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Molecular characterisation and typing the methicillin resistance of *Staphylococcus* spp. isolated from raw milk and cheeses in northwest Spain: A mini survey

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ABSTRACT

During the last decade, the occurrence of methicillin-resistant staphylococci in animals and foods derived from them has been increasingly reported, but the dairy sector still requires further surveillance. For this, 150 samples of raw bovine milk, ovine and caprine chesses were analysed for identification of *Staphylococcus* spp. isolates based on 16S rRNA gene sequencing and additionally, typing of methicillin resistance (MR) via PCR-targeting of *mec*-region genes. Accordingly, 84 staphylococci isolates were identified and their 16S *rRNA* gene sequences were deposited in GenBank. Among these, 2 isolates of each of *Staphylococcus chromogenes* and *Staphylococcus epidermidis* were methicillin-resistant. Phenotypically, a variable multidrug-resistance was noticed for the *mecA*-positive isolates against eleven selected antibiotics. Results demonstrated that coagulase-negative staphylococci can be a carrier for MR, as *Staphylococcus aureus* does, and could exist in raw milk from lactating animals; which represents a source for MR transmission to the surrounding environment.

1. Introduction

The genus *Staphylococcus* represents serious challenges for both veterinary and public health sectors. Among members of this genus, *Staphylococcus aureus* is considered as one of the leading causes of food-intoxication due to production of a wide range of heat-stable enterotoxins, as well as toxic shock syndrome toxin type-1 (TSST-1). Furthermore, *S. aureus* is a major cause of contagious mastitis in dairy livestock, and is implicated in several disorders such as bone and joint infections, and septicaemia (Juhász-Kaszanyitzky et al., 2007). On the other hand, coagulase-negative staphylococci (CoNS) also possess several threats towards humans and animals and have been documented as the most isolated minor mastitis agents, particularly responsible for subacute and chronic patterns. Another major challenge related to staphylococci is their possession for methicillin resistance (MR). MR in *Staphylococcus* spp. has been increasingly reported worldwide, which is concerning due to the resistance to first line antibiotics such as β-lactams.

As early as 1960, methicillin resistant *S. aureus* (MRSA) was first described in humans, following the introduction of antibiotics into clinical practises (Rolinson, 1961). Since then, resistance to methicillin has gradually disseminated and continued to spread in most countries. Nowadays, both MRSA as well as methicillin resistant CoNS (MR-CoNS) are critically important human pathogens due to their implication in nosocomial and hospital-linked infections, and on the other hand, are also known to be of emerging concern in veterinary medicine and animal farming. The methicillin-resistance *Staphylococcus* spp. (MRS) were frequently reported in farm animal populations, mainly in pigs (Vanderhaeghen et al., 2010; Zhang, Agidi, & LeJeune, 2009), with the sequence type ST398 found most frequently (Monecke, Kuhnert, Hotzel, Slickers, & Ehricht, 2007; Vanderhaeghen et al., 2010).

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The isolation of MRS from dairy animals was first reported in 1972, where MRSA isolates were reported in milk samples obtained from mastitic cows (Devriese, Damme, & Fameree, 1972). Since then, efforts have been carried out for surveying MRS occurrence in dairy animals and foods derived from them. In this regard, several studies have documented a remarkable isolation of MRSA from raw milk, bulk tank milk (BTM) and various dairy products (Haran et al., 2012; Hata et al., 2010; Kamal, Bayoumi, & Salah, 2013; Lee, 2003; Normanno et al., 2007; Sampimon, Lam, Mevius, Schukken, & Zadoks, 2011), and even of hospital- or community-acquired MRSA genotypes (Haran et al., 2012). MRSA was also detected in milk secreted from both clinical and subclinical-mastitis cases (Huber, Koller, Giezendanner, Stephan, & Zweifel, 2010; Juhász-Kaszanyitzky et al., 2007; Kumar, Yadav, & Singh, 2011; Monecke et al., 2007; Spohr et al., 2011; Vanderhaeghen et al., 2010). Cattle-associated-MRSA can vary in possessing common virulence factors, including those implicated in mastitis and food-poisoning, such as TSST-1, haemolysins and staphylococcal enterotoxins (SEs) (Haran et al., 2012; Monecke et al., 2007; Normanno et al., 2007). Similarly to MRSA, the possession of MR feature in CoNS isolated from healthy livestock and milk samples from both non-mastitic and mastitic dairy ruminants has been described (Feßler, Billerbeck, Kadlec, & Schwarz, 2010; Huber et al., 2011; Sampimon et al., 2011; Virgin, Van Slyke, Lombard, & Zadoks, 2009; Walther & Perreten, 2007).

The MR in staphylococci is due to the production of a specific modified penicillin-binding protein (PBP), named PBP2a, which showed a reduced affinity to β-lactam antibiotics (Feßler et al., 2010; Mevius, Sampimon, & Sol, 2005; Prèrea, Barona, Cohen, Bacriea & Fayetb, 2006). Such process is encoded by the *mecA* gene, which is conserved in all MR carriers as a part of the novel designated genetic element, staphylococcal cassette chromosome mec (SCCmec) (Huber et al., 2011; Mevius et al., 2005; Prèrea, Barona, Cohen Bacriea, & Fayetb, 2006; Sampimon et al., 2011). However, MR patterns can differ considerably between *mecA*-carrier strains (Luthje & Schwarz, 2006). This difference is greatly dependent on two regulatory genes located on *mec* DNA region, *mecI* and *mecR1*, which encode for the *mecA* repressor protein and signal transducer protein, respectively (Mevius et al., 2005; Suzuki, Hiramatsu, & Yokota, 1992). MRSA strains possessing intact *mecI* and *mecR1* genes along with *mecA* gene are called pre-MRSA, since the intact *mecI* product strongly supress the *mecA* gene expression, and thus PBP2a production. In turn, the pre-MRSA strain is apparently methicillin susceptible (Kobayashi et al., 1996).

