



Methicillin-resistant staphylococci in clinical bovine mastitis: occurrence, molecular analysis, and biofilm production

Silvia Prezioso¹ · Anna-Rita Attili¹ · Vincenzo Cuteri¹

Received: 10 May 2023 / Accepted: 23 November 2023 / Published online: 1 December 2023
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Abstract

Staphylococcus aureus is an important pathogen that causes mastitis in cattle, and the emergence of methicillin-resistant *S. aureus* (MRSA) poses a threat to veterinary and human medicine. The aims of the study were to investigate the prevalence of MRSA and methicillin-resistant coagulase-negative staphylococci (MR-CoNS) isolated from clinical mastitis, their ability to form biofilms, and the antimicrobial susceptibility of *S. aureus* strains. In addition, the Staphylococcal Cassette Chromosome mec (SCCmec) type, *spa* type and the presence of Pantone-Valentine Leucocidin in MRSA were evaluated. A total of 326 staphylococcal strains were screened by multiplex-PCR for *S. aureus* and *Staphylococcus intermedius* group (SIG) identification. The *S. aureus* strains ($n = 163$) were subjected to phenotypic testing for antimicrobial susceptibility and biofilm formation. Molecular analysis was performed on MRSA *mecA*-positive strains. Of 163 *S. aureus* isolates, 142 strains (87.1%) were resistant to at least one antibiotic, and all 19 MRSA strains were resistant to at least four out of five antibiotics tested. All *S. aureus* strains harboured the *icaA* gene and were biofilm producers. Nineteen MR-CoNS strains were also isolated. The most prevalent *spa* types among MRSA were t001 (57.9%) and t037 (31.6%), while one MRSA was type t008 and one was type t041. Most MRSA were SCCmec type I (63.2%) and III (31.6%) and only one strain was type IV. None of the MRSA isolates had the PVL gene. The prevalence of multidrug-resistant *S. aureus* in bovine mastitis is a serious concern. The finding of MRSA with *spa* types predominant in humans and infrequent in Italian cows and with SCCmec infrequently found in bovine milk or cheese suggest a human origin of these strains. The ability of MRSA and MR-CoNS involved in bovine mastitis to be transferred to humans and vice versa poses a public health concern.

Keywords *Staphylococcus aureus* · cows · Milk · MRSA · Typing · Biofilms

Introduction

Bovine mastitis is an inflammation of the mammary gland, which is the most predominant disease in the dairy industry and causes major economic losses (Zaatout et al. 2020). *Staphylococcus aureus* has been reported to be one of the most relevant causative agents of bovine mastitis (Côté-Gravel and Malouin 2019). Its main reservoir seems to be

the infected quarter, and its transmission between cows usually occurs during milking procedure (Monistero et al. 2018).

Staphylococcus spp. are common commensals of humans and animals, and important causes of opportunistic infections. In humans, *S. aureus* is the most important pathogen among the various *Staphylococcus* species and is the cause of widespread opportunistic and foodborne infections. Similarly, it is an important cause of infection of various animal species, including cattle. Other staphylococci are of varying clinical relevance, ranging from essentially harmless commensal to potentially important cause of disease. Moreover, *S. pseudintermedius* and other staphylococci of the *Staphylococcus intermedius* group (SIG) are important staphylococcal pathogens in animals and their phenotypic/genotypic resistance characterization is of great importance for dairy farming. Coagulase-negative staphylococci (CoNS), while typically considered to have limited pathogenicity, can cause disease under certain circumstances.

