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The molecular epidemiology of *Staphylococcus aureus* of bovine mastitis origin

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ABSTRACT

The present study was aimed to understand the molecular epidemiology of *Staphylococcus aureus* (54 isolates), isolated from 422 milk samples obtained from 108 subclinical mastitis affected cows (CMT positive ≥1+ in at least one quarter). The molecular epidemiology of *Staphylococcus aureus* was studied using coagulase (*coa*) gene polymorphism, 16S-23S ribosomal *spas*cer (RS-PCR) polymorphism and Staphylococcal protein A (*Spa*) typing. *Staphylococcus aureus* produced 7 coagulase genotypes and 8 RS genotypes respectively. Coagulase genotype GTIII (730 bp) was the most prevalent (35 strains) followed by GTV (900 bp, 7 strains) and GTIV (800 bp, 4 strains), whereas RS genotypes GTA accounted for the highest number of strains (31 strains), followed by GTB (11strains), GTH (4 strains) and GTE (3 strains). Coagulase genotype CTIII (730 bp) showed the highest diversity, as isolates within it produced 5 RS genotypes, the majority of them belonging to the RS genotype GTA (29 out of 31 strains). Forty out of 54 *Staphylococcus aureus* samples isolated in this study were correctly typed by *spa* typing, and were assigned to 21 known *spa* types, and one new novel *spa* type t18462. The study revealed high diversity within *Staphylococcus aureus* strains, consisting of 7 coagulase genotypes, 8 RS genotypes and 22 *spa* types.

Key words: *coa* gene polymorphism; ribosomal spacer polymorphism; *Staphylococcus aureus; Spa* typing

Introduction

Mastitis, the inflammation of mammary glands, has infectious or non-infectious aetiology. Infectious causes are mainly of bacterial origin, and are broadly divided into minor and major pathogens (EBERHART et al., 1987). The species within the genus *Staphylococcus* are classified into coagulase-negative staphylococci (CoNS) and coagulase-positive staphylococci (CoPS), based on their ability to produce coagulase enzyme. *Staphylococcus aureus*, one of the most common coagulase positive contagious pathogens, causes both clinical and subclinical bovine mastitis worldwide (KARIMURIBO et al., 2005; MOMTAZ et al., 2011; HAFTU et al., 2012; GUPTA et al., 2015), and its presence in milk is a public health threat (D'AMICO and DONNELLY, 2011).

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Different *Staphylococcus aureus* strains have been observed in bovine mastitis, varying in virulence and epidemiology, and various conventional methods, such as phage typing, biotyping and antimicrobial susceptibility testing, have been used to study the strain variation (LANGE et al., 1999; SU et al., 1999). Due to the low discriminatory power of these conventional tests, newer molecular methods have been developed to improve the typing of staphylococcal strains that exploit the variations in the arrangement of chromosomal alleles and in the content of accessory genetic elements. Different genotyping methods such as: analysis of chromosomal DNA after enzymatic restriction (BUSCH and NITSCHKO, 1999), random amplified polymorphic DNA, coagulase gene typing and polymorphism (GOH et al., 1992; ISHINO et al., 2007), *spa* (*Staphylococcus aureus* Protein A) typing (SHOPSIN et al., 1999), multilocus sequence typing (MLST) (ENRIGHT et al., 2000) and pulsed-field gel electrophoresis (PFGE) (ZADOKS et al., 2000, MELLES et al., 2007), have been used in the genetic typing of *Staphylococcus* spp.

