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Prevalence, antimicrobial susceptibility and molecular typing of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in bulk tank milk from southern Italy

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ABSTRACT

This paper assesses the prevalence of MRSA in bulk tank milk (BTM) samples from southern Italy, and the relationship between the Coagulase Positive Staphylococci count (CPS) and MRSA prevalence. Of 486 BTM samples tested, 12 samples (2.5%) resulted positive for the presence of MRSA. Great genetic diversity was found among the isolates: ST1/t127 and t174/IVa, ST5/t688/V, ST8/t unknown/IVa/V, ST45/t015/IVa, ST71/t524/V, ST88/t786/Iva, ST398/t011 and t899/IVa/V and ST2781/t1730/V. All isolates were *pvl*-negative and *ica*A positive. The majority of strains (58%) carried the *ses* (*sec*, *seh*, *seg*, *seo*, *sem* and *sen*) genes. All tested strains resulted susceptible to amikacin, cephalotin, cloramphenicol, gentamycin, trimethoprim – sulfamethoxazole, tobramycin and vancomycin, and variably resistant to ampicillin, oxacillin and tetracycline. No statistical association between the CPS count and MRSA detection was found in the MRSA-positive samples. Although some of the *spa*-types and STs detected in our survey are known to cause human infections, raw milk from Italian herds in the considered area is not a common source of MRSA. Nonetheless, it is necessary to assess the risk of foodborne infection and the risk related to the handling of milk.

1. Introduction

MRSA infections are now a serious public health concern, since the host spectrum of this pathogen and its transmission route have greatly increased in recent years. In the 1970s, MRSA infections were mainly localised in hospital environments (healthcare-associated i.e. HA-MRSA) (Wendlandt et al., 2013). Subsequently, MRSA spread into the community (community-acquired infections, i.e. CA-MRSA) among people with no risk factors for MRSA infections (Doyle et al., 2012). In recent years, other MRSA clones associated with exposure to livestock (livestock associated – LA-MRSA) have emerged in different countries worldwide (Witte et al., 2007; Köck et al., 2013). In particular, LA-MRSA – genotypically classified under Clonal Complex 398 (CC 398) – seems to have found a reservoir in animals, primarily pigs, cattle and horses, and has been shown to colonize and cause serious infections in humans in close contact with these animals (van Cleef et al., 2010; Lozano et al., 2011; Soavi et al., 2010; van Loo et al., 2007), but also in people without livestock exposure (Larsen et al., 2015). It has also been shown that the handling/consumption of food of animal origin contaminated by MRSA could provide a potential vehicle for transmission to humans (EFSA, 2009; Feingold et al., 2012). In fact, MRSA clones have been isolated from meats (Agersø et al., 2012; Lim et al., 2010; O'Brien et al., 2012), fish (Hammad et al., 2012), milk, dairy products and ice cream (Karmal et al., 2013; Normanno et al., 2007) worldwide, and these isolates have often been considered as potentially harmful for consumers.

MRSA is also an important cause of mastitis in cattle (Fessler et al., 2010; Haran et al., 2012; Vanderhaeghen et al., 2010); in the subclinical forms, MRSA could be shed in milk, without any alteration to the sensorial characteristics of the product, and thus spread through the food chain.

Italy produces about 10 millions of tons of cow's milk annually (www.agri.istat.it), and a large amount is used in the manufacture

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of typical Italian cheeses, or sold after pasteurization, sterilization or other food processing. The two regions considered in this paper (Apulia and Basilicata) produce many kinds of typical cheeses, both soft and seasoned; among the most popular are "*pasta filata*" cheeses (e.g. *mozzarella* and *caciocavallo*) and *burrata* cheese (Dambrosio et al., 2013). This fact is important for food safety, because contaminated food other than raw milk could be another source of infection for food handlers and food consumers.

Furthermore, the spread of raw milk vending machines (www.milkmaps.com) across Italy increases the possibility that people who have no direct contact with farm animals may handle a potential source of MRSA. Moreover, dairy producers often consume raw milk produced on their farms (Haran et al., 2012).

