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Detection of Virulence Genes in *Streptococcus uberis* Isolated from Bovine Mastitis in Aydın Province by Multiplex Polymerase Chain Reaction[*, **]

The objective of this study was to identify the presence and distribution of virulence genes via multiplex Polymerase Chain Reaction by identification of *Streptococcus uberis*, one of the agents which cause mastitis in dairy cows. *S. uberis* strains were identified from samples and stored at -20 °C deep freeze for molecular studies. DNA extraction of the isolated strains was performed according to the procedure with the genomic DNA extraction kit (Fermentas®). The presence of 16S rRNA and virulence-associated genes was examined in all strains identified as *S. uberis* phenotypically. *S. uberis* isolation was conducted in 35 (17.5%) of the 200 milk samples. After examination of the identified *S. uberis* strains by polymerase chain reaction, 16S rRNA gene was detected in a total of 35 isolates (100%) at 1400 bp fragment base pairs. When the virulence genes were examined individually, 32 (91%) strains expressed hasB, 32 (91%) strains expressed gapC, 32 (91%) strains expressed skc, 30 (86%) strains expressed cfu, 30 (86%) strains expressed hasC, 30 (86%) strains expressed hasA 29 (83%) strains expressed sua, 28 (80%) strains expressed oppF, 25 (71%) pauA, 9 (26%) strains expressed lbp genes. The pauB gene was not identified. When the distribution of virulence genes of *S. uberis* strains isolated from mastitis cases are examined, it has been shown that the genes which exert capsular formation in terms of the pathogenity of the agent play important role and also the genes involved in the synthesis of serine protease enzymes are distributed in significant proportion.

Key words: Streptococcus uberis, mastitis, virulence genes, mPCR

Aydın İlinde Bulunan Sığır Çiftliklerinde İzole Edilen *Streptococcus uberis* Kökenli Mastitislerin ve Virulens Genlerinin Multiplex Polimeraz Zincir Reaksiyonu ile Belirlenmesi

Sütçü ineklerde mastitise sebep olan etkenlerden olan *Streptococcus uberis*'in izolasyonu ve tür spesifik hedef genler ile identifikasyonu yapılarak, virulans genlerinin varlığı ve dağılımının multipleks Polimeraz Zincir Reaksiyonuyla ortaya konması amaçlanmıştır. Araştırmada laboratuvara getirilen süt örneklerinden elde edilen izolatlar, koloni görünümü, Gram boyama ve katalaz testi ve biyokimyasal testler ile identifikasyonları sonucu *S.uberis* suşları ayrıldı ve moleküler çalışmalar için –20 °C'de saklandı. İzole edilen suşlardan DNA ekstraksiyonu, genomik DNA ekstraksiyon kiti (Fermentas®) ile prosedüre uygun olarak yapıldı. Fenotipik olarak *S. uberis* olduğu belirlenen tüm suşlarda 16S rRNA ve virulans ilişkili genlerin varlığı incelendi. İncelenen 200 süt örneğinin 35 (%17.5)'inden *Streptococcus uberis* izolasyonu gerçekleştirildi. Polimeraz zincir reaksiyonu ile, identifiye edilmiş *S. uberis* suşları incelendiğinde toplam 35 adet izolatın tamamında (%100) 1400 bp fragment aralığında16S rRNAgeni tespit edilmiştir. Virulans genleri tek olarak incelendiğinde, 32 (%91) suştan *hasB*, 32 (%91) suştan *gapC,* 32 (%91) suştan *skc*, 30 (%86) suştan *cfu,* 30 (%86) suştan *hasC,* 30 (%86) suştan *hasA,* 29 (%83) suştan *sua,* 28 (%80) suştan *oppF,* 25 (%71) *pauA,* 9 (%26) suştan *lbp* geni identifiye edilmiştir. *pauB* genine ise rastlanmamıştır. Mastitis vakalarından izole edilen *S. uberis* etkenlerinin virulans genlerinin dağılımı incelendiğinde etkenin patojenitesi açısından kapsül formasyonunu eksprese eden genlerin önemli role sahip olduğu ortaya konulmuştur, ayrıca serin proteaz enzimi sentezinde görevli olan genlerin de dikkate değer oranda dağılım gösterdiği görülmüştür.

