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Prevalence and characterization of methicillin-resistant *Staphylococcus aureus* carrying *mecA* or *mecC* and methicillin-susceptible *Staphylococcus aureus* in dairy sheep farms in central Italy

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ABSTRACT

Between January and May 2012, a total of 286 bulk tank milk samples from dairy sheep farms located in central Italy were tested for the presence of *Staphylococcus aureus*. One hundred fifty-three samples were positive for *S. aureus* (53.5%), with an average count of 2.53 log cfu/mL. A total of 679 *S. aureus* colonies were screened for methicillin resistance by the cefoxitin disk diffusion test, and 104 selected cefoxitin-susceptible isolates were also tested for their susceptibility to other antimicrobials representative of the most relevant classes active against *Staphylococcus* spp. by using the Kirby-Bauer disk diffusion method. Two methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, carrying respectively the *mecA* and the *mecC* genes, were detected in 2 samples from 2 different farms (prevalence 0.7%). The *mecA*-positive MRSA isolate was *blaZ* positive, belonged to *spa* type t127, sequence type (ST)1, clonal complex (CC)1, carried a staphylococcal cassette chromosome *mec* (SCC*mec*) type IVa, and was phenotypically resistant to all the β-lactams tested and to erythromycin, streptomycin, kanamycin, and tetracycline. The *mec*C-positive MRSA isolate was negative for the chromosomally or plasmid-associated *blaZ* gene[AU1: Throughout, ensure genes and mRNA are italicized; leave roman for proteins.] but positive for the *blaZ* allotype associated with SCC*mec* XI (*blaZ*-SCC*mec*XI), belonged to *spa* type 843, ST(CC)130, carried a SCC*mec* type XI, and was resistant only to β-lactams. Both MRSA were negative for the presence of specific immune-evasion and virulence genes such as those coding for the Panton-Valentine leucocidin, the toxic shock syndrome toxin 1, and the immune evasion cluster genes. Regarding the presence of the major *S. aureus* enterotoxin genes, the *mec*C-positive MRSA tested negative, whereas the ST (CC)1 *mecA*-positive MRSA harbored the *seh* gene. Among the 104 methicillin-susceptible *S. aureus* isolates examined for antimicrobial susceptibility, 63 (60.58%) were susceptible to all the antimicrobials tested, and 41 (39.42%) were resistant to at least 1 antimicrobial. In particular, 23 isolates (22.12%) were resistant to tetracycline, 16 (15.38%) to sulfonomides, 14 (13.46%) to trimethoprim and sulfamethoxazole, and 9 (8.65%) to ampicillin, whereas only 1 isolate was resistant to both fluoroquinolones and aminoglycosides. The high prevalence of *S. aureus* found in bulk tank milk samples and the isolation of MRSA, although at a low prevalence, underlines the importance of adopting control measures against *S. aureus* in dairy sheep farms to minimize the risks for animal and public health. Moreover, this study represents the first report of *mecC*-positive MRSA isolation in Italy and would confirm that, among livestock animals, sheep might act as a *mecC*-MRSA reservoir. Although this lineage seems to be rare in dairy sheep (0.35% of farms tested), because *mecC*-positive MRSA are difficult to detect by diagnostic routine methods employed for *mecA*-positive livestock-associated MRSA, diagnostic laboratories should be aware of the importance of searching for the *mecC* gene in all the *mecA*-negative *S. aureus* isolates displaying resistance to oxacillin, cefoxitin, or both.

Key words: sheep milk, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *mecA*/*mecC*

INTRODUCTION

Staphylococcus aureus is involved in a wide variety of diseases in humans and animals and its pathogenicity is mainly related to a combination of genetic characteristics mediating virulence, invasive capacity, immune evasion, and antibiotic resistance (Chua et al., 2014). *Staphylococcus aureus* is a common cause of IMI in

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dairy ruminants, causing both clinical and subclinical forms accompanied with relevant economic losses due to reduced milk production and quality (Bergonier et al., 2003).