The presence of MRS in multiple microbial ecosystems such as humans, animals, and dairy products represent a potential risk, due to their dissemination through the water cycle and the food chain. Furthermore, it has been already reported the dissemination of MRS among and between dairy animals, and humans (Haran et al., 2012; O'Mahony et al., 2005). In this regard, MRSA clones were isolated from both dairy animals and carrier workers in close contact (Juhász-Kaszanyitzky et al., 2007; Spohr et al., 2011). An international survey by Wulf et al. (2008) showed that 12.5% of surveyed veterinarians were infected with- or carrier for MRSA. Additionally, Huber et al. (2011) showed the prevalence of MR-CoNS in samples from livestock, BTM as well as persons in contact. Realistic threats that follow the transmission of MRS include the complications of treatment of invasive diseases caused by such pathogens, or in the case that a horizontal transfer process of MR feature to bacteria regularly infecting human or animals occurred (Haran et al., 2012; Mevius et al., 2005).

During the last few decades, a radical replacement of traditional phenotypic methods with more accurate and rapid genotypic-based identification approaches has occurred. In particular, 16S rRNA gene sequencing (16S rRNA GS) is a gold standard to identify staphylococcal species of food safety concern. On the other hand, *mecA* gene is universally used as a reference determinant for MR phenotype, and the targeting of genes regulating *mecA* activities, such as *mecR*, *mecI* and *mec* promoter/operator is used for better characterisation and understanding of MR mechanism (Lee, 2006; Luthje & Schwarz, 2006; Pereira et al., 2009; Prèrea et al., 2006). Alternatively, many assays are recently performed to further characterise methicillin resistance strains, such as multilocus sequence typing (Enright, Day, Davies, Peacock, & Spratt, 2000; Haran et al., 2012; Vanderhaeghen et al., 2010), determination of SCC*mec* type (Feßler et al., 2010; Vanderhaeghen et al., 2010; Zhang et al., 2009), MALDI-TOF MS (Böhme et al., 2012; Du, Yang, Guo, Song, & Wang, 2002; Edwards-Jones et al., 2000), microarray (Monecke et al., 2007), and pulsed-field gel electrophoresis (Enright et al., 2000; Haran et al., 2012).

The clinical importance and the increasing dissemination of MRS in clinical and environment systems made necessary to implement surveillance programs to determine their risk, and identify potential critical control points. In addition, detailed knowledge of AR patterns will obviously contribute to treatment decisions and strategies in dairy farms. Therefore, the aim of this work was to determine the presence of MRS with potential clinical relevance in raw milk and cheeses marketed in the northwest of Spain.

2. Materials and methods

2.1. Samples and samples preparation

One hundred raw cow milk samples were randomly collected from different bovine dairy farms in the Galicia region, Spain. Each sample of approximately 500 mL of milk was transferred to a sterile capped bottle that was placed into an insulated ice box. Additionally, twenty five samples of each ovine and caprine cheeses were collected from different markets in the Galicia region, in their original packages. All samples were directed to the laboratory to be bacteriologically examined within 3 h of collection. Raw milk samples were thoroughly mixed and serially diluted using 0.1% peptone water (Merck, Darmstadt, Germany). For cheese samples, 25 g of each sample was cut into small parts and placed into a sterile plastic bag with 225 mL of sterile distilled water and mashed with automatic stomacher for 2 min. After the mixture become homogenous, 1 mL from the cheese homogenate solution was used for preparation of serial dilutions.

2.2. Isolation of staphylococci and testing of coagulase activity

Staphylococcus spp. were isolated on Baired-Parker agar medium (Biomérieux, Paris, France). Based on distinct morphological characters (black with and without opaque halo), different suspected separate colonies were picked. The preliminary differentiation of isolated staphylococci was done based on coagulase test (CT). Each suspected colony was inoculated into peptone water (Merck) at 37 °C for 24 h. Later, 0.5 mL of each tube was transferred aseptically to sterile tube contains 0.5 mL of reconstituted rabbit plasma (STAPH-ASE) (Biomérieux, France) followed by vortexing. The tubes were then incubated at 37 °C for 1–3 h to assess the coagulation activity. Additional incubation for another 24 h was done for negative reactions. The assessment of coagulation reaction was observed by gently inclining each tube to avoid breaking of any developed clot. A negative control tube was done in parallel.

2.3. Identification and phylogenetic analysis based on 16S rRNA gene sequencing

Each isolate was allowed to grow in Brain Heart Infusion broth (Merck) followed by incubation at 37 °C for 24 h. The bacterial pellets were obtained by centrifugation of 1 mL of overnight cultures at 5000× *g* for 12 min. The bacterial cells were lysed by adding 180 μL of lysis solution (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 2 h at 37 °C. The DNeasy Tissue Mini Kit (Qiagen, Valencia, CA, USA) were used for extraction and purification of total DNA. PCR-amplification of a fragment of the 16S rRNA gene was done using the universal primer pair: p8FPL (5′-AGTTTGATCCTGGCTCAG-3′) and p806R (5′-GGACTACCAGGGTATCTAAT-3′) (McCabe, Zhang, Huang, Wagar, & McCabe, 1999). All PCR assays were carried out using the Thermal Cycler, as previously described (Quintela-Baluja et al., 2013). Electrophoretic results of PCR were visualised using Chemidoc imaging system (BioRad, Hercules, CA, USA). PCR products were purified using EXOSAP-IT Kit (GE Healthcare, Uppsala, Sweden) prior to performing the sequencing with Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). The same primers used for PCR assays were also incorporated for the sequencing reactions. Analysis of sequencing reactions was done in an automatic sequencing system (ABI 3730XL DNA-Analyser, Applied Biosystems) coupled with the POP-7 polymer.

