead to a reduction in milk yield and quality [2]. Additionally, clinical mastitis affects animal welfare due to the pain it causes [3]. Research related to bovine mastitis has been conducted for more than 100 years. Mastitis is a multifaceted disease influenced by pathogens, host, and management factors that affect its prevalence and incidence. Over hundred different pathogens have been isolated from the milk of mastitic cattle. Intramammary infections are primarily caused by bacteria, but algae, yeasts, and fungi can also lead to bovine mastitis. Under similar infection pressure, some cows are affected by mastitis, while others remain healthy. For pathogens to establish an intramammary infection, they

need to penetrate the teat canal and multiply within the mammary gland. In cows with high resistance to mastitis, pathogens are less likely to penetrate the teat canal and are more effectively eliminated by mammary immunity. Management factors affect both the infection pressure and host resistance, leading to low or high mastitis prevalence and incidence [4].

Neutrophils play a crucial role in mammary gland immunity. Genes related to neutrophil function are potential genetic markers for mastitis, as the migration of neutrophils to infection sites via blood is essential for combating mastitis. Neutrophils interact via specific C-X-C motif chemokine receptors (CXCR1 and CXCR2) on their surfaces. The recognition of pathogens by CXCR1 leads to neutrophil activation and ultimately the elimination of the pathogen [5]. CXCR1 is becoming more well known for its role as a cancer stem cell identifier and therapeutic target [6].

Inflammatory cytokines and the genes that control them have been given great importance in expanded research to combat mastitis. The chemokine receptor Interleukin-8 receptor α (IL-8RA), which is found on neutrophil cells and binds with high affinity to pro-inflammatory IL-8, is encoded by the CXCR1 gene. IL8RA plays an important role in the control of mastitis by strengthening immunity and counteracting the immunosuppressive effect of *S. aureus* mastitis [7]. However mRNA transcription levels of five genes, including CXCR1, were significantly higher in the *E. coli*-induced mastitis group than in the *S. aureus*-induced group, therefor *E. coli* often causes acute mastitis, while *S. aureus* causes chronic mastitis [8]. Bacteria activate CXCR1, which regulates NF-κB signaling after interaction with TLR4. NF-κB binds to DNA and induces expression of the CXCR1 gene [9]. In addition, the interaction of IL-8 with CXCR1 or CXCR2 genes causes some changes that lead to chemotaxis of neutrophils against infection in the mammary gland and increase cell survival, migration and phagocytosis activity [10]. During predicting mastitis resistance in buffalo CXCR2 was identified as a potential gene [11].

The importance of the CXCR1 gene in this context has been highlighted in several studies suggesting that single nucleotide polymorphisms (SNPs) within this gene can affect an animal's susceptibility to mastitis. The ability to monitor and understand these SNPs may provide critical information for improving mastitis resistance in Holstein cattle [5]. The relationships of CXCR1 gene polymorphisms with clinical mastitis, reproductive disorders and performance characteristics in Hardhenu cattle were investigated, and it was reported CXCR1 gene could be associated with both increased mastitis susceptibility and high milk production [12]. Beyond CXCR mechanism other genes are also effect host resistnacy, Aksel et al.[13] reported that two SNP loci in mannose-binding lectin (MBL1) gene may lead a role in subclinic mastitis in Holstein cattle. However CXCR1 could be the highest priority gene for host resistance in mastitis among all candidate genes [14].

Holstein cattle are the most widely used breed in dairy production due to their high milk yield; however, this very characteristic also makes them more prone to diseases such as mastitis. The identification of genetic markers, such as SNPs within immune-related genes like CXCR1, has become a valuable tool in the field of animal breeding for disease resistance [15]. In particular, certain SNPs in the CXCR1 gene have been associated with variations in neutrophil function and immune response, which may influence the severity of mastitis infections. Some studies have reported a strong association between milk SCC and CXCR1 mutations in dairy cattle. Exploring the relationship between these SNPs and mastitis resistance could offer potential for selective breeding programs aimed at enhancing disease resistance without compromising milk production [15-17].

