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Molecular types and antimicrobial resistance profile of *Staphylococcus aureus* isolated from dairy cows and farm environments

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Abstract: The aim of this study was to assess the prevalence of *Staphylococcus aureus* in cows with subclinical mastitis and detect enterotoxin genes and patterns of antimicrobial resistance. Out of 180 animals, subclinical mastitis was detected in 78 cows (46.99%) and three buffalo (21.43%). The prevalence of clinical mastitis was low (3.61%). *S. aureus* could be isolated from mastitic milk (67/81), udder swabs (38/81), floor swabs (13/27), milking machine liner (5/8), and healthy animals' milk (12/22). All the strains were confirmed by PCR targeting amplification of the *femA* gene. Out of 52 PCR-confirmed *S. aureus* samples, 17 were enterotoxin-producing strains. The pattern of enterotoxin production was revealed as: *seb* (1.92%), *sec* (21.15%), *sed* (7.69%), and *see* (1.92%). The pattern of superantigens and methicillin resistance was revealed as: *etb* (1.92%), *tst1* (25%), and *mecA* (3.85%). Combination of *sec* and *tst1* and the rare distribution of *sea*, *seb*, and *sed* was observed. *S. aureus* isolates were sensitive to netilmicin (85.88%), amikacin (80%), tobramycin (76.47%), and gentamicin (68.24%) and resistant to penicillin (91.76%), oxacillin (71.76%), and kanamycin (63.53%). Antimicrobial resistant genes *blaZ*, *ermB*, and *tetK* were also detected in the isolates. This study provides a baseline for monitoring antimicrobial resistance in *S. aureus* strains associated with bovine mastitis in India.

Key words: Bovine mastitis, *Staphylococcus aureus* enterotoxins, antimicrobial resistance

1. Introduction

In tropical countries like India, bovine mastitis is a major problem and farmers incur heavy economic losses due to expenditures for treatment and reduced milk production (1). Farmers' perception about routine screening of milking animals for subclinical mastitis is poor; thus, effective control of bovine mastitis in India heavily relies on husbandry and management practices. Mastitis is a multietiological disease; however, *Staphylococcus aureus* is the predominant and most studied mastitogen. Based on the epidemiological studies and mastitis control efforts worldwide (2), *S. aureus* has been classified as a contagious pathogen with zoonotic implications. It has the potential to produce an array of extracellular toxins and virulence factors known as staphylococcal enterotoxins. At present, little is known about the occurrence of molecular types and antimicrobial resistance (AMR) profile of *S. aureus* isolates from bovine subclinical mastitis in India. Changes in mastitis isolate profiles influenced by setting have been reported earlier (3), which again emphasizes the need for periodic evaluation of *S. aureus* in terms of virulence and AMR. Currently, penicillin, erythromycin, and tetracycline are frequently used for the treatment of mastitis. Molecular studies on the detection of enterotoxigenic *S. aureus* strains isolated from bovine mastitis and their genotypic and phenotypic AMR patterns are lacking in the Maharashtra state of India. Information about genetic variability may help in the identification of the most likely source of an isolate and selection of a proper antimicrobial agent for mastitis management; therefore, this study was conducted with the aim of characterizing *S. aureus* isolates for enterotoxins and AMR.

2. Materials and methods

2.1. Sample collection and identification

A total of 180 lactating animals (166 cows and 14 buffalo) were sampled from seven farms. Before collection of clinical samples, a brief history of various animal

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husbandry practices was collected through questionnaires and personal interviews with farmers. For detection of subclinical mastitis, 720 teats of 180 animals were milked and screened by the California Mastitis Test (CMT). Bacterial culturing of 225 samples including CMT-positive subclinical mastitis milk (n = 81), clinical mastitis milk (n = 6), healthy animals' milk (n = 22), udder swabs (n = 81), floor swabs (n = 27), and milking machine liner swabs (n = 8) was done for isolation of *S. aureus* as per the methods described earlier (1).

2.2. Detection of *S. aureus* enterotoxins by PCR

Bacterial DNA was extracted by boiling and snap chilling method in all the polymerase chain reaction (PCR) assays performed. Detection of *S. aureus* enterotoxins was performed by multiplex PCR in two sets using the protocol designed by Mehrotra et al. (4). The primer sequences used are detailed in Table 1. Set A was designed to amplify *sea*, *seb*, *sec*, *sed*, *see*, and *femA*, whereas set B was designed to amplify *mecA*, *eta*, *etb*, and *tst1* with *femA* as an internal control. Multiplex primer set A contained 20 pmol each of *sea*, *seb*, *sec*, *see*, and *femA* primers and 40 pmol of *sed* primer. Set B contained 50 pmol of *eta* and 20 pmol each of *etb*, *tst*, *mecA*, and *femA* primers. PCR contents were as follows: forward and reverse primers 1 µL each; 25 µL of 2X PCR Taq Master Mix (Promega); and 5 µL of DNA template. Multiplex PCR was performed in a final reaction volume of 50 µL adjusted with nuclease-free water. DNA amplification was carried out in a Applied Biosystems thermal cycler with the following cycling conditions: an initial denaturation at 94 °C for 5 min followed by 35 cycles of amplification (denaturation at 94 °C for 2 min, annealing at 57 °C for 2 min, and extension at 72 °C for 1 min), ending with a final extension at 72 °C for 7 min and holding at 4 °C until further use. Amplified PCR products (5 µL) were separated by electrophoresis in 1.2% agarose gel stained by ethidium bromide and visualized under a UV transilluminator (G-BOX F3 Syngene). Oligonucleotide sequences were synthesized from Integrated Technologies (IDT) supplied by Bioresource Biotech Pvt. Ltd., Pune.