✉ Vincenzo Cuteri
vincenzo.cuteri@unicam.it

Silvia Prezioso
silvia.prezioso@unicam.it

Anna-Rita Attili
annarita.attili@unicam.it

¹ School of Biosciences and Veterinary Medicine - University of Camerino, Via Circonvallazione, 93/95, 62024 Matelica, MC, Italy

A common problem with all staphylococci is the tendency to acquire resistance to antimicrobials. Most of the attention has been paid to *S. aureus*, particularly with the emergence of methicillin-resistant *S. aureus* (MRSA), first in humans then in various animal species (Lee 2006). Methicillin-resistance in all staphylococci is due to the presence of the chromosomal *mecA* gene encoding a penicillin-binding protein with low affinity for β -lactams, resulting in resistance to virtually all beta-lactam antimicrobials. *mecA* resides on a Staphylococcal Cassette Chromosome *mec* (*SCCmec*), of which 8 types (I–VIII) are currently described (Rajeswari Anburaj and Raja 2020). The main route of transmission of MRSA is person-to-person, but pathogen cycling between humans and animals has been repeatedly reported (Weese et al. 2005; Rossi et al. 2016). The possibility of an interspecies transmission is a public health concern, which is clearly manifested by the increasing number of epidemiological studies of MRSA in animal populations reported in recent years (Kløve et al. 2022).

The occurrence and transmission of antimicrobial-resistant *S. aureus* itself or its genes has been suggested as one of the reasons for difficulty in antibiotic therapy. Therefore, the determination of susceptibility or resistance of strains to antibiotics is very important from a clinical and economic point of view. Moreover, this issue is of great public health importance because antibiotic treatment of infectious diseases in animals carries the risk of selection of resistant strains and introduction of these strains into the food chain and the environment, particularly the aquatic environment (White and McDermott 2001; Silva et al. 2023). MRSA detection and containment, associated with mastitis in dairy herds, is therefore essential. Epidemiological studies on MRSA associated with mastitis are documented (Lee 2006; Feltrin et al. 2016; Asadollahi et al. 2018). Likewise, reports on methicillin resistance in CoNS associated with mastitis have been demonstrated due to *S. chromogenes*, *S. epidermidis* and *S. sciuri* (Mello et al. 2020; Song et al. 2020; Silva et al. 2014; Devriese et al. 2002), and other CoNS, recently (Klibi et al. 2018; de Oliveira et al. 2022; Ibrahim et al. 2022).

Eradication of these microorganisms is also not always successful due to their ability to form biofilms. Extensive research concerning biofilm formation has been carried out using *S. epidermidis* as a model organism for staphylococci. There are several differences between *S. epidermidis* and *S. aureus*, with respect to biofilm formation. Most *S. aureus* strains reported so far contain the entire *ica* gene cluster (Cramton et al. 2001; Knobloch et al. 2002). The implication of biofilms in chronic infections has triggered an increasing interest in the characterization of genes involved in biofilm formation (Tormo et al. 2005).

The main objectives of this research work were (i) to evaluate the prevalence of methicillin-resistant staphylococci in milk sample of cows with clinical mastitis, (ii) to investigate the *icaA* gene in biofilm producer staphylococci strains, (iii)

to determine the acquired multidrug resistance in *S. aureus*, and finally, (iv) to characterize the *SCCmec* of MRSA strains.

Materials and methods

Animals, samples, and bacterial strains isolation

According to National Mastitis Council (NMC) guidelines, a total of 813 quarter milk samples were aseptically collected from Holstein–Friesian dairy cows with clinical mastitis in 8 different farms of Marche and Umbria regions, Central Italy, during 2019 – 2020, including farm 1 (in the Macerata province; herd size ≥ 100 ; milking frequency, 2 times per day; $n = 51$ samples), farm 2 (in Macerata province; herd size ≥ 100 ; milking frequency, 2 times per day; $n = 54$ samples), farm 3 (in the Macerata province; herd size ≥ 200 ; milking frequency, 3 times per day; $n = 47$ samples), farm 4 (in the Ancona province; herd size ≥ 400 ; milking frequency, 3 times per day; $n = 110$ samples), farm 5 (in the Pesaro-Urbino province; herd size ≥ 700 ; milking frequency, 3 times per day; $n = 201$ samples), farm 6 (in the Perugia province; herd size ≥ 700 ; milking frequency, 3 times per day; $n = 225$ samples), farm 7 (in the Perugia province; herd size ≥ 100 ; milking frequency, 2 times per day; $n = 43$ samples), farm 8 (in the Terni province; herd size ≥ 200 ; milking frequency, 2 times per day; $n = 82$ samples). All the randomly chosen lactating dairy cows were examined using clinical inspection of the udder as described by Quinn et al. (2002) and California Mastitis Test (CMT). For milk collection, the udders of cows with clinical mastitis were cleaned with water and dried. Cotton balls with 75% ethanol were used to disinfect the surface of the udder. The first few streams of milk were discarded. The collected milk, from each quarter, was kept in a sterile tube and transported refrigerated to the laboratory within 4 h.

