Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Daldaban F, Arslan K, Akyüz B (2023).** The Relationship Between *CACNA2D1* Gene and Subclinic Mastitis in Holstein Breed Cattle. *Van Vet J*, 34(2), 93-97. DOI: https://doi.org/10.36483/vanvetj.1242270

ISSN: 2149-3359 **Original Article** e-ISSN: 2149-8644

The Relationship Between *CACNA2D1* Gene and Subclinic Mastitis in Holstein Breed Cattle

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Received: 25.01.2023 Accepted: 17.05.2023

ABSTRACT The *CACNA2D1* gene encodes the *CACNA2D1* protein and, this protein is involved in the excitation-contraction mechanism of the muscle cells during milk withdrawal, helps the nipples to open and close. Because of this role in physiological mechanism and its relationship with quantitative trait locus (QTL) regions, *CACNA2D1* gene is known to be associated with mastitis resistance. In this study, it was aimed to investigate the relationship between different three SNP (C367400T, A496561G and G519663A) on the *CACNA2D1* gene, and subclinical mastitis in Holstein breed cattle reared in Develi district of Kayseri province. SNPs were genotyped from DNA samples by PCR-RFLP method. In the study, California mastitis test (CMT) data, and distributions of genotypes of the three SNPs on the *CACNA2D1* gene were calculated. In the study, genotype distributions were determined in terms of C367400T, A496561G and G519663A SNPs found on the *CACNA2D1* gene according to CMT status. The difference between the C367400T, A496561G and G519663A SNPs was not significant ($p>0.05$). In the study group examined the Chi-square ($\chi 2$) analysis conducted, it was observed that the Holstein cattle were in the Hardy-Weinberg equilibrium (HWE) in terms of C367400T and A496561G SNPs, deviation from HWE for the G519663A SNP ($p<0.05$). As a result, it was thought that the *CACNA2D1* gene and these SNPs should be evaluated with more samples and different mastitis indicator data in studies on mastitis resistance.

Keywords: *Bovine mastitis, CACNA2D1, Cattle, Genetic marker.*

ÖZ

Holştayn Irkı Sığırlarda *CACNA2D1* Geni ve Subklinik Mastitis Arasındaki İlişki

CACNA2D1 geni, *CACNA2D1* proteinini kodlar ve sütün indirilmesi sırasında kas hücrelerinin uyarılma-kasılma mekanizmasında görev alarak meme uçlarının açılıp kapanmasına yardımcı olur. Fizyolojik olarak üstelendiği bu görev ve kantitatif özellik lokusu (QTL) bölgelerine yakınlığı nedeni ile *CACNA2D1* geninin mastitis direnci ile ilişkili olduğu bilinmektedir. Bu çalışmada Kayseri ili Develi ilçesinde yetiştirilen sağmal Holştayn sığırlarda *CACNA2D1* geninde bulunan üç farklı SNP bölgesi ile subklinik mastitis durumları arasındaki ilişkinin araştırılması amaçlandı. Çalışma grubunu hepsi üçüncü laktasyonda 151 baş sağmal Holştayn sığır ırkı oluşturdu. Çalışmada California mastitis test (CMT) durumuna göre *CACNA2D1* geni üzerinde bulanan üç SNP (C367400T, A496561G ve G519663A) yönünden genotip dağılımları belirlendi. Yapılan istatistiki analizler sonucunda C367400T, A496561G ve G519663A SNP genotipleri arasında fark bulunmadı ($p>0.05$). Yapılan Chi-Kare analizinde incelenen çalışma grubunda C367400T ve A496561G kodlu SNP'ler yönünden Hardy-Weinberg dengesinde (HWE) oldukları, G519663A kodlu SNP yönünden ise HWE'den saptıkları ($P<0.05$) gözlemlendi. Sonuç olarak mastitis direnci ile ilgili çalışmalarda *CACNA2D1* geni ve bu SNP'lerin daha fazla örneklemle ve farklı mastitis göstergesi verilerle değerlendirilmesi gerektiği düşünüldü.

Anahtar Kelimeler: *CACNA2D1, Genetik belirteç, Sığır, Sığır mastitisi.*

INTRODUCTION

The survival of livestock enterprises depends on the continuity of production and profitability. Production and profitability in a dairy farm are ensured by having animals with high milk and reproductive efficiency. In dairy cattle breeding, the longer the productive period of the existing animal stock, the higher the enterprises will have a chance to benefit from dairy cattle (Mundan and Karabulut 2008). In recent years, innovations in the field of molecular genetics have been used in the improvement of yield characteristics such as milk and fertility, disease resistance, etc. and the data obtained from these areas are reflected in the cattle breeding. These methods have contributed to both the identification of the genetic

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structure of the animal existence and the selection of high-yielding animals in modern breeding practices (Narayana et al. 2022).