Coagulase enzymes produced by *Staphylococcus aureus* strains showed polymorphism due to heterogeneity at the 3' coding region of the coagulase gene that consisted of 81 bp tandem repeats, differing in number, and also in the location of *Alu*I and *Hae*III restriction enzyme sites (HIMABINDU et al., 2009). Moreover, bacteria possess rRNA genetic loci containing genes for all three rRNA, i.e., 16S, 23S, and 5S, which are separated from one another by spacer regions, varying in length and sequence, both at genus and species level. Also, the good diversity due to variations in the number and type of tRNA sequences found within the spacers regions, was used to discriminate between different species/ strains of prokaryotes (BARRY et al., 1991). *Spa* typing is a PCR-based single-locus sequence typing technique, based on sequencing of the polymorphic region X of the *Staphylococcus aureus* Protein A (*spa*) gene (FRENAY et al., 1996). The *spa* locus consists of 24-bp nucleotide repeats, showing diversity due to deletions and duplications, and to a lesser extent by point mutations (SHOPSIN et al., 1999). The discriminative power of *spa* typing lies between PFGE and MLST (MALACHOWA and DELEO 2010), and in contrast to MLST and PFGE, can be used to investigate molecular evolution and disease outbreaks caused by methicillin resistant *Staphylococcus aureus* (KOREEN et al., 2004). The main advantage of *spa* typing over MLST is sequencing of only a single locus, as compared to seven loci in MLST.

In the present study, we combined PCR based techniques, *i.e. coa* gene polymorphism, RS polymorphism and *spa* typing (DNA sequence based technique), to understand the molecular epidemiology of *Staphylococcus aureus* of bovine mastitis origin, from five agro climatic regions of Punjab, India.

Materials and methods

Milk sample collection. In total, 250 randomly selected dairy cows were included, 50 from each region of Punjab namely: the Central plain zone, the Sub-mountain undulating zone, the Undulating plain zone, the Western plain zone and the Western zone. The representative farms / animal herds in each region were visited during the regular evening milking hours, and animals were screened for subclinical mastitis using the California mastitis test (CMT). 422 milk samples from 108 mastitis positive dairy cows (CMT positive ≥1 + in at least one quarter) were collected for bacteriological analysis. 10 mL of fore-quarter milk samples were collected aseptically in sterile 15 mL glass test tubes and carried to the laboratory in an icebox for bacterial analysis, as per the guidelines of the National Mastitis Council (HOGAN et al. 1999).

Microbial evaluation for identification of Staphylococcus aureus. Staphylococcus aureus was presumptively identified on the basis of colony characteristics on blood agar, Gram staining, clumping factor, growth characteristics on mannitol salt agar, DNase agar, Baird parker agar, tube coagulase test and by biochemical identification using a HiStaph identification kit (HiMedia Laboratories Pvt. Ltd., Mumbai, India). *Staphylococcus aureus* (ATCC 33591) and *S. epidermidis* (MTCC 3382) were used as standard controls. Individual *Staphylococcus aureus* isolates were stored at -20 °C in trypticase soy broth containing 30% glycerol for future use.

DNA extraction. 1 mL of overnight inoculum of an individual *Staphylococcus aureus* colony in brain heart infusion broth (BHI, HiMedia) was pelleted at 7500 rpm for 5 min in refrigerated centrifuge (Heraeus Biofuge Primo R, Thermo Scientific). 180 µL lysis solution (lysozyme enzyme 20 mg mL^{-1}; Tris HCl 20 mM, pH 8; Triton X 1.2%; Tween 20 0.5% and EDTA 2 mM) was added to the pellet and incubated at 37 ºC for 30 min. Bacterial DNA was extracted using a QIAamp DNA mini kit (Qiagen) following the manufacturer's guidelines, and the eluted DNA was stored at -20 °C until further use.