2.3. Detection of AMR genes by PCR

Prevalence of antimicrobial resistant genes *blaZ*, *ermB*, and *tetK* was studied by the multiplex PCR assay developed previously (5). PCR primer sequences used for detection of AMR genes are detailed in Table 2. PCR reaction was performed using 1.5 µL of each forward and reverse primer specific for amplification of the *ermB* and *blaZ* genes, and 0.5 µL of each for amplification of the *tetK* gene. PCR was performed with a final reaction volume of

Table 1. Oligonucleotide sequences used for detection of SE genes.

Gene	Oligonucleotide sequence (5'-3')	Product size (bp)	PCR set	Reference
sea	F- GGTTATCAATGTGCGGGTGG R- CGGCACTTTTTTCTCTTCGG	102	A	(4)
seb	F- GTATGGTGGTGTAACTGAGC R- CCAAATAGTGACGAGTTAGG	164	A	
sec	F- AGATGAAGTAGTTGATGTGTATGG R- CACACTTTTAGAATCAACCG	451	A	
sed	F- CCAATAATAGGAGAAAATAAAAG R- ATTGGTATTTTTTTTCGTTC	278	A	
see	F- AGGTTTTTTCACAGGTCATCC R- CTTTTTTTTCTTCGGTCAATC	209	A	
femA	F- AAAAAAGCACATAACAAGCG R- GATAAAGAAGAAACCAGCAG	132	A/B internal control	
mecA	F- ACTGCTATCCACCCTCAAAC R- CTGGTGAAGTTGTAATCTGG	163	B	
eta	F- GCAGGTGTTGATTTAGCATT R- AGATGTCCCTATTTTTGCTG	93	B	
etb	F- ACAAGCAAAAGAATACAGCG R- GTTTTTGGCTGCTTCTCTTG	226	B	
tst	F- ACCCCTGTTCCCTTATCATC R-TTTTCAGTATTTGTAACGCC	326	B	

Table 2. Oligonucleotide sequences used for detection of AMR genes.

Target genes	Oligonucleotide sequences (5′-3′)	Product size (bp)	Reference
blaZ	F- AAGAGATTTGCCTATGCTTC R-GCTTGACCACTTTTATCAGC	517	(5)
ermB	F- ACGACGAAACTGGCTAA R- TGGTATGGCGGGTAA	409	
*tet*K	F- TCGATAGGAACAGCAGTA R- CAGCAGATCCTACTCCTT	169	

30 µL. Contents of PCR were 15 µL of PCR master mix (Promega), 5 µL of DNA template, and nuclease-free water to adjust the volume. Thermal cycling conditions were set as initial denaturation at 95 °C for 5 min followed by 38 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 35 s, and extension at 65 °C for 1.5 min. After the final cycle, the preparation was kept at 65 °C for 10 min to complete the reaction and then held at 4 °C until further use. Amplified PCR products (5 µL) were further separated by electrophoresis in 3% agarose gel stained by ethidium bromide and visualized under a UV transilluminator (G-BOX F3 Syngene).

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility of *S. aureus* isolates was evaluated by Kirby–Bauer disk diffusion method on Mueller–Hinton agar (MHA) (HiMedia Laboratories, India) according to the guidelines of the Clinical and Laboratory Standards Institute (6). Pure bacterial cultures were grown in brain–heart infusion (BHI) broth and incubated at 37 °C for 18 h. BHI broth cultures of *S. aureus* were further evenly spread on MHA plates. The inoculated agar plates with antimicrobial disks were left at room temperature for 30 min followed by incubation at 37 °C for 24 h. Strains were classified as resistant, intermediate, or susceptible on the basis of the size of the inhibition zone. The following antimicrobial agents were tested: penicillin (10 IU), oxacillin (1 μg^{-1}), ceftazidime (30 μg^{-1}), gentamicin (10 μg^{-1}), amikacin (30 μg^{-1}), kanamycin (30 μg^{-1}), netilmicin (30 μg^{-1}), and tobramycin (10 μg^{-1}).

3. Results

Out of 180 animals, 78 (46.99%) cows and 3 (21.43%) buffalo had subclinical mastitis and 6 (3.61%) cows had clinical mastitis. As shown in Table 3, farms C, G, F, and D had the highest prevalence of subclinical mastitis. Bacterial culturing of milk samples positive for subclinical mastitis and clinical mastitis revealed 73 *S. aureus* isolates. We could isolate *S. aureus* from udder and teat surfaces (46.91%), animal floor swabs (48.15%), milking machine liners (62.50%), and milk of healthy animals (54.54%). The frequency of *S. aureus* isolation on different farms is given in Table 3. Out of 225 total samples screened, 141 *S. aureus* strains were isolated with an overall prevalence of 62.66%.

The first purpose of this study was to establish the presence of staphylococcal enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *femA*, *eta*, *etb*, *tsst1*, and *mecA*) (Figures 1 and 2).

Table 3. Prevalence of subclinical and clinical mastitis on different farms.

