Methicillin-resistant *Staphylococcus aureus* CC22-MRSA-IV as an agent of dairy cow intramammary infections.

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Abstract

Methicillin-resistant *S. aureus* (MRSA) lineages have become major responsible of healthcare- and community-associated infections in human population. Bovine MRSA are sporadically detected in the dairy herd, but its presence enhances the risk of zoonosis. Some lineages are able to lose the specific host tropism, being easily transmitted from animals to humans and vice-versa. The present study aims at clarifying the epidemiology of MRSA intramammary infections in a closed dairy herd, which was running a mastitis control program since years. Quarter milk samples were collected from all lactating cows once a week for 9 weeks and bacteriologically tested. At the end of the follow-up

period, also a self-taken nasal swab of the milker was analysed. Three cows (12.5%) were MRSA positive, a fourth showed a transient infection and MRSA was isolated also from the milker's nose. Somatic cell counts of infected quarters fluctuated from 1,000 to 1,800,000 cells/mL. The isolates were genotyped using DNA microarrays and identified as the epidemic UK-EMRSA-15 grouping in CC22. All strains carried the genes for β-lactam and macrolide resistance. The milker isolate differed from cow isolates mainly for the absence of the untruncated β-haemolysin and the presence of the immune evasion cluster. The milker had been volunteering in a nursing home since months, thus playing the role of MRSA vector into the herd. Our results showed the adaptive capacity of such MRSA to the bovine host. Therefore, we suggest that CC22-MRSA should be regarded as a potential cause of reverse zoonosis in dairy cattle herds.

Introduction

Staphylooccus aureus (*S. aureus*) is widely known as the major cause of contagious bovine mastitis and an important pathogen in different livestock species (Fitzgerald, 2012). The treatment with β-lactam antibiotics resulted in a selective pressure for resistance, and the acquisition of the mobile staphylococcal cassette chromosome (*SCCmec*), carrying the *mecA* or *mecC* gene, allows the bacteria to continue the cell wall biosynthesis, nullifying the antibiotic action. Methicillin-resistant *S. aureus* (MRSA) lineages are the result of this successful evolution, becoming a major responsible of healthcare- and community-associated human infections on a global scale (Köck et al., 2010). In contrast with some of the human-associated lineages, all bovine MRSA clones are occasionally detected in the dairy herds, being mostly associated with low prevalence of subclinical mastitis. Despite that, the persistence of MRSA clones in dairy herds enhances the risk of zoonosis (Luini, et al., 2015). From the first bovine MRSA detected about 50 years ago (Devriese et al.,1972), understanding the risk of *S. aureus* cross-species transmission is still an interesting scientific field of research. The phylogenetic studies on MRSA demonstrated that bovine strains belong to a limited group of clonal complexes (CC; Enright et al., 2002; Holden et al., 2013). Human lineages of MRSA,

53 such as CC5, CC8, CC22, CC30 and CC45 are rarely found in dairy animals, suggesting host range
54 barriers (Enright et al., 2002; Sung et al., 2008). On the animal side, the most common livestock-
55 associated MRSA (LA-MRSA) isolates belong to a small number of animal-associated clones: in
56 particular bovine mastitis isolates group in few CCs, including CC9, CC97, CC130, CC133, CC398,
57 CC522 and CC705 (Fitzgerald et al., 2012). Some of these have been demonstrating their ability to
58 shift from animal to human hosts. This is the case of CC398 MRSA, that is considered the most
59 important livestock-associated complex, affecting pig, poultry and ruminant farms, but can colonize
60 and infect humans with direct or indirect livestock contact; moreover, it has been introduced in
61 healthcare settings (van Alen et al., 2018). By contrast, CC8 MSSA originated in humans and
62 emerged in the cow after ancient or recent host jumps (Sakwinska et al., 2011). The new bovine-
63 adapted genotype loses the ability to colonize humans, lacking of a human-related mobile genetic
64 element (Resch et al., 2013). As a result, some *S. aureus* clones can lose the specific host tropism and
65 be easily transmitted from animals to humans (zoonotic threat) or vice-versa (concept of reverse
66 zoonosis). This study aims at clarifying the epidemiological origin of a new MRSA intramammary
67 infection in a closed dairy cow herd, which was running a mastitis control program since years.
68
69 Material and methods
70 Herd history
71 The study was performed in a small farm located in Lombardy region. The herd is housed in freestall
72 with cubicle barns and milked in a herringbone parlour. A contagious mastitis control program has
73 been running since years, because raw milk is sold directly at the farm. Briefly, the control program
74 was based on precise and consistent milking procedure, segregation of infected cows and accurate
75 diagnostic procedures. A milking sequence was established to reduce infection risk: healthy cows
76 first, followed by cows after parturition, and then by *S. aureus* infected ones. After the initial sampling
77 of all lactating cows to segregate infected ones, the cows were tested twice after calving (at 7 and 14
78 days) and the healthy group was tested again at three-month interval until no new *S. aureus* infections