This study aims to investigate the key SNPs in the CXCR1 gene in Holstein cattle, with a focus on their association with mastitis resistance. By following the presence and frequency of these SNPs in the population, this study seeks to contribute to the growing body of knowledge on genetic markers for mastitis resistance, providing a foundation for more effective breeding strategies that combine high milk yield with enhanced disease resilience.

Material and Methods

Ethical Statement

All experimental procedures were applied in this study received approval from the the Istanbul University-Cerrahpaşa, Animal Experiments Local Ethics Committee: 2018/118480. Additionally, informed consent forms were obtained from the owners

Sampling and DNA isolation

In this study, a total of 240 blood samples from 24 different farms located within the boundaries of Kırklareli province were collected from Holstein cows using a sterile double-ended cannula from the caudal vena of the animals into vacuum sterile EDTA blood tubes. The ear tag numbers of the animals were recorded on the blood tubes. The collected blood samples were transported in insulated bags with ice packs and were kept at 4°C until they reached the laboratory within 2-3 days. Each blood sample in the EDTA vacuum tubes was aliquoted into two 1.5 mL sterile Eppendorf tubes, approximately 600 µL each, with one being a backup. The blood samples were stored at -20°C in sealed storage racks. DNA isolation from the blood samples was performed using Bioneer Exiprep 16 Plus

Genomic DNA innovation robot (Bioneer Corporation, Korea). To visualize the genomic DNA samples obtained from whole blood 0.8% agarose gel was used. For this purpose, 100 mL of TBE Buffer was measured, and 0.8 mg of agarose was weighed, dissolved with the help of a microwave and then 3 µL of Red Safe dye (iNtRON) was added. The isolated DNA samples were mixed with 6x loading dye and loaded into the gel wells. The agarose gel electrophoresis system was run at 100 volts for 35 min, allowing the genomic DNA samples to migrate within the gel.

Amplification of the CXCR1 Gene

The PCR process was conducted with using AccuPower PCR PreMix (Catalog No.pcr K-2012, Bioneer, Korea) which is a lyophilized (Taq DNA polymerase 1U, each: dNTP -dATP, dCTP, dGTP, dTTP- 250 µM Tris-HCl pH 9.0 10 mM, KCl 30 mM, $MgCl_2$ 1.5 mM, stabilizer and tracking dye) mixture ready-to-use in 0.2 mL attached 8-tubes. The PCR mixture content (20 µL) was an adequate volume of sterile bidistilled water (15 µL), 50 ng of DNA (3 µL), and 0.25 µM of each primer pair (0.5 µL each) were added. Amplification of CXCR1 gene was performed with TCCTTGATGAGAGTGATTTGGA and TTGACATGGGACTGTGAACG primer pairs under the conditions of: denaturing at 95°C for 5 min, and 35 cycles of 95°C for 30 sec, 62°C for 30 sec, 72°C for 75 sec, and final extension at 72°C for 4 min [5]. Following the amplification of the CXCR1 gene region, 1% agarose gel was used to visualize the PCR products with 1 µg of 1 kb ladder (NEB) under UV imaging system. The agarose gel electrophoresis system run at 120 volts for 30 min. PCR products bands associated with the products to be made visible using UV tools of E-Box imaging system (Vilber Lourmat).

Detection of SNPs by Sequence Analyses

In order to analyse SNP regions within the amplified PCR products were sent to Genoks Company for sequencing (ABI 3130xl DNA Analyser; Thermofisher; USA). The sequences were analyzed and aligned using CLC Genomics Workbench 10.1. 1 (QIAGEN, Hilden, Germany). Sequencing data were manually inspected and edited, and consensus sequences were generated from both forward and reverse reads. The aligned sequences were screened for SNPs by comparison with the reference genome.

Once the variants were identified, they were further analyzed using the Ensembl genome browser *(https://www.ensembl.org)*. This tool was used to annotate the SNPs and predict their potential impact on gene function.

Statistical Analyses

Genetic variation in SNP regions terms of the minor allele frequency, observed (Ho) and expected (He) heterozygosity and the probability of a deviation from Hardy-Weinberg equilibrium (HWE) were calculated using PopGene ver. 1.32 [18] program.

