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The prevalence, genetic diversity and antibiotic resistance of *Staphylococcus aureus* associated with subclinical bovine mastitis in Balikesir, Turkey

Hakan Tavsanli[1]*, and Recep Cibik[2]

[1]*Department of Public Health, Faculty of Veterinary Medicine, University of Balikesir, Balikesir, Turkey*

[2]*Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, University of Bursa Uludag, Bursa, Turkey*

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ABSTRACT

Subclinical mastitis caused by *Staphylococcus aureus* is very common in dairy cows and creates serious problems on dairy farms. In this study, we investigated the prevalence, genetic diversity and antimicrobial resistance of *S. aureus* from subclinical bovine mastitis on 12 dairy herds in Balikesir province of Turkey, by SCC*mec* and *spa* typing. Ninety-five isolates of *S. aureus* were isolated from 725 subclinical mastitic milk samples that exceeded the somatic cell count (SCC) limit of 4 x 10^5 cell/mL. The frequency of MRSA (methicillin-resistant *Staphylococcus aureus*) and MSSA (methicillin-sensitive *S. aureus*) was 6.3% (6 isolates) and 93.68% (89 isolates) respectively. SCC*mec* types of MRSA isolates were community-associated CA-MRSA type IVb (4 isolates) and type IVd (two isolates), while the *spa* types were T 005 and T 5163 (three isolates from each). The resistance rate of MRSA isolates was 100% for oxacillin and cefoxitin, 83% for penicillin, ampicillin, clindamycin, erythromycin and 66% for gentamicin and trimethoprim/sulfamethoxazole. However, compared to MRSA, the resistance of MSSA isolates was relatively lower. This study supported the scientific data on the occurrence of MRSA and MSSA in subclinical mastitis, and highlighted the need for preventive measures to eliminate or decrease *S. aureus* contamination of milk in dairy herds.

Key words: *SCCmec* types MRSA; somatic cell count; *spa* types of MRSA; subclinical mastitis

Introduction

Modern diagnostic methods have demonstrated clinical and subclinical mastitis asone of the most important causes of economic losses in dairy management. It has been known for a long time that the microorganisms responsible for mastitis in cows may cause important health problems for people consuming inadequately prepared foodstuffs (GEARY et al., 2012). In dairy, subclinical mastitis cases have special importance because 90-95% of animals present no clinical symptoms, however milking yield may be reduced in a range varying from 5-20% (JUOZAITIENE et al., 2006).

Somatic cell count (SCC) in milk is an indicator of the health status of mammalian tissue in dairy cows and is composed of leucocytes (75-85%) and mammary gland epithelial cells (15-25%) (BARRETT, 2002). A high SCC is an indicator for early diagnosis of subclinical mastitis and could play an important role in the prevention of harm (FRANZOI et al., 2020). According to epidemiological studies, subclinical mastitis could be caused either by contagious or environmental microorganisms (CERVINKOVA et al., 2013). *Staphylococcus aureus*, *Streptococcus agalactiae*,

*Corresponding author:

Assist. Prof. Dr. Hakan Tavşanlı, Department of Public Health, Faculty of Veterinary Medicine, University of Balikesir, Balikesir, Turkey, E-mail: tavsanli@balikesir.edu.tr

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Corynebacterium bovis and *Mycoplasma* spp. are amongst the most commonly identified contagious pathogens, while *Escherichia coli*, *Str. dysgalactiae*, *Str. uberis, Klebsiella* spp. and others are the environmental pathogens (HARMON, 1994). *S. aureus,* as one of the most important foodborne pathogens, might be transmitted to dairy animals through other animals, personnel and/or milking machines (KÜMMEL et al., 2016). Evidence has been found that this bacterium is responsible for subclinical mastitis cases in dairy cows in China, Iran, Finland and Kenya (BAHRAMINIA et al., 2017).