PCR confirmation of Staphylococcus aureus. Duplex PCR amplification was carried out for the detection of genus specific *16S rDNA* (STROMMENGER et al., 2003), and *nuc (Staphylococcus aureus* species specific) genes (BRAKSTAD et al., 1992). The amplification was carried out in a total reaction volume of 25 µL containing 12.5 µL qiagen PCR Master Mix (Qiagen), 10 pmol/µL of each primer set containing forward and reverse primers (Table 1), 0.01 µg-0.2 µg template and sterilized nuclease free water to make up the reaction volume. Thermocycler (Biorad®) was used to perform the amplification reaction. The cycling conditions included an initial denaturation at 94 °C for 5 minutes, followed by 30 cycles each of denaturation at 94 °C for 30 seconds; annealing at 57.7 °C for 40 seconds and extension at 72 °C for 1 minute; followed by a final extension at 72 °C for 5 minutes, and hold at 4 °C. The amplified products were electrophoresed in 1.5% agarose gel containing ethidium bromide (10 µg mL^{-1}) and visualized and imaged using the Molecular Imager® ChemiDoc™ XRS + imaging system (BioRad®).

Amplification of coagulase gene. Isolates were tested for the presence of the *coa* gene, as per the protocol of GOH et al. (1992), with slight modifications. The primer pair sequence is given in Table 1. The PCR mixture was prepared in a 25 µL reaction and amplified using the following protocol: initial denaturation at 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 54 °C for 2 min, 72 °C for 2 min; final extension at 72 °C for 10 min. The amplified products were electrophoresed in 1.5% agarose gel containing ethidium bromide (10 µg mL^{-1}) and visualized.

Ribosomal spacer PCR (RS-PCR). RS-PCR was carried out using the primers and protocol of JENSEN et al. (1993) with slight modifications. The assay involved 12.5 µL of Taq master mix, 1 µL each of two primers (primers Gl and L1; 50 pmol; Table 1) and deionized water in a total of 25 µL reaction. Reaction mixtures were amplified once at 94 ºC for 5 min followed by twenty-five amplification cycles at 94 °C for 1 min; 2-min ramp to 55 °C for 7 min; 2-min ramp to 72 °C for 2 min and a final step of 7 min at 72 °C. The band length of the genotypes was correctly noted by matching the size with an adjacently run molecular DNA marker, and any two strains with the same banding pattern were assigned as similar genotypes, while strains differing in more than one band were assigned as separate genotypes.

Table 1. Primers used in the study

Organisms	Primer designation	Oligonucleotide sequence (5`-3`)	Amplicon size	Reference
Staphylococcus spp.	*16S rDNA*-F *16S rDNA*-R	CAG CTC GTG TCG TGA GAT GT AAT CAT TTG TCC CAC CTT CG	420	Strommenger et al., (2003)
Staphylococcus aureus	*Nuc*-F *Nuc*-R	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	280	Brakstad et al., (1992)
Coa gene	*Coa*-F *Coa*-R	CGAGACCAAGATTCAACAAG AAAGAAAACCACTCACATCA	Variable	Goh et al., (1992)
RS-PCR	G1 L1	GAAGTCGTAACAAGG CAAGGCATCCACCGT	Variable	Jensen et al., (1993)
Spa gene	*Spa*-F *Spa*-R	F: AGACGATCCWTCAGTGAGC R: TAATCCACCAAATACAGTTGTACC	Variable	Shopsin et al., (1999)

PCR for spa (Staphylococcal Protein A) gene amplification. The primers and amplification conditions for *spa* were used as per protocol the given by SHOPSIN et al. (1999). For PCR amplification of the *spa* gene a 50 µL reaction was used consisting of Q5 High-Fidelity 2X master mix (New England Biolabs), 10 pmol/µL each reverse and forward primer (Table 1), DNA template 0.01 µg- 0.2 µg and sterilized nuclease free water to make up the reaction volume, along with negative (sterile deionized water) and positive controls (Standard ATCC 33591).

After visualization, PCR products were sent for sequence analysis (BioServe Pvt. Ltd. Hyderabad, India). The consensus sequences of *Staphylococcus aureus* were blasted with the BLASTN programme (http:/blast.ncbi.nim.nih.gov/Blast.cgi) to check the identity with sequences available in the NCBI database. The consensus sequences were analysed by DNA gear software, resulting in identification of unique SSR (short sequence repeats) types. The *spa* (strain) type is defined by the number and sequence of repeats revealed on this analysis. Unique sequences were submitted to an online SpaServer website (spa.ridom.de) for assignment of the strain number.