Anahtar Kelimeler: Streptococcus uberis, mastitis, virulans genleri, mPCR

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Introduction

Mastitis is defined as inflammatory changes in the mammary gland, characterized by pathological changes in the mammary tissue and an increase in the number of somatic cells in the milk and dairy products, which are very important for human and animal health, nutrition and national economy, can only be obtained from healthy animals. As a result of pathological changes caused by the inflammatory state of the mammary gland, a number of physical and chemical changes occur in the milk. These changes limit the availability of milk yield and dairy products, thus mastitis is an important problem that needs to be carefully considered (1-3).

It is possible to reduce mastitis-induced losses by applying effective control programs at the enterprise level. Implementation of mastitis control programs and ensuring the sustainability of these programs are the most important indicators of economic efficiency, especially in dairy farms with large capacity. However, due to the fact that the milk production capacity of our country is small especially for dairy farming and the productivity-centered mastitis control programs cannot be established yet, major economic losses occur in milk production. Since wholesome milk is obtained from healthy animals, it is a basic aim to maintain animal health and to diagnose mastitis cases early. Economic losses due to mastitis in the dairy industry result from reduced milk quality and milk yield, resulting in increased use of medicines and veterinary services and increased feed costs. At the same time, antibiotics used for the treatment of infections are left in the milk, antibiotic-resistant bacteria due to drug use unconsciously, and limitations of treatment possibilities. The implementation of routine and conscious control programs ensures that clinical mastitis is reduced and that subclinical infections are diagnosed early (1).

Environmental factors that cause mastitis include unsuitable environmental conditions, inadequate hygiene at stables and shelters, inadequate ventilation conditions, and stiff and dirty substances. In order to increase milk yield, protein-rich feeding increases the likelihood of mastitis. The fact that milkers do not pay attention to cleaning and disinfection also accelerates the spread of infections among animals. When looking at microbial causes; many bacteria, fungi and viral agents cause the formation of mastitis. Mastitis agents are divided into infectious and environmental pathogens, taking into account their characteristics, as well as their relation to breast and mammary ducts. Microbial pathogens multiply in the bovine mammary gland and bulldog animals during milking. The environmental pathogens *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Escherichia coli* cause environmental mastitis infections. The infectious pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae*, Mycoplasma species and *Corynebacterium bovis*. Tuberculosis agents, Proteus, Leptospira, Listeria and Brucella species also cause mastitis (4)

Streptococcal mastitis is initially insidious and mild. Clinically detectable anomalies are not seen immediately in the teat. Infection usually takes formation during the lactation period, and as time goes by, the clinical signs of the disease begin to form due to the accumulation of water in the mammary gland (5). The major strains of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* are isolated in mastitis infections, which are very common in dairy cattle and economic losses occur. Mastitis control programs, improvements in milk, post-partum hygiene practices, and common antibiotic treatments reduced mastitis infections caused by *S. agalactiae* and *S. dysgalactiae*, but no significant decrease in infections caused by *S. uberis* was identified (6).

The scope of this study was to determine the isolation of factors from mastitic milk and the role of *S. uberis* etiology in mastitis etiology, and the distribution of virulence related genes in isolated strains by means of molecular methods and to provide information about the typing of isolated strains and distribution of virulence genes.

Materials and Methods

A total of 200 milk samples were collected from Holstein cows with acute clinical mastitis found in 17 private dairy cattle farms which have machine milking systems with 20-150 head capacity in Aydın Province between June and November 2013. The animals were all in lactation, had not received antibiotic treatment in the last 1 month, had at least one calf delivery, between 2-8 year of age. Acute clinical mastitis was determined according to physical examination abnormalities (swelling and tenderness, temperature, pain, erythema in mammary lobes, coagulated milk) of the ill cows. Microbiological analyses of the milk samples for identification of *Streptococcus uberis* were conducted at the Diagnostic and Analysis Laboratory of the Department of Microbiology, Adnan Menderes University, Faculty of Veterinary Medicine. Milk samples were inoculated onto sheep blood agar and incubated for 48 hours at 37 °C for isolation of *Streptococcus* spp. At the end of the incubation period, S-type colonies 1-3 mm in diameter were selected in the media and stained by Gram staining method. Cocci that were microscopically Gram positive were tested for catalase reaction and catalase negative colonies were detected as *Streptococcus* spp (3, 7). Streptococcal colonies were inoculated onto Edward's medium and incubated at 37 ºC for 24 hours. At the end of the incubation, dark brown-black colored colonies were considered positive for *S. uberis*, light brown colored or colorless colonies were evaluated as negative for *S. uberis*. Esculin test was conducted for identification at species level (8, 9).