Farm	No. of animals	CMT positive (no. and %)	Clinical mastitis (no. and %)	Prevalence of *S. aureus* (%)			
				Milk	Udder	Floor	Milking machine
A	21	8 (38.10%)	0.00	100.00	37.50	66.66	0.00
B	14	3 (21.43%)	0.00	100.00	66.66	50.00	0.00
C	21	14 (66.66%)	0.00	57.14	28.57	0.00	100
D	22	10 (45.45%)	0.00	80.00	40.00	100	0.00
E	23	7 (30.43%)	0.00	85.71	28.57	75.00	100
F	55	27 (49.09%)	3 (5.45%)	83.33	46.66	22.22	25.00
G	24	12 (50.00%)	3 (12.50%)	100.00	90.00	100	0.00
Total	180	81 (45.00%)	6 (3.30%)	82.71	46.91	48.15	62.50

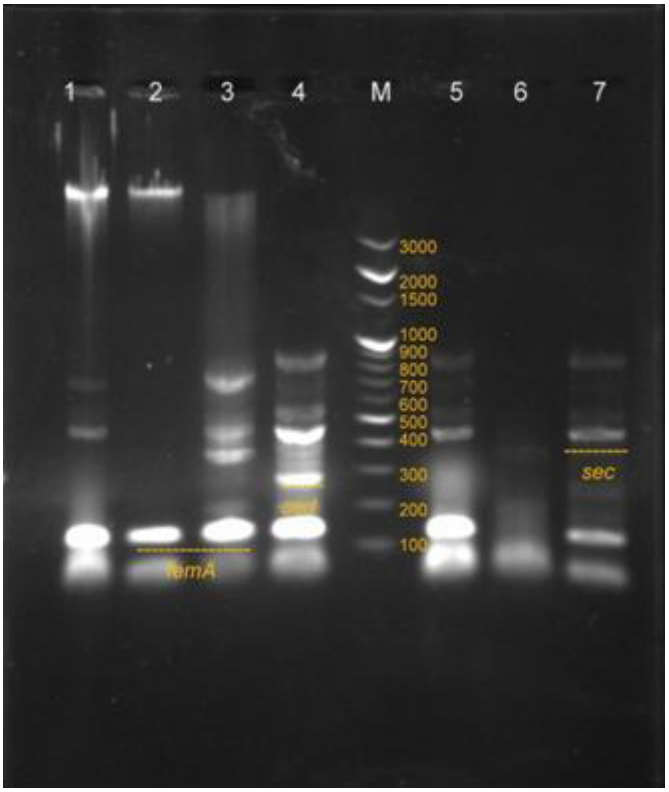

Figure 1. Detection of *S. aureus* enterotoxin genes (set A): lane M- DNA ladder (100–3000 bp); lanes 1, 2, 4, 5, and 7- *sec* (451 bp); lane 4: *sed* (278 bp); lanes 1, 2, 3, 4, 5, and 7- *femA* (132 bp).

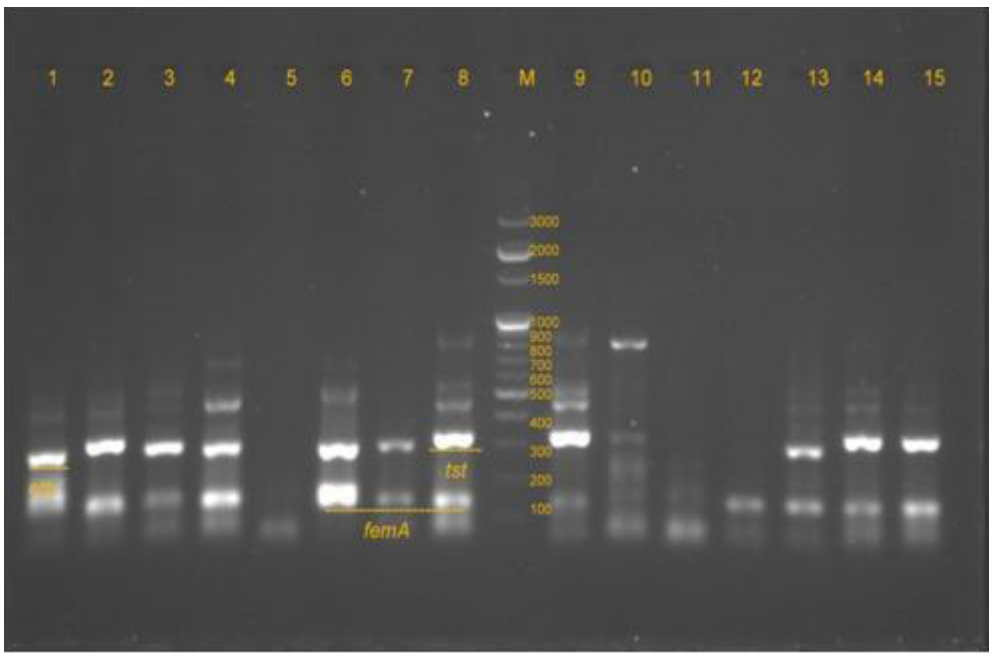

Figure 2. Detection of *S. aureus* enterotoxin genes (set B): lane M- DNA ladder (100–3000 bp); lanes 1 and 13- *etb* (226 bp); lanes 2, 3, 4, 6, 7, 8, 9, 12, 14, and 15- *tst* (326 bp); all lanes- *femA* (132 bp).