Results

54 *Staphylococcus aureus* isolated from 422 milk samples were correctly identified with the help of duplex PCR (Fig. 1). On the basis of the tube coagulase test, only 44 were coagulase positive; however, the *coa* gene was detected in 51 isolates. Genotypes for *Staphylococcus aureus* on the basis of the *coa* gene (Fig. 2) and RS-PCR (Fig. 3) were assigned, and corresponding strains belonging to different genotypes were analysed using the frequency distribution given in Table 2.

Table 2. Molecular epidemiology of *Staphylococcus aureus* based on *coa* gene and RS-PCR polymorphism

	coa gene polymorphism			RS-PCR polymorphism			
S. No.	Coagulase Genotype	PCR band size (bp)	No. of strains	RS genotype	No. of bands	Band range	No. of strains
1	CTI	405	1	GTB	7	390-620	1
2	CTII	670	1	GTB	7	390-620	1
3	CTIII	730	35	GTA	7	430-690	29
4				GTB	7	390-620	1
5				GTC	6	270-600	1
6				GTE	4	430-570	3
7				GTF	5	430-630	1
8	CTIV	800	4	GTB	7	390-620	2
9				GTC	6	270-600	1
10				GTH	5	400-550	1
11	CTV	900	7	GTB	7	390-620	4
12				GTH	5	400-550	3
13	CTVI	1400	2	GTB	7	390-620	2
14	CTVII	1000, 405	1	GTA	7	430-690	1
15		N		GTA	7	430-690	1
16		N		GTD	4	310-500	1
17		N		GTG	2	50-650	1

Table 3. Prevalence of *Staphylococcus aureus* spa strains prevalent in cows in different agro-climatic regions of Punjab

Spa type	*Spa* repeats	No. of Strains	Strains in agro-climatic regions					First Reported	
		Cows	CPZ	UPZ	SMUZ	WZ	WPZ	India	Abroad
t18462	07-16-12-23-02-02-34-34-34-34	1	1					This study	ND
t005	26-23-13-23-31-05-17-25-17-25-16-28	1	1					2007	Germany, 2001
t008	11-19-12-21-17-34-24-34-22-25	1				1		2007 Mitra et al., 2013	Germany, 2001
t091	07-23-21-17-34-12-23-02-12-23	3	2				1	Gulazar, 2017	Germany, 2014
t13078	26-23-13-23-31-17-25-17-25-16-28	1	1					Gulazar, 2017	Germany, 2013
t131	07-23-12-34-33-34	1	1					This study#	Belgium, 2004
t15515	07-16-12-23-02-02-34-34-34	4	1	3				2014	-
t15889	07-16-12-23-02-12-23-02-34-34	1	1					This study#	Denmark, 2016
t159	14-44-13-12-17-17-23-18-17	1		1				This study#	Germany, 2004
t1598	07-12-21-13-13-34-34-33-34	1		1				This study#	UK, 2006
t1659	07-16-12-23-02-02-34	1				1		This study#	Germany, 2006
t1839	26-23-13-21-17-34-34-34-33-34	1		1				2006	Germany, 2008
t3841	26-22-17-20-17-12-17-16-16	1		1				2008	Netherlands, 2014
t386	07-23-13	1	1					This study#	Sweden, 2004
t4363	26-23-13-21-17-34-34-24-33-34	1				1		This study#	China, 2008
t442	35-17-34-17-20-17-12-17-16	1	1					2016	Norway, 2008
t4812	07-16-12-23-02-34	2	2					This study#	Poland, 2009
t5919	07-21-17-13-13-13-34-33-34	1		1				This study#	UAE, 2009
t605	07-23-38-101-23-02-72-23	1	1					This study#	Sweden, 2005
t7286	07-16-12-23-02-34-34	4	2	2				2010 Mitra et al., 2013	-
t7867	07-16-12-23-02-02-34-34	11	3	2	1	4	1	2011 Mitra et al., 2013	-