Results

CXCR1 gene was obtained as 1083 bp with PCR process and visualised with 1% agarose gel electrophoresis *(Fig. 1)*.

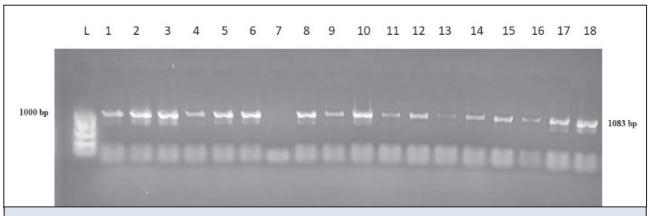

Fig 1. Visualization of PCR products of CXCR1 gene on 1% agarose gel electrophoresis in lines 1-6 and 8-18 (1083bp), line 7 is negative control. L: 1kb ladder

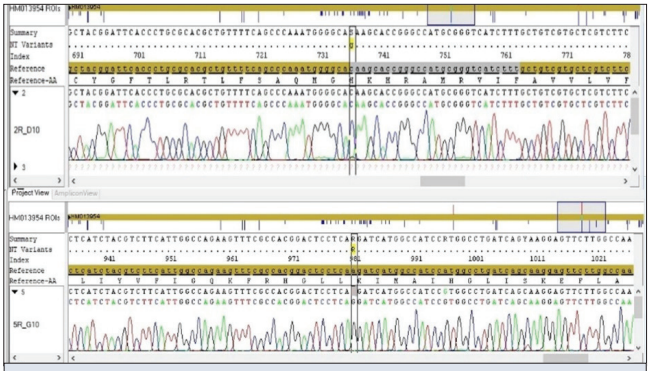

Fig 2. Two SNP loci data obtained with Sanger sequence and alligned in order to find variations of CXCR1 gene in Holstein cattle of Türkiye

Through bioinformatic mining, 16 SNPs identified in CXCR1 gene *(Fig. 2)*. The genotypic and allelic distributions of 16 SNP loci in the CXCR1 gene were evaluated in the Holstein cow population raised in Türkiye *(Table 1)*.

The frequency of the undesirable C allele in c.771C>G, associated with an incomplete immune response, was moderate (C = 0.4630), while the G allele was more prevalent (G = 0.5370). For c.1016A>G, the A allele, which contributes to mastitis resistance, was at low frequency (A = 0.1761), whereas the G allele was predominant (G = 0.8239). The loci c.606G>A (P<0.05), c.678G>A (P<0.05), c.1104G>A (P<0.05), c.1119+6C>A, c.1119+7A>C, c.1119+10A>C, and c.1119+10C>T exhibited significant deviations from HWE, indicating selection or non-random mating. In contrast, other loci, such as c.318C>T, c.369T>C, c.771C>G, c.852C>A, and c.855G>A, were in HWE (P>0.05).

Loci c.1119+6C>A, c.1119+7A>C, and c.1119+10A>C showed complete absence of heterozygotes. For c.606G>A, c.678G>A, and c.1104G>A, the observed heterozygosity was lower than expected.

Table 1. *The genotypic and allelic distributions of 16 SNP loci in the CXCR1 gene in the Holstein cow population raised in Türkiye*