The analysis of 16S rRNA gene sequences was carried out using Chromas software (Griffith University, Queensland, Australia) and the two side resulting sequences were aligned with Clustal-X software (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). Identification of resulting sequences was done using the NCBI BLAST tool (NCBI, http://blast.ncbi.nlm.nih.gov/), via sequence homology alignment among published reference sequences (Altschul, Gish, Miller, Myers, & Lipman, 1990). Later, the sequences of all studied isolates were aligned with Clustal-X software (Thompson et al., 1997), and phylogenetic clustering was conducted via MEGA 6 software (Kumar, Nei, Dudley, & Tamura, 2008) using the neighbour-joining method, "Bootstrap" to test phylogeny and the "Kimura 2-parameter model" to compute the distances. The robustness of the nodes was evaluated by bootstrapping (1000 replicates).

2.4. Determination of MR in Staphylococcus spp. isolates

All isolates were examined for the presence of MR genes; *mecA*, *mecR1*, *mecI* and *mec*-promoter as previously described by Hsueh, Teng, Ho, Hsieh, and Luh (1996) and Lee (2006). The primers used for targeting *mecA*, *mecR1*, *mecI* and the *mec* promoter region are illustrated in Table 1. PCR was done as follows: 7.5 μL of DNA was mixed with 12.5 μL of Ex Taq DNA polymerase (Takara Bio) and 2.5 μL of each primer in sterile PCR tubes. Forty cycles of amplification were performed, each consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and an extension stage at 72 °C for 1 min, followed by 5 min of final extension at 72 °C. Amplified products were visualised using Chemidoc imaging system (BioRad) after gel electrophoresis.

Table 1
Primers used in detecting MR among staphylococci isolates.

Target gene	Primer sequence	Amplicon size
mecA	5-AAAATCGATGGTAAAGGTTGGC-3 5-AGTTCTGCAGTACCGGATTTGC-3	533 bp
mecR1	5-TGGTATTTGGTTTAGTGAA-3 5-GATTAGGTTTAGGCATTGA-3	414 bp
mecI	5-AATGGCGAAAAAGCACAACA-3 5-GACTTGATTGTTTCCTCTGTT-3	480 bp
mec-promoter	5-GGAGACGAGCACTAATAACC-3 5-TCGGACGTTCAGTCATTT-3	180 bp

2.5. Antibiotic susceptibility testing

The *mecA*-positive strains were tested for susceptibility to eleven antimicrobials using the disc diffusion method on Mueller-Hinton agar (Oxoid, Basingstoke, UK), and according to standard instructions (Clinical and Laboratory Standards Institute: CLSI, 2008). The antibiotic discs (antibiotic concentration, in μg) were as follows: ampicillin (25), amoxycillin (30), tetracycline (30), gentamycin (10), oxacillin (5), erythromycin (15), ciprofloxacin (15), clindamycin, (2), kanamycin (20), lincomycin (15) and vancomycin (30). Zones of growth inhibition were measured, and the interpretation of results was accomplished following CLSI guidelines, whereby intermediate results were considered resistant.

3. Results and discussion

3.1. Identification of Staphylococcus spp. isolates based on 16S rRNA GS

All isolates were subjected to genetic analysis based on targeting their 16S rRNA gene (approximately 800-bp fragment) using a universal 16S rRNA primer pair. The resulting sequences were analysed and compared with those located in GenBank using the BLAST tool. Accordingly, 84 staphylococcal strains from examined samples were successfully identified based on 16S rRNA GS (≥98% DNA-sequence homology to reference sequences for 16S rRNA) (Table 2). The isolates comprised 11 different species of staphylococci (*S. aureus*, *Staphylococcus epidermidis*, *Staphylococcus xylosus*, *Staphylococcus chromogenes*, *Staphylococcus saprophyticus*, *Staphylococcus lentus*, *Staphylococcus haemolyticus*, *Staphylococcus equorum*, *Staphylococcus simulans*, *Staphylococcus succinus* and *Staphylococcus capitis*). The 16S rRNA GS results were uploaded to GenBank database as illustrated from accession numbers corresponding to isolates (Table 2).

All isolates (100%) were identified to the species level. Additionally, the identification was extended to subspecies level in 7 strains corresponding to (*S. saprophyticus* subsp. *saprophyticus*, *S. succinus* subsp. *succinus* and *S. succinus* subsp. *casei*). To great extent, the discrimination between different staphylococcal species was possible on phylogenetic analysis (high bootstrap values of ≥60 existed between all dendrogram clusters), and only the differentiation between *S. xylosus* and *S. saprophyticus* was not clear, as will be discussed later (Fig. 1). As shown in phylogenetic dendrogram, separate clusters were formed for 7 staphylococcal species: *S. succinus* (A), *S. equorum* (B), *S. haemolyticus* (E), *S. aureus* (F), *S. chromogenes* (I), *S. lentus* (J) *and S. simulans* (K). Meanwhile, *S. epidermidis and S. capitis* formed 2 branches from a common cluster, but their discrimination was significant (bootstrap value of 60). For the remaining *S. xylosus* and *S. saprophyticus* strains, these were joined in a common cluster in which 8 *S. xylosus* isolates formed a separate sub-cluster (LHICA61, LHICA8, LHICA131, LHICA132, LHICA12, LHICA85, LHICA70 and LHICA29), while another sub-cluster was evolved (bootstrap value of 18) that included remaining 3 *S. xylosus* isolates (LHICA71, LHICA42 and LHICA76) in one branch, and LHICA24, LHICA204 and LHICA112 *S. saprophyticus* strains in a second branch. The last evolved branch included *S. saprophyticus* subsp. *saprophyticus* (LHICA47).