As an internal control *femA* was detected in all the strains of *S. aureus*. This gene has been suggested to have a role in cell metabolism and is present in all *S. aureus* during the active growth phase. Out of 141 *S. aureus*, 52 isolates were selected for assessment of enterotoxin genes. The selected isolates represented all seven farms and met specific identification requirements such as growth in pure culture and expression of the *femA* gene. Of them, 17 were enterotoxin-producing strains showing expression of one or more staphylococcal enterotoxins (SEs). Farm-wise and isolate-wise distribution of SEs is expressed in Tables 4 and 5, respectively. The pattern of enterotoxin production was revealed as: *seb* (1.92%), *sec* (21.15%), *sed* (7.69%), and *see* (1.92%). The pattern of superantigens and methicillin resistance was revealed as: *etb* (1.92%), *tsst1* (25%), and *mecA* (3.85%). Highest numbers of enterotoxin-producing strains were detected at farm A (77.77%), followed by farms B (66.66%), F (22.22%), C (14.28%), and G (11.11%). Coexistence of *sec* and *tsst1* was detected in almost all the *sec*-positive strains. Maximum SE-producing strains were isolated from milk, udders, and milking machine liners.

The antimicrobial resistance/susceptibility pattern expressed by the *S. aureus* isolates is shown in Table 6. Most strains were resistant to kanamycin, penicillin, and oxacillin and sensitive to netilmicin and amikacin. Out of the 52 PCR-confirmed enterotoxin-producing *S. aureus* isolates, 7 (13.46%), 9 (17.31%), and 16 (30.77%) were positive for the *blaZ, ermB*, and *tetK* genes, respectively (Figure 3). Variation in the distribution of *S. aureus* strains harboring AMR genes was recorded; however, the maximum frequency of AMR genes was detected in strains obtained from milk samples (Tables 7 and 8).

4. Discussion

The presented results indicate a substantial prevalence of subclinical mastitis and greater frequency of isolation of *S. aureus* in dairy cows and their associated environment. Poor knowledge regarding mastitis management was noted in the dairy farmers included in this study. Subclinical

Table 4. Farm-wise distribution of enterotoxin types of *S. aureus* isolates.

Farm	No. of isolates	Source of sample	*S. aureus* enterotoxigenic genes									
			sea	*seb*	*sec*	*sed*	*see*	*femA*	*mecA*	*eta*	*etb*	*tst*
A	9	Mastitis milk	-	-	5	1	1	6	-	-	1	5
		Healthy milk	-	-	1	1	-	1	-	-	-	1
		Udder Swab	-	-	1	1	-	2	-	-	-	1
B	6	Mastitis milk	-	-	3	-	-	3	-	-	-	3
		Healthy milk	-	-	-	-	-	1	-	-	-	-
		Udder swab	-	-	1	-	-	2	-	-	-	1
C	7	Mastitis milk	-	-	-	-	-	4	-	-	-	-
		Udder swab	-	-	-	-	-	2	-	-	-	-
		Machine swab	-	-	-	1	-	1	-	-	-	-
E	9	Mastitis milk	-	-	-	-	-	5	-	-	-	-
		Healthy milk	-	-	-	-	-	1	-	-	-	-
		Udder swab	-	-	-	-	-	1	-	-	-	-
		Floor swab	-	-	-	-	-	1	-	-	-	-
		Machine swab	-	-	-	-	-	1	1	-	-	-
F	12	Mastitis milk	-	-	-	-	-	9	-	-	-	1
		Udder swab	-	-	-	-	-	2	1	-	-	1
		Floor swab	-	-	-	-	-	1	-	-	-	-
G	9	Mastitis milk	-	1	-	-	-	7	-	-	-	-
		Healthy milk	-	-	-	-	-	1	-	-	-	-
		Udder swab	-	-	-	-	-	1	-	-	-	-
Clinical samples	3	College laboratory	-	-	2	-	-	3	-	-	-	3

Table 5. Isolate-wise details of *S. aureus* enterotoxin.

Farm	Isolate source	Isolate no.	*S. aureus* enterotoxin genes									
			sea	*seb*	*sec*	*sed*	*see*	*fem A*	*mecA*	*eta*	*etb*	*tst*
A	Mastitis milk	A1	-	-	+	-	-	+	-	-	-	+
		A2	-	-	+	+	+	+	-	-	-	+
		A4	-	-	+	-	-	+	-	-	+	+
		A6	-	-	+	-	-	+	-	-	-	+
		A7	-	-	-	-	-	+	-	-	-	-
		A8	-	-	+	-	-	+	-	-	-	+
	Udder swab	AU1	-	-	+	-	-	+	-	-	-	+
		AU8	-	-	-	+	-	+	-	-	-	-
	Healthy milk	AH1	-	-	+	+	-	+	-	-	-	+
B	Mastitis milk	B1	-	-	+	-	-	+	-	-	-	+
		B2	-	-	+	-	-	+	-	-	-	+
		B3	-	-	+	-	-	+	-	-	-	+
	Udder swab	BU1	-	-	-	-	-	+	-	-	-	-
		BU2	-	-	+	-	-	+	-	-	-	+
	Healthy milk	BH1	-	-	-	-	-	+	-	-	-	-
C	Mastitis milk	C1	-	-	-	-	-	+	-	-	-	-
		C4	-	-	-	-	-	+	-	-	-	-
		C10	-	-	-	-	-	+	-	-	-	-
		C12	-	-	-	-	-	+	-	-	-	-
	Udder swab	CU11	-	-	-	-	-	+	-	-	-	-
		CU12	-	-	-	-	-	+	-	-	-	-
	Machine swab	CM2	-	-	-	+	_	+	_	-	-	-
E	Mastitis milk	E1	-	-	-	-	-	+	-	-	-	-
		E2	-	-	-	-	-	+	-	-	-	-
		E3	-	-	-	-	-	+	-	-	-	-
		E4	-	-	-	-	-	+	-	-	-	-
		E6	-	-	-	-	-	+	-	-	-	-
	Udder swab	EU2	-	-	-	-	-	+	-	-	-	-
	Floor swab	EF3	-	-	-	-	-	+	-	-	-	-
	Healthy milk	EH2	-	-	-	-	-	+	-	-	-	-
	Machine swab	EM1	-	-	-	-	-	+	+	-	-	-
F	Mastitis milk	F3	-	-	-	-	-	+	-	-	-	-
		F5	-	-	-	-	-	+	-	-	-	-
		F6	-	-	-	-	-	+	-	-	-	-
		F14	-	-	-	-	-	+	-	-	-	-
		F15	-	-	-	-	-	+	-	-	-	-
		F19	-	-	-	-	-	+	-	-	-	-
		F22	-	-	-	-	-	+	-	-	-	-
		F28	-	-	-	-	-	+	-	-	-	-
		F29	-	-	-	-	-	+	-	-	-	+
	Udder swab	FU2	-	-	-	-	-	+	+	-	-	+
		FU20	-	-	-	-	-	+	-	-	-	-
	Floor swab	FF2	-	-	-	-	-	+	-	-	-	-