Strains of *S. aureus* showing resistance to methicillin (MRSA) were first characterized in the 1980s, and since then attention has been focused on this microorganism (BURNETT et al., 2016). Resistance to methicillin is genetically conferred by expression of the *mecA* gene, which is frequently carried on a mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) (PATERSON et al., 2014). This element is largely distributed between coagulase positive and negative staphylococci. The expression of *mecA* results in the production of PBP2a, a special penicillin binding protein, harbouring a transpeptidase domain. In the presence of β-lactam antibiotics, normal PBPs are blocked, but PBP2a precedes transpeptidation allowing normal cell wall synthesis (GOFFIN and GHUYSEN, 1998).

According to their SCC*mec* types, MRSA strains are classified in three groups: hospital associated (HA-MRSA), community associated (CA-MRSA) and livestock associated (LA-MRSA). HA-MRSA isolates characteristically belong to SCC*mec* types I to III and are associated with a high mortality rate, while types IV and V are generally related to CA-MRSA isolates expressing some virulence factors such as Panton-Valentine-Leukocidin. For LA-MRSA, several sequence types and clonal complexes (CC) have been identified from different animal associated strains (ANJUM et al., 2019). The European Food Safety Authority (EFSA) reported the role of animal originated foods as possible sources of MRSA (EFSA, 2008). In 2009, the necessity was expressed to clarify the epidemiology and prevalence of MRSA in human and animals, as well as in food and environmental samples (EFSA, 2009).

Antibiotic resistance developed by bacteria is one of the most important public health concerns, and is becoming a worldwide threat. According to reports by the Centre for Disease Control and Prevention (CDC), in the United States, nearly three million people became sick and 50 000 died due to antibiotic-resistant bacteria in 2018 (CDC 2019). It is predicted that mortality will increase considerably in the next 30 years (TAGLIABUE and RAPPUOLI, 2018). As one of the bacteria that shows high resistance to several antibiotics, *S aureus* may carry multiple antibiotic resistance traits responsible for resistance development. In the present research, the prevalence of *S. aureus* in subclinical mastitis, SCC*mec* and staphylococcal protein A (*spa*) sequence types of MRSA and their antibiotic resistance were characterized to better understand their role in mastitis and its pathogenesis.

Materials and methods

Collection of milk samples. Milk samples were taken from 2165 animals with no signs of clinical mastitis from 12 dairy farms in Balikesir province. This region is ranked in fourth place in terms of milk production in Turkey. The farms were selected from those equipped with a proper Cleaning in Place (CIP) system integrated into the automatic milking unit. It was confirmed that each of the selected farms have applied regular preventive vaccination (Starvac®, Spain) against contagious and environmental mastitis. The farms were visited twice with an interval of 15 days. Three doses of vaccine were administered intramuscularly: the first and second vaccinations were 45 and 10 days before the expected parturition date, respectively, while the booster vaccination was 62 days after the second vaccination.

Milk Samples were collected by trained farm workers. The teat ends were cleaned, disinfected, and wiped with commercial towels (Iomin D Plus®, Deosan, USA). The first streaks of milk were discarded. Approximately 100 mL of morning milk taken from the sampling unit were transferred into sterile tubes. The tubes were kept at +4 °C during transport and analysed within 4 hours.

