Antibiotic resistance and pathogenicity factors in *Staphylococcus aureus* isolated from mastitic Sahiwal cattle

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Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a serious problem in dairy animals suffering from mastitis. In the present study, the distribution of mastitic MRSA and antibiotic resistance was studied in 107 strains of *S. aureus* isolated from milk samples from 195 infected udders. The characterizations pathogenic factors (adhesin and toxin genes) and antibiotic susceptibility of isolates were carried out using gene amplification and disc diffusion assays, respectively. A high prevalence of MRSA was observed in the tested isolates (13.1%). The isolates were also highly resistant to antibiotics, i.e. 36.4% were resistant to streptomycin, 33.6% to oxytetracycline, 29.9% to gentamicin and 26.2% each to chloramphenicol, pristinomycin and ciprofloxacin. A significant variation in the expression of pathogenic factors (*Ig*, *coa* and *clf*) was observed in these isolates. The overall distribution of adhesin genes *ebp*, *fib*, *bbp*, *fnbB*, *cap*5, *cap*8, *map* and *cna* in the isolates was found to be 69.1, 67.2, 6.5, 20.5, 60.7, 26.1, 81.3 and 8.4%, respectively. The presence of *fib*, *fnb*B, *bbp* and *map* genes was considerably greater in MRSA than in methicillin-susceptible *S. aureus* (MSSA) isolates. The proportions of toxin genes, namely, *hlb*, *seb*, *sec*, *sed*, *seg* and *sei*, in the isolates were found to be 94.3, 0.9, 8.4, 0.9, 10.2 and 49.5%, respectively. The proportions of *agr* genes I, II, III and IV were found to be 39.2, 27.1, 21.5 and 12.1%, respectively. A few isolates showed similar antibiotic-resistance patterns, which could be due to identical strains or the dissemination of the same strains among animals. These findings can be utilized in mastitis treatment programmes and antimicrobials strategies in organized herds.

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1. Introduction

Mastitis is an infectious disease that is associated with massive financial losses in the dairy sector. Among the various causative agents, *Staphylococcus aureus* is one of the most prevalent and contagious pathogens of intra-mammary infections in dairy cattle globally. The evolution of antibiotic resistance in *S. aureus* strains is a serious cause of concern in dairy animals (Wang *et al.* 2008). Strains of *S. aureus* resistant to β-lactam antibiotics are known as methicillin-resistant *S. aureus* (MRSA). These strains in intra-mammary dissemination often produce incurable severe intra-herd infections (Moon *et al.* 2007; Kumar *et al.* 2010). MRSA strains have been observed to be multi-drug resistant, such as aminoglycosides, macrolides, lincosamides, streptogramins, tetracyclines, etc., which are often used in the treatment of mastitis (Wang *et al.* 2008; Kumar *et al.* 2010). The transmission of bovine MRSA to humans is possible and may contribute to outbreaks in animal and human populations (Lee 2003). Hence, it is necessary to know which endemic strains of *S. aureus* in dairy cattle populations are highly pathogenic and methicillin-resistant.

MRSA strains show pathogenic and epidemiological characteristics in various ways such as mutation, clonal

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evolution (Fitzgerald *et al.* 2001) and horizontal gene transfer (Brody *et al.* 2008). These evolutionary processes enhance the pathogenic and antimicrobial-resistant properties of *S. aureus* strains. However, a limited diversity of *S. aureus* strains or clones cause most of the mastitic infections in each geographical region, as these isolates are better adapted to infect animals (Annemüller *et al.* 1999; Salasia *et al.* 2004; El-Sayed *et al.* 2006; Moon *et al.* 2007). Various molecular techniques have been explored and used to analyse the pathogenesis and distribution of pathogenic genes in strains of *S. aureus* (Fitzgerald *et al.* 2001; Peacock *et al.* 2002; Løvseth *et al.* 2004). Identification and early elimination of pathogenic MRSA strains at the herd level is possible by the use of different molecular microbiology tools. The available information is limited regarding the genetic heterogeneity of MRSA strains in mastitic cattle under subtropical conditions i.e. in the Indian environment. The present investigation was carried out with the objective to determine the distribution of MRSA genetic variants of *S. aureus* isolates from mastitic cattle in a closed herd located in northwest India.

2. Materials and methods

2.1 *Identification and biochemical characterization*

Milk samples (195) from animals of the Sahiwal herd suffering from mastitis were screened using the California mastitis test. The isolation of *S. aureus* stains was carried out using the standard method (Kumar *et al.* 2010). Briefly, an aliquot of 100 µl of aseptically collected milk samples from each infected animal was spread over a Baird Parker agar plate and incubated at 37°C for 24 h. After incubation, colonies were selected and subjected to Gram staining, catalase test and morphological identification. The Gram- and catalase-positive cocci were characterized for carbohydrates fermentation (Hicarbohydrate™ kit containing 34 different carbohydrate; Himedia, India), which was followed by urease, ortho-nitrophenyl-β-galactoside (ONPG), Vogues-Proskauer, arginine utilization, lysostaphin sensitivity, coagulase, clumping factor, thermonuclease, haemolysin, capsule, bio-film and slime production tests as explained by Kumar *et al.* (2010).

2.2 *Antibiotic susceptibility*

The antibiotic-susceptibility profile of isolates was prepared using the disk diffusion method on Mueller-Hinton agar as recommended by Clinical and Laboratory Standards Institute (2008). In brief, *S. aureus* isolates were grown overnight on blood agar at 37°C, and the colonies were suspended in sterile saline water equivalent to a 0.5 McFarland standard. The suspension (100 µl) was spread over the medium plate. Then, the antibiotic disk was transferred aseptically on to the surface of the inoculated medium, and methicillin was incubated further at 30°C, and other antibiotics at 35°C, for a period of 24 h. *Staphylococcus aureus* ATCC 25923 and *S. aureus* NCDC 110 were used as controls. The antibiotics and their concentrations used are as follows: amikacin (30 µg), amoxicillin (10 µg), amoxicillin-sulbactum (20 µg), amoxicillin-clavulanate (20 µg), ampicillin (25 µg), cephalexin (30 µg), chloramphenicol (30 µg), ciprofloxacin (30 µg), clindamycin (10 µg), cloxacillin (30 µg), gentamicin (20 µg), kanamycin (20 µg), cefixime (5 µg), lincomycin (15 µg), methicillin (5, 10 and 15 µg), ofloxacin (5 µg), oxacillin (5 µg), oxytetracycline (30 µg), penicillin-G (10 units), pristinomycin (15 µg), rifampicin (20 µg), vancomycin (10 µg) and streptomycin (20 µg).

2.3 *Partial amplification of 16S rDNA, mecA and nuc genes*

Subsequent to biochemical characterizations, staphylococcal isolates were further subjected to species-specific gene amplification (16S rDNA). DNA from the isolates was extracted as per procedure reported previously (Kumar *et al.* 2010). Information about annealing temperatures and oligonucleotide primers used is given in table 1. The reaction mixture (25 µl) used for gene amplification contained: 2 µl dNTPs (200 µm/µl), 2.5 µl of 10× *Taq* buffer consisting of 15 mM $MgCl_2$ (Banglore Genei, India), 1 µl each forward and reverse oligonucleotide primers (10 pm/µl; Sigma Aldrich, USA), 0.35 µl *Taq* DNA polymerase (3 U/µl; Banglore Genei, India), 1 µl DNA (30 ng/µl) and 17.2 µl distilled water. The amplification was carried out in 0.2 ml PCR tubes in thermal cycler (Løvseth *et al.* 2004). Isolates were tested for the presence of *nuc* (Brakstad *et al.* 1992) and *mec*A genes (Murakami *et al.* 1991). Segments of *mec*A and *nuc* genes were amplified using primers and annealing temperatures as listed in table 1. The amplified products were imaged by running them in 1.5% agarose containing 0.5 µg/ml ethidium bromide.