were evidenced in the herd. The control program was concluded when the remaining *S. aureus* positive animals were culled. Dry cow therapy was applied on all the cows; in lactating animals, intramammary infections by *S. aureus* were treated only in the first month after calving. One year and half before our study, the routine bacteriological analysis of bulk tank milk had evidenced the presence of *S. aureus*, with a value of 40 CFU/mL. Quarter milk samples were collected from all the cows and the new infected ones were milked after the healthy animals, but not physically segregated. After 6 months, *S. aureus* count had increased to 140 CFU/mL. Therefore, the owner decided to cull part of the infected animals, so that 6 months before the beginning of the present study the bulk milk concentration of *S. aureus* had decreased to 73 CFU/mL. New cows were not introduced into the herd and the total number of lactating animals was 24.

Sampling and bacteriological analysis

Quarter milk samples of all the lactating animals were aseptically collected once a week for 9 weeks (T1 to T9) during milking in the months of April to June, and immediately delivered to the laboratory. Bacteriological analysis was performed as previously indicated (Hogan et al., 1999) and somatic cells (SCC) were counted using a Bentley Somacount 150 (Bentley, USA).

At the end of the follow-up period, we also analysed a self-taken nasal swab of the milker.

The isolates were presumptively identified as *S. aureus* according to the following scheme: Gram-positive cocci, haemolytic on blood agar, catalase positive, and coagulase positive in 4–24 h.

The antibiotic resistance of all *S. aureus* isolates to the drugs mostly used in mastitis therapy (penicillin, ampicillin, amoxicillin/clavulanate, oxacillin, 1st, 3rd and 4th generation cephalosporins, tylosin, spiramycin, kanamycin, rifaximin, quinolones, thiamphenicol, trimethoprim/sulfamethoxazole) was tested by disk-diffusion following Clinical and Laboratory Standards Institute guidelines (2017).