In conclusion, the phylogenetic analysis based on targeting 16S rRNA gene allowed for high species identity and for a good differentiation for almost all dairy staphylococcal isolates recovered in the current study. Several studies showed the potential of using 16S

Table 2
Staphylococcus spp. isolates and their identification based on 16S rRNA GS.[a]

Code	Sequence ID (gi)	GenBank Accession No.	Species	Sample	Code	Sequence ID (gi)	GenBank Accession No.	Species	Sample
LHICA 109	1033837713	KX348312.1	*S. aureus*	BM	LHICA 9	1033837759	KX348358.1	*S. equorum*	BM
LHICA 120	1033837714	KX348313.1	*S. aureus*	BM	LHICA 17	1033837760	KX348359.1	*S. equorum*	BM
LHICA 219	1033837715	KX348314.1	*S. aureus*	BM	LHICA 20	1033837761	KX348360.1	*S. equorum*	BM
LHICA 220	1033837716	KX348315.1	*S. aureus*	BM	LHICA 25	1033837762	KX348361.1	*S. equorum*	BM
LHICA 221	1033837717	KX348316.1	*S. aureus*	BM	LHICA 33	1033837763	KX348362.1	*S. equorum*	BM
LHICA 82	1033837718	KX348317.1	*S. epidermidis*	BM	LHICA 35	1033837764	KX348363.1	*S. equorum*	BM
LHICA 113	1033837719	KX348318.1	*S. epidermidis*	BM	LHICA 48	1033837765	KX348364.1	*S. equorum*	OC
LHICA 114	1033837720	KX348319.1	*S. epidermidis*	BM	LHICA 104	1033837766	KX348365.1	*S. equorum*	OC
LHICA 116	1033837721	KX348320.1	*S. epidermidis*	BM	LHICA 99	1033837767	KX348366.1	*S. equorum*	CC
LHICA 155	1033837722	KX348321.1	*S. epidermidis*	OC	LHICA 206	1033837768	KX348367.1	*S. equorum*	CC
LHICA 103	1033837723	KX348322.1	*S. epidermidis*	CC	LHICA 10	1033837769	KX348368.1	*S. haemolyticus*	BM
LHICA 121	1033837724	KX348323.1	*S. epidermidis*	CC	LHICA 90	1033837770	KX348369.1	*S. haemolyticus*	BM
LHICA 85	1033837725	KX348324.1	*S. xylosus*	BM	LHICA 87	1033837738	KX348337.1	*S. chromogenes*	BM
LHICA 75	1033837726	KX348325.1	*S. xylosus*	OC	LHICA 15	1033837739	KX348338.1	*S. chromogenes*	BM
LHICA 76	1033837727	KX348326.1	*S. xylosus*	OC	LHICA 18	1033837740	KX348339.1	*S. chromogenes*	BM
LHICA 8	1033837728	KX348327.1	*S. xylosus*	BM	LHICA 19	1033837741	KX348340.1	*S. chromogenes*	BM
LHICA 12	1033837729	KX348328.1	*S. xylosus*	BM	LHICA 49	1033837742	KX348341.1	*S. chromogenes*	BM
LHICA 27	1033837730	KX348329.1	*S. xylosus*	BM	LHICA 72	1033837743	KX348342.1	*S. chromogenes*	BM
LHICA 29	1033837731	KX348330.1	*S. xylosus*	BM	LHICA 88	1033837744	KX348343.1	*S. chromogenes*	BM
LHICA 42	1033837732	KX348331.1	*S. xylosus*	BM	LHICA 96	1033837745	KX348344.1	*S. chromogenes*	BM
LHICA 61	1033837733	KX348332.1	*S. xylosus*	BM	LHICA 101	1033837746	KX348345.1	*S. chromogenes*	BM
LHICA 70	1033837734	KX348333.1	*S. xylosus*	BM	LHICA 102	1033837747	KX348346.1	*S. chromogenes*	BM
LHICA 71	1033837735	KX348334.1	*S. xylosus*	BM	LHICA 213	1033837748	KX348347.1	*S. chromogenes*	BM
LHICA 131	1033837736	KX348335.1	*S. xylosus*	CC	LHICA 2	1033837782	KX348381.1	*S. succinus*	BM
LHICA 132	1033837737	KX348336.1	*S. xylosus*	CC	LHICA 13	1033837783	KX348382.1	*S. succinus*	BM
LHICA 7	1033837771	KX348370.1	*S. simulans*	BM	LHICA 14	1033837784	KX348383.1	*S. succinus*	BM
LHICA 22	1033837772	KX348371.1	*S. simulans*	BM	LHICA 21	1033837785	KX348384.1	*S. succinus*	BM
LHICA 28	1033837773	KX348372.1	*S. simulans*	BM	LHICA 26	1033837786	KX348385.1	*S. succinus*	BM
LHICA 60	1033837774	KX348373.1	*S. simulans*	BM	LHICA 81	1033837787	KX348386.1	*S. succinus*	OC
LHICA 69	1033837775	KX348374.1	*S. simulans*	BM	LHICA 98	1033837788	KX348387.1	*S. succinus*	OC
LHICA 73	1033837776	KX348375.1	*S. simulans*	BM	LHICA 107	1033837789	KX348388.1	*S. succinus*	OC
LHICA 95	1033837777	KX348376.1	*S. simulans*	BM	LHICA 111	1033837790	KX348389.1	*S. succinus*	CC
LHICA 115	1033837778	KX348377.1	*S. simulans*	OC	LHICA 78	1033837791	KX348390.1	*S. succinus* subsp. *casei*	BM
LHICA 122	1033837779	KX348378.1	*S. Simulans*	OC	LHICA 89	1033837792	KX348391.1	*S. succinus* subsp. *casei*	BM
LHICA 126	1033837780	KX348379.1	*S. simulans*	CC	LHICA 94	1033837793	KX348392.1	*S. succinus* subsp. *casei*	BM
LHICA 127	1033837781	KX348380.1	*S. simulans*	CC	LHICA 79	1033837794	KX348393.1	*S. succinus* subsp. *succinus*	BM
LHICA 86	1033837749	KX348348.1	*S. capitis*	BM	LHICA 91	1033837795	KX348394.1	*S. succinus* subsp. *succinus*	BM
LHICA 50	1033837750	KX348349.1	*S. lentus*	OC	LHICA 130	1033837796	KX348395.1	*S. succinus* subsp. *succinus*	BM
LHICA 63	1033837751	KX348350.1	*S. lentus*	OC	LHICA 24	1033837755	KX348354.1	*S. saprophyticus*	BM
LHICA 43	1033837752	KX348351.1	*S. lentus*	BM	LHICA 112	1033837756	KX348355.1	*S. saprophyticus*	BM
LHICA 44	1033837753	KX348352.1	*S. lentus*	BM	LHICA 204	1033837757	KX348356.1	*S. saprophyticus*	CC
LHICA 67	1033837754	KX348353.1	*S. lentus*	CC	LHICA 47	1033837758	KX348357.1	*S. saprophyticus* subsp. *saprophyticus*	BM