Each specimen was cultured according to standard protocols plating 10 μ l on Columbia Blood agar and Mannitol Salt agar (Liofilchem®, Italy), and incubated at 36 ± 1 °C in aerobic atmosphere for 24–48 h. All presumptive staphylococci were tested for coagulase (Staphylase Test kit, Oxoid) and identified by using the MALDI-TOF MS (SOP Direct Transfer Procedure Revision.4; Bruker Microflex Lt®, Bruker Daltonics, Germany).. Mass spectra were processed using Flex Analysis (version 3.4; Bruker Daltonics, Germany) and BioTyper software (version 3.1; Bruker Daltonics, Germany); The row spectra obtained, were compared with those in the Biotyper database and a log (score) of ≥ 2.0 indicated a secure genus identification and a highly probable species-level identification.

Genotypic identification

A molecular identification of strains was performed by amplifying the *nuc* gene of *S. aureus* and SIG using

species-specific oligonucleotide primers (Baron et al. 2004). The *mecA* (Strommenger et al. 2003) and *icaA* (Zmantar et al. 2008) primers were used to detect the methicillin-resistant gene and a biofilm gene, respectively, by PCR. The oligonucleotide primers were used together with an internal positive control that targets a highly conserved region of 16S rDNA in a multiplex PCR (Baron et al. 2004). ATCC 33591 was used as MRSA control strain.

The oligonucleotide primer sequences, and PCR programs are summarized in Table 1. For DNA preparation, three colonies of freshly sub-cultured strains were suspended in 50 µl lysozyme 5 U/µl (Sigma, Germany). After incubation for 1 h at 37 °C, 0.75 µl proteinase K 20 µg/µl (Sigma, Germany) was added, and the suspension was re-incubated for 1 h at 56 °C. Finally, the proteinase K was inactivated through boiling of the mixture for 10 min followed by 5 min cooling on ice. After centrifugation at 8000×g for 10 min the supernatant was used for PCR.

The PCR reaction mixture (30 µl) contained 1.4 µl, 1.7 µl, 1.6 µl, 1.5 µl and 0.3 µl of *SInuc*, *SANuc*, *mecA*, *icaA* and *16S* primers (10 pmol/µl) respectively (Table 1), 15 µl of Taq PCR mastermix 2x (Qiagen, Germany), 2 µl of MgCl₂ 25 mM (Sigma, Germany). Finally, 1 µl DNA preparation was added to each reaction tube. The tubes were then subjected to thermal cycling with the following programs: 1 × (95 °C 300 s), 35 × (94 °C 60 s, 55 °C 60 s, 72 °C 60 s), 1 × (72 °C 420 s). The presence of PCR products was determined by electrophoresis of 10 µl of reaction products in a 3% agarose gel (Qbiogene, Germany) with Tris–acetate EDTA buffer (TAE, 4.0 mmol/l Tris–HCl 1 mmol/l EDTA, pH 8.0) and visualized under UV light (Gel Logic 100, Imaging system). The primers used in this study are reported in Table 1.

Panton Valentine leukocidin (PVL) gene screening Real time PCR for PVL with primers PVLSC-F, GCTCAGGAG ATACAAG and PVLSC-R, GGATAGCAAAGCAATG

was performed on MRSA strains as described previously (Roberts et al. 2005). The Sybr Green PCR reaction was carried out by using the kit Premix Ex Taq DNA Polymerase (Takara Bio, Japan). According to the manufacturer's instructions, the 20 µl mixture contained 10 µl of Premix Ex Taq 2x, 1 µl of each 10 pmol/µl primer, 6 µl of PCR water and 2 µl of DNA. The cycling conditions were as follows: 98 °C for 5 min, 30 cycles of amplification (15 s of denaturation at 95 °C, 5 s of annealing at 55 °C and 10 s of extension at 72 °C). The melt curve analysis was carried out as follows: 5 s at 95 °C, 15 s at 65 °C, then ramp from 65 to 95 °C at 0.1 8C/s.