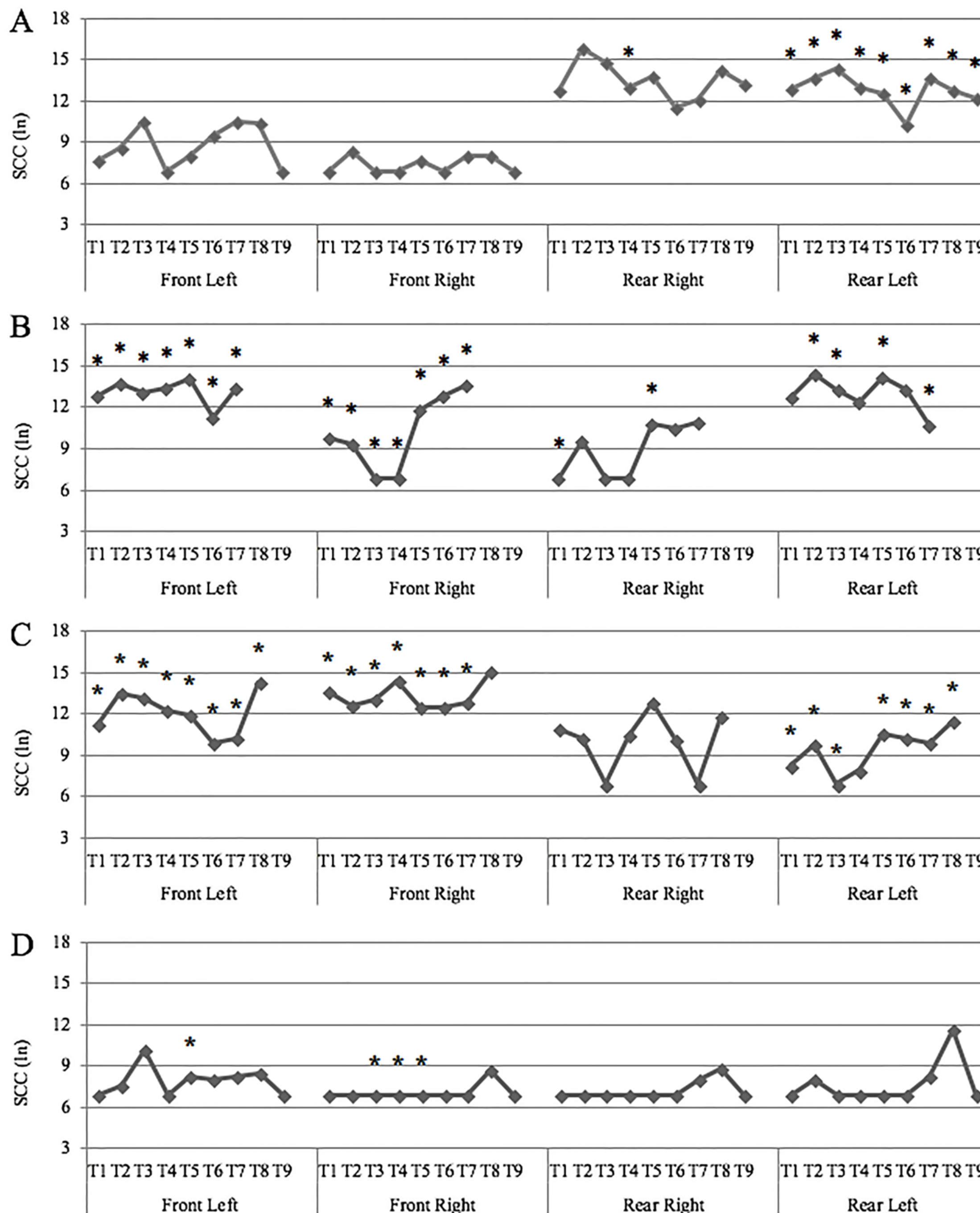
Molecular analysis

The DNA of coagulase-positive strains was extracted using DNeasy kit (QIAgen, Hilden, Germany), with the addition of lysostaphin (5 mg/mL; Sigma-Aldrich, St. Luis, MO, USA) for bacterial lysis.

105 Amount and quality of DNA samples were measured on a NanoDrop ND-1000 spectrophotometer
106 (Nano-Drop Technologies, Wilmington, DE, USA).They were confirmed as *S. aureus* by a duplex
107 real-time PCR assay, following Pilla et al. (2013).
108 Genotyping was performed by DNA microarrays using Alere StaphyType DNA microarray (Alere
109 Technologies Gmbh, Jena, Germany). The microarray covers approximately 170 distinct genes and
110 their allelic variants for a total of 330 target sequences including accessory gene regulator alleles,
111 genes coding for virulence factors and for microbial surface components recognizing adhesive matrix
112 molecules (MSCRAMMs), capsule type-specific genes, and numerous antimicrobial resistance genes
113 (Monecke et al., 2007). Probes for the methicillin-resistance genes *mecA* and *mecC* are also included.
114 The overall pattern was analyzed automatically for the presence or absence of specific genes and
115 compared to a database of strain profiles allowing the assignment to Clonal Complexes (CC). The
116 genotyping service was performed at Alere Technologies (Jena, Germany).
117
118 Results
119 The results of bacteriological analysis of quarter milk samples collected at the first sampling showed
120 that 3 out 24 lactating cows (12.5%) had 2 up to 3 quarters infected by coagulase-positive
121 Staphylococci. At the third sampling, another animal tested positive in one quarter, but cured
122 spontaneously within 3 weeks and remained negative in the following two months (the quarter was
123 tested repeatedly until the end of August). A coagulase-positive Staphylococcus was recovered also
124 from the milker's nasal swab. PCR assay confirmed the identification of the isolates as *S. aureus*.
125 The disk diffusion test showed the same pattern of antibiotic resistance for all *S. aureus* isolates: they
126 were susceptible to macrolides, rifaximin, thiamphenicol and trimethoprim/sulfamethoxazole but
127 resistant to penicillin, ampicillin, amoxicillin/clavulanate, oxacillin, 1st, 3rd and 4th generation
128 cephalosporins, kanamycin and quinolones. Therefore, the isolates were classified as MRSA.
129 During the follow-up period, SCC of the infected quarters of three cows fluctuated from extremely
130 low values (1,000 cells/mL) to values exceeding one million cells/mL. To the contrary, the transient

