Methicillin-resistant *Staphylococcus aureus* (MRSA) is associated with low within-herd prevalence of intra-mammary infections in dairy cows: genotyping of isolates.

Luini, M.[*], Cremonesi, P.[†], Magro, G.[‡], Bianchini, V.[*], Minozzi, G.[‡], Castiglioni, B.[†], Piccinini R.[‡1]

[*]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna - IZSLER, via Einstein, 26900 Lodi, Italy

[†]Institute of Agricultural Biology and Biotechnology, National Research Council, via Einstein, 26900 Lodi, Italy

[‡]Department of Veterinary Science and Public Health, University of Milan, Via Celoria 10, 20133 Milan, Italy.

[1]Corresponding author:

Renata Piccinini

renata.piccinini@unimi.it

Phone: +39 02 503 18069

Fax: +39 02 503 18079

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Abstract

Staphylococcus aureus is one of the most common mastitis-causing pathogens worldwide. In the last decade, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) infections have been described in several species, included the bovines. Hence, this paper investigates the diffusion of MRSA within Italian dairy herds; the strains were further characterized using a DNA microarray, which detects 330 different sequences, including the methicillin-resistance genes *mecA* and *mecC* and SCC*mec* typing. The analysis of overall patterns allows the assignment to Clonal Complexes (CC). Overall 163 *S. aureus* isolates, collected from quarter milk samples in 61 herds, were tested. MRSA strains were further processed using *spa* typing. Fifteen strains (9.2%), isolated in 9 herds (14.75%), carried *mecA*, but none harboured *mecC*. MRSA detection was significantly associated (P <0.011) with a within-herd prevalence of *S. aureus* intra-mammary infections (IMI) ≤5%. Ten MRSA strains were assigned to CC398, the remaining ones to CC97 (n=2), CC1 (n=2) or CC8 (n=1). In 3 herds, MRSA and MSSA co-existed: CC97-MRSA with CC398-MSSA, CC1-MRSA with CC8-MSSA and CC398-MRSA with CC126-MSSA. The results of *spa* typing showed an overall similar profile of the strains belonging to the same CC: t127-CC1, t1730-CC97, t899 in 8 out of 10 CC398. In the remaining 2 isolates a new *spa* type, t14644, was identified. The single CC8 was a t3092. The SCC*mec* cassettes were classified as type IV, type V or type IV/V composite. All or most strains harboured the genes encoding the β-lactamase operon and the tetracycline resistance. Streptogramin resistance gene was related to CC398. Enterotoxin and leukocidin genes were carried only by CC1, CC8 and CC97-MRSA. The persistence of MRSA clones characterized by broader host range, in epidemiologically unrelated areas and in dairy herds with low prevalence of *S. aureus* IMI, might enhance the risk for adaptation to human species.

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most common pathogens causing intra-mammary infections (IMI) in the dairy cow. The microorganism produces not only a wide range of virulence