spa typing

The *S. aureus* isolates resulted MRSA, were used for *spa* typing. The SSR region of the *spa* gene was amplified and sequenced according to a method described by Shopsin et al. (1999). Sequences were analyzed by the eGenomics software (<http://tools.egenomics.com>) and were reported using a numerical system that corresponded to a *spa* type sequence. Ridom database equivalents were identified by the Ridom Spaserver website (<http://www.spaserver.ridom.de>) and were reported using a numerical coding system preceded by a 't'.

Characterization of SCCmec

The presence of *SCCmec* types I – IV was determined in MRSA by multiplex PCR as previously described (Zhang et al. 2005). In particular, the PCR reaction mix included two pairs of primers each for characterization of the *mec* gene (*mecI*-F, *mecI*-R, IS1272-F and *mecR*1-R) and of the *ccr* gene (*ccrAB*-2, *ccrAB*-3, and *ccrAB*-4) (Zhang et al. 2005). The PCR reaction mixture contained 25 µl of Taq PCR mastermix 2x (Qiagen, Germany), 0.08 µM of each primer, 2 µl of DNA and PCR water up to a final volume of 50 µl. The reaction

Table 1 Primers used in this study for identification of *S. aureus* and *SIG* and for detection of *mecA* and *icaA* genes

Oligonucleotide primer	Sequence	Size of the PCR Product	Genbank Accession no. (reference)
<i>SANuc1</i>	5' TGCTATGATTGTGGTAGCCATC 3'	420	CP001844, bp1356038-1356059
<i>SANuc 2</i>	3' TCTCTAGCAAGTCCCTTTTCCA 5'		CP001844, bp 1356457–1356436
<i>SInuc 1</i>	5' CAATGGAGATGGCCCTTTTA 3'	125	X67678, bp 558–577
<i>SInuc 2</i>	3' AGCGTACACGTTTCATCTTG 5'		X67678, bp 687–669
<i>16S1</i>	5' GGACGGGTGAGTAACACGTGG 3'	250	GQ406605, bp 50–70
<i>16S2</i>	3' TCCCGTAGGAGTCTGGACCGT 5'		GQ406605, bp 301–281
<i>mecA1</i>	5' AAAATCGATGGTAAAGGTTGGC 3'	532	Y00688, bp 1282–1303
<i>mecA2</i>	3' AGTTCTGCAGTACCGGATTTGC 5'		Y00688, bp 1814–1793
<i>icaAF</i>	5' ACACTTGCTGGCGCAGTCAA 3'	188	AF086783, bp 2906–2925
<i>icaAR</i>	3' TCTGGAACCAACATCCAACA 5'		AF086783, bp 3093–3074

condition were: 94 °C for 5 min followed by 10 cycles of 94 °C for 45 s, 65 °C for 45 s, and 72 °C for 90 s and another 25 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1.5 min, ending with a final extension step at 72 °C for 10 min and followed by a hold at 4 °C. The PCR amplicons were visualized using a UV light box after electrophoresis on a 2% agarose gel.

Congo red agar method

The Congo Red Agar method (CRA) for phenotypic identification of biofilm production was done according to the Freeman protocol (Knobloch et al. 2002). Studies have demonstrated that this method has low accuracy, but it was chosen as support for PCR test (*icaA* gene detection) because is cheap and easy to perform and the evaluation criteria is based on visual analysis of the colour of the colonies that grow on the agar. The constituents of media were, Brain Heart Infusion broth (Oxoid, Milan, Italy) 37 g/l, sucrose (Carlo Erba Reagents, Milan, Italy) 50 g/l, Agar N. 1 (Oxoid, Milan, Italy) 10 g/l and Congo Red stain (Carlo Erba Reagents, Milan, Italy). A quantity of 0.8 g/l Congo Red stain was prepared as a concentrated aqueous solution, autoclaved separately and added to the media when the agar had cooled to 55 °C. Plates of the medium were inoculated and incubated aerobically for 24 h at 37 °C. Biofilm-producing strains generated black colonies, which correspond to high biofilm production or brown colonies, which was considered evidence of low biofilm production, while biofilm-negative strains were red or pink.