The data about the presence of MRSA in BTM produced in Italy are scarce and fragmentary. Therefore, this paper aimed to assess the prevalence of MRSA in bulk tank milk (BTM) produced in two regions of southern Italy, and to describe the characteristics of the isolates, in order to lay the groundwork for further investigations on the risks of occupational and foodborne transmission linked to handling or consumption of raw milk in Italy. An additional objective of the study was to assess the relationship between the Coagulase Positive Staphylococci (CPS) level and MRSA prevalence in BTM.

2. Materials and methods

2.1. Bulk tank milk sample collection

BTM samples were collected between September 2012 and April 2013 from 486 dairy farms in southern Italy. In detail, the samples were from 398 dairy farms in Apulia and from 88 in Basilicata. They represent about 16% of the total dairy farms in Apulia and 9% of those in Basilicata (ISMEA, 2013). Each herd had an average number of 40 milking cows, with average milk production of 229.840 Kg per year (ISTAT, 2011). The sampled herds represented approximately 4.4% of the herds in Apulia and Basilicata regions and 1% of total Italian milk production (ISTAT, 2011).

One milk sample per farm was aseptically collected and immediately transported under refrigeration to the laboratory, where it was stored at −80 °C before testing.

2.2. Isolation of MRSA

Samples were thawed at room temperature for approximately one hour, and then 1 ml of milk was added to Mueller-Hinton broth (BiolifeItaliana, Milan, Italy) supplemented with 6.5% (W/v) NaCl (Sigma Aldrich, St Louis MO, USA). After incubation for 24 h at 37 °C, 20 μl of each culture was spread onto a MRSA-SELECT® plate (Bio-Rad, Marnes la Coquette, France) and incubated at 37 °C for 24-48-72 h (Nahimana et al., 2006). Suspected MRSA colonies (pink colonies) were subcultured on a Columbia Sheep Blood Agar plate (Oxoid, Basingstoke, Hampshire, UK) for purification, then screened for methicillin resistance and characterized by genetic and phenotypic analysis as reported below.

2.3. Molecular confirmation of methicillin resistance and genetic characterization of MRSA

Genomic DNA was extracted from the presumptive MRSA isolates using a GenomicPrep® cell and tissue isolation kit (Amersham, Piscataway, NJ, USA) according to the manufacturer's instructions. DNA concentration was determined at a wavelength of 260 nm using a DU 640 spectrophotometer (Beckman, Fullerton, CA, USA) and adjusted with distilled water to 10 ng/μl.

2.3.1. Real time PCR

Real-time PCR for the detection of the *mecA* gene contained a pair of primers (*mec*A147-F and *mec*A147-R) previously described (Zhang et al., 2005). Real-time PCR for the detection of *Staphylococcus aureus*-specific sequences contained a pair of primers (*sau1* and *sau2*) previously described (Strommenger et al., 2003). Conditions for both real-time PCR assays were optimized in a gradient cycler (Mastercyclerep Gradient, Eppendorf, Germany). Both assays were performed in a 25 μL reaction containing 1 μM of each primer, 200 μM each of dATP, dTTP, dGTP, and dCTP (dNTP Mix 5-prime Eppendorf), 1U of Taq DNA polymerase (5-prime Eppendorf), 10X Taq buffer (5-prime Eppendorf), 1X EvaGreen (20X concentrateBiotium, Hayward, CA, USA) and 2 μL template DNA. The procedure consisted of an initial step of 2 min at 94 °C, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and a final extension step at 72 °C for 45 s, with a single fluorescence measurement during the annealing step. The procedure ended with a melting curve program 50–95 °C with a heating rate of 20 min and a continuous fluorescence measurement. One isolate per positive sample identified as MRSA was characterized in the following way.

2.3.2. MLST analysis of MRSA

Alleles at the seven loci, *arc*, *aro*E, *glp*F, *gmk*, *pta*, *tpi* and *yqi*L, were assigned by comparing the sequences at each locus with those of the known alleles in the *S. aureus* MLST database. The allele numbers at each of the seven loci define the allelic profile of each isolate, and an allelic profile is defined as a sequence type (ST). The eBURST program was used to determine the group of each ST based on the MLST database. Grouping was carried out using an analysis panel that selects six minimum numbers of identical loci out of seven loci for group definition, and three minimum single locus variant contents for subgroup definition (Kwon et al., 2005).