UPZ: Upper plain Zone, CPZ: Central Pain Zone, SMUZ: Sub-Mountainous Undulating Zone, WZ: Western Zone, WPZ: Western plain Zone; ND: not reported in any other country (new *spa* strain); # Strains reported first time from India in this study (n = 10)

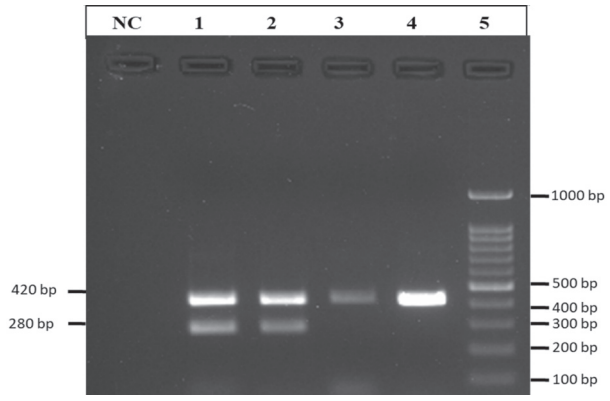

Fig. 1. Electrophoresis image of mPCR for *16SrDNA* (420bp) and *nuc* (280np) genes. Lanes indicate NC: negative control, 1: *Staphylococcus aureus* 2: *Staphylococcus aureus* positive control (ATCC 33591) 3: *Staphylococcus* spp. 4: *S. epidermidis* positive control (MTCC 3382). M indicate ExcelBand 100 bp DNA ladder (DM2300, SMOBio).

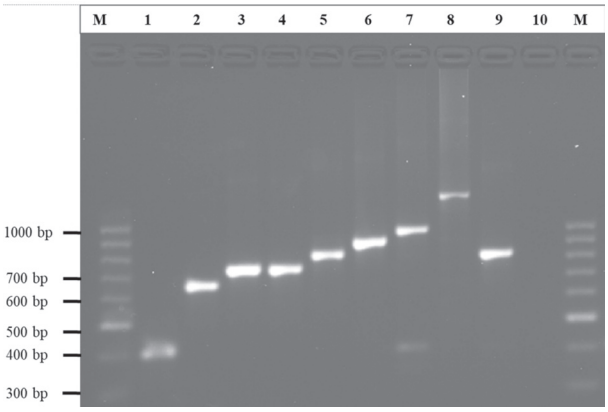

Fig. 2. Electrophoresis image of *coa gene* polymorphism of *Staphylococcus aureus.* Isolates from cows (lane 1-8). M indicate GeneRuler 100 bp DNA ladder (SM0243, Thermo Scientific) and lanes 9 and 10 indicate *Staphylococcus aureus* positive control (ATCC 33591) and *S. epidermidis* negative control (MTCC 3382).

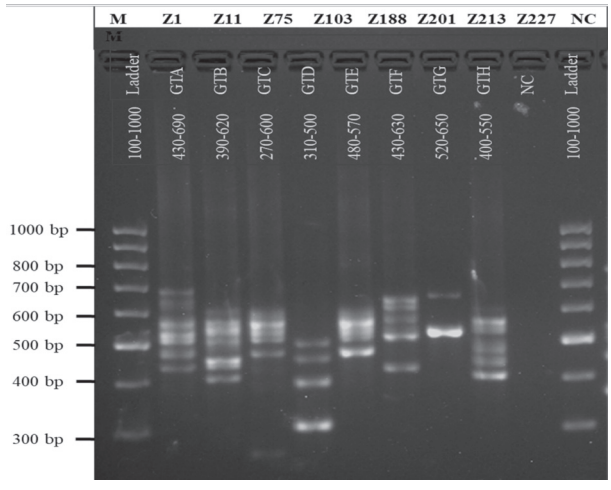

Fig. 3. Electrophoresis image of RS-PCR polymorphism of *Staphylococcus aureus.* RS genotypes of *S. aureuas* (lanes 2-9). NC indicate negative control (nuclease free water). M indicate SMOBio ExcelBand 100 bp DNA ladder (DM2100)