2.4 *PCR amplification of genes encoding putative pathogenic factors*

Identified *S. aureus* isolates were studied for putative pathogenic factors (adhesins, enterotoxins, toxic shock syndrome toxin and exfoliative toxins). All the oligonucleotide primers used were selected from earlier reports as mentioned in table 1. Amplifications of genes encoding the

Table 1. Oligonucleotide primers and amplification conditions

Genes	Oligonucleotide Sequences	Location of primer	Accession number	Annealing and other temperature conditions*	Product size (bp)	References
16S rDNA F 16S rDNA R	GTAGGTGGCAAGCGTTACC CGCACATCAGCGTCAG	545–564 773–758	X68417	64°C	228	Løvseth *et al.* (2004)
nuc F *nuc* R	GCGATTGATGGTGATACGGTT ACGCAAGCCTTGACGAACTAAAGC	511532 786–766	V01281	55°C	280	Brakstad *et al.* (1992)
mecA F *mecA* R	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTTGC	1282–1303 1814–1793	Y00688	55°C	533	Murakami *et al.* (1991)
spa F *spa* R	TCAAGCACCAAAAGAGGAAGA GTTTAACGACATGTACTCCGTTG	1522–1544 1806–1784	X61307	60°C	Variable	Montesinos *et al.* (2002)
Ig F *Ig* R	CACCTGCTGCAAATGCTGCG GGC TTGTTGTTG TCT TCC TC	789–808 1698–1679	M18264	58°C	Variable	Seki *et al.* (1998)
clf F *clf* R	GGCTTCAGTGCTTGTAGG TTTTCAGGGTCAATATAAGC	354–372 1329–1309	Z18852	57°C	Variable	Stephan *et al.* (2001)
coa F *coa* R	AACAAAGCGGCCCATCATTAAG TAAGAAATATGCTCCGATTGTCG	1303–1325 2176–2153	X17679	50°C 8 cycles 55°C 25 cycles	Variable	Montesinos *et al.* (2002)
*fnb*A F *fnb*A R	GCGGAGATCAAAGACAA CCATCTATAGCTGTGTGG	524–540 1802–1785	J04151	50°C	1280	Booth *et al.* (2001)
*fnb*B F *fnb*B R	GGAGAAGGAATTAAGGCG GCCGTCGCCTTGAGCGT	1132–1149 1944–1928	X62992	50°C	820	Booth *et al.* (2001)
cna F *cna* R	AGTGGTTACTAATACTG CAGGATAGATTGGTTTA	1719–1735 3457–3441	M81736	55°C	Variable	Peacock *et al.* (2002)
bbp F *bbp* R	AACTACATCTAGTACTCAACAACA ATGTGCTTGAATAACACCATCATCT	524–549 1098–1073	Y18653	55°C	575	Tristan *et al.* (2003)
eno F *eno* R	ACG TGCAGCAGCTGACT CAACAGCATYCTTCAGTACCTTC	464–481 766–743	AF065394	55°C	302	Tristan *et al.* (2003)
ebp F *ebp* R	CATCCAGAACCAATCGAAGAC CTTAACAGTTACATCATCATGTTTATCTTTG	384–405 570–539	U48826	55°C	186	Tristan *et al.* (2003)
fib F *fib* R	CTACAACTACAATTGCCGTCAACAG GCTCTTGTAAGACCATTTTCTTCAC	180–205 585–560	X72014	55°C	404	Tristan *et al.* (2003)
cap 5 F *cap* 5 R	ATGACGATGAGGATAGCG CTCGGATAACACCTGTTGC	7621–7638 8501–8483	U81973	57°C	880	Moore and Lindsay (2001)
cap 8 F *cap* 8 R	ATGACGATGAGGATAGCG CACCTAACATAAGGCAAG	7691–7708 8838–8821	U73374	52°C	1147	Moore and Lindsay (2001)
map F *map* R	TAACATTTAATAAGAATCAA CCATTTACTGCAATTGT	128–147 1076–1060	AJ223806	45°C	940	Peacock *et al.* (2002)
agr-1 F *agr*-1R	ATGCACATGGTGCACATGC GTCACAAGTACTATAAGCTG CGAT	1990–2008 2428–2405	X52543	55°C	439	Lina *et al.* (2003)
agr-2 F *agr*-2R	ATGCACATGGTGCACATGC TATTACTAATTGAAAAGTGCCATAGC	215–233 786–761	AF001782	55°C	572	Lina *et al.* (2003)
agr-3 F *agr*-3R	ATGCACATGGTGCACATGC GTAATGTAATAGCTTGTATAATAATACCCAG	215–233 535–505	AF001783	55°C	321	Lina *et al.* (2003)
agr-4 F *agr*-4R	ATGCACATGGTGCACATGC CGATAATGCCGTAATACCCG	932–950 1588–1569	AF288215	55°C	657	Lina *et al.* (2003)
hla F *hla* R	GGTTTAGCCTGGCCTTC CATCACGAACTCGTTCG	55–71 589–573	X55185	53°C	550	Booth *et al.* (2001)
hlb F *hlb* R	GCCAAAGCCGAATCTAAG GCGATATACATCCCATGG C	286–303 1116–1101	X61716	62°C	840	Booth *et al.* (2001)
eta F *eta* R	GCAGGTGTTGATTTAGCATT AGATGTCCCTATTTTTGCTG	775–794 867–848	M17347	57°C	93	Mehrotra *et al.* (2000)
etb F *etb* R	ACAAGCAAAAGAATACAGCG GTTTTTGGCTGCTTCTCTTG	509–528 734–715	M17348	57°C	226	Mehrotra *et al.* (2000)

Table 1. (continued)

Genes	Oligonucleotide Sequences	Location of primer	Accession number	Annealing and other temperature conditions*	Product size (bp)	References
sea F	GCAGGGAACAGCTTTAGGC	126–144	M18970	68°C 15 cycles	521	Løvseth *et al.*
sea R	GTTCTGTAGAAGTATGAAACACG	646–624		64°C 20 cycles		(2004)
seb F	ACATGTAATTTTGATATTCGCACTG	267–291	M11118	Same as for *sea*	667	Løvseth *et al.*
seb R	TGCAGGCATCATGTCATACCA	934–913				(2004)
*sec*F	CTTGTATGTATGGAGGAATAACAA	524–547	X05815	Same as for *sea*	284	Løvseth *et al.*
sec R	TGCAGGCATCATATCATACCA	807–787				(2004)
sed F	GTGGTGAAATAGATAGGACTGC	659–680	M28521	Same as for *sea*	385	Løvseth *et al.*
sed R	ATATGAAGGTGCTCTGTGG	1043–1025				(2004)
see F	TACCAATTAACTTGTGGATAGAC	446–468	M21319	Same as for *sea*	171	Løvseth *et al.*
see R	CTCTTTGCACCTTACCGC	616–599				(2004)
seg F	CGTCTCCACCTGTTGAAGG	317–335	AF064773	Same as for *sea*	328	Løvseth *et al.*
seg R	CCAAGTGATTGTCTATTGTCG	644–624				(2004)
seh F	CAACTGCTGATTTAGCTCAG	452–471	U11702	Same as for *sea*	359	Løvseth *et al.*
seh R	GTCGAATGAGTAATCTCTAGG	810–790				(2004)
sei F	CAACTCGAATTTTCAACAGGTACC	325–347	AF064774	Same as for *sea*	466	Løvseth *et al.*
sei R	CAGGCAGTCCATCTCCTG	790–773				(2004)
sej F	CATCAGAACTGTTGTTCCGCTAG	1381–1403	AF053140	Same as for *sea*	142	Løvseth *et al.*
sej R	CTGAATTTTACCATCAAAGGTAC	1522–1500				(2004)
tsst -1 F	GCTTGCGACAACTGCTACAG	63–82	J02615	Same as for *sea*	559	Løvseth *et al.*
tsst-1 R	TGGATCCGTCATTCATTGTTAT	623–601				(2004)

*Initial denaturation step (5 min at 94°C) followed by 30 cycles of amplification (denaturation for 30 s at 94°C, annealing temperature [given in table] for 30 s and elongation for 1 min at 72°C) terminated with a 5 min incubation step at 72°C.

X-region of protein A (*spa*), immunoglobulin-binding region (*Ig*), clumping factor (*clf*), collagen-binding protein (*cna*), capsular polysaccharide 5 and 8 (*cap* 5 and *cap* 8), major histocompatibility complex class II analogue protein (*map*), fibronectin-binding proteins A and B (*fnb*A and *fnb*B), accessory gene regulator alleles (*agr* I-IV) and α- and β-haemolysin (*hla* and *hlb*) were carried out using PCR. Oligonucleotide primers and annealing temperatures used for these genes are presented in table 1. The composition of the reaction mixture was the same in all PCR amplifications as explained for 16S rDNA gene.

The genes encoding elastin-binding protein (*ebp*), laminin-binding protein (*eno*), bone-sialoprotein-binding protein (*bbp*), fibrinogen-binding protein (*fib*), enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei* and *sej*), toxic shock syndrome toxin (*tsst*-1) and exfoliative toxins (*eta* and *etb*) were amplified using multiplex PCR (Mehrotra *et al.* 2000; Tristan *et al.* 2003; Løvseth *et al.* 2004). Strains of *S. aureus*, namely, *S. aureus* NCDC 109, *S. aureus* NCDC 110, *S. aureus* NCDC 133 and *S. aureus* NCDC 237, were used as controls for toxin genes. The annealing temperatures and conditions used are presented in table 1. The reaction mixture (25 µl) for multiplex PCR consisted of 2 µl dNTPs (200 µm/µl), 2.5 µl 10× *Taq* buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl of each forward and reverse oligonucleotide primer (25 pm/µl), 0.75 µl of *Taq* DNA polymerase (3 U/µl), 5 µl DNA (30 ng/µl) and the final volume was made with distilled water. The amplified PCR products were subjected to electrophoresis in 1.5% agarose gel containing 0.5 µg/ml ethidium bromide.