2.3.3. Spa-typing

The x region of the *spa* gene was amplified by PCR using primers spa-1113f (5′ TAA AGA CGA TCC TTC GGT GAG C 3′) and spa-1514r (5′ CAG CAG TAG TGC CGT TTG CTT 3′) (Strommenger et al., 2006). DNA sequences were obtained using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, Calif.) with BigDye 3.1 Ready reaction mix (Applied Biosystems) according to the manufacturer's instructions. *Spa*-types were determined using BioNumerics 7.1 (Applied Maths, Belgium) software.

2.3.4. SCC-mec characterization

Staphylococcal cassette chromosome *mec* element (SCC-*mec*) typing was carried out as described by Zhang (Zhang et al., 2005).

2.3.5. Detection of PVL encoding gene

All the MRSA strains were tested by PCR for *luk*S-*luk*F-PV, encoding Panton-Valentine leukocidin (PVL), as described elsewhere (Hesje et al., 2011).

2.3.6. Detection of icaA gene encoding for polysaccharide intercellular adhesin (PIA)

All the MRSA strains were tested by PCR for the *ica*A gene (intercellular adhesion) as described elsewhere (Zmantar et al., 2008).

2.3.7. Detection of staphylococcal enterotoxin encoding genes

MRSA isolates were tested by PCR for *sea* to *seg, seh, sei, sej, sen, seo and sem* genes encoding staphylococcal enterotoxins as described elsewhere (Boerema et al., 2006).

Table 1
Genotypic characteristics of MRSA isolates from bulk tank milk.

Isolates	*mec*A	ST	*spa*-type	Repeat succession	SCC-*mec*	*se*
1	+	1	t174	14-21-16-34-33-13	IVa	*seh*
2	+	1	t127	07-23-21-16-34-33-13	IVa	*seh*
3	+	1	t127	07-23-21-16-34-33-13	IVa	*seh*
4	+	5	t688	26-23-17-34-17-16	V	*seg/sem/sen/seo*
5	+	8	Unknown	04-12-22-25-25	IVa	*seh/seo*
6	+	8	Unknown	11-21-12-21-17-34-24-34-22-25-25	V	*seo*
7	+	45	t015	08-16-02-16-34-13-17-34-16-34	IVa	*sec/seg*
8	+	71	t524	04-17	V	_
9	+	88	t786	07-12-21-17-13-34-34-33-34	IVa	_
10	+	398	t899	07-16-23-02-34	IVa	_
11	+	398	t011	08-16-02-25-34-24-25	V	_
12	+	2781	t1730	26-23-101-21-17-34-34-34-34-34-33-34	V	_

3. Microbiological confirmation of methicillin resistance and detection of MRSA antimicrobial resistance pattern

3.1. Oxacillin and cefoxitin disc diffusion test

Oxacillin and cefoxitin disc diffusion susceptibility tests were performed with 1 μg oxacillin and 30 μg cefoxitin discs (Rosco-Diagnostica, Taastrup, Denmark), following CLSI (Clinical and Laboratory Standards Institute, 2012) recommendations. Mueller-Hinton agar plates (Biolife) were inoculated with a suspension (equivalent to a 0.5 McFarland standard) of each MRSA considered. Plates were incubated at 37 °C and zone diameters were read after 18–24 h. The following breakpoints were considered: oxacillin: resistant ≤10 mm, intermediate 11–12 mm, susceptible ≥13 mm; cefoxitin: resistant ≤21 mm, susceptible ≥22 mm (Shariati et al., 2010).

3.2. Agar screening method

The MRSA suspension (adjusted to match 0.5 McFarland turbidity standard) was inoculated on Oxacillin Salt Screen Agar® (Mueller-Hinton agar containing 4% NaCl and 6 μg oxacillin/ml-Biolife). Plates were incubated at 37 °C for 24 h and any growth on the plate was considered as resistant to methicillin (Shariati et al., 2010).

3.3. E-test

Mueller-Hinton agar plates supplemented with 2% NaCl (Biolife) were inoculated by streaking the standardized inoculums (equivalent to a 0.5 McFarland standard) with a sterile swab. Oxacillin E-test strips (bioMèrieux, Marcy l'Etoile, France) were placed on the plates, followed by incubation at 37 °C for 18–24 h. The minimum inhibitory concentration (MIC) for each isolate was read at the intersection point of the zone of growth inhibition with the graduated strip (resistant ≥ 4 μg/ml; susceptible: ≤ 2 μg/ml) (Shariati et al., 2010).