Table 5. (Continued).

G	Mastitis milk	G2	-	-	-	-	-	+	-	-	-	-
		G3	-	+	-	-	-	+	-	-	-	-
		G6	-	-	-	-	-	+	-	-	-	-
		G7	-	-	-	-	-	+	-	-	-	-
		G10	-	-	-	-	-	+	-	-	-	-
		G13	-	-	-	-	-	+	-	-	-	-
		G15	-	-	-	-	-	+	-	-	-	-
	Udder swab	GU7	-	-	-	-	-	+	-	-	-	-
	Healthy milk	GH2	-	-	-	-	-	+	-	-	-	-
Clinical samples	Mastitis milk	CA	-	-	-	-	-	+	-	-	-	+
		CD	-	-	+	-	-	+	-	-	-	+
		CM	-	-	+	-	-	+	-	-	-	+

Table 6. Farm-wise AMR pattern of *S. aureus* isolates (n = 90).

Farm	Total no. of samples	AMR pattern	Penicillin (10 U)		Oxacillin (1 µg)		Gentamicin (10 µg)		Amikacin (30 µg)		Kanamycin (30 µg)		Netilmicin (30 µg)		Tobramycin (10 µg)		Ceftazidime (30 µg)	
			No	%	No.	%	No.	%	No	%	No.	%	No.	%	No.	%	No.	%
A	8	S	0	0.0	2	25.0	8	100	8	100	5	62.50	8	100	8	100	2	25.0
		I	0	0	0	0	0	0	0	0	2	25.0	0	0	0	0	1	12.50
		R	8	100	6	75.0	0	0	0	0	1	12.50	0	0	0	0	5	62.50
B	6	S	0	0	1	16.66	6	100	6	100	5	83.33	6	100	6	100	1	16.67
		I	0	0	0	0	0	0	0	0	1	16.67	0	0	0	0	2	33.33
		R	6	100	5	83.33	0	0	0	0	0	0	0	0	0	0	3	50
C	5	S	0	0	1	20	4	80	5	100	2	40	5	100	4	80	3	60
		I	0	0	0	0	1	20	0	0	3	60	0	0	0	0	0	0
		R	5	100	4	80	0	0	0	0	0	0	0	0	1	20	2	40
E	16	S	2	12.50	6	37.50	12	75	15	93.75	3	18.75	16	100	11	68.75	7	43.75
		I	0	0	0	0	2	12.50	1	6.25	1	6.25	0	0	0	0	2	12.5
		R	14	87.50	10	62.50	2	12.50	0	0	12	75	0	0	5	31.25	7	43.75
F	35	S	6	17.14	15	42.86	12	34.29	23	65.71	8	22.86	22	62.86	23	65.71	15	42.86
		I	0	0	0	0	2	5.71	2	5.71	2	5.71	1	2.86	0	0	4	11.43
		R	29	82.26	20	57.14	21	60	10	28.57	25	71.43	12	34.29	12	34.29	16	45.71
G	20	S	0	0	0	0	19	95	16	80	0	0	20	100	17	85	0	0
		I	0	0	0	0	0	0	2	10	2	10	0	0	3	15	0	0
		R	20	100	20	100	1	5	2	10	18	90	0	0	0	0	20	100

S- Sensitive; I- intermediate; R- resistant.

mastitis is difficult to detect and was found to be the main form of mastitis in the animals studied. In India, endemicity of subclinical mastitis in the range of 10%–70% has been recorded (1,7). In this study a greater frequency of *S. aureus* isolation was recorded, which is in compliance with the findings of other investigators (8). Isolation of *S. aureus* strains from udder surfaces, floors, and milking machine liners indicate that these factors are crucial in the spread of *S. aureus* at farm level. A very high proportion of animals harboring the predominant genotypes of *S. aureus*