In the last years, the emergence of livestock-associated (**LA**) methicillin-resistant *Staphylococcus aureus* (**MRSA**) has been increasingly reported worldwide, with a raising concern for the risks of zoonotic transmission, especially for people with occupational livestock exposure (Hanselman et al., 2006; Vanderhaeghen et al., 2010; Fessler et al., 2012; Guardabassi et al., 2013), but also for the possible introduction of these strains in the community through the food chain (Kluytmans, 2010). Clonal complex (**CC**)398 is the most prevalent LA-MRSA lineage in Europe, although in Italy other major LA-MRSA lineages, such as CC1 and CC97, have spread and have also been found to colonize and cause infections in livestock (Alba et al., 2015; Feltrin et al., 2015; Luini et al., 2015; Carfora et al., 2016). In the last decade, MRSA clones with a divergent *mecA* homolog, named *mec*C (formerly *mecA*LGA251), have been detected in different animal species and human beings in different European countries, with isolates mainly belonging to CC130, CC1943, and CC425 (García-Álvarez et al., 2011; Paterson et al., 2014; Angen et al., 2017). Zoonotic transmission of *mecC*-MRSA has been previously reported (Harrison et al., 2013; Petersen et al., 2013), although data on the prevalence, animal reservoir, and epidemiology of *mecC*-MRSA are still limited (Harrison et al., 2013; Petersen et al., 2013).

In recent years, our research group has been investigating the presence and the characteristics of *S. aureus*, particularly MRSA, from sheep dairy products and sheep farms of central Italy (Carfora et al., 2015; Carfora et al., 2016), an area where the milk and cheese manufacturing industry is well developed and the consumption of raw milk dairy products of ovine origin is quite popular.

In this paper we report data on the prevalence of *S. aureus* in the bulk tank milk (**BTM**) samples collected from dairy sheep farms located in central Italy. The antimicrobial resistance profiles of methicillin-susceptible *S. aureus* (**MSSA**) isolates are also reported, together with the genotypic characteristics of *mecA* and *mecC*-positive MRSA strains.

MATERIALS AND METHODS

Sample Collection

Between January and June 2012, a total of 286 BTM samples were collected from 286 dairy sheep farms located in central Italy (Lazio region). The milk samples, collected by trained technicians, were transported to the laboratory in ice-cooled containers and analyzed within 24 to 48 h after collection.

S. aureus Isolation and Identification

All collected samples were analyzed for the enumeration of coagulase-positive staphylococci using Baird-Parker agar with rabbit plasma fibrinogen supplement according to ISO 6888–2: 1999 and Amd1: 2003 (ISO 6888–2: 1999/Amd1; ISO, 2003). Coagulase-positive colonies were identified as *Staphylococcus* spp. by microscopic observation, Gram staining, and catalase determination. Considering the composite nature of BTM samples, multiple suspected colonies (up to 5) were further analyzed from each positive sample. Genomic DNA was obtained from *Staphylococcus* spp. colonies previously subcultured on blood agar (5% defibrinated bovine blood) by using InstaGene Matrix (Bio-Rad, Milano, Italy), as reported by Bianchi et al. (2014). *Staphylococcus aureus* identification was performed by a modified species-specific PCR, using primers targeting the *femA* gene (Mehrotra et al., 2000).

Screening for Methicillin Resistance by Cefoxitin Disk Diffusion Test

A total of 679 *S. aureus* colonies were screened for methicillin resistance by the cefoxitin disk diffusion test according to the criteria of Clinical Laboratory Standards Institute (**CLSI**). The results were interpreted following the Performance Standards for Antimicrobial Susceptibility Testing, Twenty-Third Informational Supplement (CLSI, 2013a).

Molecular Characterization

Cefoxitin-resistant isolates were tested for the presence of the *mec*A/*mec*C and *bla*Z genes by PCR assays using primers and protocols described by Stegger et al. (2012) and Martineau et al. (2000), respectively. The MRSA isolates were further genotyped by *spa* typing, multilocus sequence typing (**MLST**) and by typing/subtyping of the staphylococcal cassette chromosome *mec* (**SCC*mec***) using multiplex PCR methods as previously described (Battisti et al., 2010; Shore et al., 2011). The *MecC*-positive isolates were also tested by PCR analysis for the presence of the *blaZ* allotype associated with SCC*mec* XI (*blaZ*-SCC*mec*XI), as reported by García-Álvarez et al. (2011).