Mastitis is a disease characterized by inflammation in the mammary tissue, causing major economic losses in dairy cattle enterprises and affecting all farm animals raised for milk yield worldwide (Bronzo et al. 2020). The financial loss in severe mastitis cases in dairy cattle farms in Türkiye is equivalent to 710 L of milk per infected animal, and this rate corresponds to 22.6% of lactation milk yield (Sarıözkan 2019). The financial loss due to mild/moderate mastitis in dairy cattle farms is reported to be equivalent to 310 L of milk per infected animal and this rate corresponds to 9.9% of lactation milk yield (Sarıözkan 2019). Subclinical mastitis is a form of mastitis in which there is no visible change in the breast tissue and milk (Alaçam 1997; Krishnamoorthy et al. 2021). For this reason, subclinical mastitis, which is difficult to diagnose, persists in the infected animal for a long time and causes the spread of the disease in the herd (Baştan 2019). On the other hand, the cost of treatment of sick animals, the decrease in milk yield of sick animals, the destruction of milk obtained from treated animals and the removal of high-yielding animals from the herd cause significant economic losses in dairy cattle enterprises (Sabuncuoğlu and Çoban 2006; Sarıözkan 2019).

The researchers on the genetic basis of mastitis resistance in dairy cattle reported an association between some genes and polymorphisms with mastitis resistance which can use in breeding studies on mastitis resistance (Youngerman et al. 2004; Asaf et al. 2014a; Asaf et al. 2014b; Tolone et al. 2016; Jacob et al. 2020; Kirsanova et al. 2020). However, these genes and single nucleotide polymorphisms (SNPs) need to be validated in different breeding herds. One of these genes is the *CACNA2D1* gene, which encodes the calcium channel voltage-dependent alpha-2/delta subunit 1 protein, which is involved in the excitation-contraction mechanism in muscle and glial cells and neurons (Yuan et al. 2011a; Yuan et al. 2011b; Magotra et al. 2017; Magotra et al. 2019). *CACNA2D1* protein, which is involved in the excitation-contraction mechanism of the muscle cells during milk withdrawal, helps the nipples to open and close (Gabashvili et al. 2007). The *CACNA2D1* gene mapped in cattle was reported to be associated with 7 different quantitative trait locus (QTL) regions, including the somatic cell score (SCS), an indicator of mastitis (Buitkamp et al. 2003; Rupp and Boichard 2003; Zhang et al. 1998). Moreover, a QTL region was also identified near this gene, associated with somatic cell number (SCC) (Longeri et al. 2006). Also, Zhang et al. (2021) suggested that the *CACNA2D1* gene may be a candidate gene for mastitis in cattle.

This study aimed to investigate the relationship between subclinical mastitis and three SNP (C367400T, A496561G and G519663A) found on the *CACNA2D1* gene, which is reported as a candidate gene for mastitis in Holstein cattle.

MATERIAL AND METHODS

Animal Material

Animal materials used in the study were approved by the Animal Experiments Local Ethics Committee (11.12.2013 date and 13/157 number). The animal material consisted of 151 Holstein cattle breed with an average age of 5.9 years which were raised in a dairy cattle farm in Develi district of Kayseri province. Blood and milk samples were collected from animals, all in their third lactation. Subclinical mastitis status of animals was determined by from milk samples and total DNA was isolated from blood samples by phenol: chloroform: isoamyl extraction method.

Subclinical Mastitis Test

Subclinical mastitis status of the cattle was determined by the California Mastitis Test (CMT) by the farm's responsible veterinarian from the milk samples. Mastitis status of animals was noted as suggested by Daldaban et al. (2021) and Fthenakis (1995).

DNA Isolation and PCR-RFLP

Genomic DNA was extracted from whole blood using the phenol: chloroform: isoamyl extraction method (Puttaraju et al. 2020). DNA concentration was quantified by using a Nano Drop (Synergy H1Hybrid MultiMode Microplate Reader, BioTek, USA) and stored at −20 °C until use. Primer pairs and restriction enzymes were obtained from Yuan et al. (2011b) (Table 1). Polymerase chain reaction (PCR) analysis was performed for C367400T, A496561G and G519663A SNPs with DNA samples.