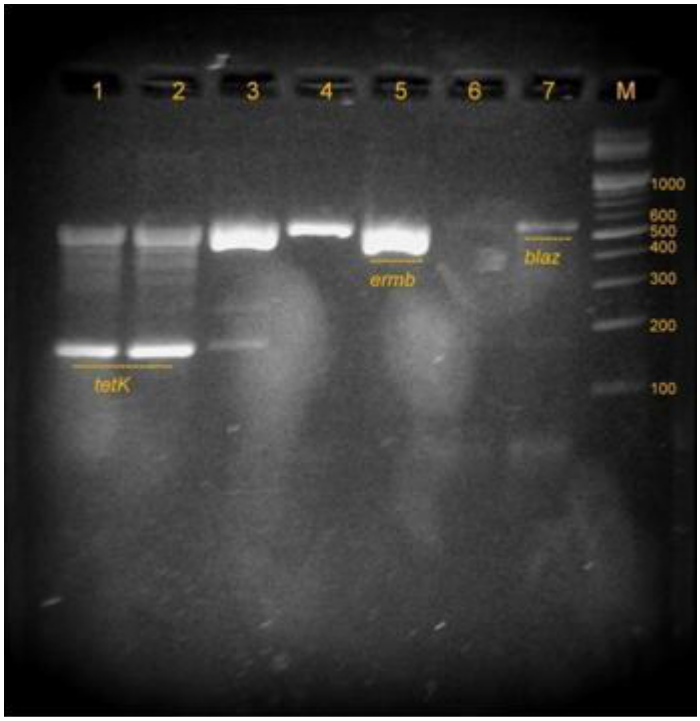

Figure 3. Detection of antimicrobial resistance genes in *S. aureus* isolates by multiplex PCR. Lane M- 100-bp DNA ladder; lanes 1, 2, and 3- *tetK* gene (169 bp); lanes 3 and 5- *ermB* gene (409 bp); lanes 4 and 7- *blaZ* gene (517 bp).

on udder skin and intramammary sites was revealed in a Finnish dairy herd (2). A high prevalence of mastitis could be attributed to lack of mastitis awareness in farmers. Lower prevalence of mastitis in buffalo may be due to the smaller sample size and thus it is not critically discussed. The high prevalence of *S. aureus* in milk and other sources indicates that virtually all cows in the herds could have had exposure to this pathogen via udders, floors, and milking machines. The observations of this study revealed lack of use of disinfectant for cleaning operations, lack of use of pre- and postmilking teat dipping, lack of teat sealing, and lack of routine testing of subclinical mastitis by CMT as the most substantial risk factors. These observations are well supported by earlier studies conducted globally (9,10).

In agreement with earlier studies globally (8,11) and in India (12,13), the highest rate of resistance was detected for beta-lactam antibiotics penicillin, oxacillin, and kanamycin. Very high resistance to penicillin G (82.72%) in *S. aureus* isolated from cows with subclinical mastitis was also reported by Behiry et al. (14). Resistance to penicillin is attributed to its widespread use in intramammary preparations and expression of β-lactamases encoded by the *blaZ* gene, which causes hydrolysis of the β-lactam ring of penicillin (15). In the present study, oxacillin was included for detection of methicillin-resistant *S. aureus* (MRSA) and 71.76% of strains were resistant to it. Oxacillin resistance is reported as resistance to all β-lactam antimicrobial agents. The high degree of resistance to oxacillin observed in this study is alarming. Phenotypic AMR by Kirby–Bauer disk diffusion method was studied in India by many investigators and most of the *S. aureus* strains isolated from bovine mastitis cases were found resistant to penicillin, amoxicillin, cephalexin, oxytetracycline, and methicillin and sensitive to gentamicin, ceftriaxone, chloramphenicol, and enrofloxacin (12,13,16). AMR with special reference to β-lactams is common in *S. aureus* strains and netilmicin, tobramycin, and gentamicin-containing antimicrobials could be better alternatives for treating *S. aureus*-associated subclinical mastitis if used judiciously.

The presence of classical enterotoxin genes in *S. aureus* isolates recovered from cows with mastitis has been investigated in previous studies outside of India (17,18); however, scanty reports are available from India. *S. aureus* can produce more than 30 virulence factors and SE a, b, and c are the most common in food poisoning. We could not detect *sea*, but the detection of *seb* and *sec* highlights the risk associated with SE production in raw milk. Kalorey et al. (1) isolated coagulase-positive *S. aureus* harboring virulent genes encoding clumping factor

Table 7. Isolate-wise phenotypic and genotypic antimicrobial resistance pattern of *S. aureus* (n = 52).