The *Mec*A/*mec*C-positive isolates were also screened by PCR analysis for the presence of specific immune evasion and virulence genes. These included the genes

coding for the Panton-Valentine leucocidin (**PVL**) and the toxic shock syndrome toxin 1 (**TSST-1**; Fueyo et al., 2005), the immune evasion cluster (**IEC**) genes *sak* (staphylokinase) and *scn* (staphylococcal complement inhibitor precursor), as reported by van Wamel et al. (2006). The presence of 9 selected *S. aureus* enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *ser*) and 2 staphylococcal-like enterotoxin (*selj*, *selp*) genes was assessed in the 2 MRSA isolates by using 2 multiplex PCR protocols, as previously described (Kérouanton et al., 2007; Bianchi et al., 2014).

Antimicrobial Susceptibility Testing

Selected MSSA isolates were tested for phenotypic susceptibility to antimicrobials representative of the most relevant classes active against *Staphylococcus spp.* by using the Kirby-Bauer disk diffusion method (CLSI, 2013a,b). Isolates selection was performed by choosing one single isolate from each positive sample/farm, when still available/vital. The results were interpreted according to the criteria of CLSI (2013a,b). The following antimicrobials were tested: ampicillin, amoxicillin/clavulanic acid, cefotaxime, tetracycline, sulfonamides, chloramphenicol, gentamicin, trimethoprim/sulfamethoxazole, clindamycin, enrofloxacin, erythromycin, cephalothin, and kanamycin.

The *Mec*-positive isolates were also tested for their antimicrobial susceptibility by the broth microdilution method (Trek Diagnostic Systems, Westlake, OH). The following drugs were tested: penicillin, cefoxitin, ciprofloxacin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, linezolid, quinopristin/dalfopristin, fusidic acid, mupirocin, rifampicin, tetracycline, tiamulin, sulfamethoxazole, trimethoprim, and vancomycin. Minimum inhibitory concentrations were determined, and results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org), using epidemiological cut-offs for the categorization of "microbiological resistance" or "non-wild-type" isolates. Results for the quality control documents were within published ranges.

RESULTS

S. aureus and MRSA Detection

Staphylococcus aureus was detected in 153 out of 286 BTM samples tested (53.5%, 95% CI: 47.54–59.37). The average *S. aureus* count was 2.53 log cfu/mL, with counts ranging from 1.26 to 4.63 log cfu/mL. Two out of the 679 *S. aureus* colonies screened were resistant to cefoxitin. These isolates were from 2 samples collected in 2 different farms and carried the *mecA/blaZ* and *mecC* genes, respectively. Both MRSA-positive farms consisted of semi-extensive dairy sheep herds with an average of 500 lactating ewes mechanically milked and no other livestock animals reared. The overall estimated between-herd MRSA prevalence was 0.70% (2/286, 95% CI: 0.12–2.78).

Characterization of the MRSA Isolates

The *mecA*-positive isolate belonged to *spa* type t127, Sequence Type (**ST**) (CC)1, harbored a SCC*mec* type IVa, carried also the *blaZ* gene encoding for β-lactamase, and was phenotypically resistant to all the β-lactams tested, and to erythromycin, streptomycin, kanamycin, and tetracycline. The isolate was detected in the same holding where previous investigations had already reported the presence of similar *spa* type t127, ST1, *mecA*-positive MRSA (Carfora et al., 2016). The *mecC*-positive isolate belonged to *spa* type t843, ST(CC)130, harbored a SCC*mec* type XI, was negative for the chromosomally or plasmid-associated *blaZ* gene with primers and conditions as described by Martineau et al. (2000), and was positive, as expected, to the *blaZ* allotype associated with SCC*mec* XI (*blaZ*-SCC*mec*XI). It was phenotypically resistant to all the β-lactams, but susceptible to all the other antimicrobials tested. Both ST1 and ST130 MRSA were negative for the genes coding PVL, TSST-1, and IEC. Regarding the presence of the major *S. aureus* enterotoxin genes, the ST130 *mec*C-positive MRSA tested negative, whereas the ST1 *mecA*-positive MRSA harbored the *seh* gene, as expected (Alba et al., 2015).