51 factors, but it can also acquire methicillin resistance, giving rise to methicillin-resistant
52 *Staphylococcus aureus* (MRSA). This characteristic is conferred by *mec*A gene, which is included in
53 a mobile staphylococcal cassette chromosome (SCC*mec*) and encodes for PBP2A, an alternative
54 penicillin-binding protein characterized by low affinity for beta-lactam antibiotics. Recently *mecC*, a
55 new *mecA* homologue, has been identified and its occurrence has been described in livestock, in
56 companion animals and wildlife, as well as in humans (Paterson et al., 2014). Initially, MRSA strains
57 were involved only in nosocomial infections (HA-MRSA); later, a new MRSA group referred to as
58 community-associated MRSA (CA-MRSA), emerged in healthy individuals with no links to hospital
59 settings. In companion animals, MRSA infections were rarely detected and were assumed to be of
60 human origin. In recent years, MRSA infections have been arising in food production animals and a
61 third epidemiological form of MRSA was recognized, the livestock-associated MRSA (LA-MRSA)
62 (Paterson et al., 2014). While humans could represent an important source of new pathogenic strains
63 affecting livestock (Cuny et al., 2013), animals have the potential to act as a source of *S. aureus*
64 zoonotic infections, especially for those clones seeming to lack specific host tropism (Peton and Le
65 Loir, 2014). The literature is rich of papers regarding the detection of MRSA in bovine species and
66 in the milk (Haran et al., 2012, Kreausukon et al., 2012), but data on within-herd prevalence of MRSA
67 in dairy cows are still scarce (Spohr et al, 2011, van Duijkeren et al., 2014). Therefore, understanding
68 the diffusion of MRSA in dairy herds is important from public health perspective, to prevent the
69 transmission of the bacteria to humans. The present paper aimed to investigate the diffusion and the
70 within-herd prevalence of MRSA in dairy herds in Italy: to that end, MRSA strains were characterized
71 by microarray and assigned to Clonal Complexes (CC), and further genotyped by *spa* typing.
72
73 **Material and methods**
74 Overall 163 *S. aureus* isolates, collected in 61 dairy herds between 2006 and 2013, were considered.
75 The herds were located in different Italian regions (Lombardy, Trentino, Emilia-Romagna, Lazio,
76 Puglia and Calabria) and had a size range of 38 – 285 milking cows. They were undergoing a control

77 program for *S. aureus* IMI, or they had been enrolled in the study after a double sampling. In each
78 herd, quarter milk samples were aseptically taken from all lactating cows and bacteriological analysis
79 was performed following Hogan et al. (1999). The samples were processed either at the Department
80 of Veterinary Sciences and Public Health (DIVET), or at the Regional Public Health Veterinary
81 Laboratories (IZSLER).
82 Coagulase-positive staphylococcal strains were confirmed as *S. aureus* by a duplex real-time PCR
83 assay, following Pilla et al. (2013), or by a PCR assay targeting the *nuc* gene (Cremonesi et al. 2005).
84 Depending on the number of *S. aureus* isolates and on colony morphology on blood agar plate, 1 to
85 4 isolates per herd were included in the study. Essentially, in 6 herds all strains were analyzed; in the
86 remaining 3 herds (Co, Dz and herd 18), 2-3 isolates were considered. The antibiotic resistance of
87 such isolates to the drugs mostly used in mastitis therapy (penicillin, ampicillin,
88 amoxicillin/clavulanate, oxacillin, 1^{st}, 3^{rd} and 4^{th} generation cephalosporins, tylosin, streptomycin,
89 rifaximin, quinolones, thiamphenicol, trimethoprim/sulfamethoxazole) was tested by disk-diffusion
90 method. The isolates were then frozen at -80°C in Microbank bacterial preservation system (Thermo
91 Fisher Scientific Inc, Waltham, MA, USA).
92 Bacterial DNA was extracted using DNeasy kit (QIAgen, Hilden, Germany), with the addition of
93 lysostaphin (5 mg/mL; Sigma-Aldrich, St. Luis, MO, USA) for bacterial lysis, or applying the
94 protocol described by Cremonesi et al. (2006). The amount and quality of all DNA samples was
95 measured on a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE,
96 USA).
97 All *S. aureus* strains were genetically characterized using a DNA microarray (*S. aureus* Genotyping
98 Kit 2.0; Alere Technologies GmbH, Jena, Germany) that detects a total of 330 different sequences,
99 including accessory gene regulator alleles, genes coding for virulence factors and for microbial
100 surface components recognizing adhesive matrix molecules (MSCRAMMs), capsule type-specific
101 genes, and numerous antimicrobial resistance genes. Probes for the methicillin-resistance genes *mecA*
102 and *mecC* were also included. The overall pattern was analyzed automatically for the presence or

absence of specific genes and compared to a database of strain profiles allowing the assignment to Clonal Complexes (CC). The genotyping service was performed at Alere Technologies (Jena, Germany).

All MRSA strains were further processed using *spa* typing, following Shopsin et al. (1999) and *spa* types were assigned using the Ridom SpaServer (http://www.spaserver.ridom.de).