Farm	Isolate source	Isolate no.	Phenotypic AMR pattern		Genotypic AMR pattern		
			Sensitive	Resistance	*blaZ*	*ermB*	*tetK*
A	Mastitis milk	A1	Ak, Ctx, G, K, Net, Tob	P	+	-	+
		A2	Ak, G, K, Net, Tob	Ctx, Ox, P	-	-	-
		A4	Ak, Ctx, G, K, Net, Tob	Ox, P	-	-	-
		A6	Ak, Ctx, G, K, Net, Tob	Ox, P	-	-	-
		A7	Ak, G, K, Net, Tob	Ctx, Ox, P	-	-	-
		A8	Ak, Ctx, G, Net, Ox, Tob	K, P	-	-	-
	Udder swab	AU1	Ak, G, K, Net, Tob	Ox, P	+	-	-
		AU8	Ak, G, K, Net, Tob	Ctx, Ox, P	-	-	-
	Healthy milk	AH1	Ak, G, Net, Tob	Ctx, Ox, P	-	-	-
B	Mastitis milk	B1	Ak, G, Net, Tob	Ctx, Ox, P	-	-	-
		B2	Ak, G, K, Net, Tob	Ctx, Ox, P	-	-	-
		B3	Ak, Net, Tob	Ctx, Ox, P	-	-	-
	Udder swab	BU1	Ak, G, K, Net, Tob	Ox, P	-	-	+
		BU2	Ak, Ctx, G, K, Net, Ox, Tob	P	-	+	-
	Healthy milk	BH1	Ak, G, K, Net, Tob	Ox, P	-	-	-
C	Mastitis milk	C1	Ak, Caz, Net, Tob	Ox	-	+	-
		C4	Ak, Caz, Net, Tob	Ox	-	-	-
		C10	Ak, G, Net, Tob	Caz, Ox, P	-	-	-
		C12	Ak, Caz, G, K, Net, Ox, Tob	P	-	-	-
	Udder swab	CU11	Ak, G, K, Net, Ox, Tob	Caz, P	-	+	+
		CU12	Ak, G, Net, Tob	Caz, Ox, P	+	-	-
	Machine swab	CM2	Ak, Caz, G, K, Net, Tob	Ox, P	-	-	+
E	Mastitis milk	E1	Ak, G, Net, Tob	Caz, K, Ox, P	-	-	-
		E2	Ak, Net, Tob	Caz, K, Ox, P	+	-	-
		E3	Ak, Caz, G, Net, Ox, Tob	K, P	-	-	+
		E4	Ak, G, Net, Tob	Caz, K, Ox, P	-	+	+
		E6	Ak, Caz, G, K, Net, Ox, P, Tob	-	+	-	-
	Udder swab	EU2	Ak, Caz, G, K, Net, Ox, P, Tob	-	-	-	+
	Floor swab	EF3	Ak, Caz, G, Net, Ox, Tob	K, P	-	-	-
	Healthy milk	EH2	Ak, Net, Tob	Caz, K, Ox, P	-	-	-
	Machine swab	EM1	Ak, Caz, G, Net	Ox, P, Tob	-	-	-
F	Mastitis milk	F3	Ak, Caz, G, K, Net, Tob	Ox, P	-	-	+
		F5	-	Ak, Caz, G, K, Net, Ox, P, Tob	-	-	+
		F6	Ak, Caz, G, Net, Ox, P, Tob	K	-	-	+
		F14	Ak, Net, Tob	Caz, Ox, P	-	-	-
		F15	Ak, Net, Tob	Caz, Ox, P	-	-	-
		F19	Caz, Ex, Met	Ak, G, K, Net, Tob	-	+	+
		F22	Caz, Ox, Tob	G, Net, K, P	-	-	-
		F28	Caz, Ox, Tob	Ak, G, Net, K, P	-	-	+
		F29	Ak, K, Net, Tob	Caz, G, Ox, P	-	-	-
	Udder swab	FU2	Ak, Z, G, Net, Tob	Caz, Ox, P	+	-	-
		FU20	-	Ak, Caz, G, K, Net, Ox, P, Tob	-	+	+
	Floor swab	FF2	Net	Ak, Caz, G, K, Ox, P, Tob	-	+	_

Table 7. (Continued).

G	Mastitis milk	G2	Ak, G, Net, Tob	Caz, Ox, P	-	-	-
		G3	Ak, G, Net, Tob	Caz, K, Ox, P	-	-	-
		G6	Ak, G, Net, Tob	Caz, K, Ox, P	-	+	+
		G7	Ak, G, Net, Tob	Caz, K, Ox, P	-	-	-
		G10	Ak, G, Net, Tob	Caz, K, Ox, P	-	+	+
		G13	G, Net, Tob	Caz, K, Ox, P	+	-	+
		G15	Ak, G, Net, Tob	Caz, Ox, P	-	-	+
	Udder swab	GU7	Ak, G, Net	Caz, K, Ox, P	-	-	-
	Healthy milk	GH2	Ak, G, Net, Tob	Caz, K, Ox, P	-	-	-
Clinical samples	Mastitis milk	CA	Ak, G, Net, Tob	Caz, Ox, P	-	-	-
		CD	Ak, G, K, Net, Tob	Caz, Ox, P	-	-	-
		CM	Ak, G, K, Net, Tob	Caz, Ox, P	-	-	-

Ak- Amikacin, Caz- ceftazidime, G- gentamicin, K- kanamycin, Net- netilmicin, Ox- oxacillin, P- penicillin, Tob- tobramycin.

Table 8. Farm-wise genotypic antimicrobial resistance pattern of *S. aureus* isolates.