2.5 *PCR-RFLP of coagulase gene*

Primers used for *coa* gene analysis were selected from a previous study (Montesinos *et al.* 2002). The mixture for PCR was prepared in the same manner as for 16S rDNA gene amplification *vide supra*. Amplicons were digested with *Hae*III (Fermentas, India). In brief, PCR products (10 µl) were incubated with 10 units of restriction enzyme in 0.2 ml tubes at 37°C for 3 h. The generated fragments were separated by electrophoresis in 2.75% agarose containing 0.5 µg/ml ethidium bromide.

2.6 *Statistical analysis*

The Pearson χ^2 test was used to analyse the results of pathogenic genes amplification and antimicrobial resistance

Table 2. Antibiotic resistance in MRSA and MSSA isolates

Antibiotic groups	Antibiotic	Resistance (%) in MRSA (n=14)	Resistance (%) in MSSA (n=93)	Resistance (%) in total (n=107)	*agr* variants				*coa* variants					
					I (n=42)	II (n=29)	III (n=23)	IV (n=13)	A (n=38)	B (n=8)	C (n=13)	D (n=33)	E (n=2)	F (n=2)
β-Lactam	Methicillin	100	0.0	13.1	4.8	13.8	26.1	15.4	5.3	12.5	15.4	15.2	100.0	0.0
	Penicillin-G	100	18.3	28.9	23.8	34.5	30.4	30.8	34.2	37.5	30.8	21.2	100.0	0.0
	Cloxacillin	100	15.1	26.2	23.8	34.5	21.7	23.1	28.9	37.5	30.8	18.2	100.0	0.0
	Ampicillin	100	19.4	29.9	23.8	34.5	39.1	23.1	28.9	37.5	30.8	33.3	100.0	0.0
	Amoxicillin	100	12.9	24.3	23.8	27.6	21.7	23.1	34.2	25.0	38.5	9.1	100.0	0.0
Amino penicillin	Amoxicillin-clavulanate	71.4	10.8	18.7	16.7	20.7	21.7	15.4	28.9	12.5	23.1	12.1	50.0	0.0
	Amoxicillin-sulbactum	100	15.1	26.2	19.1	34.5	30.4	23.1	34.2	37.5	30.8	18.2	100.0	0.0
Cephalosporins	Cephalexin	71.4	7.5	15.9	14.3	10.3	26.1	15.4	23.7	12.5	23.1	9.1	50.0	0.0
	Cefixime	64.2	11.8	18.7	16.7	13.8	26.1	23.1	21.1	25.0	15.4	18.2	50.0	0.0
Aminoglycosides	Gentamicin	78.5	22.6	29.9	21.4	27.6	43.5	38.5	47.4	37.5	23.1	15.2	100.0	50.0
	Streptomycin	78.5	30.1	36.4	35.7	27.6	47.8	38.5	39.5	37.5	53.8	33.3	100.0	0.0
	Amikacin	71.4	21.5	28.0	21.4	20.7	39.1	46.2	34.2	37.5	38.5	18.2	100.0	0.0
	Kanamycin	71.4	31.2	36.4	38.1	31.0	43.5	30.8	44.7	25.0	69.2	21.2	100.0	50.0
Fluoroquinolones	Ciprofloxacin	71.4	19.4	26.2	19.1	20.7	43.5	30.8	39.5	37.5	23.1	12.1	50.0	50.0
	Ofloxacin	78.5	9.7	18.7	14.3	10.3	34.8	23.1	23.7	12.5	23.1	15.2	100.0	0.0
Lincosamide	Clindamycin	71.4	10.8	18.7	14.3	17.2	30.4	15.4	26.3	12.5	30.8	6.1	100.0	50.0
	Lincomycin	85.7	16.1	25.2	21.4	17.2	34.8	38.5	31.6	12.5	23.1	24.2	50.0	0.0
Streptogramins	Pristinomycin	71.4	19.4	26.2	23.8	17.2	39.1	30.8	34.2	50.0	30.8	15.2	50.0	0.0
Tetracyclines	Tetracycline	78.5	26.9	33.6	28.6	37.9	43.5	23.1	39.5	37.5	30.8	33.3	100.0	0.0
Rifampin	Rifampicin	85.7	16.1	25.2	19.1	20.7	39.1	30.8	34.2	37.5	23.1	18.2	50.0	0.0
Amphenicols	Chloramphenicol	85.7	17.2	26.2	21.4	20.7	39.1	30.8	31.6	37.5	23.1	21.2	50.0	0.0
Macrolide	Erythromycin	85.7	23.7	31.8	28.6	37.9	26.1	38.5	42.1	25.0	38.5	24.2	100.0	0.0
Glycopeptides	Vancomycin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 3. Distribution of pathogenic genes among the isolates

Genetic determinant of *S. aureus*	Genes studied		Toxin genes distribution									Haemolysin		Accessory gene regulation (*agr*)				MRSA isolates (*n*=14)	Total observations (*n*=107)
	Gene	Pattern	*seb* (*n*=1)	*sec* (*n*=6)	*sed* (*n*=1)	*seg* (*n*=5)	*sei* (*n*=46)	*sec*/*seg* (*n*=1)	*sec*/*sei* (*n*=2)	*seg*/*sei* (*n*=5)	* (*n*=0)	*hla* (*n*=107)	*hlb* (*n*=101)	I (*n*=42)	II (*n*=29)	III (*n*=23)	IV (*n*=13)		
Protein A (X-region)	*spa*	3 R	100.0	0.0	0.0	0.0	4.3	0.0	0.0	0.0	0.0	2.8	2.9	2.3	0.0	8.7	0.0	0.0	2.8
		4 R	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9	0.0	3.4	0.0	0.0	0.0	0.9
		7 R	0.0	0.0	0.0	20.0	15.2	0.0	0.0	20.0	0.0	17.8	14.8	9.5	27.6	13.0	30.8	14.3	17.8
		8 R	0.0	66.7	100.0	40.0	58.7	100.0	100.0	60.0	0.0	57.9	60.3	66.7	51.7	52.2	7	57.1	57.9
		9 R	0.0	33.3	0.0	40.0	19.6	0.0	0.0	0.0	0.0	15.9	14.5	16.7	17.2	17.4	7.7	28.6	15.9
		10 R	0.0	0.0	0.0	0.0	2.2	0.0	0.0	20.0	0.0	2.8	2.9	4.8	0.0	0.0	7.7	0.0	2.8
		11 R	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	1.9	0.0	0.0	8.7	0.0	0.0	1.9
Ig-binding protein	*Ig*	a	0.0	0.0	0.0	0.0	17.4	0.0	0.0	0.0	0.0	14.9	12.8	19.0	13.8	13.0	7.7	14.3	14.9
		b	100.0	66.7	0.0	80.0	56.5	100.0	100.0	80.0	0.0	55.1	56.4	47.6	62.1	52.2	69.2	42.9	55.1
		c	0.0	33.3	100.0	20.0	26.1	0.0	0.0	20.0	0.0	29.9	30.7	33.3	24.1	34.8	23.1	42.9	29.9
Clumping factor	*clf*	I	0.0	0.0	0.0	0.0	2.2	0.0	0.0	0.0	0.0	3.7	3.9	2.3	0.0	8.7	7.7	0.0	3.7
		II	0.0	66.7	0.0	60.0	76.1	100.0	100.0	100.0	0.0	70.1	71.3	71.4	82.8	52.2	69.2	64.3	70.1
		III	100.0	33.3	100.0	40.0	17.4	0.0	0.0	0.0	0.0	20.6	20.8	19.0	13.8	34.8	15.4	28.6	20.6
Coagulase	*coa*	A	0.0	16.7	0.0	60.0	50.0	0.0	0.0	60.0	0.0	35.5	37.6	26.2	48.3	30.4	46.2	28.6	35.5
		B	0.0	33.3	0.0	20.0	8.7	0.0	100.0	0.0	0.0	7.5	7.9	7.1	6.9	13.0	0.0	7.1	7.5
		C	100.0	33.3	0.0	0.0	8.7	0.0	0.0	0	0.0	12.1	11.9	11.9	10.3	17.4	7.7	14.3	12.1
		D	0.0	16.7	0.0	0.0	23.9	0.0	0.0	20.0	0.0	30.8	30.7	38.1	27.6	21.7	30.8	35.7	30.8
		E	0.0	0.0	100.0	0.0	2.2	0.0	0.0	0.0	0.0	1.9	1.9	0.0	0.0	8.7	0.0	14.3	1.9
		F	0.0	0.0	0.0	0.0	0.0	100.0	50.0	0.0	0.0	1.9	1.9	2.3	0.0	4.3	0.0	0.0	1.9
Elastin-binding protein	*ebp*		0.0	66.7	0.0	60.0	80.4	100.0	100.0	80.0	0.0	69.2	71.3	29	75.9	60.9	69.2	64.3	69.2
Laminin-binding protein	*eno*		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Fibrinogen-binding protein	*fib*		100.0	50.0	0.0	60.0	78.3	100.0	100.0	60.0	0.0	67.3	69.3	69.0	79.3	52.2	53.8	50.0	67.3
Bonesialo-binding protein	*bbp*		0.0	0.0	0.0	0.0	4.3	0.0	0.0	0.0	0.0	6.5	6.9	11.9	6.9	0.0	0.0	0.0	6.5
Fibronectin-binding proteins A	*fnb*A		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Fibronectin-binding proteins B	*fnb*B		100.0	0.0	0.0	20.0	23.9	0.0	0.0	20.0	0.0	20.6	21.8	16.7	13.8	30.4	30.8	28.6	20.6
Capsular polysaccharide 5	*cap5*		0.0	83.3	100.0	80.0	60.9	0.0	100.0	40.0	0.0	60.7	62.4	73.8	55.2	43.5	61.5	50.0	60.7
Capsular polysaccharide 8	*cap8*		100.0	16.7	0.0	0.0	40.4	100.0	0.0	20.0	0.0	26.2	25.7	14.3	27.6	43.5	30.8	42.9	26.2
MHC class II analogue protein	*map*		100.0	100.0	100.0	100.0	84.8	100.0	50.0	100.0	0.0	81.3	83.2	81.0	75.9	82.6	92.3	85.7	81.3
Collagen-binding protein	*cna*		0.0	16.7	0.0	0.0	8.7	0.0	0.0	40.0	0.0	8.4	8.9	4.8	3.4	21.7	7.7	7.1	8.4