PCR was performed to determine the genotypes of cattle in C367400T, A496561G and G519663A SNPs. PCR was carried out using 60 ng of genomic DNA in a total reaction volume of 20 µL containing 10× PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 10 pM of forward and reverse primers (Yuan et al. 2011b), and 0.5 U of Taq DNA polymerase (Thermo Fisher Scientific, WA, USA). Amplification reactions were performed 5 min at 94^0C, followed by 35 cycles of 30 s at 94^0C, 30 s of Table 1 annealing temperature and 30 s at 72^0C with an extension step of 8 min at 72^0C. The amplicons obtained at the end of PCR were screened by a 1.5% agarose gel (Prona, Biomax, Ankara, Türkiye) and visualized (Kodak Gel Logic Imaging System, New York, ABD, USA).

These amplicons obtained with PCR were analysed by Restriction Fragment Lenght Polymorphism (RFLP). Restriction endonuclease enzymes (Thermo Fisher Scientific, WA, USA) were applied to identify genotype. All enzymes were incubation PCR device at a temperature of suitable temperature for 10 h. Enzymes and incubation times used for the PCR-RFLP are indicated in Table 1. The RFLP digestion products were checked with 2% agarose gel electrophoresis (120 V for 40 min).

Table1: Primer and amplicon sizes of *CACNA2D1* gene SNP regions.

SNP	Primer Sequence	AT	C	A	RE	RET
C367400T	F TGAAAGGGTTGTCCTGCCATC R GTGCTTGTTGTTCCCATTGCC	61	35	322	*Rsa*I	37
A496561G	F CCATATCTGTGTCCTGTGTCT R GGTAAAGTAAAGTGGAAGTCG	59	35	386	*Taq*I	65
G519663A	F TCTAACGCCTTATTGACATC R CTTACTGTTTCCTTTGGTTC	54.6	35	269	*Hpa*II	37

F: Forward, R: Reverse, AT: Anneling temperature, C: Cycle, A: Amplicon RE: Restriction endonulease enzyme, RET: Restriction endonulease enzyme digestion temperature.

Statistical Analysis

Proportional distributions of *CACNA2D1* gene C367400T, A496561G and G519663A coded SNP loci were calculated according to the subclinical mastitis status of the cows used in the study [no mastitis, M (-); and existence of mastitis, M (+)]. Statistical significance-control was

performed with Pearson Chi-square test. IBM SPSS program was used for statistical analysis.

RESULT

The PCR-RFLP analysis performed for the *CACNA2D1* gene, PCR bands of 322 bp, 386 bp, and 269 bp were observed for C367400T, A496561G and G519663A respectively (Figure 1).

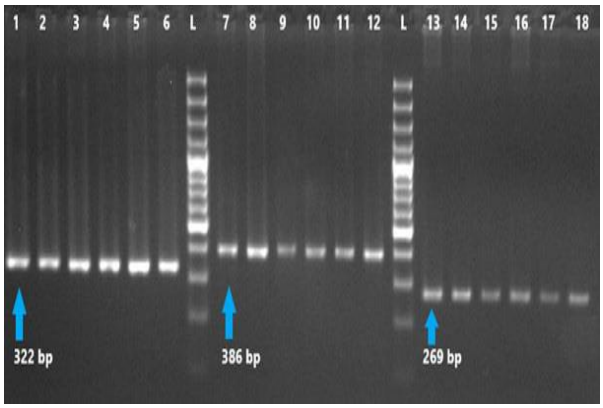

Figure 1: PCR amplicon sizes of C367400T, A496561G and G519663A SNPs. (1-6: 322 bp (C367400T SNP), 7-12: 386 bp (A496561G SNP) and 13-18: 269 bp (G519663A SNP); L: 100 bp ladder; bp: base pair).

Three genotypes for the C367400T SNP with *Rsa*I restriction enzyme digestion were detected: 322 bp for the TT genotype; 322, 236 and 86 bp for the CT genotype; 236 and 86 bp for the CC genotype (Figure 2a). After digestion with the *TaqI* restriction enzyme digestion, the AA (386 bp), AG (386, 229 and 157 bp) and GG (229 and 157 bp) genotypes were observed for the A496561G SNP (Figure 2-b). The polymorphism in the G519663A SNP was identified by digestion of the PCR product with *HpaII* restriction enzyme. After digestion with restriction enzyme digestion, the AA (269 bp), AG (269, 175 and 64 bp) and GG (175 and 64 bp) genotypes were observed for the G519663A SNP (Figure 2-c).