Farm	No. of isolates	Source of sample	AMR genes detected		
			blaZ	*ermB*	*tetK*
A	9	Mastitis milk	1	-	1
		Healthy milk	-	-	-
		Udder swab	1	-	-
B	6	Mastitis milk	-	-	-
		Healthy milk	-	-	-
		Udder swab	-	1	1
C	7	Mastitis milk	-	1	-
		Udder swab	1	1	1
		Machine swab	-	-	1
E	9	Mastitis milk	2	1	2
		Healthy milk	-	-	-
		Udder swab	-	-	1
		Floor swab	-	-	-
		Machine swab	-	-	-
F	12	Mastitis milk	-	1	4
		Udder swab	1	1	1
		Floor swab	-	1	-
G	9	Mastitis milk	1	2	4
		Healthy milk	-	-	-
		Udder swab	-	-	-
Clinical mastitis samples	3	College laboratory	-	-	-

and coagulase activity from cows with subclinical mastitis from the Vidarbha region of India; however, they could not detect *sea*-producing strains. Similarly, a low proportion of *seb* (0.9%), *sec* (8.4%), *sed* (0.9%), and *seg* (10.2%) and a high proportion of pathogenic genes in MRSA isolates from Sahiwal cows was recorded from India (16). Our findings are more consistent with the observations of Karahan et al. (17) from Turkey, in which 29.3% of *S. aureus* strains isolated from bovine mastitis were enterotoxigenic. A positive correlation between *sec* and *tst1* genes was also reported by them, as observed in this study. Coexistence of *sec* and *tst1* was detected in almost all the *sec*-positive strains. One *S. aureus* strain carried *sec*, *sed*, *see*, and *tst1* genes, which is a unique finding. Strains carrying multiple genes may have higher virulence than a strain carrying one gene. Occurrence of SEs a, c, d, *tst1*, g, i, and j with the combination of *sec* and *tst1* and rare distribution of exfoliative toxin in *S. aureus* isolated from mastitis cattle in Germany has been documented previously (19). We have also revealed expression of *tst1* in *sec*-positive stains and *eta* could not be detected. Variability in the prevalence of *S. aureus* enterotoxin genotypes among farms and dairy products from different countries has been documented earlier (20). Thus, the design and implementation of region-specific surveillance programs on the molecular epidemiology and resistance pattern of *S. aureus* associated with bovine mastitis is essential in India. No such surveillance program is in practice at present in Maharashtra. Our findings indicated that *sec* and *tst1* SE-producing *S. aureus* strains are predominant in this region. Identification of specific virulence factors is essential in the control of *S. aureus* intramammary infection.

Exact correlation in the phenotypic and genotypic determination of methicillin and β-lactam resistance could not be established since the frequency of detection of *mecA* (3.84%) and *blaZ (*13.46%) was poor in the phenotypically confirmed resistant isolates. In contrast, close correspondence between phenotypic and genotypic tests was recorded in a previous study (15). Methicillin resistance is heterogeneous in nature and the level of resistance varies according to the culture conditions and β-lactam antibiotics used. An extensive study from India on Sahiwal cows revealed 13.08% of *S. aureus* strains to be resistant to methicillin, out of which 71.43% MRSA amplified the *mecA* gene (17).

The majority of the isolates expressed a single AMR gene, except for the combination of *blaZ* + *tetK* and *tetK* + *ermB* in 3.84% and 11.53% of isolates, respectively. Resistance to penicillin is closely associated with the *blaZ* gene, which encodes β-lactamases. Resistance to erythromycin is predominantly mediated by the *erm* genes. The resistance to tetracycline is presented by active efflux pump and protection of the tetracycline target sites. These mechanisms are determined by the *tet* genes such as *tetM* and *tetK*, which are most common in conferring tetracycline resistance in *S. aureus* (21).

The link between genotype and AMR in *S. aureus*-associated bovine mastitis was studied by Sakwinska et al. (22) and their observations revealed a strong association of penicillin-resistant clusters, highlighting the fact that knowledge of local epidemiology is essential for accurate treatment by veterinarians. They further emphasized the need of more research and knowledge of local and global epidemiology of *S. aureus* clones.

Out of 52 *S. aureus* isolates, 46 (88.46%) exhibited resistance to penicillin and only seven (13.46%) isolates expressed the *blaZ* gene. Similarly, only nine (17.30%) strains expressed the *ermB* gene. Out of these nine isolates, only five (55.55%) isolates displayed both the presence of *ermB* and phenotypic resistance to erythromycin. Genotypic resistance to *tetK* was recorded in 16 (32.69%) isolates, out of which 15 (93.75%) were resistant to oxytetracycline. Such variations in phenotypic and genotypic AMR patterns in the staphylococci from bovine mastitis milk have been documented earlier (23), whereby 47.4% of coagulase-negative staphylococci (CNS) harbored the *tetK* gene and 11.1% of macrolide-resistant CNS strains harbored the *ermB* gene.

All the animals studied were lactating and milk procured from them was sold either in retail or to the dairy cooperatives. Food Safety and Standards Act 2006, Regulations 2011, promulgates legal standards for the selling of milk and milk products. Accordingly, the sale of raw, unpasteurized, contaminated, and adulterated milk is prohibited. However, testing for somatic cell count, microbial counts, etc. is not practiced at farms. These parameters are monitored for the bulk milk tanks finally delivering milk to the processing plant. From a public health point of view, raw milk containing antimicrobial-resistant enterotoxigenic strains of *S. aureus* definitely poses a health risk. Therefore, farmers' awareness of food safety issues of mastitis milk is needed. In conclusion, the high prevalence of subclinical mastitis and *S. aureus* found in this study suggests that farm environments could be an important source of enterotoxin-producing *S. aureus* strains with the potential for causing mastitis. There is a need to improve the knowledge level of farmers towards mastitis management. *S. aureus* strains are highly resistant to the beta-lactam group of antimicrobials. Results of this study clearly highlight the role of toxigenic and multidrug-resistant *S. aureus* strains in bovine mastitis. Effort is needed to strengthen the molecular surveillance of *S. aureus* associated with bovine mastitis.

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