SNP Locus	Variant Type	Variant Code	Alleles	Allele Frequency	Genotypes	Obs_ Gen	Obs_ Hom	Obs_Het	Exp_ Hom^{+}	Exp_ Het^{+}	Nei^{++}	Ave_ Het	HWE
c.318C>T	Missense	rs208795699	C	0.9978	CC	229	0.9957	0.0043	0.9957	0.0043	0.0043	0.0043	NS
			T	0.0022	CT	1							
					TT	0							
c.369T>C	Synonymous	rs209401160	T	0.9978	TT	229	0.9957	0.0043	0.9957	0.0043	0.0043	0.0043	NS
			C	0.0022	TC	1							
					CC	0							
c.606G>A	Synonymous	rs137262611	G	0.8478	GG	180	0.8696	0.1304	0.7414	0.2586	0.2580	0.2580	*
			A	0.1522	GA	30							
					AA	20							
c.678G>A	Synonymous	rs109587131	G	0.8652	GG	183	0.8609	0.1391	0.7663	0.2337	0.2332	0.2332	*
			A	0.1348	GA	32							
					AA	15							
c.771C>G	Missense	rs43323012	C	0.4630	CC	51	0.5174	0.4826	0.5016	0.4984	0.4973	0.4973	NS
			G	0.5370	CG	111							
					GG	68							
c.852C>A	Synonymous	rs210501501	C	0.7935	CC	147	0.6913	0.3087	0.6715	0.3285	0.3277	0.3277	NS
			A	0.2065	CA	71							
					AA	12							
c.855G>A	Synonymous	rs134348371	G	0.4717	GG	56	0.5435	0.4565	0.5005	0.4995	0.4984	0.4984	NS
			A	0.5283	GA	105							
					AA	69							
c.1016A>G	Synonymous	rs134348371	A	0.1761	AA	3	0.6739	0.3261	0.7092	0.2908	0.2902	0.2902	NS
			G	0.8239	AG	75							
					GG	52							
c.1031A>G	Missense	rs43323013	A	0.2391	AA	18	0.6783	0.3217	0.6353	0.3647	0.3639	0.3639	NS
			G	0.7609	AG	74							
					GG	138							
1044C>T	Missense / Synonymous	rs43323014	C	0.7761	CC	143	0.6913	0.3087	0.6517	0.3483	0.3476	0.3476	NS
			T	0.2239	CT	71							
					TT	16							
c.1054T>C	Synonymous	rs722933381	T	0.9978	TT	229	0.9957	0.0043	0.9957	0.0043	0.0043	0.0043	NS
			C	0,0022	TC	1							
					CC	0							
c.1104G>A	Synonymous	rs132989373	G	0.6826	GG	129	0.7565	0.2435	0.5657	0.4343	0.4333	0.4333	*
			A	0.3174	GA	56							
					AA	45							
c.1119+6C>A	-	rs446949386	C	0.8522	CC	196	1.0000	0.0000	0.7475	0.2525	0.2519	0.2519	*
			A	0.1478	CA	0							
					AA	34							
c.1119+7A>C	-	rs446949386	A	0.8565	AA	197	1.0000	0.0000	0.7537	0.2463	0.2458	0.2458	*
			C	0.1435	AC	0							
					CC	33							

Table 1. *The genotypic and allelic distributions of 16 SNP loci in the CXCR1 gene in the Holstein cow population raised in Türkiye (continued)*

SNP Locus	Variant Type	Variant Code	Alleles	Allele Frequency	Genotypes	Obs_ Gen	Obs_ Hom	Obs_Het	Exp_ Hom⁺	Exp_ Het⁺	Nei⁺⁺	Ave_ Het	HWE
c.1119+10A>C	-	rs452865404	A	0.9957	AA	229	1.0000	0.0000	0.9913	0.0087	0.0087	0.0087	*
			C	0.0043	AC	0							
					CC	1							
c.1119+10C>T	-	rs452865404	C	0.8674	CC	199	0.9957	0.0043	0.7695	0.2305	0.2300	0.2300	*
			T	0.1326	CT	1							
					TT	30							
						Mean	0.8291	0.1709	0.7495	0.2505	0.2499	0.2499	
						St.Dev.	0.1770	0.1770	0.1690	0.1690	0.1687	0.1687	

⁺ *Expected homozygosty and heterozygosity were computed using Levene (1949),* ⁺⁺ *Nei's (1973) expected heterozygosity, NS: non-significant, *P<0.001*