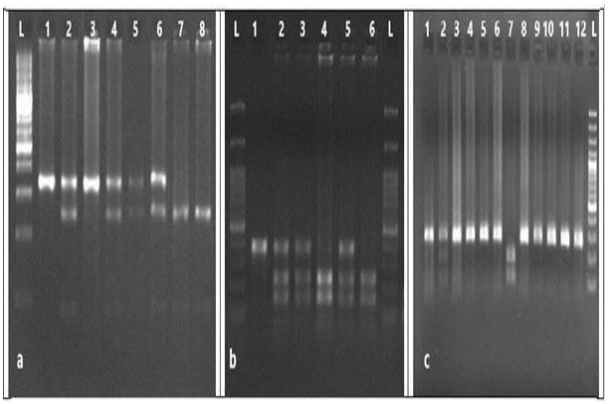

Figure 2: PCR-RFLP results of C367400T, A496561G and G519663A SNPs. a: PCR-RFLP results of C367400T (lanes 1,3: genotype TT; lanes 2, 4, 5 and 6: genotype CT; lanes 7 and 8: genotype CC); b: PCR-RFLP results of A496561G *(*lanes 1: genotype AA; lanes 2, 3, 5: genotype AG; lanes 4 and 6: genotype GG*)*; c: PCR-RFLP results of G519663A (lanes 1,3-6 and 8-12: genotype AA; lanes 2: genotype AG; lanes 7: genotype GG)*;* Lanes L: 100 bp ladder.

The difference between the subclinical mastitis status of the examined Holstein breed milk cows and the proportional distributions of the genotypes for C367400T, A496561G and G519663A coded SNPs in the *CACNA2D1* gene was not found to be significant (p>0.05). However, 49.4% (37/75) of subclinical mastitis positive (M+) cattle were found in CT genotype in terms of C367400T polymorphism; 54.6% (71/130) were in GG genotype in terms of G519663A polymorphism, and 53.4% (71/133) were in the GG genotype in terms of A496561G polymorphism (Table 2).

The most common genotypes were CT (0.497) for the C367400T SNP region, GG (0.861) for the A496561G SNP region, and AA (0.881) for the G519663A SNP region in the examined Holstein dairy cows. The examined Holstein cattle were in HWE in terms of C367400T and A496561G coded SNP regions, and they deviated from HWE (p<0.05) in terms of G519663A coded SNP region (Table 3).

Tablo 2: Proportional distribution of C367400T, A496561G and G519663A SNPs.

SNP	Genotype	M (-)	M (+)	n	χ2	p value
C367400T	CC	10 (45.4%)	12 (54.6 %)	22	1.756 *NS*	0.416
	CT	38 (50.6%)	37 (49.4%)	75		
	TT	21 (38.9%)	33 (61.1%)	54		
G519663A	AA	0 (0 %)	1 (100.0 %)	1	0.996 *NS*	0.608
	AG	10 (50.0 %)	10 (50.0%)	20		
	GG	59 (45.4%)	71 (54.6)	130		
A496561G	AA	62 (46.6 %)	71 (53.4%)	133	0.426 *NS*	0.808
	AG	6 (40.0 %)	9 (60.0%)	15		
	GG	1 (30.0 %)	2 (60.0%)	3		

M (-): Mastitis positive, M (+): Mastitis negative, NS: Not Not significant, χ2: Chi-square.

Table 3: Allele and genotype frequency of C367400T, A496561G and G519663A SNPs.

SNP	Genotype	n	Genotype Frequency	Allele	Allele Frequency	X^2
C367400T	CC	22	0.146	C	0.39	0.243^{NS}
	CT	75	0.497	T	0.61	
	TT	54	0.357			
G519663A	AA	133	0.881	A	0.93	**8.151***
	AG	15	0.099	G	0.07	
	GG	3	0.02			
A496561G	AA	1	0.007	A	0.07	0.057^{NS}
	AG	20	0.132	G	0.93	
	GG	130	0.861			

X^2: Chi-Square value, *NS*: Not significant, *:p<0.05.

DISCUSSION AND CONCLUSION

Mastitis is one of the most common health problems in dairy farms. Delay in diagnosing the disease along with the difficulties in diagnosing the animals with subclinical mastitis, place mastitis in the disease group with high treatment costs for dairy cattle farms. Therefore, it is important for dairy cattle enterprises to prevent economic losses due to cause by mastitis. Identifying and keeping mastitis-resistant animals in the herd has important advantages in preventing losses caused by subclinical mastitis in farms. Thanks to the development of molecular genetic methods, it has become easier to search for genes or SNPs associated with mastitis resistance. A number of genes, including the *CACNA2D1* gene, was reported in studies conducted for this purpose (Yuan et al. 2011a; Yuan et al. 2011b).