**sea*, *see*, *seh*, *sej*, *tsst*-1, *eta* and *etb* genes; a, 500 bp; b: 1,000 bp; c, 1,100 bp; I, 950 bp; II, 1,000 bp; III, 1,100 bp; R=repeats in X-region; A, B, C, D, E and F=RFLP patterns of coagulase genes.

for significant association using SYSTAT 12. P-value≤0.05 was considered significant.

3. Results

3.1 *Identification and biochemical characterization*

Biochemical tests revealed 107 isolates of *S. aureus*. All these isolates were positive (100.0%) for catalase, bio-films and the presence of capsules. The isolates that were also positive for urease, the Vogues-Proskauer test, arginine hydrolysis, lysostaphin sensitivity, clumping factor, DNase, coagulase, β-haemolysin, α-haemolysin, slime production and TNase were in the proportions of 98.1, 63.5, 59.8, 96.2, 90.6, 86.9, 78.5, 77.5, 22.4, 65.4 and 57.0%, respectively.

3.2 *Antibiotic susceptibility*

Antibiotic-susceptibility assays revealed that, among the 107 isolates, 34 were susceptible to all the antibiotics used in this study. All the isolates (100.0%) were also susceptible to vancomycin. Higher resistance was observed to streptomycin (36.4% of the isolates), oxytetracycline (33.6%) gentamicin and ampicillin (29.9%), penicillin-G (28.9%), chloramphenicol, pristinomycin, ciprofloxacin (26.2% each), and rifampicin and lincomycin (25.2% each). However, some isolates were highly susceptible to cephalexin (84.1%), amoxicillin-clavulanate, ofloxacin and clindamycin (81.3%). Fourteen isolates were found to be methicillin-resistant, while the remaining (93) were methicillin-susceptible. Of the 14 MRSA isolates, only 10 were resistant to amoxicillin-clavulanate. The majority of the MRSA isolates showed resistance to cloxacillin, penicillin-G, chloramphenicol and rifampicin (table 2). MSSA isolates were also resistant to streptomycin (30.1% of the isolates), followed by oxytetracycline (26.9%), gentamicin (22.6%) and amikacin (21.5%), and they were also highly susceptible to cephalexin (92.5%), ofloxacin (90.3%), amoxicillin-clavulanate and clindamycin (89.2%). MRSA isolates were significantly (P<0.05) more resistant to different antibiotics than MSSA isolates. Statistical analysis revealed that the association between the antibiotics was highly significant (P<0.01).

3.3 *Detection of 16S rDNA, mecA and nuc genes*

The partial amplification of specific 16S rDNA confirmed that all the 107 isolates belonged to *S. aureus* and were also positive for the *nuc* gene. Of the 14 methicillin-resistant isolates, only 10 gave the band for *mec*A gene.

3.4 *Molecular characterization for pathogenic factors*

Molecular characterization revealed that the recovered isolates were positive for *fnb*A, *hla*, *eno*, *spa*, and *Ig*-binding genes (table 3). Amplification of the *spa* gene (X-region) showed seven amplicons of 3, 4, 7, 8, 9, 10 and 11 repeats (R). The most frequent number of repeats was eight (in 57.9% of the isolates) followed by seven (17.7%) and nine (15.8%). Polymorphic band patterns were also observed in *Ig*-binding (figure 1) and *clf* genes. The sizes of the amplicons for *Ig*-binding genes were 500 (in 14.9% of the isolates), 1000 (55.1%) and 1100 bp (29.9%) and for *clf*, 950 (3.7%), 1000 (70.0%) and 1100 bp (20.5%). All the isolates with more than 7R were found to express a large number of pathogenic factors when compared with other categories (table 4). Isolates with 3R, 4R, 7R, 8R, 9R, 10R and 11R were positive (100.0%) with four (*eno*, *fnb*A, *cap*8 and *map*), three (*eno*, *fnb*A and *cap5*), two (*eno* and *fnb*A), two (*eno* and *fnb*A), two (*eno* and *fnb*A), five (*eno*, *ebp*, *fib, fnb*A and *map*) and four (*eno*, *fnb*A, *cap*8 and *map*) genes, respectively. In addition, isolates consisting 3R, 9R, 10R and 11R were not positive for two (*bbp* and *cap*5), one (bbp), two (*cap*8 and *cna*) and five (*ebp*, *bbp*, *fnb*B, *cap*5 and *cna*) genes, respectively. The details of *spa* repeats and associations with different genes are presented in table 4. The majority of MRSA isolates revealed more than seven repeats in the *spa* gene (table 3).

The isolates were positive in high proportion for *ebp*, *eno* and *fib* as compared with those for *bbp* (table 3; figure 2). The distribution of accessory gene regulators I, II, III and IV in isolated bacterial strains was found to be 39.2, 27.1, 21.5 and 12.1%, respectively. The occurrence of pathogenic genes between MRSA and MSSA isolates was uneven (table 3). The percentage-wise distribution of pathogenic genes *cap*8 (42.9, 23.7), *fnb*B (28.6, 19.4), *map* (85.7, 80.6) and *coa* (100.0, 88.2) was greater in MRSA than in MSSA isolates. However, the occurrence of *bbp* (0.0, 7.5), *ebp* (64.3, 69.9), *fib* (50.0, 69.9), *clf* (92.9, 94.6), *cna* (8.6, 7.1) and *cap*5 (50.0, 62.4) genes was slightly lower in MRSA than in MSSA isolates. Table 5 shows that the antibiotic resistance was high in isolates that had the *hlb*, *ebp*, *fib* and *map* genes; whereas the proportion was less in isolates showing the presence of *cna*, *fnb*B and *bbp* genes. The overall presence of capsular genes (*cap*5 and *cap*8) in isolates was significantly associated with antibiotic resistance. However, category-wise antibiotic-resistance differences in isolates with *cap*5 and *cap*8 were non-significant (table 5). The presence of genes, namely, *fnb*A, *eno*, *hla* and *nuc*, did not show any relation to antibiotic resistance, because all the tested isolates were positive for these genetic determinants.