Discussion

This study analyzed genetic variation in the CXCR1 gene in Holstein cattle raised in Türkiye, focusing on the association of 16 SNP loci with mastitis resistance. The findings obtained from this study were found to be consistent with previous studies. Loci c.606G>A, c.678G>A and c.1104G>A ($p < 0.05$), which had significant deviations from the Hardy-Weinberg equilibrium (HWE), indicate that they probably experienced strong selection pressures. The absence of heterozygosity at c.1119+6C>A, c.1119+7A>C and c.1119+10C>T loci may suggest genetic drift or the presence of population substructure. A higher prevalence of the G allele (0.5370) at c.771C>G locus, may indicate a selection advantage for this allele in the population, as it is potentially associated with improved immune response efficiency [16]. Besides that, the frequency of the C allele (0.4630) which has been associated with a deficiency in neutrophil response and is therefore less desirable [5], is concerning. On the contrary, it is also thought-provoking that the A allele in the c.1016A>G locus which is defined to have a protective effect against mastitis [16,19], is found at low frequency (0.1761) in the relevant population.

Previous studies have emphasized the need to increase the frequency of resistance alleles through breeding programs to increase host resistance to combat mastitis in dairy cattle [20]. Nei's gene diversity (0.2499) and mean heterozygosity across all loci (0.2505) indicate moderate genetic variability. In order to increase mastitis resistance in Holstein cattle, it is important to adopt marker-assisted selection (MAS) programs to reduce the frequency of the C allele in c.771C>G and increase the prevalence of advantageous genotypes such as c.1016AA and c.1016GG. These findings, together with previous studies, highlight the impact of CXCR1 SNP variations on disease resistance and the essential role of genetic diversity in improving herd health and productivity.

In this study, it was found that c.606G>A, c.678G>A, c.1104G>A, c.1119+6C, c.1119+7A and c.1119+10 loci in the Holstein cattle population raised in Türkiye deviated significantly from Hardy-Weinberg equilibrium (HWE) ($P<0.0001$). HWE based on the principles of random mating, the absence of mutation, migration or selection, and a sufficient population size, helps maintain constant allele and genotype frequencies across generations. Significant deviations from HWE of c.606G>A, c.678G>A ve c.1104G>A ($P<0,0001$) loci and the complete absence of heterozygosity at the c.1119+6C, c.1119+7A and c.1119+10 ($P<0.0001$) loci indicate significant population substructure or genetic drift. Genetic diversity may be reduced due to small effective population sizes or controlled matings [21]. Understanding the genetic diversity and evolutionary forces that influence traits such as disease resistance and milk production heavily relies on deviations from HWE. For marker-assisted selection (MAS) programs we gain valuable insights by identifying loci that are under selection. MAS can improve breeding strategies for economically important traits and also maintain the genetic diversity essential for the long-term health of the population.

There are synonymous mutations in c.606G>A, c.678G>A and c.1104G>A that do not affect the amino acid sequence but cause specific changes in the coding region. These variations do not cause any changes in protein structure, but may affect protein synthesis through changes in mRNA stability, splicing, translation efficiency, protein folding and quantity. On the other hand, c.1119+6C, c.1119+7A and c.1119+10 mutations are located in the untranslated regions and can regulate gene expression by affecting mRNA stability, localization or translation initiation, thus not changing the protein structure but may affect the amount of protein produced. Therefore, while protein structure is unaffected by synonymous mutations, its expression and regulation may be influenced.

This study examined 16 SNP loci in the CXCR1 gene of Holstein cattle in Türkiye, considering their relationship with mastitis resistance. The moderate occurrence of the undesirable C allele at c.771C>G and the low presence of the protective A allele at c.1016A>G, as well as the absence of heterozygotes at certain loci, highlight the effects of population substructure and genetic drift in this managed breeding program. These results provide important information about the genetic structure of the CXCR1 gene in Holstein cattle raised in Türkiye and the potential role of selective breeding strategies in increasing disease resistance.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (K. Avanus) on reasonable request.

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Author Contributions: KA, AY, AA, HG: design of the study, AY, BE, HY, DK, NÖ: determine the farms, BE, HY, DA, NÖ: funding acquisition, KA, AY: field work and sample collection, KA: laboratory analyze, KA, HG, AA: statistical analyze, KA, AY, HG, AA: writing the manuscript. All authors have read and agreed to the published version of the manuscript.

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