Table 1. Oligonucleotides used in the present study and their specifications

Target gene	Oligonucleotide sequence	(bp)	Target gene	Oligonucleotide sequence	(bp)
nuc 1	F:GCGATTGATGGTGATACGGTT R:AGCCAAGCCTTGACGAACTAAAGC	279	SCCmec IVa	F:GCCTTATTCGAAGAAACCG R:CTACTCTTCTGAAAAGCGTCG	776
mecA	F: AAAATCGATGGTAAAGGTTGGC R: AGTTCTGCAGTACCGGATTTGC	533	SCCmec IVb	F:TCTGGAATTACTTCAGCTGC R:AAACAATATTGCTCTCCCTC	493
spa	F:TAAAGGCGATCCTTCGGTGAGC R:CAGCAGTAGTGCCGTTTGCTT	220	SCCmec IVc	F:ACAATATTTGTATTATCGGAGAGC R:TTGGTATGAGGTATTGCTGG	200
SCCmec I	F:GCTTTAAAGAGTGTCGTTACAGG R:GTTCTCTCATAGTATGACGTCC	613	SCCmec IVd	F:CTCAAAATACGGACCCCAATACA R:TGCTCCAGTAATTGCTAAAG	881
SCCmec II	F:CGTTGAAGATGATGAAGCG R:CGAAATCAATGGTTAATGGACC	398	SCCmec V	F:GAACATTGTTACTTAAATGAGCG R:TGAAAGTTGTACCCTTGACACC	325
SCCmec III	F:CCATATTGTGTACGATGCG R:CCTTAGTTGTCGTAACAGATCG	280			

Table 2. Somatic cell counts, subclinical mastitis rates, MSSA and MRSA percentages

Farm No	Number of cows tested	Average SCC	Number and percentage of cows with SM	Average SCC of SM Cows	MSSA (%)	MRSA (%)
1	196	451.107	98 (50%)	696.405	5 (5.26%)	-
2	682	303.098	225 (32.9%)	475.043	-	-
3	119	780.731	31 (26.0%)	1.817.250	1 (1.05%)	-
4	192	288.731	30 (15.6%)	677.822	2 (2.10%)	-
5	94	759.000	30 (31.6%)	1.007.150	2 (2.10%)	-
6	131	337.328	39 (29.7%)	408.714	18 (18.94%)	1 (1.05%)
7	108	246.638	30 (27.7%)	442.310	17 (17.89%)	2 (2.10%)
8	219	325.602	52 (23.7%)	944. 152	2 (2.10%)	-
9	176	1.338.557	111 (63%)	1.619.530	37 (38.94%)	3 (3.15%)
10	101	660.222	42 (41.5%)	1.722.970	1 (1.05%)	-
11	76	367.260	25 (32.8%)	906.200	3 (3.15%)	-
12	71	269.732	12 (16.9%)	895.870	1 (1.05%)	-
Total	2165	510.667	725 (33.4%)	969.933	89 (93.68%)	6 (6.31%)

SCC: somatic cell counts; SM: subclinical mastitis, MSSA: Methicillin susceptible *S. aureus*, MRSA: Methicillin resistant *S. aureus*

Somatic cell count (SCC) analysis. A Bentley® Combi FTS (USA) milk analyser was used for SCC determination. Samples with SCC higher than 4 x 10^5 cells/ml were regarded as subclinical mastitis, as previously stated (TAHAWY and EL-FAR, 2010; KASWAN et al., 2012; HISIRA et al., 2019).

Isolation and Identification S. aureus. Samples regarded as having subclinical mastitis were subsequently used for isolation of *S. aureus* according to VIÇOSA et al. (2010). Typical colonies were tested for coagulase using a Staphytect Plus kit (Oxoid-DR0850, Basingstoke, UK). A latex agglutination test SLIDEX® (bioMérieux, France) was used for determination of MRSA.

Table 3. SCC*mec* and *Spa* types of MRSA isolates

Farm no	SCC*mec* type		*Spa* type	
	IVb	IVd	T005	T5163
6	1	-	-	1
7	-	2	-	2
9	3	-	3	

Table 4. Antibiotic resistance of MSSA and MRSA isolates

	OX	CEF	P	CN	AML	DA	SXT	E
MSSA	-	-	67 (75.2%)	12 (13.4%)	5 (5.6%)	16 (17.9%)	8 (8.9%)	16 (17.9%)
MRSA	6 (100%)	6 (100%)	5 (83.3%)	4 (66.6%)	5 (83.3%)	5 (83.3%)	4 (66.6%)	5 (83.3%)