Statistical analysis

Data analysis was carried out using SPSS (version 22, SPSS Inc., Chicago, IL, USA). The prevalence of *S. aureus* IMI in each herd was defined as the number of cows affected by MSSA or MRSA IMI, over the overall number of lactating cows. The association between herd prevalence of *S. aureus* IMI and MRSA was analyzed by a binary logistic regression analysis, using the MRSA presence/absence as dependent variable, and the prevalence groups (low, ≤5% IMI; intermediate, 5%-40%; high, ≥40%) as independent variables. The region of herd location, the number of animals in the herd and the year of strain isolation were also included in the model. Results were considered as statistically significant at P values <0.05.

Results

Overall 1025 cows were sampled twice and quarter milk samples were subjected to bacteriological analysis. Out of 163 *S. aureus* strains tested, 148 (90.8%) were MSSA and 15 (9.2%) were MRSA. The most frequently identified CC among tested isolates was CC8, which included 44.6% of MSSA strains from 22 out of 61 herds. CC97-MSSA and CC398-MSSA were less diffused, but still detected in roughly 10% of tested strains; 11 more different genotypes were identified with variable frequency (Table 1). When considering MRSA, 66.7% of strains belonged to CC398 and the remaining ones were assigned to CC97 (2 strains), to CC1 (2 strains), or to CC8 (1 strain; Table 1).

Table 1. Distribution of MSSA and MRSA strains in Clonal Complexes (CC) among herds, grouped according to *S. aureus* intra-mammary infection (IMI) prevalence.

IMI Prevalence (%)	Herds (n.)	Strains tested (n.)	CC-MSSA (n.)	CC- MRSA (n.)
<= 2,5%	8	13	CC133 (3), CC398 (3), CC5 (1), CC126 (1), CC705 (1)	CC398 (3), CC1 (1)
> 2,5 - 5%	8	19	CC479 (3), CC20 (3), CC398 (2), CC151 (1)	CC398 (7), CC97 (2), CC8 (1)
> 5 - 10%	5	11	CC398 (3), CC1 (2), CC8 (2), CC522 (2), CC5 (1), CC72 (1)	-
> 10 - 20%	6	16	CC8 (6), CC97 (6), CC151 (3), CC1 (1)	-
> 20 - 30%	8	24	CC8 (12), CC126 (5), CC151 (3), CC97 (2), CC1 (1), CC479 (1)	-
> 30 - 40%	9	27	CC8 (9), CC5 (4), CC126 (4), CC151 (4), CC97 (3), CC20 (1), CC398 (1), CC479 (1)	-
> 40 - 50%	8	25	CC8 (15), CC97 (5), CC398 (4), CC126 (1)	-
> 50%	9	28	CC8 (22), CC1 (2), CC101 (1), CC126 (1), CC398 (1)	CC1 (1)
Total	**61**	**163**	CC8 (66, 44.6%), CC97 (16, 10.8%), CC398 (14, 9.5%), CC126 (12, 8.1%), CC151 (11, 7.4%), CC1 (6, 4.1%), CC5 (6, 4.1%), CC479 (5, 3.4%), CC20 (4, 2.7%), CC133 (3, 2.0%), CC522 (2, 1.4%), CC72 (1, 0.7%), CC101 (1, 0.7%), CC705 (1, 0.7%)	**15** CC398 (10, 66.7%), CC1 (2, 13.3%), CC97 (2, 13.3%), CC8 (1, 6.7%)

MRSA strains were detected in 9 out of 61 tested herds (14.75%). In 6 out of these 9 herds, a unique circulating MRSA clone was demonstrated, but in the remaining 3 herds MSSA and MRSA grouping in different CC coexisted (Table 2). When the herds were grouped according to the prevalence of *S. aureus* IMI, in 16 (26.2%) the prevalence was ≤5%, in 19 (31.1%) it was ≥40%, and in the remaining 26 herds (42.6%) the prevalence ranged from 5.1 to 38.5% (Table 1). All MRSA were detected in low-prevalence herds: the association between IMI prevalence ≤5% and MRSA isolation was statistically significant (P <0.011), while region, number of animals in the herd and year of isolation were not significant. The ODDs ratio was of 15.39 for cases to be found in the low-prevalence group compared with group 2 and 3 (80% C.I. 3.22-72.9).