Among the 67 enterotoxin-producing isolates, 59 were involved in the production of only one type of enterotoxin

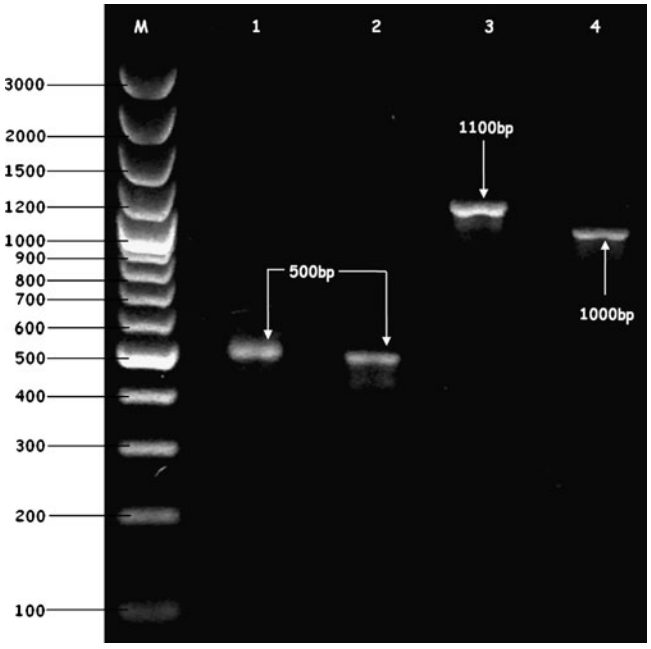

Figure 1. PCR amplicons of genes encoding for immunoglobulin-binding gene (*Ig*-binding). Lane M: 100-bp-molecular-size DNA ladder; lane 1 and 2: 500 bp amplicons; lane 3: amplicons of 1100 bp; lane 4: amplicons of 1050 bp.

(*seb* – 1, *sec* – 6, *sed* – 1, *seg* – 5 and *sei* – 46 isolates) as shown in figure 3. Only eight isolates amplified more than one enterotoxin gene (*sec*/*seg* – 1, *sec*/*sei* – 2 and *seg*/*sei* – 5 isolates). The enterotoxin *sei* was the most frequent among these isolates (42.9%). All the isolates lacked amplification of *sea*, *see*, *seh*, *sej*, *tsst*-1, *eta* and *etb* genes (table 3). Statistical analysis for association was found to be significant ($P<0.05$) between *fnb*B and *cna*, *hlb* and *map*, *ebp* and *cap5*, *hlb* and *ebp*, *clf* and *hlb*, *hlb* and *coa*, *fib* and *coa*, *ebp* and *sei*, *fib* and *sei*, *sei* and *mecA*, and *cna* and *seg*/*sei*.

3.5 *PCR-RFLP of coagulase gene*

The amplicons digested with *Hae* III generated six different RFLP patterns (A–F) as shown in figure 4. The number of fragments produced during digestion varied in size (50 to 850 bp). The RFLP patterns A and D (both account for 66.3%) dominated within the isolated population (table 3). The isolates with RFLP pattern F showed susceptibility for methicillin, penicillin-G, oxacillin, amoxicillin-clavulanate, amoxicillin, cloxacillin, lincomycin, pristinomycin and cephalexin, while isolates revealing RFLP pattern E were resistant to these antibiotics. The antibiotic-resistant isolates showed RFLP patterns A (38.4% of the isolates), C (10.9%) and D (35.6%). Statistical analysis showed that the association between coagulase and antibiotics, namely, amoxicillin, clindamycin, gentamicin, oxytetracycline, amoxicillin-clavulanate and ofloxacin was significant ($P<0.05$). The patterns A, C and E were associated with the pathogenic factors; whereas the B, E and F patterns were negative for the factors (table 4).

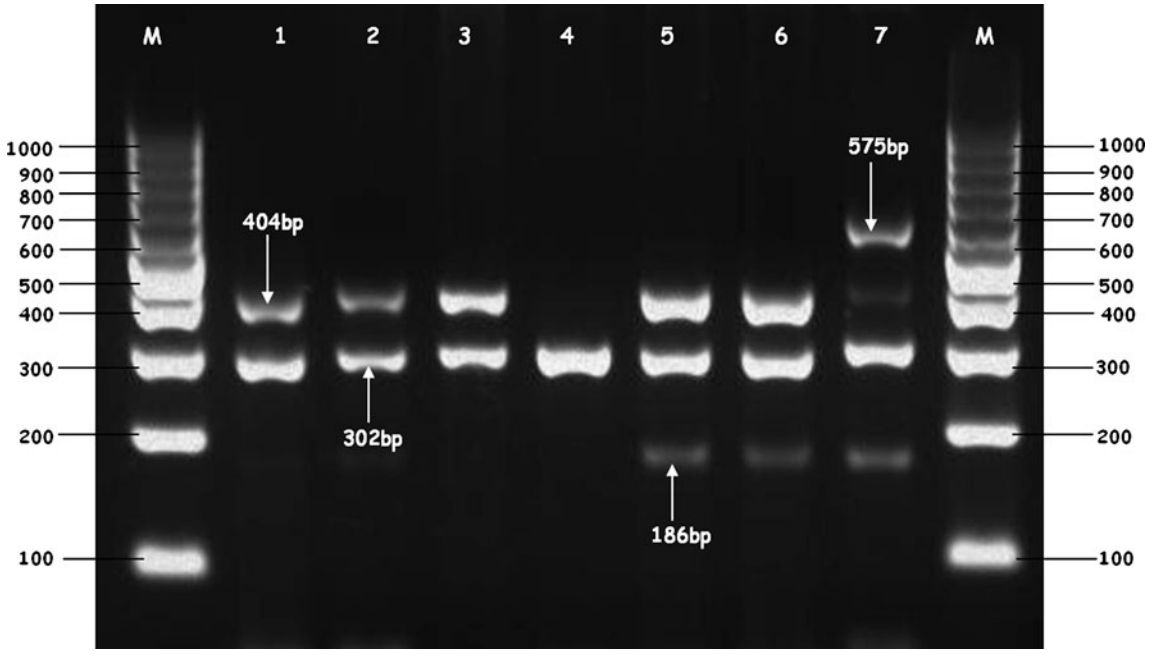

Figure 2. Multiplex PCR amplicons of genes encoding for *ebp* (186 bp), *eno* (302 bp), *bbp* (575 bp) and *fib* (404 bp). Lane M: 100-bp-molecular-size DNA ladder; lane 1–7: example of amplification of above mentioned genes.

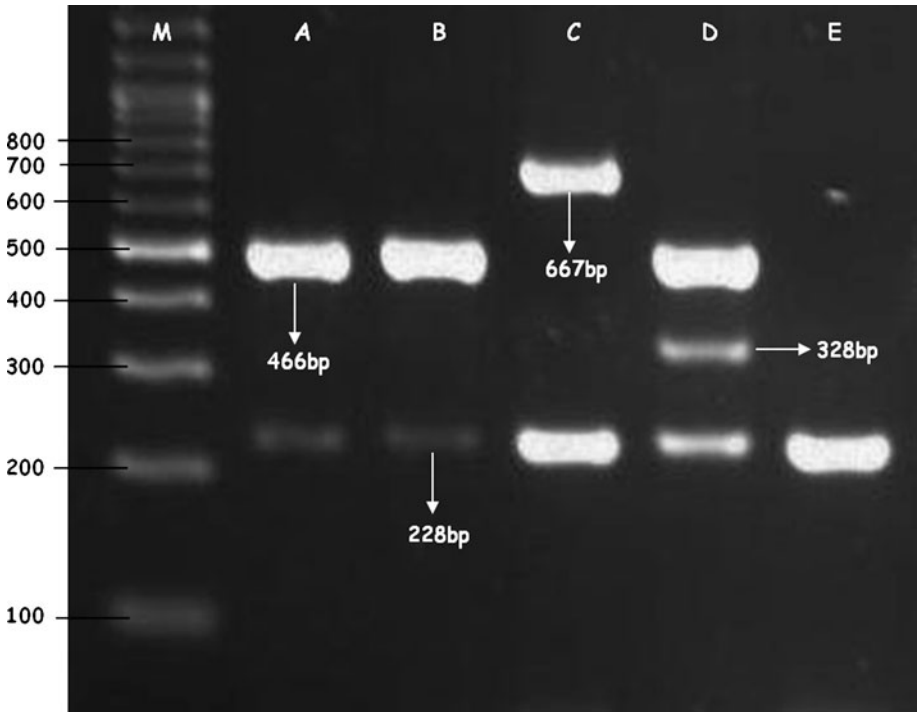

Figure 3. Multiplex PCR amplicons of toxin genes. Lane M: 100-bp-molecular-size DNA ladder; lane A–E: example of amplification of above toxin genes in isolated population. 228 bp, 16S-rDNA; 466 bp-*sei*; 667 bp, *seb*; 328 bp, *seg*.

4. Discussion

The present investigation on the distribution of MRSA, putative pathogenic genes and antibiotic-resistance of *S. aureus* isolates from mastitic cattle showed diverse biochemical characteristics. Mastitic *S. aureus* strains have been reported as expressing atypical characteristics (Aarestrup *et al.* 1999). The distribution of α, β-haemolysin and coagulase was in agreement with earlier findings (Aarestrup *et al.* 1999; Boerlin *et al.* 2003; Kumar *et al.* 2010). Identification of isolates was also confirmed by molecular assay because biochemical tests sometimes give false results or identities.