Tested by the Disc Diffusion Test, OX: oxacillin (1 µg), CEF: cefoxitin (30 µg), P: penicillin (10 IU), CN: gentamicin (10 µg), AML: ampicillin (10 µg), DA: clindamycin (2 µg), SXT: trimethoprim/sulfamethoxazole (1.25µg / 23.75 µg) and E: erythromycin (15 µg)

Molecular methods. DNA was extracted from bacteria using GeneJET Genomic DNA Purification (K0722, Fermentas®, Lithuania) as recommended by the manufacturer. Confirmation of *S. aureus* and MRSA was performed by PCR on *Nuc 1* and *mec A* genes respectively (MAES et al., 2002). SCC*mec* typing was performed using specific primers for SCC*mec* I / SCC*mec* II / SCC*mec* III / SCC*mec* IVa / SCC*mec* IVb / SCC*mec* IVc / SCC*mec* IVd / SCC*mec* V (ZHANG et al., 2012). *Spa* sequence typing was performed as outlined by ERDEM (2011). The StaphType software program (Genometr Biotechnology, Turkey) was used for this purpose, and the results were analysed on a Ridom SpaServer (http://www.spaserver.ridom.de/). Primers and PCR conditions are summarized in Table 1.

Antibiotic Resistance. Antibiotic resistance of MSSA and MRSA isolates was performed using the Kirby-Bauer disc diffusion method in accordance with the guidelines of the Clinical and Laboratories Standards Institute (CLSI, 2012). Penicillin (10 IU), gentamicin (10 µg), clindamycin (2 µg), trimethoprim/sulfamethoxazole (1.25µg / 23.75 µg), ampicillin (10 µg), oxacillin (1 µg), cefoxitin (30 µg) and erythromycin (15 µg) were used as test antibiotics and the strain *S. aureus* ATCC 25923 as the positive control.

Results

Among the tested farms, the seventh farm had the lowest SCC, with an average value of 246,638 cells/ml while the ninth farm had the highest value, with an average of 1,338,557 cell/ml. The calculated average SCC value for all 2165 cows was 510,667 cell/ml (Table 2). It is worth noting that 725 cows had SCC higher than the accepted upper limit of $4x10^5$ cell/ml. The overall prevalence of subclinical mastitis cases was 33.48%, nevertheless remarkable variations were observed among the farms. The lowest prevalence was obtained from the fourth farm with a percentage of 15.62%, while the highest percentage was 63.06% on the ninth farm. By microbiological analysis, 95 isolates of *S. aureus* were isolated from these 725 samples: 89 (93.68%) and 6 (6.3%) of which were thereafter characterized as MSSA and MRSA, respectively (Table 2). A significant number of these isolates (37 MSSA and 3 MRSA) were from the ninth farm, which indicates the poor hygiene status of this herd. As expected, relatively higher SCC values were obtained for herds where MSSA and MRSA were isolated (1.284.561 and 1.054.831 cells / ml respectively). This suggests a positive correlation between high SCC and *S. aureus* related subclinical mastitis cases.

The data related to SCC*mec* types of MRSA isolates are presented in Table 3 and shown in Fig.