Out of the 2 CC1-MRSA strains, one was the unique *S. aureus* isolated in the herd, the other was detected in a herd with a prevalence of *S. aureus* IMI higher than 50%. Notably, in such herd all isolates were tested, given the preliminary results, and a single CC1-MRSA strain was detected, while all the other *S. aureus* isolates belonged to CC8-MSSA (Table 2).

The characterization of the SCC*mec* cassettes revealed 3 different types in CC398: a type IV, a type V and a type IV/V composite SCC*mec*. The other strains carried mostly the type V cassette (Table 2). In the array analysis, *mecC* gene was never detected.

Table 2. Dairy herds and *S. aureus* intra-mammary infection (IMI) prevalence; genotyping of MRSA and MSSA strains from MRSA affected herds.

Herd	**Cows (n.)**	***S. aureus* infected cows (n.)**	**IMI Prevalence (%)**	**MSSA**	**MRSA**		
				CC (n.)	**CC (n.)**	**SCC*mec***	***spa* type**
Co	142	5	3.5	n.d.	CC398 (2)	IV	t14644
Dg	40	2	5	-	CC398 (2)	IV/V composite	t899
Dz	216	3	1.4	CC126 (1)	CC398 (1)	IV	t899
Ma	45	1	2.2	-	CC1 (1)	IV	t127
Mi	35	1	2.9	-	CC8 (1)	V	t3092
18	267	10	3.7	n.d.	CC398 (3)	V	t899
25	76	41	53.9*	CC8 (3)	CC1 (1)	IV	t127
27	85	4	4.7**	CC398 (3)	CC97 (2)	V	t1730
30	119	2	1.7	-	CC398 (2)	V	t899

n.d., no data. All *S. aureus* isolates tested were MRSA.

* The high prevalence of IMI was caused by CC8-MSSA, a unique MRSA strain was isolated.

** Two morphologically different isolates were collected from the same cow.

The results of *spa* typing showed overall little variability in the strains belonging to the same CC: both CC1-MRSA were assigned to t127, both CC97-MRSA to t1730 and 7 out of 9 CC398-MRSA to t899 (Table 2). A new *spa* type, t14644, was identified in herd Co and was shared by both CC398-MRSA strains. The single CC8-MRSA was a t3092.

All but 2 MRSA strains were phenotypically resistant to all β-lactams: CC8 and both CC398 from Dg herd were susceptible to the association amoxicillin/clavulanate and to cephalosporins (data not shown). All MRSA strains showed an intermediate or complete resistance to thiamphenicol, while the other antibiotics displayed different patterns of sensitivity, independently of the CC. The arrays showed that all strains harboured the genes coding for the β-lactamase operon (including repressor and regulatory genes), but rarely those for macrolide and aminoglycoside, while vancomycin resistance was never detected. All but the CC8 and CC1 strains, also carried the tetracycline resistance gene and the CC398-MRSA-IV strains harboured in addition the florfenicol exporter gene. The gene conferring resistance to streptogramin was observed in 11 strains (Table 3).

Table 3. Virulence characteristics of MRSA strains, including antibiotic-resistance genes, enterotoxins and leukocidins.

CC, *spa* type	***blaZ, I, R***	***erm* (B)**	***erm* (C)**	***vga* (A)**	***vga* (A, BM3327)**	***aadD***	***aphA3***	***tet* (M)**	***fexA***	***tst1***	***sec***	***seh***	***sel***	**egc**	***luk F***	***luk D***	***luk E***
CC1-IV, t127	+		+				+					+				+	+
CC1-IV, t127	+		+				+					+				+	+
CC8-V, t3092	+									+	+		+	+	+	+	+
CC97-V, t1730	+	+			+	+		+								+	+
CC97-V, t1730	+				+			+								+	+
CC398-IV, t14644	+			+				+	+								
CC398-IV, t14644	+			+				+	+								
CC398-IV, t899	+							+	+								
CC398-IV/V, t899	+				+			+									
CC398-IV/V, t899	+				+			+									
CC398-V, t899	+				+			+									
CC398-V, t899	+				+			+									
CC398-V, t899	+				+			+									
CC398-V, t899	+				+			+									
CC398-V, t899	+				+			+									