DNA isolation of the isolates identified as *S. uberis* was made according to the procedure with the genomic DNA extraction kit (Fermentas®). The extracted DNAs were stored in cryo-tubes in −20 ºC freezer until PCR runs. The presence of 16S rRNA and virulence related genes in all strains determined to be *S. uberis* phenotypically was examined by multiplex PCR method. The preparation of the master mix was as followed; 20 ng of DNA sample was used in a total volume of 50 µL using 1 µM of each primer, 0.4 mM dNTP, 1.5 mM MgCl2, 1X reaction buffer, 1.5 U Taq DNA polymerase (10). In PCR for identification of virulence related genes, primer mixes were prepared by combining each of the primers of 100 mM cfu, hAs, hasB, hasC, gapC, lbp, oppF, pauA / pauB, skc and sua to be 40 µM each. The primers of *S. uberis* virulence associated genes used in our study are shown in Table 1.

Table 1. Oligonucleotide primer sequences used in the multiplex PCR

S. uberis pathogenicity factor	Virulence genes	Primer Pairs (5'-3')	Target region (bp)
-	16S rRNA gene	GAGAGTTTGATCCTGGCTCAGGA CGGGTGTTACAAACTCTCGTGGT	1400
CAMP	*cfu*	TATCCCGATTTGCAGCCTAC CCTGGTCAACTTGTGCAACTG	205
Hyaluronic acid	*hasA*	GAAAGGTCTGATGCTGAT TCATCCCCTATGCTTACAG	600
	hasB	TCTAGACGCCGATCAAGC TGAATTCCYATGCGTCGATC	300
	hasC	TGCTTGGTGACGATTTGATG GTCCAATGATAGCAAGGTACAC	300
Surface dehydrogenase protein	*gapC*	GCTCCTGGTGGAGATGATGT GTCACCAGTGTAAGCGTGGA	200
Lactoferrin binding protein	*lbp*	CGACCCTTCAGATTGGATTC TAGCAGCATCACGTTCTTCG	698
Solvent active transfer	*oppF*	GGCCTAACCAAAACGAAACA GGCTCTGGAATTGCTGAAAG	419
Plasminogen activator	*pauA/pauB*	GAGATTCCTCTCTAGATATCA GGGCTGCAGATCCGTTAAAAAATGACATTAATAT	1200
Serine protease	*skc*	CTCCTCTCCAACAAAGAGG GAAGGCCTTCCCCTTTGAAA	800
Epithelial cell invasion	*sua*	ACGCAAGGTGCTCAAGAGTT TGAACAAGCGATTCGTCAAG	776

After the preparation of mastermix, the mixture was added to 0.2 mL PCR tubes, numbered up to the sample number and completed up to 48 µL of volume. Then, 2 µL of the extracted DNA was added, added into the relevant tubes, and the mouths were tightly closed. The prepared tubes were then loaded into the thermal cycler. The thermal cycle and time diagram used in the PCR analyzes of the mastermixes prepared for the identification of S*. uberis* and virulence associated genes was performed as previously described (11).

6X of the loading dye was added in the volume of 3 µL on the pipette tip and mixed with 10 µL of PCR products. The mixture was added to the appropriate position on the gel. After the preparation of the samples and markers, the electrophoresis gel was run for 40 min at 100 volts.

Results

Biochemical tests, isolation and identification of 35 (17.5%) strains of *S. uberis* were identified from 200 milk samples examined in our study. Biochemically identified *S. uberis* strains were examined via Multiplex polymerase chain reaction, and 16S rRNA gene was detected in a total of 35 isolates (100%) with 1400 bp fragment length (Figure 1).