3.4. Antimicrobic susceptibility testing of MRSA

MRSA isolates were tested for susceptibility to a panel of 21 antimicrobial agents using the disc agar diffusion method on Mueller-Hinton agar, following the guidelines of the CLSI (CLSI, 2012). The antibiotic discs (antibiotic concentration in μg) from Liofilchem (Liofilchem s.r.l, Roseto d. A., Italy) were as follows: amikacin (30), amoxicillin/clavulanic acid (30), ampicillin (10) cephalotin (30), cefotaxime (30), cefoxitin (30), clindamycin (2), choramphenicol (30), doxycycline (30), enrofloxacin (5), erythromycin (15), gentamicin (10), kanamicin (30), oxacillin (1), penicillin (10), streptomycin (10), sulfisoxazole (250), tetracycline (30), trimethoprim-sulfamethoxazole (25), tobramycin (10), and vancomycin (30). The results were recorded after 24 h incubation at 37 °C, and interpreted according to charts supplied with the discs.

4. Detection and enumeration of Coagulase Positive Staphylococci and the relationship with MRSA prevalence

Detection and enumeration of Coagulase Positive Staphylococci (CPS) were performed on all milk samples according to ISO 6888-2, with some modifications (ISO 6888-2, 1999). The enumeration of CPS (CFU per ml of milk) was performed as a weighted mean from two successive dilutions according to ISO 7218:2007/Amd 1:2013. The CPS count was expressed as follows: low level: <100 CFU/ml; medium level: 100–800 CFU/ml; high level: > 800 CFU/ml.

The relationship between MRSA detection and the level of CPS contamination of milk samples (low: <100 CFU/ml; medium: 100–800 CFU/ml; high > 800 CFU/ml) was calculated using the Chi-square test (χ^2, $P < 0.05$) with Epi Info 3.3.2 software.

5. Results

Out of 486 samples of bulk tank milk analysed, 12 (2.5%) were positive for MRSA. The *mec*A gene was detected in all MRSA isolates (Table 1). MLST analysis and SCC-*mec* typing of the MRSA isolates identified the following genotypes: ST1/IVa (3 isolates; 25%), ST8/IVa/V (2 isolates; 16.6%), ST398/IVa/V (2 isolates; 16.6%), ST5/V, ST45/IVa, ST71/V, ST88/IVa, ST2781/V (one isolate, 8.3%) (Table 1). ST1 isolates showed two *spa*-types (t) (t127 and 1 t147); ST8 isolates had unknown *spa*-types; ST5, ST45, ST71, ST88 and ST2781 isolates showed t688, t015, t524, t786 and t1730 *spa*-types, respectively; ST398 isolates showed t011 and t899 *spa*-types (Table 1).

None of the 12 MRSA strains carried *pvl* genes; conversely, the *ica*A gene was detected in all MRSA isolates (Table 1).

With regard to staphylococcal enterotoxin encoding genes, 7 (58.3%) of the 12 MRSA isolates, carried enterotoxin genes and 3 carried more than one gene. Of the 13 investigated enterotoxin genes, *seh* was the most prevalent, since it was recovered in 4 (57.1%) isolates. The genes encoding enterotoxins O and G were detected in 3 (42.8%) and 2 (28.6%) isolates respectively; *sem*, *sen*, *sec* were expressed once in separate single strains (Table 1).

In the microbiological confirmation assays of methicillin resistance, the disc diffusion test with cefoxitin discs, oxacillin agar screen test and oxacillin E-test confirmed all 12 (100%) isolates as MRSA. The disc diffusion test with oxacillin discs confirmed 11 (92%) of the 12 isolates as MRSA (Table 2).

The results of antimicrobial susceptibility testing of MRSA isolates are shown in Table 2. Testing was performed on only 11 MRSA

Table 2
Phenotypic characteristics and antimicrobial resistance profile of MRSA isolates.