[a] Abbreviations are: BM, bovine milk; OC, ovine cheese; CC, caprine cheese.

rRNA GS for identification of *Staphylococcus* spp. of dairy and food safety concerns (Böhme et al., 2012; Haran et al., 2012; Pereira et al., 2009). For instance, 16S rRNA GS was more conclusive for identification of *S. equorum*, whereas *rpoB* and *cpn60* data were noisy (Sampimon et al., 2009).

Statistical analysis showed that 61 out of 100 (61%), 12 out of 25 (48%) and 11 out of 25 (44%) samples of raw milk, ovine and caprine cheeses, respectively, were contaminated with staphylococci (Table 3). In raw milk, 11 staphylococcal species were identified in the following order: *S. chromogenes* and *S. succinus* (n = 11 of each), *S. xylosus* (n = 9), *S. simulans* (n = 7), *S. equorum* (n = 6), *S. aureus* (n = 5), *S. epidermidis* (n = 4), *S. saprophyticus* (n = 3), *S. lentus* and *S. haemolyticus* (n = 2 of each), and *S. capitis* (n = 1). The results reported previously (Feßler et al., 2010; Sampimon et al., 2009, 2011) for isolated CoNS from bovine milk show great similarity to our results. In ovine cheese, 6 staphylococcal species were isolated corresponding to *S. succinus* as the most identified (25%) followed by *S. lentus*, *S. equorum*, *S. simulans* and *S. xylosus* (16.67% of each) and finally *S. epidermidis* (8.33%). Caprine cheese harboured the same species as ovine cheese in addition to *S. saprophyticus*.

The results of CT were not surprising since only *S. aureus* strains were coagulase-positive and none of other staphylococcal strains showed abnormal coagulase activities. In turn, the CT was carried out as a confirmatory step since coagulase-negative strains of *S. aureus* do occur (Fox, Besser, & Jackson, 1996), and on the other hand, some CoNS identified in the veterinary field may also be coagulase-positive (Taponen & Pyörälä, 2009). Coagulase-positive *S. aureus* constituted 8.20% of overall staphylococci from raw milk, while interestingly, were not identified in either ovine or caprine cheese samples. Contrary to our findings, *S. aureus*, even of toxigenic type, were recovered from cheeses in several studies