In our study, it is observed that 68, 5% of strains were identified in E, D, B, Q, AH, AN patterns, respectively. 32 (91%) strains expressed *hasB*, 32 (91%) strains expressed *gapC*, 32 (91%) strains expressed *skc*, 30 (86%) strains expressed *cfu*, 30 (86%) strains expressed *hasC*, 30 (86%) strains expressed *hasA* 29 (83%) strains expressed *sua*, 28 (80%) strains expressed *oppF*, 25 (71%) *pauA*, 9 (26%) strains expressed *lbp* genes. The pauB gene was not identified.
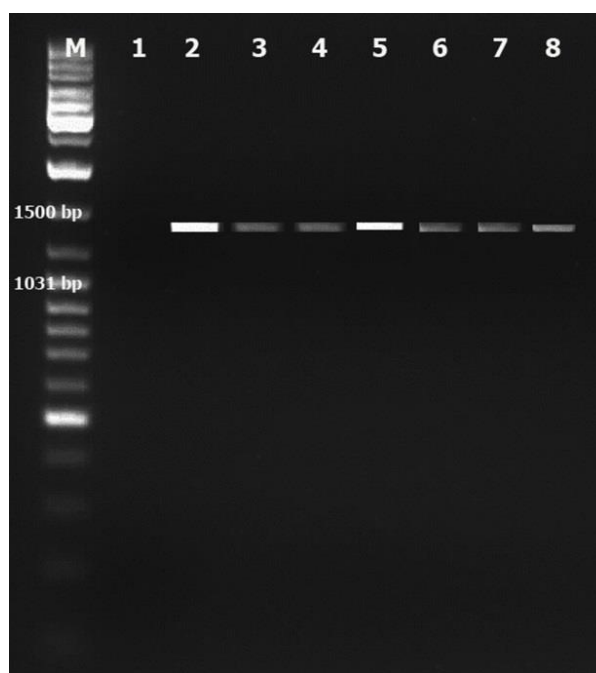

Figure 1. *S. uberis* spesific 16S rRNA gene identification M: Marker (100 bp), 1: Negative control (*Escherichia coli* ATCC® 25922), 2: Positive control (*Streptococcus uberis* ATCC® 700407), 3-8: *S. uberis* positive samples

When the virulence-related genes of *S. uberis* isolates were examined, 3 (8.57%) isolates were identified in pattern B (*cfu, hasA, hasB, hasC, gapC, pauA/B, skc, sua*), 4 (11.4%) isolates in pattern D (*cfu, hasA, hasB, hasC, gapC, lbp, oppF, pauA/B, skc, sua*), 8 (22.8%) isolates in pattern E (*cfu, hasA, hasB, hasC, gapC, oppF, pauA/B, skc, sua*), 2 (5.7%) isolates in pattern G (*cfu, hasA, hasB, hasC, gapC, pauA/B, skc,*

sua), 2 (5.7%) isolates in pattern L (*hasA, hasB, hasC, gapC, oppF, pauA/B, skc, sua*), 2 (5.7%) isolates in pattern N (*hasA, hasB, hasC, gapC, oppF, pauA/B, skc, sua*), 1 (2.85%) isolte in pattern P (*hasA, hasB, hasC, oppF, pauA/B, skc*), 3 (8.57%) isolates in pattern Q (*cfu, hasA, hasB, hasC, gapC, oppF, sua*), 3 (8.57%) isolates in pattern AH (*cfu, gapC, lbp, oppF, pauA/B, skc*), 3 (8.57%) isolates in pattern AN (*cfu, hasA, hasB, hasC, gapC, oppF, skc, sua*), 2 (5.7%) isolates in pattern AT (*cfu, hasB, hasC, lbp, oppF, skc*), 2 (5.7%) isolates in pattern AX (*cfu, hasA, hasB, gapC, skc, sua*) (Table 2).

Table 2. Distribution of virulence patterns of *S. uberis* strains (n= 35)

Isolates	Virulence patterns										
	B	D	E	G	L	N	P	Q	AH	AN	AT	AX
S. uberis	3	4	8	2	2	2	1	3	3	3	2	2

Discussion

Mastitis is the most important problem of dairy production. In cows, mastitis is an infection that occurs in all farms, including modern farms, and causes significant economic loss. It has been shown that economic losses in cows are more frequent in subclinical mastitis than in clinical mastitis. Mastitis is the leading cause of economic loss in milk production when yield, product losses and treatment costs are evaluated together. Mastitis frequency can be observed up to 40%, especially in modern intensive farms. Despite all hygienic practices, the continuation of the mastitis problem has brought medical preventive measures to the agenda and the development of control programs, including vaccines, has become unavoidable. However, in order to be able to effectively combat an infection, definite etiopathogenesis and its associated epidemiology must be known on the basis of region and herd (10).

In cattle, mastitis is widespread all over the world and leads to significant economic losses. Pathogens of mastitis usually exhibit a contagious course during transport, and 2 groups are separated as infectious agents from environmental reservoirs. Many streptococcal species are among those isolated as mammary pathogens. *S. uberis* is an important pathogen in bovine mastitis, and is associated mostly with subclinical and clinical intramammary infections in both culled and untreated cows. These species are often a problem because they are ubiquitous around the dairy farms. Increasing the hygiene procedures against these opportunist pathogens results in the reduction of consequent mastitis, however does not yield an effective result in environmental contamination (12).