Antimicrobial susceptibility testing

S. aureus strains were tested for susceptibility to a panel of five antimicrobials using the disc agar diffusion (Kirby-Bauer) method on Müeller Hinton agar (Liofilchem®, Italy) following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. The selected antibiotics, the most widely used for the treatment of clinical mastitis in the regions where the farms were located, were as follows: oxytetracycline (30 µg), gentamicin (10 µg), erythromycin (10 µg), clindamycin (10 µg) and ceftiofur (30 µg). The strains were classified as susceptible or resistant according to Clinical Breakpoint EUCAST Guidelines (EUCAST version 12.0, 2022).

Results

Totally, 326 *Staphylococcus* spp. were identified from the collected bovine milk samples; of these, 163 *S. aureus* (50%) and 19 SIG (5.8%) were identified. The remaining 144 staphylococci (44.2%), even if were speciated, were all considered coagulase-negative staphylococci (Table 2).

Nineteen (11.7%) out of 163 *S. aureus* isolates possessed *mecA* gene and were all phenotypically resistant to ceftiofur. All SIG isolates were *mecA* negative and 19 (13.2%) of the CoNS strains were *mecA* positive and considered methicillin-resistant (MR-CoNS). All *S. aureus* strains isolated from clinical mastitis harboured the *icaA* gene.

In CRA, 129 (79.1%) *S. aureus* produced black colonies, which correspond to high biofilm production and 34 (20.9%) produced brown colonies, which is considered evidence of weak biofilm production. The *icaA* gene was detected in 30 (20.8%) CoNS isolates. Twenty-five of them belong to the black colonies group and 5 to the brown colonies group. None of the SIG strains showed the *icaA* gene. All 19 MRSA isolates and 18 out 19 MR-CoNS produced black colonies on CRA.

S. aureus susceptibility data were presented in Table 3. Only 21 strains (12.9%) were susceptible to all the antibiotics. All 19 MRSA isolates were resistant to at least 4 of the tested antimicrobials and 9 (47%) were resistant to 5. Four isolates were resistant to clindamycin, erythromycin, gentamicin and oxytetracycline and one isolate to clindamycin, erythromycin, and gentamicin (Table 3).

Eleven MRSA isolates were *spa* type 385/t001 and *SCCmecI*, 6 were *spa* type 3/t037 and *SCCmec III*, 1 was *spa* type 388/t041 and *SCCmec I* and 1 strain was *spa* type 1/t008 and *SCCmecIV* (Table 4). All the MRSA strains were negative for the PVL toxin encoding gene.

Discussion

Staphylococcus spp. play an important role in causing clinical mastitis in cattle. Understanding prevalence and mechanisms of antimicrobial resistance and biofilm production is

Table 2 Frequency of coagulase-negative *Staphylococcus* spp. (CoNS) isolated during 2019–2020 in Central Italy

CoNS	Frequency N (%)
<i>Staphylococcus chromogenes</i>	38 (26.4%)
<i>Staphylococcus haemolyticus</i>	27 (18.7%)
<i>Staphylococcus arlettae</i>	23 (15.94%)
<i>Staphylococcus sciuri</i> ssp. <i>sciuri</i>	8 (5.52%)
<i>Staphylococcus simulans</i>	7 (4.9%)
<i>Staphylococcus sciuri</i> ssp. <i>carnaticus</i>	6 (4.16%)
<i>Staphylococcus xylosus</i>	6 (4.16%)
<i>Staphylococcus succinus</i> ssp. <i>succinus</i>	6 (4.16%)
<i>Staphylococcus hyicus</i>	6 (4.16%)
<i>Staphylococcus equorum</i> ssp. <i>equorum</i>	5 (3.5%)
<i>Staphylococcus epidermidis</i>	3 (2.1%)
<i>Staphylococcus auricularis</i>	3 (2.1%)
<i>Staphylococcus capitis</i> ssp. <i>capitis</i>	3 (2.1%)
<i>Staphylococcus hominis</i>	3 (2.1%)
Total	144