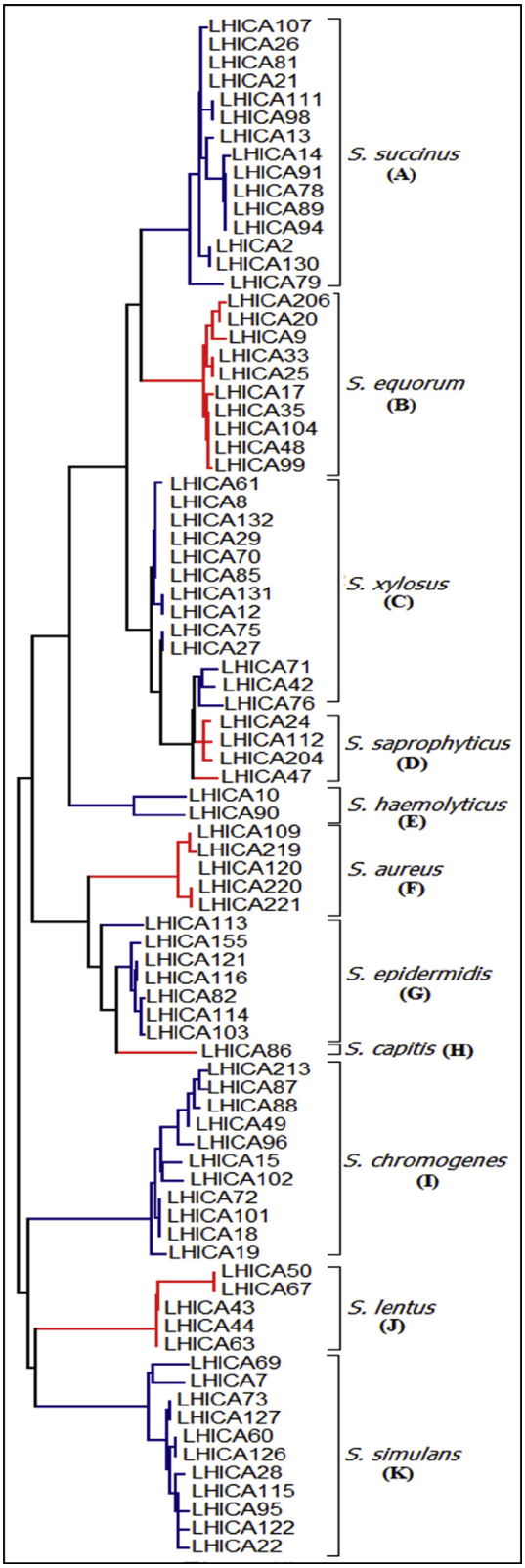

Fig. 1. Phylogenetic dendrogram of *Staphylococcus* spp. isolated from raw milk and cheese samples.

(Böhme et al., 2012; Jakobsen, Heggebø, Sunde, & Skjervheim, 2011; Normanno et al., 2007).

The rapid and accurate identification of *Staphylococcus* spp. is a fundamental issue, since several staphylococcal species are responsible for serious problems in human and veterinary fields. The traditional phenotypic tools involved in staphylococcal diagnosis have a lot of drawbacks attributed to their time-consuming nature, amount of required materials and poor diagnostic accuracy compared with molecular approaches. For example, API Staph-ID 32 and Staph-Zym phenotypic assays correctly identified only 41% and 31%, respectively, of the CoNS isolates from bovine milk samples compared with *rpoB* GS (Sampimon et al., 2009). Similarly, the differentiation of certain staphylococci as *S. haemolyticus*, *S. chromogenes* and *Staphylococcus warneri* was questionable when using API Staph-ID 32 (Feßler et al., 2010). Thus, relying on these assays for CoNS identification was problematic. Therefore, laboratories greatly rely on DNA-sequencing, being used several housekeeping genes for staphylococci identification, such as 16S rRNA, *rpoB*, *cpn60*, *dnaJ* or *tuf* (Capurro et al., 2009; Sampimon et al., 2009; Shah et al., 2007). This study used 16S rRNA GS to identify *Staphylococcus* spp. isolates from bovine milk and cheeses of ovine and caprine origins.

Table 3
Occurrence and incidence of *Staphylococcus* spp. isolates in examined samples.

Isolates	Raw milk		Ovine cheese		Caprine cheese	
	No	%	No	%	No	%
S. aureus	5	8.20	0	0	0	0
S. epidermidis	4	6.56	1	8.33	2	18.18
S. simulans	7	11.5	2	16.67	2	18.18
S. xylosus	9	14.8	2	16.67	2	18.18
S. saprophyticus	3	4.92	0	0	1	9.09
S. capitis	1	1.64	0	0	0	0
S. chromogenes	11	18	0	0	0	0
S. equorum	6	9.84	2	16.67	2	18.18
S. haemolyticus	2	3.28	0	0	0	0
S. lentus	2	3.28	2	16.67	1	9.09
S. succinus	11	18	3	25	1	9.09

3.2. Occurrence of MR in Staphylococcus spp.

Based on PCR-targeting, results showed that 4 out of 84 (4.76%) staphylococcal isolates carried the *mecA* gene (Table 4). These isolates were all CoNS corresponding to *S. epidermidis* and *S. chromogenes* (2 of each). There was a considerable variation in the presence of the other *mec* genes involved in regulation of MR processing and expression. All 4 *mecA*-positive strains carried the *mecR* gene, and none of them carried the repressor *mecI* gene. Only 2 strains (*S. epidermidis*) contained the *mec*-promoter, comprising 2.38% of total staphylococci (Table 4).

Compared with our findings, Moon et al. (2007) reported the occurrence of MR in 2.4% of CoNS, but in contrast, a higher percentage (62%) of MR-CoNS positive BTM samples was reported by Huber et al. (2011), although in parallel a low MRSA prevalence was

Table 4
Occurrence of MR genes among *Staphylococcus* spp. isolates.

Gene	Carrier strains	No.	% from total Staphylococci
mecA	LHICA82 LHICA116 LHICA101 LHICA102	4	4.76
mecR	LHICA82 LHICA116 LHICA101 LHICA102	4	4.76
mecI	–	0	0.00
mec promoter	LHICA82 LHICA116	2	2.38

noted (Huber et al., 2010). In accordance with our findings, methicillin resistant *S. epidermidis* "MRSE" and *S. chromogenes* isolated from bovine milk were found to harbour multiple AR genes including *mecA* (Sampimon et al., 2011). In another study, *S. epidermidis* and *S. haemolyticus* represented the most frequently observed MR-CoNS from bovine milk (Feßler et al., 2010). Moreover, other species of CoNS of dairy and human concerns were shown to carry the *mecA* gene, and thus MR feature, such as *S. simulans* (Suzuki et al., 1992; Ubukata, Nonoguchi, Song, Matsuhashi, & Konno, 1990), *S. haemolyticus* (Feßler et al., 2010; Huber et al., 2011; Prèrea et al., 2006; Suzuki et al., 1992; Ubukata et al., 1990), *S. saprophyticus* (Suzuki et al., 1992), *S. equorum* (Sampimon et al., 2011), *Staphylococcus sciuri* (Couto et al., 1996; Huber et al., 2011; Sampimon et al., 2011; Suzuki et al., 1992), *Staphylococcus hominis* (Prèrea et al., 2006; Suzuki et al., 1992), *Staphylococcus fleurettii* (Huber et al., 2011; Sampimon et al., 2011), *S. capitis* (Suzuki et al., 1992), *S. warneri* (Prèrea et al., 2006; Suzuki et al., 1992), *Staphylococcus cohnii* (Huber et al., 2011) and *Staphylococcus caprae* (Suzuki et al., 1992).