The isolation rates of streptococci from milk samples vary. In a previous research (13), *S. dysgalactiae* was isolated from 2.3% of the milk samples examined, *S. uberis* and *S. agalactiae* was ddetected in the ratio of 0.3% on the farm to have a low isolation rate. Sampimon et al. (14) found the prevalence of *S. uberis* at 1.1%, *S. dysgalactiae* at 0.9%, and did not encounter

S. agalactiae. Tenhagen et al. (15) have identified *S. agalactiae* as 0.1%, *S. dysgalactiae* and *S. uberis* as 2.3% from cow milk. In a study conducted in UK (16), bacteriological sampling in the milk resulted in the isolation of *S. uberis* with ratio of 23.5%, whereas *S. agalactiae* and *S. dysgalactiae* were not identified.

Bentley et al. (17) reported that only three of the 206 gram-positive cocci isolated from mastitic milk were *S. parauberis*. Researchers have indicated that it is difficult to distinguish *S. parauberis* agents from *S. uberis* and that there is limited information on epidemiology. Pitkala et al. (18) identified two *S. uberis* isolates as *S. parauberis* in their study in Finland and found that the antibiotic resistance of *S. parauberis* strains resembled that of *S. uberis*. Devriese et al. (19) reported that catalase-negative and esculin-positive Gram-positive cocci isolated from clinical and subclinical mastitis were mostly identified as *S. uberis* and that they were not correctly identified.

In studies conducted in Turkey, Dakman (20) reported that 12.5% of cow mastitis cases were caused by *Streptococcus* spp. and that 4.5% of these Streptococci were *S. agalactiae*. Karahan (21) examined 24.3% of mastitic milk samples from *Streptococcus* spp. 10.4% of isolates were *S. agalactiae* and 13.9% of other isolates were identified as other Streptococci.

Şahin et al. (22) found that Streptococci isolation rate from milk was 29.82% and 14.03% of *S. agalactiae*, 8.77% of *S. dysgalactiae* and 7.02% of *S. uberis* were identified.

The average isolation rate obtained in this study was found to be correlated with the findings obtained from previous studies conducted in Turkey. These findings may arise from the lack of application of mastitis control programs in dairy cows when assessed based on the mean Streptococcal isolation rate. The isolation rates obtained in other studies are at the same level.

Jayarao and Oliver (23) identified 11 different enzymes in the RFLP-PCR method to isolate mastitic Streptococci at the species level by single enzyme cuts. McDonald et al. (9) performed identification of Streptococci at the species level by using dual enzyme combinations in a single reaction in the RFLP-PCR method.

Different molecular methods have been used for species-level identification of streptococci. These methods are used especially in the typing of *S. uberis* (2).

Duarte et al. (24) identified RFLP-PCR as an efficient method for diagnosing in a single reaction with shorter time than conventional methods. Odierno et al. (25) reported that RFLP-PCR was 100% accurate after RFLP-PCR, but that conventional test results did not fully support RFLP-PCR.

Jayarao and Oliver (23) compared the results with classical biochemical results in RAPD-PCR evaluation and distinguished near genotypic factors by conventional method.

Duarte et al. (24) showed that this method could be used in typing microorganisms in the RAPD PCR procedure they applied for group B Streptococci, but the use of additional typing methods in the interpretation of the results increased the reliability.

Chatellier et al. (26) identified 71 profiles in 114 strains of RAPD-PCR for *S. agalactiae* and found that these profile differences could be due to the geographical areas where the farms were located.

In our study, 35 isolates identified as *S. uberis* by bacteriological culture and biochemical identification method were all identified as *S. uberis* in PCR result using 16S rRNA gene specific primer. The fact that the primer used has a high specificity and that it is derived from the 16S rRNA gene, which is more stable than other gene regions, indicates that PCR identification can be performed as a reliable and rapid method in routine diagnostic laboratories. The use of molecular methods those are more specific and more sensitive than those of conventional methods with lower sensitivity and would be more beneficial.

In a study conducted by Hassan et al. (27), positive for *cfb* gene was detected in *S. agalactiae* isolates, phenotypically negative for CAMP, by PCR. This suggests that the *cfb* gene-specific PCR assay can avoid false negative conditions that may arise from the CAMP reaction, which is considered to be one of the key criteria in the biochemical characterization of streptococci.