Table 3 Frequency of antibiotic resistance and susceptibility in bovine *S. aureus* isolated during 2019–2020 in Central Italy

No	Antibiotics	Resistance		Susceptibility		Total No
		No	%	No	%	
1	Clindamycin	140	85.9	23	14.1	163
2	Erythromycin	161	98.7	2	1.3	163
3	Gentamicin	140	85.8	23	14.2	163
4	Cefoxitin	19	11.7	144	88.3	163
5	Oxytetracycline	141	86.5	22	13.5	163

important to develop and assess measures to control or treat clinical mastitis and evaluate the potential for horizontal gene transfer of antimicrobial resistance genes.

MRSA is not commonly reported in bovine mastitis, but this has recently been identified, typically at a low prevalence. In this study, the prevalence of MRSA (11.7%) was remarkable compared to some previous reports (Bengtsson et al. 2009; Alves et al. 2009). Direct comparison of the prevalence of MRSA from clinical mastitis observed in this study with other studies should be performed with care because of potential differences in study population. Limited clinical data were available to determine whether the MRSA isolates were from infections that were chronic and/or had been previously treated with antimicrobials, but regardless, the data indicate that MRSA is common in bovine mastitis in these regions. These results are concordant with a survey carried out in European countries of prevalence of

antimicrobial resistance in cattle, which reported that antimicrobial resistance was greater (> 10%) in Italy than European countries like Denmark and Sweden (Hendriksen et al. 2008). Resistance of MRSA to antimicrobials that are used in cattle, such as macrolides and lincosamides, is not particularly surprising. While the origin of MRSA in these cattle cannot be determined, the presence of strains commonly found in humans and resistance to a drug not used in cattle suggests that investigation of human-cattle transmission of MRSA is required. MRSA can be transmitted between animal species (Kaszanyitzky et al. 2007; Rossi et al. 2016) and given the close contact between human hands and the bovine udder during milking, human-animal transmission is certainly plausible.

Genes conferring resistance to one of the Macrolide-Lincosamide-Streptogramin B (MLSB) antibiotics may confer cross-resistance to others, because they have similar effects

Table 4 Resistance phenotypes and PCR results in MRSA strains

Strain No	Phenotypic Resistance	CRA	PCR			spa type	mec type
			mec A	PVL	ica A		
49	DA,E,CN,FOX,OT	Bl	+	-	+	3/t037	Type III
50	DA,E,CN,FOX,OT	Bl	+	-	+	3/t037	Type III
51	DA,E,CN,FOX,OT	Bl	+	-	+	3/t037	Type III
56	DA,E,CN,FOX,OT	Bl	+	-	+	1/t008	Type IV
57	DA,E,CN,FOX,OT	Bl	+	-	+	385/t001	Type I
62	DA,E,CN,FOX,OT	Bl	+	-	+	3/t037	Type III
63	DA,E,CN,FOX,OT	Bl	+	-	+	3/t037	Type III
167	DA,E,CN,FOX	Bl	+	-	+	385/t001	Type I
218	DA,E,CN,FOX,OT	Bl	+	-	+	385/t001	Type I
221	DA,E,CN,FOX	Bl	+	-	+	385/t001	Type I
262	DA,E,CN,FOX,OT	Bl	+	-	+	3/t037	Type III
267	DA,E,CN,FOX	Bl	+	-	+	385/t001	Type I
270	DA,E,CN,FOX	Bl	+	-	+	385/t001	Type I
285	DA,E,CN,FOX	Bl	+	-	+	385/t001	Type I
290	DA,E,CN,FOX,OT	Bl	+	-	+	388/t041	Type I
327	DA,E,CN,FOX	Bl	+	-	+	385/t001	Type I
328	DA,E,CN,FOX,OT	Bl	+	-	+	385/t001	Type I
333	DA,E,CN,FOX,OT	Bl	+	-	+	385/t001	Type I
337	DA,E,CN,FOX,OT	Bl	+	-	+	385/t001	Type I