131 infected cow (cow D) showed always very low SCC, never exceeding 7,000 cells/mL. Two infected
132 animals were culled before the end of the study, i.e. after the 7th or 8th sampling respectively. Somatic
133 cell count values and *S. aureus* shedding by the infected quarters of the 4 cows are presented in Figure
134 1.

135 Figure 1. Quarter milk Somatic Cell Counts and MRSA shedding by infected cows during the study.
136 The capital letters A-D indicate the four cows. The symbol * represents the recovery of *S. aureus* in
137 the milk.



138

139 Microarray genotyping evidenced the *mecA* gene in all the 5 isolates, including the human one. They
140 were identified as epidemic MRSA-15 (also known as UK-EMRSA-15 or Barnim EMRSA) and
141 grouped in CC22. The microarray results showed minor differences among the isolates, as reported
142 in Table 1.

143 Table 1. Main results of microarray analysis showing the differences among the MRSA strains
144 isolated during the study.

Virulence factor	**Cow A**	**Cow B**	**Cow C**	**Cow D**	**Milker's nose**
β-haemolysin probe 1, 2, 3 (*hlb*)	POS	POS	POS	POS	POS
Un-truncated β-haemolysin (*hlb*)	POS	POS	POS	POS	NEG
Staphylokinase, *sak*	NEG	NEG	NEG	NEG	POS
Chemotaxis-inhibiting protein, *chp*	NEG	NEG	NEG	NEG	POS
Staph. complement inhibitor, *scn*	NEG	NEG	NEG	NEG	POS
γ- haemolysin, component A, *hlgA*	POS	POS	POS	POS	POS
γ- haemolysin, component B, *lukF*	POS	POS	POS	POS	POS
γ- haemolysin, component C, *lukS*	NEG	NEG	NEG	POS	AMB
Panton-Valentine leucocidin, component F, *lukF-PV*	NEG	NEG	NEG	NEG	NEG
Panton-Valentine leucocidin, component S, *lukS-PV*	NEG	NEG	NEG	NEG	NEG
Ruminant hypothetical leukocidin, component F, *lukF-PV (P83)*	NEG	NEG	NEG	NEG	NEG
Ruminant hypothetical leukocidin, component S, *lukM*	NEG	NEG	NEG	NEG	NEG
Leukocidin D, *lukD*	NEG	NEG	NEG	NEG	NEG
Leukocidin E, *lukE*	NEG	NEG	NEG	NEG	POS
Leukocidin/haemolysin toxin, *lukX*	POS	POS	POS	POS	POS
Leukocidin/haemolysin toxin, *lukY*	POS	POS	POS	POS	NEG

145

146 All cow isolates carried the γ-haemolysin genes *hlgA* and *hlgB*, only the strain isolated from the last
147 infected cow carried *hlgC*. All isolates were Panton-Valentine leucocidin (PVL) negative, but positive
148 for the enterotoxin genes *seg*, *sei*, *sem*, *sen*, *seo* and *seu* an allelic variant of von Willebrand factor
149 (vvb-RF122). They harboured also the protease genes encoding aureolysin or staphopain A, B, in
150 accordance with typical CC22 profile (Albrecht et al., 2011; data not shown). Human and cow isolates
151 differed basically for the absence of the untruncated β-haemolysin and the presence of *sak*, *chp* and