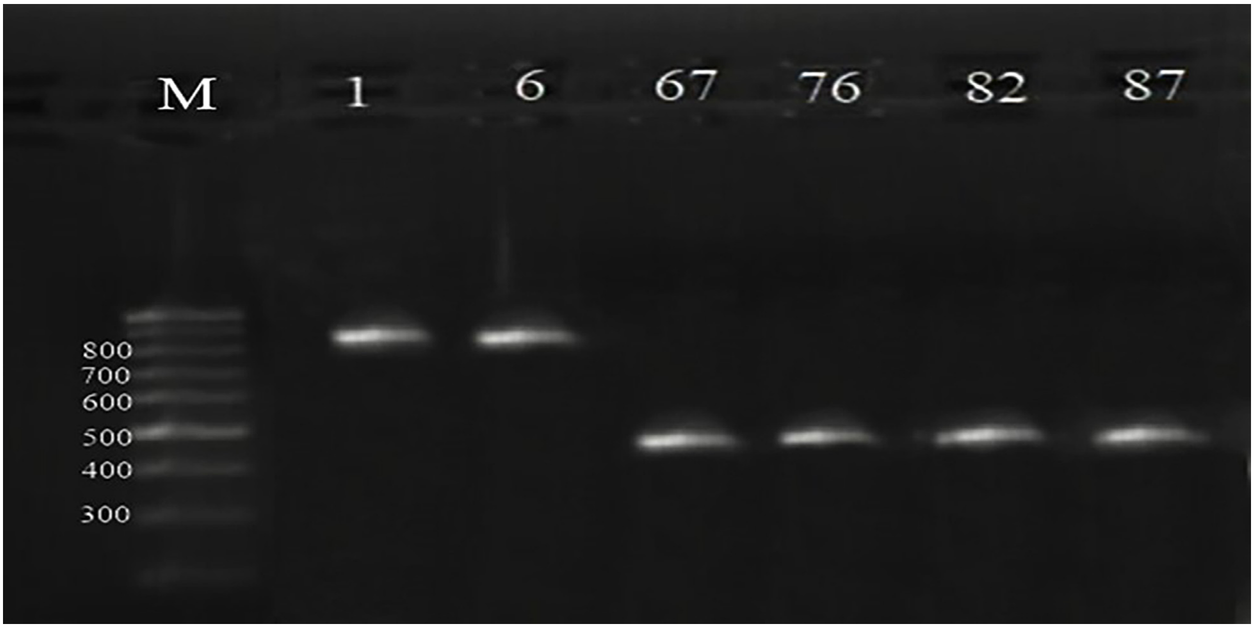

Fig. 1. SCCmec types of MRSA isolates

***Spa* type T005**

CGATGCTCAAGCACCAAAAGAGGAAGACAACAAAAAACCTGGTAAAGAAGACG
GCAACAAACCTGGCAAAGAAGACAACAACAAACCTGGTAAAGAAGACGGCAAC
AAACCTGGTAAAGAAGACGGCAACAAACCTGGCAAAGAAGACAACAAAAAGCC
TGGCAAAGAAGACGGCAACAAGCCTGGTAAAGAAGATGGCAACAAACCTGGTAA
AGAAGACGGCAACAAGCCTGGTAAAGAAGATGGCAACAAACCTGGTAAAGAAG
ACGGCAACAAACCTGGTAAAGAAGATGGTAACAAACCTGGCAAAGAAGACGGC
AACGGGGTACATGTCGTTAAACCTGGTGATACAGTAAATGACATTGCAAAAGCA
AACGG

Repeats: 26-23-13-23-31-05-17-25-17-25-16-28

***Spa* type T5163**

GCACCAAAAGAGGAAGACAACAACAAACCTGGTAAAGAAGACGGCAACAAACC
TGGTAAAGAAGACAACAAAAAACCTGGTAAAGAAGATGGCAACAAGCCTGGCA
AAGAAGACAACAAAAAACCTGGTAAAGAAGACGGCAACGGAGTACATGTCGTTA
AACCTGGTGATACAGTAAATGACATTGCAAAAGCAAACGGCACTACTGCTGA

Repeats: 07-12-21-17-13-13-13-13-33-34

Fig. 2. Spa sequence and repeats of MRSA isolates

1. The isolates were classified as CA-MRSA type IVb (four isolates) and type IVd (two isolates). As to *spa* typing, three isolates were typed as T005, while the other three were T 5163 (Fig. 2). All of the IVb typed isolates were from the ninth farm and typed as T005 by *spa* typing.