DA-clindamycin, E-erythromycin, CN-gentamicin, FOX-cefoxitin, OT-oxytetracycline. Bl-Black colour on CRA

on bacterial protein synthesis inhibition. Bengtsson et al. (2009) reported only 1.9% MRSA were resistant to one or more antimicrobials, but in this study MRSA strains were all shown a multidrug resistance. All the *S. aureus* strains showed highly resistance to erythromycin, gentamicin, oxytetracycline and clindamycin and were probably related to the large use in veterinary medicine. Out of 163 isolates, 127 (77.9%) were resistant to one or more antimicrobials, and, like our results, Gentilini et al. (2000) and Wang et al. (2008) reported that high percentage (64% and 93.1%) of *S. aureus* isolates were resistant to one or more antimicrobials, respectively. Susceptibility to erythromycin (1.3%) in all tested strains was very low.

Kaszanyitzky et al. (2007) reported *S. aureus* isolated from subclinical mastitis was high susceptible to gentamicin, while our result showed high resistance to gentamicin. Overall, resistance rate against one of the antibiotics included in the group MLSB is high in our isolates and is like the resistance found in *S. aureus* isolates from clinical mastitis in China (Wang et al. 2008). The chance that the large use of some macrolide (e.g., erythromycin) and lincosamide (e.g., clindamycin) antibiotics in animals increases the rate of *S. aureus* resistant to other MLSB should be better evaluated to prevent the selection of multidrug resistant strains. There is the risk that livestock becomes a reservoir of *S. aureus* resistant to some streptogramin, that are used very carefully in humans as the last chance for the treatment of infections not responding to other antibiotics.

Information of biofilm production by coagulase positive *S. aureus* of intramammary origin is largely lacking. The *ica* locus has been detected in majority of the mastitis *S. aureus* isolates indicating its potential role as a virulence factor in the pathogenesis of mastitis in ruminants and all identified *S. aureus* in this study were harboured *icaA* gene. Similarly, Cramton et al. (1999) observed that *icaA* was present in all *S. aureus* strains studied. Similar percentages were observed by Pérez et al. (2020), Salina et al. (2020) and Felipe et al. (2017) reporting rates of 98.5%, 82%, and 100%, respectively. In contrast, Bouzidi et al. (2023) reported that the *icaA* gene was detected in 32.2% ($n=20$) of *S. aureus* strains isolated from subclinical bovine mastitis in Algeria.

Spa typing is a cheaper and faster typing method than MLST that is currently used for typing *S. aureus* isolates. Although *spa* typing relies only on the assessment of the number of and sequence variation in repeats at the X region of the *spa* gene, this method exhibits excellent discriminatory power and is useful for epidemiological studies and exchanging data between laboratories. In this study, four different *spa* types were found in the MRSA strains: t001 (11 out of 19 strains), t037 (6 out of 19), t041 (1 out of 19) and t008 (1 out of 19). These *spa* types have been not so far described in MRSA strains isolated from bovine milk or cheeses in Italy (Tomao et al. 2020; Jöhler et al. 2018;

Thiran et al. 2018; Basanisi et al. 2017; Luini et al. 2015). *Spa* types t0037, t008 and t001 are highly spread among human patients in different continents (Asadollahi et al. 2018). In particular, t0037 is the most prominent *spa* type found in Africa and is the second one found in Asia and in Australia, while it is not included in the group of the predominant *spa* types detected in Europe and America. The *spa* type t008 is the most predominant *spa* type in America and the second one detected in Europe. The *spa* type t001, which is the predominant type detected in our MRSA strains isolated from bovine, is not included among the most predominant *spa* types in Europe but is one of the most predominant types detected in Asia. All *spa* types found in the bovine MRSA isolates included in the present study have been already detected in human patients during a first nationwide survey about *S. aureus* epidemiology in 2012 in Italy (Campanile et al. 2015). These data shows that MRSA isolated from bovine milk with the same *spa* types of human strains are spread in Italy and attention should be paid about transmission of these strains from cattle to human and vice versa.