Ekin et al. (28) reported a positive phenotype in 73.3% of *S. agalactiae* isolates obtained from cattle milk in terms of CAMP test in our country. In another study 26.3% of cattle-born Streptococcal isolates were positive for CAMP test (29). Although this test is generally considered to be a determinant test for the identification of Streptococci, it is likely that CAMP testing cannot be used effectively for identification from earlier studies. Reinoso et al. (10) found that the *cfu* gene distribution in their study was 76.9%.

The *cfu* gene responsible for the CAMP factor in *S. uberis* was identified in 30 (86%) strains in our study. These results were in parallel with findings in our study. Thus, the result that *cfu* gene identification can be used for identification in *S. uberis* quickly and reliably.

The operon in the capsule formation consists of the *hasA*, *hasB* and *hasC* gene loci, which are essential genes for capsule formation (11). HasA, *hasB* and *hasC* gene identification rates were 83%, 91% and 86%, respectively, in our study. Reinoso et al. (10) identified the *hasA* gene as 74.3%, the *hasB* gene as 66.6% and the *hasC* as 89.7%. According to the findings obtained, capsule formation in Streptococcal mastitis cases seems to play an important role in the development of infection.

The *oppF* gene plays an important role in the milk production of *S. uberis* agents (30). In our study, *oppF* gene identification rate was 80%. Reinoso et al. (10) identified the *oppF* gene by 64.1%. Zadoks et al. (4) reported that this gene could not be isolated from all

strains. The findings obtained correlate with the data in our study.

Serine protease enzymes that convert plasminogenetic plasmids are essential components for the degradation of extracellular matrix proteins and the colonization of bacterial agents in tissues. In addition, the milk proteins that are responsible for the activation of endogenous plasminogenes in the milk are hydrolyzed, thus forming the molecules necessary for *S. uberis* (11). The *skc* gene responsible for the synthesis of these enzymes has been identified in 91% of our studies. Reinoso et al. (10) identified 63% of the *skc* gene in their study.

The *pauA* gene (31) that was detected in association with plasminogen activation and intramammary colonization was isolated in 71% of our study. The *pauB* gene has not been identified in our study. In the study conducted by Smith et al. (30), only one strain of *S. uberis* was identified as *pauB*. Expression of *pauB* genes has not been shown to play a role in the formation of intramammary infections in the direction of the obtained data.

Multiplex PCR-based molecular assays have been found to be reliable for detecting the genotypic relationships of the virulence genes at the genus level of the *S. uberis* agent, a common effect of subclinical bovine mastitis cases. In addition, in this study, the close proximity of the homozygous for the identification rates as a result of PCR amplification of the *hasA*, *hasB* and *hasC* gene operon used for the molecular identification of *S. uberis* has shown that this gene region allows rapid and reliable identification of cattle origin *S. uberis* agents. It has been found that the molecular methods used in this study may be useful for detecting virulent types among strains of *S. uberis* which are encountered as an important problem in subclinical mastitis cases and may contribute to putting the pathogen of infection in the basis of field strains. Significant control and strategies to be undertaken in the face of these findings are expected to provide a significant reduction in the incidence of *S. uberis*-induced mastitis. Thus, effective control programs against *S. uberis* mastitis, which are very difficult to eradicate or control due to various factors that are effective in the host environment and active agent triplet, will provide important advances both in terms of animal health and public health. It is also believed that the identification of additional genotypic methods and methods for detection of new virulence genes of *S. uberis* strains as well as speeding up detailed genetic analysis studies will be useful in obtaining more specific results and more detailed information on the epidemiological characteristics of these factors. As a result, when the distribution of virulence genes of *S. uberis* isolates isolated from mastitis cases is investigated, it has been shown that the genes which exert capsular formation in pathogenicity of the agent play an important role and the genes involved in the synthesis of serine protease enzyme are distributed in a remarkable manner. It is also evaluated that the identification of additional genotypic methods and methods for detection of new virulence genes of *S.*

uberis strains as well as speeding up detailed genetic analysis studies will be useful in obtaining more specific results and more detailed information on the epidemiological characteristics of these factors. The *S. uberis* factors identified in our study were at the same level as the other studies in our country. The presence of

the bacterial agent has been demonstrated in the Aydın region and with the help of regular preventive control strategies it will become possible that the incidence of *S. uberis*-induced mastitis cases can be lowered and therefore economic losses can be minimized.

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