The antibiotic resistance of MSSA and MRSA is presented in Table 4. Resistance to oxacillin and cefoxitin was 100% for MRSA isolates, while it was 83% for penicillin, ampicillin, clindamycin, erythromycin and 66% for gentamicin and trimethoprim/sulfamethoxazole. MSSA isolates showed relatively lower resistance compared to MRSA isolates.

Discussion

Decreased SCC has several advantages for the dairy industry and food safety: i) increased milk yield and reduced milk production costs ii) decreased medication/antibiotics costs iii) higher quality and prolonged shelf life for dairy products, and iv) less public health concerns for consumers (BARBANO, 2017). In many countries, SCC is accepted as an imperative criterion in determining milk quality and milk price (SANT'ANNA and PARANHOS DA COSTA, 2011). Taking this fact into consideration, subclinical evaluation can be made using different categories: i) low risk group with SCC ranging between $2x10^5$-$3x10^5$ cells/ml, ii) risky group with SCC between $3x10^5$-$4x10^5$ cell/ml and iii) the group exceeding the $4x10^5$ cells/ml limit. This group is clinically considered as having mastitis although no clinical symptoms may be observed (TAHAWY and EL-FAR, 2010; KASWAN et al., 2012). In the present study, subclinical mastitis rates showed variations among the farms (15.6 to 63%) and the average level was 33.48%. These variations might be linked to several factors, such as: training of personnel in farm management, milking systems, milking hygiene, mastitis treatment, vaccination etc. In the literature, authors reported similar subclinical mastitis rates ranging from 20 to 80% (ABEBE et al., 2016; ZHANG et al., 2016). A strong relationship between SCC and mastitis associated pathogens has been evidenced. HISIRA et al. (2019) and DALEN et al. (2019) reported isolating *S. aureus* in samples having SCC of 200,000, 357,000, 355,000 and 200,000 to 2,000,000 cells/ml. In our study, the averages of SCC for MSSA and MRSA isolated samples were relatively high and indicated poor hygienic status and/or a subclinical mastitis problem on those farms.

The *mecA* gene found in MRSA is responsible for the synthesis of penicillin-binding protein, and such strains may be responsible for infections with high morbidity and mortality in humans and animals (HARTMAN and TOMASZ, 1984). Among 95 *S. aureus* isolates, 6 (6.3%) were identified as MRSA. This result is consistent with that of KAYNARCA and TÜRKYILMAZ (2010), but lower than that of TURUTOGLU et al. (2006) (23.1%). SCC*mec* typing is an important characteristic for epidemiological studies and may be used in determination of the contamination routes of pathogenic strains (PATERSON et al., 2014). The SCC*mec* types of our MRSA isolates were community-associated type IVb-IVd. However, different types, including HA-MRSA type I-II and CA-MRSA type IV-V (RIVA et al., 2015), HA-MRSA type II-III (ERDEM, 2011) and HA-MRSA type III (HATA et al., 2010) were identified from the milk. MRSA infections associated with SCC*mec* type IV have been increasing and are becoming progressively more important in dairy and public health (KLUYTMANS-VANDENBERGH and KLUYTMANS, 2006). Another important feature related to MRSA isolates is staphylococcal protein A, a surface protein found in the cell wall. This protein has high affinity to immunoglobulins and in consequence, bacteria could interrupt opsonization and phagocytosis (LOEFDAHL et al., 1983). Even though ERDEM (2011) and HATA et al. (2010) reported that they had already identified t030, t459, t660, t542 t002 and t179 s*pa* types, t005 and t5163 types were reported for the first time in Turkey. Genetic typing and diversity analysis might be used for epidemiological studies and comparison of isolates with newly emerged isolates.