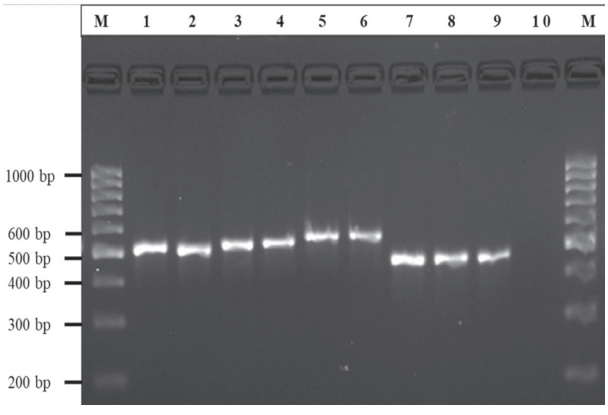

Fig. 4. Electrophoresis image of *Spa* gene. Lanes 1-9 indicate amplified products of *spa* gene of *Staphylococcus aureus* of varied sizes ranging from 450 to 580 base pairs and lane 10 indicate *S. epidermidis* negative control (MTCC 3382). M indicate SMOBio ExcelBand 100 bp DNA ladder (DM2100).

The prevalence of *spa* strains of *Staphylococcus aureus* (Fig. 4) in the different agro-climatic regions of Punjab is given in Table 3. Forty out of 54 *Staphylococcus aureus* were correctly typed by *spa* typing and assigned to 21 known *spa* types, and one novel *spa* type, t18462, was assigned on 17th December 2018 (online SpaServer website - available at: http://spa.ridom.de/frequencies.shtml). t18462 is a *mecA* negative strain, isolated from a SCM cow. *Spa* strain t7867 was observed to be most diverse in the present study, and was prevalent in cows in all five agro-climatic regions of the Punjab.

Discussion

The molecular epidemiology of *Staphylococcus aureus* was studied using *coa* gene polymorphism, 16S-23S ribosomal spacer polymorphism and *Spa* typing. Coagulase production is an important phenotypic feature of *Staphylococcus aureus,* and coagulase gene polymorphism has been used to understand epidemiology, due to its high reproducibility and good discriminatory power (GOH et al., 1992; SU et al., 1999; KARAHAN and CETINKAYA, 2007). The predominance of strains in fewer coagulase clusters had been reported by other workers as well (HIMABINDU et al., 2009; MOMTAZ et al., 2011). *Coa* gene amplification in all but one strain produced a single band, one strain produced a double band (1000, 405) and, as has also been reported earlier (ASLANTAS et al., 2007), were attributed to different allelic forms of the *coa* gene (GOH et al., 1992). FOURNIER et al. (2008) reported high diversity within 16S-23S rRNA spacer regions, reporting 17 RS genotypes, out of which 2 genotypes were predominant, comprising 80.2% of the isolates that were also positive for virulence genes. GRABER (2016), proved the superiority of RS-PCR over *spa* typing and MLST, with resolution comparable to *spa* typing or better than MLST or PFGE. The predominance of strains into fewer genotypes has been attributed to resistance to phagocytosis by neutrophils (SU et al., 1999), or coevolution of the pathogens and their host, herd management, and differences in the reservoirs and environment of each geographical area (ASLANTAS et al., 2007). The predominance of one of the genotypes/strains confirms high contagiousness, and the dissemination of predominant strains of *Staphylococcus aureus* within dairy herds. *Staphylococcus aureus* showed high diversity by *spa* typing, assigning 40 isolates into 22 *spa* types, out of which 10 *spa* types were reported for the first time in India, and one novel *spa* type t18462 was also reported. A high diversity in *spa* types of *Staphylococcus aureus* isolated from healthcare and community-acquired infections has been reported in India (GULZAR, 2017; SINGH et al., 2018) and abroad (HARASTANI et al., 2014; MOHAMMADI et al., 2014; KHADEMI et al., 2016), and has been attributed to deletions or duplications, or more seldomly, to point mutations (SHOPSIN et al., 1999). In India, *spa* types t359, t6877, t008 have been reported as predominant bovine mastitis strains, and t267 as an endemic clone responsible for subclinical mastitis (MITRA et al., 2013). SINGH et al. (2018) reported t021 (14.1%), t127 (9.6%), t657 (9.2%), t3841 (8.8%), t1149 (6.0%) and t309 (4.0%) as the most prevalent *spa* types of *Staphylococcus aureus* obtained from various human clinical samples from Haryana, India, a neighbouring state, sharing a geographical boundary with Punjab. *Spa* types, t091 and t13078 observed in the present study were reported by GULZAR (2017) from bovine milk and a telephone surface (community associated). However, observation of t13078 in milk has only been reported in the present study. *Staphylococcus aureus* showed good diversity that may be helpful in understanding the epidemiology and clonal relationships in investigating disease outbreaks. The varied types of *spa* types observed in the present study, and the observation that they were also isolated from community sources, indicate the possible transfer of these strains from community associated sources to animals or vice versa, and indicates their zoonotic potential.