In the present study, the *S. aureus* isolates were typed for antibiotic resistance to obtain vital information that could help evolving a strategy for prevention and treatment of mastitis in cattle. A large number of isolates were observed to show resistance to multiple antibiotics (table 2). Appearance of resistance against a particular antibiotic in a specific region may be due to its frequent and long-term use (Sabour *et al.* 2004; Moon *et al.* 2007; Kumar *et al.* 2010). The results of the present study revealed that a significant number of isolates showed resistance to antibiotics (penicillin-G, gentamicin, streptomycin, ampicillin, ciprofloxacin, oxytetracycline, etc.) that are frequently used in mastitic animals (table 2). The proportion of penicillin-G-resistant isolates (29.0%) in this study was closer to those in American herds (Erskine *et al.* 2002; Makovec and Ruegg 2003) than European herds (Vintov *et al.* 2003). The occurrence of isolates resistant to streptomycin, lincomycin, cloxacillin, ciprofloxacin, pristinomycin and clindamycin (table 2) was less frequent than that observed by Wang *et al.* (2008) in Chinese bovine herds. However, it was slightly higher than that reported from India (Kumar *et al.* 2010), Argentina (Gentilini *et al.* 2000), Europe and the United States (De Oliveira *et al.* 2000). Such differences can be attributed to diverse antibiotics administrated during mastitis infection. There was a higher prevalence of MRSA (13.0%) as compared with those in similar reports in the literature (Lee 2003; Moon *et al.* 2007; Van den Eede *et al.* 2009; Kumar *et al.* 2010). Among MRSA isolates, four showed poor expression of *mec*A genes or production of methicillinase (alteration of PBP subtypes) or seemed to be over-producing β-lactamase, as these isolates remained susceptible to amoxicillin-clavulanate (Moon *et al.* 2007; Turutoglu *et al.* 2009; Kumar *et al.* 2010). Moreover, the resistant proportion was higher in MRSA than in MSSA isolates for various antibiotics, as the MRSA generally express resistance to multiple drugs (Wang *et al.* 2008; Kumar *et al.* 2010).

The molecular characterization of isolates also showed variations in pathogenic genes. Polymorphic patterns in protein A, *Ig*-binding, coagulase and clumping factor genes revealed significant genetic heterogeneity among *S. aureus*

Table 4. Association of protein-A and coagulase with other pathogenic genes

	spa gene repeats							*coa* variants					
Adhesins genes	3R (*n*=3)	4R (*n*=1)	7R (*n*=19)	8R (*n*=62)	9R (*n*=17)	10R (*n*=3)	11R (*n*=2)	A (*n*=38)	B (*n*=8)	C (*n*=13)	D (*n*=33)	E (*n*=2)	F (*n*=2)
ebp	33.3 §	0.0#	68.4‡	72.6‡	70.6‡	100.0*	0.0#	81.6†	62.5‡	76.9†	57.6‡	0.0#	100.0*
eno	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*
fib	66.7‡	0.0#	47.4 §	72.6‡	70.6‡	100.0*	50.0‡	86.8†	50.0‡	76.9†	51.5‡	0.0#	100.0*
bbp	0.0#	0.0#	15.8 §	4.8 §	0.0#	33.3 §	0.0#	7.9 §	0.0#	7.7 §	6.1 §	0.0#	0.0#
*fnb*A	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*	100.0*
*fnb*B	66.7‡	0.0#	15.8 §	17.8 §	23.5 §	66.7‡	0.0#	23.7 §	0.0#	38.4 §	18.2 §	50.0	0.0#
cap5	0.0#	100.0*	47.4 §	67.7‡	64.7‡	66.7‡	0.0#	65.8‡	50.0‡	61.5‡	57.6‡	100.0*	50.0‡
cap8	100.0*	0.0#	5.3 §	25.8 §	35.3 §	0.0#	100.0*	26.3 §	25.0	23.1 §	76.9†	0.0#	50.0‡
map	100.0*	0.0#	63.2‡	82.3†	94.1†	100.0*	100.0*	92.1†	87.5†	76.9†	72.7‡	100.0*	50.0‡
cna	33.3 §	0.0#	10.5 §	6.5 §	11.8 §	0.0#	0.0#	10.5 §	0.0#	30.8 §	3.0 §	0.0#	0.0#

*100% of the isolates of 3R, 4R, 7R, 8R, 9R, 10R and 11R of *spa* and A, B, C, D, E and F of *coa* were positive with 4, 3, 2, 2, 2, 5, 4, 2, 2, 2, 2, 4 and 4 different genes, respectively.

† 75–100% of the isolates of 8R and 9R of *spa* and A, B, C and D of *coa* were positive with 1, 1, 3, 1, 3 and 1 different genes, respectively.

‡ 50–75% of the isolates 3R, 7R, 8R, 9R, 10R and 11R of *spa* and A, B, C, D, E and F of *coa* were positive with 2, 2, 3, 3, 2, 1, 1, 3, 1, 4, 1 and 3 different genes, respectively.

§ 1–50% of the isolates 3R, 7R, 8R, 9R and 10R of *spa* and A, B, C and D of *coa* were positive with 2, 6, 4, 3, 1, 4, 1, 4 and 3 different genes, respectively.

0% of the isolates 3R, 4R, 9R, 10R and 11R of *spa* and B, E and F of *coa* were positive with 2, 7, 1, 2, 5, 3, 5 and 3 different genes, respectively.

Table 5. Association between antibiotic-resistant and pathogenic genes

Antibiotic	*hlb* (*n*=101)	*ebp* (*n*=74)	*fib* (*n*=72)	*map* (*n*=87)	*bbp* (*n*=07)	*cna* (*n*=09)	*cap*5 (*n*=65)	*cap*8 (*n*=28)	*fnb*B (*n*=22)	*eno* & *fnb*A (*n*=107)	Total observations (*n*=107)
Methicillin	13	9	07	12	00	01	07	06	04	14	14
Penicillin-G	29	20	18	28	02	02	18	11	08	31	31
Cloxacillin	26	18	16	25	02	02	16	11	07	28	28
Ampicillin	31	21	20	27	02	02	19	10	07	32	32
Amoxicillin	24	18	17	25	02	03	15	10	08	26	26
Amoxicillin-clavulanate	19	13	11	18	02	02	12	07	08	20	20
Amoxicillin-sulbactum	27	20	17	24	01	02	14	13	08	28	28
Cephalexin	16	10	09	16	02	02	10	06	07	17	17
Cefixime	19	13	13	18	00	03	11	07	06	20	20
Gentamicin	31	22	21	28	02	03	20	10	11	32	32
Streptomycin	37	25	24	33	03	03	25	10	13	39	39
Amikacin	28	18	16	24	01	02	17	10	10	30	30
Kanamycin	38	27	25	32	02	03	23	10	13	39	39
Ciprofloxacin	26	18	16	24	02	02	16	09	07	28	28
Ofloxacin	19	10	09	19	02	02	11	07	08	20	20
Clindamycin	20	13	12	18	01	02	13	06	07	20	20
Lincomycin	25	17	16	25	02	04	15	10	09	27	27
Pristinomycin	26	20	18	25	02	03	16	10	09	28	28
Tetracycline	35	25	23	30	03	02	23	11	10	36	36
Rifampicin	25	18	17	24	02	03	14	12	09	27	27
Chloramphenicol	26	19	17	24	02	02	16	11	08	28	28
Erythromycin	32	24	21	28	01	02	21	10	10	34	34
Vancomycin	00	00	00	00	00	00	00	00	00	00	00

isolates. Genetic variations in pathogenic genes were found in isolates of *S. aureus* in the herd studied, and similar observations within a single herd have also been reported earlier (Sabour *et al.* 2004). The variants of *spa* have been used to discriminate epidemic and non-epidemic MRSA and MSSA strains (Montesinos *et al.* 2002; Reinoso *et al.* 2008; Kumar *et al.* 2010). In the present study, *spa* variants also showed utility in distinguishing the isolates (table 4). Fragments of different sizes in *Ig* and *clf* were observed, which differed from those in earlier reports (Akineden *et al.* 2001; Salasia *et al.* 2004; Reinoso *et al.* 2008). Coagulase genotype differs with geographic area, and polymorphic patterns have been reported to discriminate the pathogenicity of strains of *S. aureus* (Phonimdaeng *et al.* 1990; Annemüller *et al.* 1999; Montesinos *et al.* 2002; Moon *et al.* 2007; Kumar *et al.* 2010). In the present study, six different RFLP patterns were observed, whereas in the study by Montesinos *et al.* (2002) only four patterns were reported. In addition, like the *spa* genes, *Ig*-binding, RFLP variants of coagulase genes were quite useful in distinguishing the isolates on the basis of pathogenesis and antibiotic resistance (tables 2 and 4).