The targeting of *mec*-regulatory genes was necessary to classify *mecA*-carrier strains as methicillin-resistant or -susceptible. Despite the variation in harbouring *mec*-regulatory genes among *mecA*-carriers, none of the isolates carried the *mecI* gene. The absence of repressor products encoded by the *mecI* gene or deletion/mutation of the repressor function of *mecI* is considered a prerequisite for constitutive expression of MR and PBP2a production (Suzuki, Kuwahara-Arai, Richardson, & Hiramatsu, 1993; Weller, 1999). Even at high concentrations of β-lactam antibiotics, PBP2a allows cell wall synthesis in MRSA strains, in contrast to methicillin-susceptible strains with normal PBPs (Kobayashi, Taniguchi, & Urasawa, 1998). The variance in *mec*-DNA region between carrier strains was reported in several studies. For example, *mecR1-mecI* were present in 60–95% of *mecA*-positive *S. aureus* (Kobayashi et al., 1996; Suzuki et al., 1993). Similarly, *mecI* was detected in several species of *mecA*-positive CoNS (e.g., *S. epidermidis*, *S. hominis*, *S. haemolyticus*, *S. sciuri*, *S. caprae*, *S. capitis*, and *S. warneri*; Suzuki et al., 1993), which is contradictory to our results. On the other hand, mutation or deletion in *mecI* as well as *mec*-promoter/operator regions has been reported for some MRS isolates (Hiramatsu, 1995; Kobayashi et al., 1996; Kobayashi et al., 1998).

According to our findings, no MRSA or pre-MRSA strains have been detected among *S. aureus* isolates. Raw milk and cheeses devoid of MRSA have been reported in previous studies (Anderson, Lyman, Bodeis-Jones, & White, 2006; Virgin et al., 2009) in US (Peles et al., 2007), in Hungary, and by Huber et al. (2010) in Switzerland. Meanwhile, other investigations reported occurrence of MRSA in bovine milk or milk products at various rates: 0.68% (1/148 isolate) in Portugal (Pereira et al., 2009), 1.4% (2/142 isolates) in Switzerland (Huber et al., 2010), 1.5% (4/265 isolates) in Japan (Hata et al., 2010), 2.8% (21/835 isolates) in Korea (Moon et al., 2007), 3.15% (3/95 isolates) in Egypt (Kamal et al., 2013), 3.75% (6/160 isolates) in Italy (Normanno et al., 2007), 0.6%–4% in different studies in US (Anderson et al., 2006; Erskine, Walker, Bolin, Bartlett, & White, 2002; Haran et al., 2012), 9.3% (11/118 isolates) in Belgium (Vanderhaeghen et al., 2010), 5.1 (4/78 isolates) to 16.7% (7/42 isolates) and 1.4 (1/74 isolates) to 10% (4/40 isolates) in several dairy herd investigations in Germany (Spohr et al., 2011) and 17.5% (18/105 isolates) in Turkey (Turutoglu, Ercelik, & Ozturk, 2006).

Several phenotypic assays were developed for the detection of MR such as disk diffusion test, MIC determination and automated assays, MRSA latex agglutination test, Microscan and Vitek-2 system (Felten, Grandry, Lagrange, & Casin, 2002; Feßler et al., 2010; Kamal et al., 2013; Moon et al., 2007; Turutoglu et al., 2006). However, these methods are often not sufficiently sensitive or specific, and ambiguous results may be obtained. Thus, it is necessary to investigate heterogeneous resistance as well as to discriminate between MR and borderline resistance. Some staphylococcal strains not carrying *mecA* gene can show phenotypic resistance similar to MRS (Feßler et al., 2010; Lee et al., 2004), and on the other hand, some *mec-A* carriers can show susceptibility to common antibiotics using disk diffusion assay (Normanno et al., 2007) due to heterogeneous resistance. In the latter case, lowered AR phenotypic expression occurs if few cells of a clone are able to express it (Chambers, 1997). Also, phenotypic assays fail to detect low levels of PBP2a, the modified PBP responsible for MR (Prèrea et al., 2006). For these reasons, genotypic targeting of *mecA* gene, which encodes for PBP2a, as well as other genes that regulate *mecA* activities (*mecR*, *mecI* and *mec* promoter/operator) (Lee, 2006; Luthje & Schwarz, 2006; Pereira et al., 2009; Prèrea et al., 2006) is considered the reference standard for determination of MR, and should be compiled before identifying MR in a strain.

3.3. The phenotypic AR in methicillin resistant staphylococci

The phenotypic AR of CoNS strains harbouring the *mecA* gene was checked against eleven selected antibiotics (Table 5). A remarkable variation in phenotypic AR was noted in *mecA*-positive strains. None of the strains was resistant to all antibiotics, but all of them were resistant to at least two antibiotics, and higher multidrug resistance was recorded for *mecA*-positive *S. epidermidis* compared with *S. chromogenes mecA* counterparts. The AR percentage was recorded for tested antibiotics as follows: ampicillin (50%), amoxycillin (75%), tetracycline (25%), gentamycin (75%), oxacillin (100%), erythromycin (50%), ciprofloxacin (25%) and clindamycin, (25%), while no resistance by any strains was found towards kanamycin, lincomycin and vancomycin. In similar studies (Huber et al., 2011; Sampimon et al., 2011), bovine milk was shown to contain MR-CoNS strains that presented phenotypic resistance in variable percentages to several antibiotics such as oxacillin, penicillin, ampicillin, erythromycin, tetracycline, clindamycin, ciprofloxacin, cefoxitin, and gentamycin. A wide spectrum phenotypic-AR has been described previously for methicillin-resistant *S. chromogenes* and *S. epidermidis* of dairy origin (Feßler et al., 2010; Sampimon et al., 2011; Sawant, Gillespie, & Oliver, 2009).