In this study, different groups of antibiotics were tested to determine the resistance of *S. aureus*. All the MRSA isolates were resistant to oxacillin and cefoxitin. Furthermore, higher resistance was observed for penicillin, gentamicin, ampicillin, clindamycin, trimethoprim/sulfamethoxazole and erythromycin as well, indicating the multi-drug resistance characteristics of the isolates. The multidrug resistance of MRSA was also reported by others (AKLILU and YING, 2020; TURUTOGLU et al., 2006; VITALE et al., 2019; CHITSAI et al., 2020). A possible explanation for the higher resistance of MRSA and MSSA isolates to penicillin could be due to frequent use of this antibiotic in dairy herds (OLIVER and SHELTON, 2012).

The data obtained from this study highlight the need for preventive measures to eliminate or decrease *S. aureus* contamination of milk in dairy herds with subclinical mastitis. Therefore, the presence of staphylococci in cow's milk represents not only an important concern for dairy farming, but also a high risk to public health due to the occurrence of food poisoning through the consumption of milk and milk products.

Conclusions

S. aureus is one of the important causes of subclinical mastitis in cows and is not only a major economic burden for the dairy industry, but also creates serious public health risk. The percentage distribution of subclinical mastitis in the selected herds was very high and varied between 15.6 to 63 %. Six MRSA isolates obtained from subclinical mastitis cases were classified as community-associated CA-MRSA type IVb and type Ivd, and their *spa* types were T005 and T 5163. The strong resistance of MRSA isolates to oxacillin, cefoxitin, penicillin, ampicillin, clindamycin, erythromycin, gentamicin and trimethoprim/sulfamethoxazole highlights the need to control animal associated MRSA to avoid nosocomial disease. The One Health approach is a crucial step to control the spread of the pathogen.

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TAVSANLI, H., R. CIBIK: Prevalencija, genetska raznolikost i antibiotička rezistencija bakterije *Staphylococcus aureus* **kao uzročnika supkliničkoga goveđeg mastitisa u Balikesiru, Turska. Vet. arhiv 92, 17-25, 2022.**

SAŽETAK

Supklinički mastitis uzrokovan bakterijom *Staphylococcus aureus* čest je u mliječnih krava i to izaziva znatne poteškoće u proizvodnji mlijeka. U ovom je radu istražena prevalencija, genetska raznolikost i antimikrobna rezistencija bakterije *S. aureus* u životinja sa supkliničkim mastitisom. Uključene su krave iz 12 mliječnih stada u pokrajini Balikesir (Turska). Ukupno 95 izolata bakterije *S. aureus* dobiveno je od 725 uzoraka mlijeka sa supkliničkim mastitisom, pri čemu je broj somatskih stanica (SCC) bio veći od 4 x 10^5 stanica/mL. Genetska raznolikost izolata je analizirana primjenom SCC*mec* i *spa* tipizacije. Učestalost MRSA-e (*Staphylococcus aureus* rezistentan na meticilin) bila je 6,3 % (6 izolata), a MSSA-e (*S. aureus* osjetljiv na meticilin) 93,68 % (89 izolata). SCC*mec* tipovi MRSA izolata bili su iz zajednice CA-MRSA tip IVb (četiri izolata) i tip IVd (dva izolata), dok su tipovi *spa* bili T005 i T 5163 (po tri izolata od svakoga). Stopa rezistencije MRSA izolata bila je 100 % za oksacilin i cefoksitin, 83 % za penicilin, ampicilin, klindamicin, eritromicin i 66 % za gentamicin i trimetoprim-sulfametoksazol. U usporedbi s MRSA-om, rezistencija MSSA izolata bila je relativno niža. Ovo istraživanje potkrijepljuje znanstvene podatke o pojavnosti MRSA-e i MSSA-e kod supkliničkog mastitisa, te naglašava potrebu za preventivnim mjerama kojima bi se u stadima mliječnih krava spriječila ili smanjila kontaminacija mlijeka bakterijom *S. aureus*.

Ključne riječi: *SCCmec* tipovi MRSA-e; broj somatskih stanica; *spa* tipovi MRSA-e; supklinički mastitis