In conclusion, the study revealed high diversity within *Staphylococcus aureus* strains, consisting of 7 coagulase genotypes, 8 RS genotypes and 21 known *spa* types, and one novel *spa* type (t18462). *Spa* typing was found to be the most discriminatory technique, followed by RS PCR and *coa* polymorphism, in this study. The predominance of one of the genotypes/strains in this study confirmed the high contagiousness and dissemination of predominant strains of *Staphylococcus aureus* within dairy herds.

Conflict of Interest

The authors declare that they have no conflict of interest.

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SAŽETAK

Istraživanje je provedeno kako bi se razumjela molekularna epidemiologija bakterije *Staphylococcus aureus*. Ukupno 54 izolata iz 422 uzorka mlijeka dobivena su od 108 krava sa supkliničkim mastitisom (CMT pozitivni ≥ 1+ u barem jednoj četvrtini vimena). Molekularna epidemiologija S. aureus analizirana je upotrebom polimorfizma koagulaza-gena (coa), polimorfizma 16S-23S ribosomske regije razdvajanja (RS-PCR) i tipiziranjem stafilokoknog proteina A (*Spas*). Bakterija S. aureus proizvela je 7 genotipova koagulaza i 8 RS genotipova. Koagulaza genotip GTIII (730 bp) bio je najčešći (35 sojeva), zatim GTV (900 bp, 7 sojeva) i GTIV (800 bp, 4 soja), dok je kod RS genotipova najveći broj sojeva sadržavao GTA (31 soj), zatim GTB (11 sojeva), GTH (4 soja) i GTE (3 soja). Koagulaza genotip CTIII (730 bp) pokazao je najveću raznolikost jer su izolati unutar njega proizveli 5 RS genotipova, a većina njih pripadala je RS genotipu GTA (29 od 31 soja). Četrdeset od 54 uzorka bakterije S. aureus izolirana u ovom istraživanju bilo je ispravno tipizirano *spas* tipiziranjem, i pripisano 21 poznatom *spas* tipu te jednom novom *spas* tipu, t18462. Istraživanje je pokazalo veliku raznolikost sojeva bakterije S. aureus s obzirom na postojanje 7 koagulaza genotipova, 8 RS genotipova i 22 *spas* tipa.

Ključne riječi: polimorfizam gena coa; polimorfizam ribosomske regije razdvajanja; *Staphylococcus aureus*; *spas* tipiziranje