2

blaZ, β-lactamase; *blaI*, β-lactamase repressor; *blaR*, β-lactamase regulatory protein
erm, macrolide resistance; *vga*, streptogramin resistance; *aadD*, tobramycin resistance; *aphA3*, kanamycin resistance; *tetM*, tetracycline resistance; *fexA*, florfenicol resistance
tst1, toxic shock syndrome toxin 1; *sec*, enterotoxin c; *seh*, enterotoxin h; *sel*, enterotoxin l; egc, egc cluster (*seg+sei+sem+sen+seo+seu*)
lukF, *lukF-PV (83)*, F component from hypothetical leukocidin from ruminant

Both Panton Valentine leukocidin components were overall absent, while CC8-MRSA strain, both CC1 and CC97-MRSA harboured the leukocidin D/E (*luk D/E*). The CC8 also carried the F component, but not the M, of the ruminant leukocidin (Table 2). Enterotoxin (*se*), including toxic shock syndrome toxin 1 *(tst1*) were mostly absent, only the CC1 and CC8 harboured one or more genes: the CC1 strains carried uniquely *seh*, while the CC8 harboured the *egc* cluster (*seg+sei+sem+sen+seo+seu*) in addition to *sec, sel* and the human-related allele of *tst1* (Table 3).

Discussion

The results of the present study show that the prevalence of MRSA among dairy cows is still low, even though 9% of *S. aureus* isolates considered carried the *mecA* gene and roughly 15% of tested herds were affected by the pathogen. Such prevalence was higher than those reported in previous papers (Vanderhaeghen et al., 2010; Haran et al., 2012; van Duijkeren et al., 2014; Tenhagen et al, 2014), which detected the pathogen in less than 10% of tested herds. However, the criteria of herd inclusion and/or the analytical methods were different, since we considered only *S. aureus* infected herds but did not use selective enrichment media for MRSA. The herds considered in the present study were epidemiologically unrelated, as they are located in different Italian regions over the Country. Even more, the particular distribution of MRSA among herds should be carefully considered. On one hand, all strains were detected in herds with IMI prevalence caused by MRSA not higher than 5%. On the other hand, the milk samples were collected from all lactating cows, independently of the presence of clinical signs of mastitis, during control programs for *S. aureus* IMI. Therefore, if the low within-herd prevalence of MRSA could reduce the zoonotic potential of these strains, yet the presence of MRSA infected cows in dairy farms could remain unobserved, since bacteriological analysis of milk is usually performed only when a clear problem of mammary infections affects the herd.

The phenotypic antibiotic resistance of MRSA mostly reflected the strain genomic pattern, excepted for macrolides: half of the strains showed intermediate or complete resistance, without carrying the gene. The reason could be related to the different drug tested: spiramycin and tylosin were assessed by plate diffusion, while probes for erythromycin, clindamycin and virginiamycin were included in the arrays. Also, it is known that mutational changes in the 50S ribosomal subunit of the microorganism can lead to unsuccessful binding of the drug.