Characterization of the MSSA Isolates

Among the 104 MSSA isolates examined for antimicrobial susceptibility, 63 (60.58%) were susceptible to all the 13 antimicrobials tested, and 41 (39.42%) were resistant to at least 1 antimicrobial. The resistance profiles of the MSSA are reported in Table 1. Overall, 22 isolates (21.15%) were resistant to 1 single antimicrobial drug, 16 (15.38%) to 2 drugs, whereas a multidrug resistance pattern (resistance to at least 3, and up to 4 antimicrobial drugs) was observed in 3 isolates (2.88%). In particular, 23 isolates (22.12%) were resistant to tetracycline, 16 (15.38%) to sulfonomides, 14 (13.46%) to trimethoprim and sulfamethoxazole, and 9 (8.65%) to ampicillin. One isolate showed resistance to both enrofloxacin and kanamycin. None of the 104 MSSA isolates showed resistance to amoxicillin-clavulanic acid, cefotaxime, cephalothin, chloramphenicol, gentamicin, clindamycin, and erythromycin.

Table 1. Antimicrobial resistance profiles of the methicillin-susceptible *Staphylococcus aureus* (MSSA) isolated from sheep bulk tank milk samples tested by the disk diffusion method[1]

Resistance profile[2]	No. of resistant isolates
TE	16
AM	6
TE-AM	2
TE-SUL	2
SUL-SXT	12
TE-SUL-SXT	2
TE-ENR-AM-K	1
Total	41

[1]Forty-one out of 104 MSSA isolates tested were resistant to at least 1 antimicrobial.

[2]TE = tetracycline; AM = ampicillin; SUL = sulfonamides; SXT = trimethoprim-sulfamethoxazole; ENR = enrofloxacin, K = kanamycin.

DISCUSSION

Although *S. aureus* is frequently isolated from sheep raw milk and dairy products (Morandi et al., 2009; Spanu et al., 2014; Carfora et al., 2015; Jamali et al., 2015), the detection of MRSA in ovine milk (Ariza-Miguel et al., 2014; Caruso et al., 2015; Pexara et al., 2015; Carfora et al., 2016) and derived dairy products (Normanno et al., 2007; Shanehbandi et al., 2014; Carfora et al., 2015) is sporadic.

In this survey, conducted in dairy sheep farms located in central Italy, we found a high prevalence of *S. aureus* contamination in ovine BTM (53.5% of samples tested). Other studies conducted on BTM in different countries have reported lower prevalence rates, ranging from 9.6 to 33.3% (Muehlherr et al., 2003; Scherrer et al., 2004; de Garnica et al., 2013; Pexara et al., 2015; Zdragas et al., 2015).

Despite this high overall *S. aureus* prevalence, the estimated MRSA prevalence was low (0.7%; 2/286). This finding is in agreement with those of other recent investigations carried out on small ruminant BTM in Italy (Caruso et al., 2015; Cortimiglia et al., 2015), Spain (Ariza-Miguel et al., 2014), and Greece (Pexara et al., 2015; Zdragas et al., 2015), which reported prevalence rates ranging from 0 to 2%.

Interestingly, 1 of the 2 MRSA isolates identified carried a *mecC* gene. The presence of *mecC*-positive MRSA has been already reported in ruminant farms in different European countries (García-Álvarez et al., 2011; Eriksson et al., 2013; Petersen et al., 2013; Unnerstad et al., 2013; Ariza-Miguel et al., 2014; Loncaric et al., 2014; Paterson et al., 2014), meaning a wide geographical spread of these strains. However, to date, *S. aureus* carrying the *mecC* gene have never been isolated from humans or animals in Italy. Similarly to *mecA*-MRSA lineages, *mecC*-MRSA strains are highly versatile pathogens able to cause a wide range of infections in several host species (Paterson et al., 2014). The *MecC*-MRSA have been regarded as animal-adapted lineages, probably arisen in animals, possibly ruminants, and subsequently spread to humans (Harrison et al., 2013; Petersen et al., 2013). In this regard, although it was detected in only 1 of the 286 farms tested, the isolation of a *mecC*-positive strain from an ovine BTM sample is of public health relevance because of its zoonotic potential (Harrison et al., 2013; Petersen et al., 2013). The MLST analysis revealed that the *mecC* isolate belonged to *spa* type t843, ST(CC)130, SCC*mec* type XI, which is a common lineage of *mecC*-MRSA reported in cattle and sheep in Europe, and is capable of causing infections in humans (García-Álvarez et al., 2011; Eriksson et al., 2013; Petersen et al., 2013; Ariza-Miguel et al., 2014; Loncaric et al., 2014; Kerschner et al., 2015). As expected, our ST130 *mecC*-positive isolate was negative for the presence of specific phage-borne virulence and immune evasion genes such as PVL, TSST-1, and IEC, often harbored by certain human-adapted community-acquired (**CA**) MRSA lineages. This finding is consistent with an animal-host origin of this clone (Cuny et al., 2011; Paterson et al., 2014). As for the antimicrobial resistance, whereas the ST(CC)1 *mecA*-positive isolate was also resistant to tetracycline, erythromycin, streptomycin, and kanamycin, the ST(CC)130 *mecC*-positive isolate was resistant only to β-lactams, as already reported for other similar *mecC* isolates (Petersen et al., 2013; Ariza-Miguel et al., 2014; Loncaric et al., 2014). The observation that the ST130 *mecC* strain isolated in this study did not acquire further resistance determinants, suggests that this lineage may not have been extensively subjected to multiple antibacterial selection pressure (Ariza-Miguel et al., 2014).