This study investigated the relationship between C367400T, A496561G and G519663A SNPs in the *CACNA2D1* gene and subclinical mastitis in Holstein cattle. The Chi-square (χ2) analysis performed at the end of the study showed that Holstein cattle were HWE regarding the SNPs coded C367400T and A496561G but deviated from HWE regarding the SNP coded G519663A. The results obtained in the study showed that genetic variation continued regarding the studied SNPs. The genotypes with high rates in terms of mastitis resistance of the studied SNPs were TT genotype in C367400T, AA genotype in A496561G and GG genotypes in G519663A, respectively.

Yuan et al. (2011b) found that the frequency of T allele frequence (0.55) was high in cattle in which *CACNA2D1* gene C367400T SNP was analyze. Bagheri et al. (2013) reported that T allele frequency (0.94) was high in C367400T SNP in Holstein breed in Iran. Similarly in this study, T allele frequency (0.61) was found to be high in Holstein cattle examined. The study findings are consistent with Yuan et al. (2011b) and Bagheri et al. (2013).

Yuan et al. (2011b) in A496561G coded SNP in the *CACNA2D1* was reported to be the AG least common genotype (0.16 in Holstein breed; 0.22 in Sanhe breed; 0.22 in Simmental breed). Bagheri et al. (2013) reported that AA was the least common genotype (0.31) in the A496561G SNP in their study on Holstein breed in Iran. This study found the AA genotype (0.007) as the least common genotype. The study findings are consistent with Bagheri et al. (2013). However, the literature review revealed that the number of studies was quite limited that examined the A496561G coded SNP in the *CACNA2D1* gene for Holstein and other cattle breeds. Therefore, more studies are needed to reveal the general status of A496561G SNP in the Holstein cattle breed and to obtain more reliable results.

Yuan et al. (2011b) investigated the genotype and allele frequencies of G519663A coded SNP and they reported that AA was the most common genotype. Magotra et al. (2018) was reported by that the GG genotype for G519663A coded SNP was the most common genotype in Karan Fries cross-breed cattle, a native Sahiwal (*Bos taurus indicus*) and a Holstein×Tharparkar (*Bos taurus indicus*) cross-breed in India. In this study, the most observed genotype in Holstein (*Bos taurus typicus*) cattle breed was AA (0.881). The study findings are consistent with Yuan et al. (2011b). On the other hand, the reason the results of this study differ from Magotra et al. (2018) is thought to be due to the origin of the cattle breeds.

Various studies were conducted to investigate the relationship between SNPs on the *CACNA2D1* gene and mastitis in different cattle breeds. In one of these studies, Yuan et al. (2011a) investigated the relationship between *A526745G* coded SNP in the *CACNA2D1* gene and mastitis and reported a significant relationship between this SNP and SCS. Another study reported a statistically significant correlation between another SNP coded *C367284A* in the *CACNA2D1* gene and SCS (Deng et al. 2011). A study by Magotra et al. (2019) reported a relationship between mastitis and another SNP coded *G38819398A* in the *CACNA2D1* gene and argued that cattle with GG genotype were more resistant to mastitis.

As a results, there are various studies investigating the relationships between mastitis and different SNPs on the *CACNA2D1* gene in different cattle breeds (Deng et al. 2011; Yuan et al. 2011a; Yuan et al. 2011b; Magotra et al. 2019). In this study, the relationship between subclinical mastitis and C367400T, A496561G and G519663A SNPs in the *CACNA2D1* gene were investigated for Holstein cattle raised in Türkiye. However, no relationship was found between the mentioned SNPs and subclinical mastitis in the examined samples. The results obtained from this study may have resulted from the number of samples examined. Considering the QTL region where the *CACNA2D1* gene is located and the physiological processes in which this gene is involved, it is suggested to conduct comprehensive studies on mastitis resistance in which this gene and the SNPs in this gene are evaluated with larger samples.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ACKNOWLEDGMENT

We would like to thank Specialist Veterinarian Murat AKKAYA for taking blood samples of the animals used in the study and applying the California Mastitis Test (CMT) test.

In this study, blood samples taken in the TYL-2017-7703 coded project conducted by Bilal AKYÜZ were used.

AUTHOR CONTRIBUTIONS

Idea / Concept: FD
Supervision / Consultancy: KA, BA
Data Collection and / or Processing: FD
Analysis and / or Interpretation: FD, KA, BA
Writing the Article: FD
Critical Review: BA

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