152 *scn* uniquely in the milker *S. aureus*. The demonstration of the genes for β-lactams resistance in all
153 isolates explained the phenotypic resistance observed. Conversely, *ermC*, one of the genes encoding
154 macrolide resistance, did not express resistance to tylosin or spiramycin in the susceptibility test.
155
156 Discussion
157 Methicillin-resistant *S. aureus* (MRSA) strains are a major cause of healthcare- and community-
158 associated infections on a global scale (Köck et al., 2010). Different LA-MRSA lineages, are
159 implicated in farm animal infections. The possible transmission of human lineages to companion
160 animals, as well as livestock and wildlife, is widely demonstrated (Messenger et al., 2014): in such
161 cases the infection is regarded as a reverse zoonosis. In dairy cattle, MRSA is usually considered as
162 a marginal problem in terms of herd contagiousness but at the same time, a possible reservoir of new
163 human infection (Juhász-Kaszanyitzky et al., 2007; Aslantaş and Demir, 2016; Luini et al., 2015).
164 Conversely, the concept of reverse zoonosis is still poorly considered (Messenger et al., 2014). The
165 reason behind this underestimation is probably due to the difficult demonstration of the
166 epidemiological chain leading to the infection in the intensive dairy herd, what makes the distinction
167 between zoonosis and reverse zoonosis a complicated problem. In the last decades, several studies
168 focused on the possible transmission of LA-MRSA to human population, demonstrating the zoonotic
169 role of some lineages in pig, cattle, and poultry farm workers (Morgan, 2008; Springer et al., 2009).
170 CC398 is the most important group and the possible colonization of cattle farm personnel has been
171 considered as a potential MRSA vector into different compartment of the farm (Feβler et al., 2012)
172 or into hospital (Graveland et al., 2011). The results of the present study led us to consider the
173 subclinical intramammary infections of the dairy cows in the farm as a reverse zoonosis, since all *S.*
174 *aureus* isolates from quarter milk and the isolate from the milker's nose belonged to the same clonal
175 lineage, i.e. the epidemic UK-EMRSA-15. It should be highlighted that the milker volunteered since
176 months in a nursing home. Such lineage is largely diffused in pets: dogs and cats acquire the infection
177 by their owners or veterinarian (Wipf and Perreten, 2016). The genome comparison of CC22-MRSA

178 isolated from humans and pets demonstrated a few differences, mostly in the carriage of mobile
179 genetic elements (MGEs) rather than in core genes (Loeffler et al., 2013). Indeed, the lineage is
180 characterized by a flexible MGEs profile, associated with a quick ability of MGEs loss and
181 acquisition, which might explain its success in dissemination and persistence in different hosts
182 (Jamrozy et al., 2017). In our study, the major genetic difference between cow and human strains was
183 the presence of the immune evasion cluster (IEC) only in the milker's nose: the β-haemolysin
184 converting prophage carrying human-specific host immune evasion genes (*sak-scn-chp*) had been lost
185 in the cow jump, suggesting an adaptation of the lineage to the bovine host. This finding is similar to
186 the case of CC8 human-to-animal jump (Satwinska et al., 2011; Retsch et al., 2013). Analogously to
187 CC8, the loss of the prophage might help the establishment of infection in the dairy cow. A further
188 result strengthening our hypothesis is the presence of the untruncated β-haemolysin uniquely in the
189 bovine MRSA isolates, probably because the gene is necessary in ungulates for the different structure
190 of erythrocyte membranes. The outbreak and dissemination of CC22-MRSA infection in the herd
191 before our monitoring support the hypothesis that the adaptation of the lineage to this new host should
192 not be underestimated. The isolate from the cow with transient infection differed from the other
193 bovine isolates for the carriage of the *hlgC/lukS* gene, which in turn gave an ambiguous result in the
194 human isolate. We would like to highlight this result, because MRSA intramammary infections of all
195 the other cows were persistent. We could speculate that the pathogenicity island carrying γ-
196 haemolysin might have been lost in the adaptation to the bovine host. All the isolates harboured the
197 allelic variant of the Von Willebrand binding protein gene (*vvb -RF122*), which is considered one of
198 the mechanisms associated to *S. aureus* pathogenicity in the cow and a specific marker of host
199 adaptation (Viana et al., 2010). At the light of these results, we strongly suggest that CC22-MRSA
200 be regarded as a potential cause of reverse zoonosis in dairy cattle herds.
201
202 Conclusions

203 The present study provides evidence for the importance and impact of the UK-EMRSA-15 as a cause
204 of mastitis in the dairy cow, demonstrating the adaptive capacity of the lineage to the bovine host.
205 The transmission of MRSA CCs between different hosts revoke the concept of "One Health": the true
206 scale of the overall problem is still unknown, and further studies addressing both animals and farm
207 personnel are required, in order to monitor the possible emergence of new lineages among the dairy
208 cattle.
209

210 Conflict of interest statement

211 The authors declare no competing interests.
212

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216

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