Isolates	Disc diffusion test		Oxacillin agar screen test	oxacillin E-test	Antimicrobial resistance profile
	Oxacillin	Cefoxitin			
1	S	R	Growth	R	AUG-AMP-CTX-CF-E-K-P-S-TE
2	R	R	Growth	R	AUG-AMP-CTX-CF-DXT-E-K-OX-P-S-ST-TE
3	R	R	Growth	R	AUG-AMP-CTX-CF-DXT-E-K-OX-P-S-ST-TE
4	R	R	Growth	R	AMP-CTX-CF-DXT-E-OX-P-S-ST-TE
5	R	R	Growth	R	AUG-AMP-CTX-CF-DXT-E-K-OX-P-S-TE
6	R	R	Growth	R	AUG-AMP-CTX-CF-DA-DXT-OX-P-S-TE
7	R	R	Growth	R	–
8	R	R	Growth	R	AUG-AMP-CTX-CF-DA-DXT-OX-P-TE
9	R	R	Growth	R	AUG-AMP- CTX-CF-E-OX-P-ST-
10	R	R	Growth	R	AMP-CTX-CF-DA-DXT-OX-P-ST
11	R	R	Growth	R	AUG-AMP-CTX-CF-DXT-E-OX-P-S-TE-
12	R	R	Growth	R	AUG-AMP-CTX-CF—DA-DXT-ENR-OX-P-S-TE

Abbreviations.
R: resistant (Disc diffusion test- Oxacillin: $\leq$ 10 mm, Cefoxitin: $\leq$ 21 mm; Oxacillin E-test: $\geq$ 4 μg/ml).
AUG, amoxicillin/clavulanic acid; AMP, ampicillin; CTX, cefotaxime; CF, cefoxitin; DA, clindamycin; DXT, doxycycline; ENR, enrofloxacin; E, erythromycin; K, kanamicin; OX, oxacillin; P, penicillin; S, streptomycin; ST, sulfisoxazole; TE, tetracycline.
Isolate numbers are in accordance with those in Table 1.

Table 3
Coagulase Positive Staphylococci (CPS) and MRSA prevalence in the 486 bulk tank milk samples analysed.

Detection		
CPS		MRSA
CPS (CFU/ml)	Samples N (%)	Samples N (%)
Not detectable	121 (25)	/
< 100	138 (28)	4 (0.8)
100–800	145 (30)	6 (1.2)
>800	82 (17)	2 (0.4)
Total samples	**486**	
Total positive samples	**365 (75)**	**12 (2.46%)**

isolates because one was lost. In detail, 11/11 (100%) strains proved to be resistant or intermediate to ampicillin, cefotaxime, cefoxitin, and penicillin, but no strain was resistant to amikacin, cephalotin, choramphenicol, gentamicin, trimethoprim-sulfamethoxazole, tobramycin or vancomycin. Moreover, 10/11 (91%) strains showed resistance to oxacillin and tetracycline, 9/11 (82%) strains showed resistance to amoxicillin/clavulanic acid, and 1/11 (9%) strains demonstrated resistance to enrofloxacin. Susceptibility to clindamycin, doxycycline, erythromycin, kanamicin, streptomycin and sulfisoxazole was variable among strains (Table 2).

The results of detection and enumeration of CPS are shown in detail in Table 3.

Four/486 (0.8%) of the 12 MRSA isolates were detected in milk samples with low CPS levels (<100 CFU/ml), 6/486 (1.2%) in samples with medium CPS levels (100–800 CFU/ml) and 2/486 (0.4%) in samples with high CPS levels (>800 CFU/ml) (Table 3).

Statistical analysis revealed no relationship between the detection of MRSA and the level of CPS contamination of milk samples.

6. Discussion

Methicillin-resistant *S. aureus* (MRSA) can cause a number of human diseases, ranging in severity from minor to life-threatening infections. Handling/consumption of food contaminated with MRSA are potential vehicles of colonization or infections for humans (EFSA, 2009), thus the monitoring of food for the presence of MRSA is mandatory in order to better assess the foodborne risk. Our survey assessed the prevalence of MRSA in bulk tank milk produced in two large regions of southern Italy and described the characteristics of the isolates. The overall prevalence of MRSA in BTM was 2.5%. Our findings are consistent with the low prevalence reported by other authors in Europe and the USA. Studies from Hungary, UK, Germany and Belgium have revealed a prevalence of 0%, 0.3%, 4.4% and 7.4% respectively (Peles et al., 2007; Kreausukon et al., 2012; Paterson et al., 2012; Vanderhaeghen et al., 2010). A study on Minnesota (USA) dairy farms reported two MRSA-positive samples out of 150 pooled BTM samples (Haran et al., 2012). Similar findings were reported by other authors investigating the presence of MRSA in raw cow's milk in other US States (Erskine et al., 2002; Virgin et al., 2009). A low prevalence (1.4%) of MRSA in raw cow's milk was also reported in China (Wang et al., 2014). Researchers from Africa reported a prevalence of MRSA in raw milk ranging from 4.8% in Nigeria to 8.6% in Egypt (Karmal et al., 2013; Umaru et al., 2013). In addition to MRSA prevalence in BTM, numerous reports are available on the prevalence and characterization of MRSA from mastic milk worldwide; also in this case, the prevalence of MRSA as cause of bovine mastitis resulted low (Nam et al., 2011; Vanderhaeghen et al., 2010; Luini et al., 2015; Türkyılmaz et al., 2009).