Adhesin genes of *S. aureus* play a significant role in causing infection, colonization and invasion in the host (Peacock *et al.* 2002). All the isolates studied were found to be positive for *hla*, *eno*, *fnb*A and *nuc* genes expression. Similar observations have been reported for their significant role in *S. aureus* pathogenicity in cattle (Salasia *et al.* 2004; El-Sayed *et al.* 2006). Binding of *S. aureus* to fibroblasts and epithelial cells increase significantly with *fib* and *map*, while *ebp* enhances binding with peptides and tropoelastin (Palma *et al.* 1999; Zecconi *et al.* 2005). A significant number of isolates were positive for *ebp*, *fib* and *map* genes (table 3), and possibly these are essential for colonization and pathogenesis in mastitis. In these observations, only seven *S. aureus* isolates showed the *bbp* gene. Only a few isolates revealed genetic determinants of *fnb*B and *cna* genes (table 3); however, their role in mastitis could not be established. Earlier reports also did not show the influence of *fnb*B and *cna* in bovine mammary gland infection (Lammers *et al.*

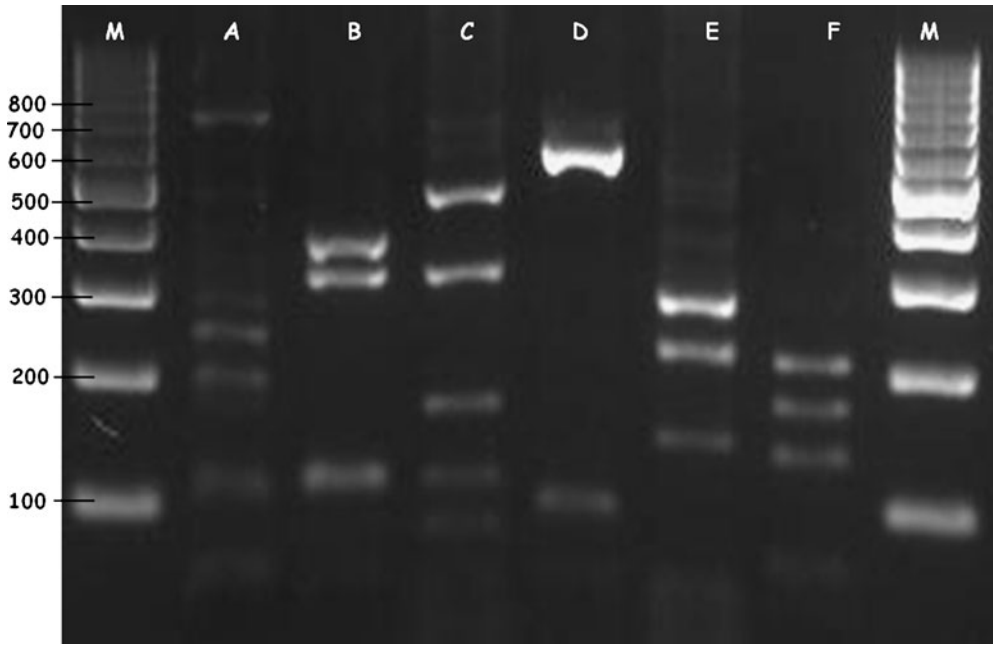

Figure 4. PCR-RFLP fragments of coagulase gene. Lane M: 100-bp-molecular-size DNA ladder; lanes show A–F patterns.

1999; Salasia *et al.* 2004; El-Sayed *et al.* 2006). Capsular types 5 and 8 predominated in isolates, and these results are in agreement with earlier studies (El-Sayed *et al.* 2006; Reinoso *et al.* 2008). The findings are also comparable with different forms of *agr* I to IV gene distribution as reported earlier (Gilot and van Leeuwen 2004; Reinoso *et al.* 2008). The relation of *agr* gene distribution to mastitic isolates still remains unclear. Significant association of *agr* variants with antibiotic resistance was not found in the present investigation (table 2).

The antibiotic-resistant isolates showed the presence of *hlb*, *ebp*, *fib* and *map*. A few pathogenic factors (*cna*, *fnb*B, *bbp*) were not significantly associated with the antibiotic resistance or were less frequent. Conversely, the antibiotic-susceptible isolates were also found to reveal these pathogenic factor genes (table 5). The presence or absence of these genes may have no relation to antibiotic-resistant or antibiotic-susceptibile aspects. Genetic determinants of antibiotics and pathogenic factors have not been reported to reside on the same loci (Brody *et al.* 2008; Fournier 2008). The expressions of pathogenic factors depend on the accessory gene regulator (*agr*) and the staphylococcal accessory regulator. The majority of antibiotic-resistant genes have been shown on reside mobile plasmids (Brody *et al.* 2008; Kumar *et al.* 2010). Capsular formations have been reported to contribute in pathogenesis in order to reduce antibiotic susceptibility (Seaman *et al.* 2004). The observations in the present study revealed the presence of capsular genes in both antibiotic-resistant and antibiotic-susceptible isolates. The association of antibiotics with *agr* and *coa* has been reported in isolates (Moon *et al.* 2007). The coagulase gene RFLP patterns (A, D and E) were found to be significantly associated with antibiotic resistance (table 2). Moreover, the expression of some pathogenic factors (*nuc*, *fnb*A and *hla*) did not show any correlations with antibiotics, as all isolates were positive for the presence of these genes. Similarly, the uneven presence of pathogenic factors in MRSA and MSSA indicated non- significant correlation (table 3).

The isolates tested for super-antigen toxins showed only enterotoxin genes (*seb*, *sec*, *sed*, *seg* and *sei*), and *sei* was most frequently encountered in this study. Toxins are supposed to modulate immune response through super-antigen activity and give rise to various diseases (Peacock *et al.* 2002). However, information is limited on the role of staphylococcal toxins in mastitis pathogenicity. Various enterotoxins, namely, *sea*, *seb*, *sec*, *sed*, *see*, *seh*, *seg*, *sei*, *sej* and *tsst*-1, have been reported among the *S. aureus* populations isolated from bovines (Akineden *et al.* 2001; Stephan *et al.* 2001; Salasia *et al.* 2004; Zschöck *et al.* 2004). All the isolates tested failed to amplify the *tsst*-1 gene, although its prevalence in bovine isolates has been reported in association with *sec* and *sed* (Akineden *et al.* 2001; Stephan *et al.* 2001; Salasia *et al.* 2004). Isolates also lacked the amplification *eta* and *etb* genes. The occurrences of exfoliative toxins have been rarely reported among *S. aureus* isolates from mastitic cattle (Akineden *et al.* 2001; Salasia *et al.* 2004; El-Sayed *et al.* 2006). Expression of toxin genes varies with location, and no specific reason has been reported

(Akineden *et al.* 2001; Stephan *et al.* 2001; Salasia *et al.* 2004; Zschöck *et al.* 2004).

In conclusion, the present study generated information on genotypic and antimicrobial typing of MRSA and MSSA isolates. The investigations revealed considerable variations in the prevalence of different adhesin and toxin genes among isolates. The unusual prevalence of bone-sialoprotein-binding protein and the absence of toxic shock syndrome toxins showed atypical characteristics of mastitic isolates. High prevalence of MRSA among mastitic cases is another significant finding. The uneven distribution of pathogenic factors between MRSA and MSSA emphasized the fact that molecular characterization is equally important in conjunction with antibiotic-susceptibility tests in order to distinguish the isolates. The prevalence of multiple-drug-resistant isolates of *S. aureus* among the mastitic cases needs special attention for the eradication of such infection. These findings could be useful in the treatment and segregation or culling in the case of mastitic animals.

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References

Aarestrup FM, Lartsen HD, Eriksen NH, Elsberg CS and Jensen NE 1999 Frequency of alpha- and beta-haemolysin in *Staphylococcus aureus* of bovine and human origin. A comparison between pheno- and genotype and variation in phenotypic expression. *APMIS* **107** 425–430

Akineden Ö, Annemüller C, Hassan AA, Lämmler C, Wolter W and Zschöck M 2001 Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. *Clin. Diagn. Lab. Immunol.* **8** 959–964

Annemüller C, Lammler CH and Zschöck M 1999 Genotyping *Staphylococcus aureus* isolated from bovine mastitis. *Vet. Microbiol.* **69** 217–224

Boerlin P, Kuhnert P, Hüssy D and Schaellibaum M 2003 Methods for identification of *Staphylococcus aureus* isolates in cases of bovine mastitis. *J. Clin. Microbiol.* **41** 767–771

Booth MC, Pence LM, Mahasreshti P, Callegan MC and Gilmore MS 2001 Clonal associations among *Staphylococcus aureus* isolates from various sites of infection. *Infect. Immun.* **69** 345–352

Brakstad OG, Aasbakk K and Maeland JA 1992 Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. *J. Clin. Microbiol.* **30** 1654–1660

Brody T, Yavatkar AS, Lin Y, Ross J, Kuzin A, Kundu M, Fann Y and Odenwald WF 2008 Horizontal gene transfers link a human MRSA pathogen to contagious bovine mastitis bacteria. *PLoS ONE* **3** e3074

Clinical and Laboratory Standards Institute 2008 *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals*.