In 6 herds, out of 9 affected by MRSA IMI, a unique circulating clone was demonstrated, while in the remaining 3 herds different *S. aureus* types could be isolated. CC97, t1730 MRSA co-existed with CC398-MSSA; CC1, t127 MRSA with CC8-MSSA and CC398, t899 MRSA with CC126-MSSA. CC398-MRSA has been widely reported in dairy animals (Feßler et al., 2012), and according to Price et al. (2013), it originated in humans as MSSA; the jump to livestock was accompanied by the acquisition of methicillin-resistance, probably as a consequence of widespread antibiotic use in food animal production. CC8-MSSA was recently reported as a frequent isolate from bovine mastitis in Western Switzerland (Sakwinska et al., 2011). To the contrary, CC126-MSSA has a rather limited distribution, as it was mainly reported in Brazil (Rabello et al., 2007; Silva et al., 2013), and the corresponding MRSA has not yet described. The large prevalence of CC398 among MRSA-causing bovine mastitis, and the two SCC*mec* types (IV and V) identified, are in accordance with the literature (Feßler et al., 2010; Bardiau et al., 2013). Our data on *spa* types, i.e. the 80% frequency of t899 among CC398-MRSA, differ from what reported in a large German study of MRSA in cattle food chain, where most isolates belonged to t011 or t034, independently of the origin (Tenhagen et al., 2014). Interestingly, the new CC398 t14644 identified in both strains of one herd, was likely the prevalent agent of contagious IMI. The molecular characteristics of antibiotic susceptibility and enterotoxin carriage of CC398-MRSA object of the present study were mostly similar to those described by Feßler et al. (2010). The major difference was the presence of the genes encoding macrolide resistance in part of the German strains, but not in ours. The broader host range of CC398, affecting different animals and also humans, represents a potential threat to milkers and farm personnel, according to

the significantly higher rates of CC398-MRSA nasal carriage by humans in contact with livestock (Cuny et al., 2013; Spohr et al., 2011). Accordingly, the results obtained by Feßler et al. (2012) demonstrated a possible interspecies exchange of the same MRSA CC398 subtype between dairy cattle, humans, pigs and/or sheep, suggesting a clone diversification during colonization of different hosts on the same farm. Similarly, CC1 and CC97 seem to have a wide host range, as they have been isolated from bovine subclinical mastitis, pigs and also humans (Cuny et al., 2013). Both CC1-MRSA shared t127, a *spa* type rather common in human infections, with a 2.2% frequency reported in the Ridom SpaServer database. It is plausible, that both IMIs were of human origin. Regarding CC97, 2 emergent clones of human epidemic CA-MRSA were discovered, originating from independent CC97 livestock-to-human jump (Spoor et al., 2013).

Recently, a close genetic relationship was demonstrated between MSSA isolated from dairy cow mastitis and the prominent human CC8, suggesting human-to-bovine jump (Resch et al., 2013). In the present study, 44.3% of all MSSA belonged to such group and a CC8-MRSA was also detected. Notably, CC8-MRSA is seldom recovered from livestock: a single isolation was reported in a veal farm (Nemeghaire et al., 2014), one case of bovine mastitis by ST8 was described in Belgium (Bardiau et al., 2013) and 1 out of 95 *S. aureus* strains collected from bulk tank milk in Minnesota was a ST8, t121 (Haran et al., 2012). If the latter profile is typically reported in community-associated MRSA lineages, the CC8-MRSA detected in the present study showed a different *spa* type, a t3092, which is reported with a low frequency (0.04%) in the Ridom SpaServer database. Nevertheless, the carriage of factors such as different *se*s including *tst1*, might confer this strain virulence properties relevant for public health.

A final remark regards *mecC*, a recently identified *mecA* homolog, which was absent in all tested strains. The allele has been detected in a wide range of domestic and wild animals in different countries across Europe, and in humans. Notably, *mecC*-MRSA isolates belonged to typical LA-MRSA lineages (Schlotter et al., 2014), but none of them was identified in our study.

In conclusion, the results of the study show that different MRSA groups characterized by broader host range, affect a relevant number of dairy herds throughout Italy. The herds location in epidemiologically unrelated areas and the low prevalence of subclinical IMI, might increase the potential zoonotic risk for milkers and farm personnel. Indeed, if MRSA IMIs could be regarded as spillover events, yet the persistence of MRSA clones in dairy herds might enhance the risk for adaptation to human species. Therefore, a wider surveillance of LA-MRSA among humans is needed, involving close cooperation between experts in animal, human and public health sciences.

Conflict of interest statement

The authors declare no competing interests.

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