With regard to strain characterization, our investigation revealed the presence of great genetic diversity, including LA-MRSA and strains of human origin; 8 STs and 9 *spa* types were detected. ST1(t127), known as CA-MRSA, has been associated with serious human infections in the United States and in Germany, and was first reported in Italian pigs by Battisti (Battisti et al., 2010). This *spa*-type was also identified as responsible for mastitis in Italian dairy cows (Benedetti et al., 2010). In our survey, 3 out 12 (25.0%) MRSA strains were identified as ST1-SCC*mec*-IVa-t127. According to data from these authors, the ST1 (t127) MRSA strains detected were PVL-negative. ST1-SCC*mec*-IVa-t286-PVL^{-} strains were also detected in bovine mastitis milk in Korea; the authors underline that the

genotypic characteristics of this strain are the same as those found in CA-MRSA strains prevalent in humans in Korea (Nam et al., 2011). Among our isolates, ST5 isolate was t688, SCC*mec*-IV and both ST8 strains had an unknown *spa*-type and carried SCC*mec*-IVa and V respectively. ST5 PFGE USA100 and ST8 PFGE USA300 were recently detected in BTM from Minnesota dairy farms, thus the authors concluded that genotypes associated with hospitals and the community can also be isolated from milk (Haran et al., 2012). ST398, the most known animal-associated MRSA type, was found in both companion and food-producing animal species, and its zoonotic potential is well documented (Witte et al., 2007; Soavi et al., 2010; Köck et al., 2013). Two (16.6%) ST398 MRSA strains (SCC*mec*V/(t-011)/PVL$^-$ and SCC*mec*IVa/(t899)/PVL$^-$) were isolated from our samples. These results are in contrast with data reported by Tavakol, who detected only ST398 strains (t011, t108 and t889/PVL$^-$) in 38,000 milk samples collected in the Netherlands (Tavakol et al., 2012). The *spa*-types isolated are commonly detected in pigs, thus the authors concluded that MRSA 398 is transmitted between various animal species and can be considered as an etiological agent of mastitis in cows (Tavakol et al., 2012). In addition, Paterson detected 7 MRSA isolates from 1500 BTM samples collected in the United Kingdom, and all resulted ST398 (SCC*mec*IVa and V, t011, t2546/PVL$^-$). The authors stated that workers in dairy farms or dairy plants, or people in regular contact with dairy cows are likely to have a higher risk of colonization or infection with LA-MRSA compared to the general population in the UK (Peterson et al., 2012). Recently, Tenhagen has isolated many ST398 isolates (t011 and t034) from the cattle chain in Germany, including samples from BTM (Tenhagen et al., 2014).

With regard to the risk of staphylococcal food poisoning (SFP) due to the presence of SEs, 58.3% of the strains in our survey resulted potentially enterotoxigenic. Other authors have also reported that MRSA isolates from raw milk carried the genes for the synthesis of SEs (Haran et al., 2012; Normanno et al., 2007; Wang et al., 2014). The most frequent gene encoding for SEs was *seh*, followed by *seo*, *seg*, *sec*, *sem*, and *sen*. The SE most frequently involved in SFP outbreaks is *sea*, in association or not with other staphylococcal enterotoxins (Balaban and Rasooly, 2000), but the role of the recently-discovered SEs in food safety is largely unknown. However, *seh* producing strains have been involved in SFP outbreaks (Ikeda et al., 2005), thus the risk of foodborne intoxication linked to our findings must be carefully assessed.