As for the antimicrobial susceptibility testing of the MSSA isolates, we found that 60.58% of them were susceptible to all the drugs tested. Lower rates of antimicrobial resistance have been reported in other studies conducted in Greece (Pexara et al., 2015; Zdragas et al., 2015) and Brazil (Martins et al., 2015). The most common resistance was that to tetracycline, observed in 22.12% of the isolates, a rate higher than that described in other studies in Italy (Lollai et al., 2008), Turkey (Ünal et al., 2012), and Brazil (Martins et al., 2015), but lower than that reported in Iran by Jamali et al. (2015). A relatively high level of resistance to sulfonamides (15.38%) and trimethoprim-sulfamethoxazole (13.46%) was also observed, a result in contrast with other studies conducted in Iran (Jamali et al., 2015), Greece (Pexara et al., 2015; Zdragas et al., 2015), and Brazil (Martins et al., 2015), reporting resistance rates ranging from 0 to 1.2%. On the other hand, the ampicillin resistance rate (8.65%) was similar to those

reported in other studies conducted in Slovenia (Pengov and Ceru, 2003) and Greece (Zdragas et al., 2015). In agreement with other studies conducted in Italy (Spanu et al., 2014), Greece (Pexara et al., 2015), and Brazil (Martins et al., 2015), multidrug resistance (≥3 antimicrobial drugs) was observed only in a small proportion of our MSSA isolates (2.88%), supporting the view that it still represents an occasional occurrence in MSSA of ovine origin (Spanu et al., 2014; Pexara et al., 2015). The relatively low rate of multi-drug resistance observed could be probably ascribed to the extensive or semi-extensive farming systems often associated with sheep breeding, and the consequent limited use of antimicrobials in this species, as compared with other intensive farming ones.

In conclusion, this study represents the first report of *mecC*-positive MRSA isolation in Italy and would confirm that, besides CC1-*mecA*-positive-LA-MRSA, sheep might act as a reservoir of *S. aureus* clones, such as CC130, prone to harbor *mecC* genes. In this regard, it should be considered that the prevalence of *mecC*-positive LA-MRSA could be underestimated because they are difficult to identify by diagnostic methods routinely employed for the detection of *mecA*-mediated methicillin resistance. Indeed, *mecC*-positive MRSA show low-level oxacillin resistance with negative PBP2a detection, if not tested after induction by cefoxitin, and are not detected by *mecA* PCR (García-Álvarez et al., 2011; Deplano et al., 2014). In addition, it has been reported that MRSA CC130 strains grow insufficiently on commercial selective chromogenic agar plates for the detection of MRSA (Cuny et al., 2011). The high prevalence of *S. aureus* found in BTM samples and the isolation of MRSA, although at a low prevalence, underlines the importance of adopting control measures against *S. aureus* in dairy sheep farms to minimize the risks for both animal and public health. In this regard, the implementation of specific monitoring/surveillance programs at the national or regional level (or both) would help in better understanding the epidemiology and trends of LA-MRSA in dairy small ruminant herds, along with specific studies aimed at investigating the risk factors involved in sheep and goat colonization/infection and between-herd transmission.

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