The association of the *spa* types found in our samples with SCCmec types is perfectly concordant with the data reported in literature (Asadollahi et al. 2018). Indeed, most strains with *spa* type t0037 isolated worldwide were associated with SCCmec type III, as in the six MRSA strains isolated in this study from bovine. The *spa* type t001 is most frequently associated with SCCmec type I in Asia, the *spa* type t041 is most frequently associated with SCCmec type I in Europe, and the *spa* type t008 is most frequently associated with SCCmec type IV worldwide. Although the most frequent SCCmec types found in MRSA strain isolated from bovine milk or cheese in Italy are types IV and V (Tomao et al. 2020; Basanisi et al. 2017; Luini et al. 2015), our MRSA strains showed mainly the SCCmec types I and III, suggesting that these strains are different than the strains previously found in dairy farms in Italy. None of the *Staphylococcus* isolates from bovine milk showed the PVL gene. The PVL gene is predominant in the community acquired MRSA and is frequently found in association with more severe diseases and a significant increased risk of sepsis (Ahmad et al. 2020). In the few studies carried out previously in Italy on MRSA in bovine milk, only one MRSA strain isolated from bulk tank milk from farms located in north-eastern Italy showed the PVL gene (Tomao et al. 2020), while 6 out of 16 MRSA isolated from milk samples and 14 out of 24 MRSA isolated from cheese in South Italy showed the PVL gene (Basanisi et al. 2017). Monitoring of PVL gene presence in *S. aureus* infecting food producing animals is required to prevent transmission of highly pathogenic strains from animals to humans.

Very little is known about SIG in bovine mastitis. A low prevalence (1–2%) has been identified in previous reports (Capurro et al. 1999). The 5.8% prevalence identified here

is therefore somewhat higher than previous reports, although the reasons and relevance are unclear.

The literature often refers to CoNS as minor mastitis pathogens, and determination of the relevance of CoNS in clinical specimens can be challenging because of their commonness as commensals and the potential for contamination. MR-CoNS have been isolated from domestic animals although they are often found as commensals. The presence of methicillin-resistance does not increase the inherent virulence of CoNS, so determination of the clinical relevance of MR-CoNS remains problematic. The ability of some CoNS strains to produce biofilm raises concern about the potential for virulence, and the treatment of infections would be complicated by the presence of methicillin-resistance. Further study of the true role of MR-CoNS in mastitis is needed, as the investigation of the role of biofilm in the pathophysiology of disease.

To our knowledge this is the first report of *icaA* gene in CoNS isolated from bovine milk in Italy.

Conclusion

The present study revealed the high prevalence of *Staphylococcus* spp. from bovine mastitis samples, something that was expected. The high prevalence of biofilm production by *S. aureus* and the ability of some CoNS (including MR-CoNS) to produce biofilms raise questions about the potential role of these organisms in disease. While uncommon, SIG were isolated from clinical mastitis samples, and the potential role of these pathogenic staphylococci in mastitis requires study. While previous reports have typically indicated a low prevalence of methicillin-resistance in mastitis isolates, these data indicate that studies must be focused on evaluating the scope, epidemiology, and control of MRSA mastitis in cattle. The finding of MRSA with *spa* types more prevalent in humans than in cattle suggests that animal-to-human transmission and vice versa may occur and that biosecurity measures are required to prevent MRSA spread. Further studies should clarify the role of farm workers in spreading MRSA in dairy herds.

Author contributions All authors have made substantial contributions to the following:

- (1) ARA, SP and VC, the conception and design of the study, acquisition of data, analysis, and interpretation of data.
- (2) ARA, SP and VC drafting the article and revising it critically for important intellectual content.
- (3) ARA, SP and VC approved the final version to be submitted.

Funding This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data Availability The data that support the findings of this study are available from the corresponding author, VC, upon reasonable request.

Declarations

Animal ethics Since cattle captures and milk sampling were conducted as part of regular milk control measures in accordance with Annex IV of Regulation (EC) 854/04, no ethics committee approval was required.

Competing interests The authors declare no competing interests.

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