It is known that bacteria capable of forming biofilms on a wide range of abiotic surfaces such as plastic, rubber, cement, glass, and stainless steel (the most common materials in food processing environments) are well protected against environmental stresses such as disinfectants (Steenackers et al., 2012). The *ica*ADBC genes, encoding for polysaccharide intercellular adhesin (PIA), are present in *S. aureus* strains capable of forming biofilm (Cramton et al., 1999). In a recent paper, Mirani demonstrated that 98.3% of 180 foodborne MRSA isolates carried the *ica*A/SCC*mec* IV profile (Mirani et al., 2013). The author noticed a strong association of *ica*A/SCCmec IV/*agr type II* and biofilm formation in foodborne MRSA isolates. In our study, all the isolates had the genotypic profile *ica*A/SCC*mec* IV or V, thus they have the ability to form biofilm in dairy processing plants, meaning that bacteria are likely to persist in this environment.

With regard to the methods used in our survey, the phenotypic assays used for the isolation and the identification of MRSA from the BTM samples revealed a high level of specificity. The chromogenic medium (MRSA-SELECT® Bio-Rad) used for the detection and the presumptive identification of MRSA was able to reveal correctly all the MRSA strains isolated from the milk samples analysed. Similar results were obtained with the cefoxitin disc diffusion test, considered the preferred phenotypic method to predict the presence of *mec*A-mediated oxacillin resistance in *S. aureus* (CLSI, 2012). The oxacillin agar screen test and oxacillin E-test also confirmed methicillin resistance in all the isolates. The further genotypic characterization confirmed the identity of the strains as MRSA, thus the use of these methods is very useful as a first step in the isolation and presumptive identification of MRSA from raw milk samples. The disc diffusion test with oxacillin confirmed 11 (92%) of the 12 isolates as MRSA. The limited sensitivity of this phenotypic method is well documented; for this reason, according to other authors (Alipour et al., 2014), in our study we combined both genotypic and phenotypic methods for more accurate MRSA detection.

It is well known that the antimicrobial susceptibility of MRSA isolated from human and environmental sources is very variable, and this is of concern in human and veterinary therapy. Moreover, MRSA strains are frequently multidrug-resistant. In this study, all MRSA isolates were resistant to between 6 and 11 antimicrobials, revealing a variable rate of resistance to ampicillin, cefotaxime, cefoxitin, penicillin, amoxicillin/clavulanic acid, oxacillin, tetracycline, clindamycin, doxycycline, erythromycin, kanamicin, streptomycin, sulfisoxazole and enrofloxacin. All isolates were susceptible to amikacin, cephalotin, choramphenicol, gentamicin, trimethoprim-sulfamethoxazole, tobramycin and vancomycin. Our findings are comparable with those of other authors, revealing the widespread diffusion of multidrug-resistant MRSA strains of bovine origin worldwide (Haran et al., 2012; Nam et al., 2011; Wang et al., 2014). As expected, the LA-MRSA isolates (ST398) displayed resistance to tetracycline (Wendlandt et al., 2013).

Interest in raw milk consumption has increased over the past decade; however, the presence of antimicrobial-resistant bacteria in raw milk may be of significant concern for public health (EFSA, 2015). In Italy, the sale of raw milk from vending machines has been legal since 2004, and in 2013, there were 1066 raw milk vending machines throughout the country (EFSA, 2015). The Italian law governing the production and direct marketing of raw milk requires the Coagulase Positive Staphylococci (*S. aureus*) count, but not MRSA detection, for quality control (Italian Republic, 2007). Our survey found no statistical association between the Coagulase Positive Staphylococci count and MRSA detection in the MRSA positive samples, therefore this parameter cannot be used as a microbiological indicator to predict the presence of MRSA in BTM. In addition, although the pasteurization of milk should ensure that MRSA does not enter the food chain (Paterson et al., 2012), the fate of MRSA in dairy products from raw milk appears largely unknown.

In conclusion, the presence of MRSA in food of animal origin poses a potential risk of infection and/or colonization to humans, both through direct contact and through food consumption. In our survey, although the prevalence of MRSA in BTM from southern Italy was low, evaluation of the foodborne and occupational risks for MRSA carriage and infection should be carefully considered.

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