De Oliveira AP, Watts JL, Salmon SA and Aarestrup FM 2000 Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and the United States. *J. Dairy Sci.* **83** 855–862

El-Sayed A, Alber J, Lämmler C, Jäger S, Wolter W and Castañeda-Vázquez H 2006 Comparative study on genotypic properties of *Staphylococcus aureus* isolated from clinical and sub clinical mastitis in Mexico. *Vet. Mex.* **37** 165–179

Erskine RJ, Walker RD, Bolin CA, Bartlett PC and White DG 2002 Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. *J. Dairy Sci.* **85** 1111–1118

Fitzgerald JR, Sturdevant DE, Mackie SM, Gill SR and Musser JM 2001 Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proc. Natl. Acad. Sci. USA* **98** 8821–8826

Fournier B 2008 Global regulators of *Staphylococcus aureus* virulence genes; in *Staphylococcus molecular genetics* (ed.) JA Lindsay (Norfolk: Caister Academic Press) pp 131–183

Gentilini E, Denamiel G, Llorente P, Godaly S, Rebuelto M and DeGregorio O 2000 Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Argentina. *J. Dairy Sci.* **83** 1224–1227

Gilot P and van Leeuwen W 2004 Comparative analysis of agr locus diversification and overall genetic variability among bovine and human *Staphylococcus aureus* isolates. *J. Clin. Microbiol.* **42** 1265–1269

Kumar R, Yadav BR and Singh RS 2010 Genetic Determinants of antibiotic resistance in *Staphylococcus aureus* isolates from milk of mastitic crossbred cattle. *Curr. Microbiol.* **60** 379–386

Lammers A, Nuijten JM and Smith E 1999 The fibronectin binding proteins of *Staphylococcus aureus* are required for adhesion to and invasion of bovine mammary gland cells. *FEMS Microbiol. Lett.* **180** 103–109

Lee JH 2003 Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Appl. Environ. Microbiol.* **69** 6489–6494

Lina G, Boutite F, Tristan A, Bes M, Etienne J and Vandenesch F 2003 Bacterial competition for human nasal cavity colonization: role of staphylococcal agr alleles. *Appl. Environ. Microbiol.* **69** 18–23

Løvseth A, Loncarevic S and Berdal K G 2004 Modified multiplex PCR method for detection of pyrogenic exotoxin genes in staphylococcal isolates. *J. Clin. Microbiol.* **42** 3869–3872

Makovec JA and Ruegg PL 2003 Antimicrobial resistance of bacteria isolated from dairy cow milk samples submitted for bacterial culture: 8905 samples (1994–2001). *J. Am. Vet. Med. Assoc.* **222** 1582–1589

Mehrotra M, Wang G and Johnson WM 2000 Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins

exfoliative toxins toxic shock syndrome toxin 1 and methicillin resistance. *J. Clin. Microbiol.* **38** 1032–1035

Montesinos I, Salido E, Delgado T, Cuervo M and Sierra A 2002 Epidemiological genotyping of methicillin resistant *Staphylococcus aureus* by pulsed field gel electrophoresis at a university hospital and comparison with antibiotyping and protein A and coagulase gene polymorphisms. *J. Clin. Microbiol.* **40** 2119–2125

Moon JS, Lee AR, Kang HM, Lee ES, Kim MN, Paik YH, Park YH, Joo YS and Koo HC 2007 Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. *J. Dairy Sci.* **90** 1176–1185

Moore PCL and Lindsay JA 2001 Genetic variation among hospital isolates of methicillin-sensitive *Staphylococcus aureus*: evidence for horizontal transfer of virulence genes *J. Clin. Microbiol.* **39** 2760–2767

Murakami K, Minamide W, Wada K, Nakamura E, Teraoka H and Watanabe S 1991 Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. *J. Clin. Microbiol.* **29** 2240–2244

Palma M, Haggar A and Flock JI 1999 Adherence of *Staphylococcus aureus* Is enhanced by an endogenous secreted protein with broad binding activity. *J. Bacteriol.* **181** 2840–2845

Peacock SJ, Moore CE, Justice A, Kantzanou M, Story L, Mackie K, O'Neill G and Day NPJ 2002 Virulent combinations of adhesins and toxin genes in natural populations of *Staphylococcus aureus*. *Infect. Immun.* **70** 4987–4996

Phonimdaeng P, O'Reilly M, Nowlan P, Bramley AJ and Foster TJ 1990 The coagulase of *Staphylococcus aureus* 8325–4 sequence analysis and virulence of site-specific coagulase deficient mutants. *Mol. Microbiol.* **4** 393–404

Reinoso EB, El-Sayed A, Lämmler C, Bognia C and Zschöck M 2008 Genotyping of *Staphylococcus aureus* isolated from humans bovine subclinical mastitis and food samples in Argentina. *Microbiol. Res.* **163** 314–322

Sabour PM, Gill JJ, Lepp D, Pacan JC, Ahmed R, Dingwell R and Leslie K 2004 Molecular typing and distribution of *Staphylococcus aureus* isolates in Eastern Canadian dairy herds. *J. Clin. Microbiol.* **42** 3449–3455

Salasia SIO, Khusnan Z, Lämmler C and Zschöck M 2004 Comparative studies on pheno- and genotypic properties of *S. aureus* isolated from bovine subclinical mastitis in central java in Indonesia and Hesse in Germany. *J. Vet. Sci.* **5** 103–109

Seaman P, Day M, Denver Russell A and Ochs D 2004 Susceptibility of capsular *Staphylococcus aureus* strains to some antibiotics, triclosan and cationic biocides. *J. Antimicrob. Chemother.* **4** 696–698

Seki K, Sakurada J, Seong HK, Murai M, Tachi H, Ishii H and Masuda S 1998 Occurrence of coagulase serotype among *Staphylococcus aureus* strains isolated from healthy individuals-special reference to correlation with size of protein-A gene. *Microbiol. Immunol.* **42** 407–409

Stephan R, Annemüller C, Hassan AA and Lämmler C 2001 Characterization of enterotoxigenic *Staphylococcus aureus* strains isolated from bovine mastitis in north-east Switzerland. *Vet. Microbiol.* **78** 373–382

Tristan A, Ying L, Bes M, Etienne J, Vandenesch F and Lina G 2003 Use of multiplex PCR to identify *Staphylococcus aureus* adhesins involved in human hematogenous infections. *Clin. Microbiol.* **41** 4465–4467

Turutoglu H, Hasoksuz M, Ozturk D, Yildirim M and Sagnak S 2009 Methicillin and aminoglycoside resistance in *Staphylococcus aureus* isolates from bovine mastitis and sequence analysis of their *mec*A genes. *Vet. Res. Commun.* **33** 945–956

Van den Eede A, Martens A, Lipinska U, Struelens M, Deplano A, Denis O, Haesebrouck F, Gasthuys F and Hermans K 2009 High occurrence of methicillin-resistant *Staphylococcus aureus* ST398 in equine nasal samples. *Vet. Microbiol.* **133** 138–144

Vintov J, Aarestrup FM, Zinn CE and Olsen JE 2003 Association between phage types and antimicrobial resistance among bovine *Staphylococcus aureus* from 10 countries. *Vet. Microbiol.* **95** 133–147

Wang Y, Wu CM, Lu LM, Ren GWN, Cao XY and Shen JZ 2008 Macrolide-lincosamide-resistant phenotypes and genotypes of *Staphylococcus aureus* isolated from bovine clinical mastitis. *Vet. Microbiol.* **130** 118–125

Zecconi A, Binda E, Borromeo V and Piccinini R 2005 Relationship between some *Staphylococcus aureus* pathogenic factors and growth rates and somatic cell counts. *J. Dairy Res.* **72** 203–208

Zschöck M, Risse K and Sommerhauser J 2004 Occurrence and clonal relatedness of sec/tst-gene positive *Staphylococcus aureus* isolates of quarter milk samples of cows suffering from mastitis. *Lett. Appl. Microbiol.* **38** 493–498

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