The use of antibiotics in agriculture farming is mostly for therapeutic purposes, and as a preventive measure during dry cow therapy, and being used in lesser extent as growth promoters (Butaye, Devriese, & Haesebrouck, 2003). The overuse and misuse of antibiotics in the last two decades increased the abundance of AR bacteria, which elevated the risk of emergence of resistant zoonotic bacterial pathogens (Haran et al., 2012; Mevius et al., 2005). Furthermore, the presence of mobile genetic elements (e.g., plasmids and transposons) allowed bacteria to interchange resistance genes between different bacterial species without a consideration to phylogenetic, ecological or geographical boundaries (Leonard & Markey, 2008; Mevius et al., 2005). Therefore, foods of animal origin are significantly linked to the spread of potential resistance bacteria to consumers, particularly raw milk and non-thermally

Table 5
Antibiotic susceptibility profiles of *mecA*-positive strains.[a]

mecA-carriers	Amp	Amo	Tet	Gen	Oxa	Ery	Cip	Cli	Kan	Lin	Van
LHICA 82	R	R	S	R	R	R	S	R	S	S	S
LHICA 116	R	R	R	R	R	S	R	S	S	S	S
LHICA 101	S	R	S	S	R	S	S	S	S	S	S
LHICA 102	S	S	S	R	R	R	S	S	S	S	S

[a] Abbreviations are: R, resistant; S, sensitive; Amp, ampicillin; Amo, amoxicillin; Tet, tetracycline; Gen, gentamycin; Oxa, oxacillin; Ery, erythromycin; Cip, ciprofloxacin; Cli, clindamycin; Kan, kanamycin; Lin, lincomycin; Van, vancomycin.

treated dairy products, where bacteria carrying AR genes can survive. Recently, the occurrence of AR in dairy animals has been greatly linked to mastitis as being the main reason for using antibiotics (Lee, 2003; Prèrea et al., 2006). According to US Centre for Disease control and Prevention (CDC), at least 2 million people in USA were infected with antibiotic-resistant bacteria and at least 23,000 die each year as a direct result of these infections.

Although the prevalence of MR-CoNS in milk samples investigated in this study was low, a potential risk of MR dissemination still exists. Dissemination of MR to humans is more possible in situations where raw milk is consumed directly or incorporated for manufacture of some artisanal non-pasteurised milk products, particularly cheeses. In this sense, many food-borne MRSA outbreaks were reported (Jones, Kellum, Porter, Bell, & Schaffner, 2002; Kluytmans et al., 1995). Our assumption was supported by several reports that presented information for the recovery of indistinguishable MRS strains (MRSA and MRSE) between farm animals and human-in-contact, which strongly suggested human/animal transmission; however, they did not clearly indicate the route of transmission (Baptiste et al., 2005; Haran et al., 2012; Huber et al., 2011; Juhász-Kaszanyitzky et al., 2007; Kamal et al., 2013; O'Mahony et al., 2005; Sampimon et al., 2011). Molecular investigations of bovine *mecA*-positive *S. epidermidis* and *S. haemolyticus* and human MR-CoNS showed high similarities (Feßler et al., 2010). However, variable findings were reported regarding animal/human transmission direction. A study by Thorberg et al. (2006) suggested that *S. epidermidis* is more likely to spread from humans to dairy cattle than vice versa. Likewise, food handlers were identified to be the source of contamination of the incriminated food in two food-borne associated MRSA outbreaks (Jones et al., 2002; Kluytmans et al., 1995). Meanwhile, it seemed from previous MRSA dairy sector surveillance studies that raw milk was the main and relevant source of MRSA in the dairy environment (Haran et al., 2012; Juhász-Kaszanyitzky et al., 2007; Lee, 2003; Lee et al., 2004). Moreover, it has been illustrated in these studies that only *mecA*-carrier *S. aureus* were isolated from milk, and none of *S. aureus* strains from other specimens in the same dairy animal (feed, faeces, trachea, uteri, joints and meat) harboured the *mecA* gene.

As shown from both genotypic and phenotypic assays for AR in the current and previous studies, MRS are usually multidrug-resistant, which offers limited choices for their control, obligating longer treatment periods with higher costs and elevating rates of co-morbidities (Feßler et al., 2010; Haran et al., 2012; Pereira et al., 2009; Sampimon et al., 2011; Sawant et al., 2009). Additionally, this could be higher risk for immunocompromised persons, where specific and non-specific immune responses are not combating well, and ingestion of food contaminated by MRS may be lethal (Kluytmans et al., 1995; Normanno et al., 2007).

Furthermore, MR- CoNS can act as a reservoir for emergence of MR in non-carrier *S. aureus* and CoNS via horizontal transmission (Archer & Niemeyer, 1994; Mevius et al., 2005). *S. sciuri* and *S. fleurettii* particularly are considered as the natural reservoir of the *mecA* gene, and it was assumed that MR was horizontally transferred from them to other staphylococcal species (Archer & Niemeyer, 1994; Couto et al., 1996; Huber et al., 2011; Tsubakishita, Kuwahara-Arai, Sasaki, & Hiramatsu, 2010). There is evidence that *S. aureus* acquired the SCC-*mec* from CoNS by such a mechanism (Leonard & Markey, 2008), and that *S. sciuri* was the main source for *mecA* in MRSA during evolution of MR (Archer & Niemeyer, 1994; Couto et al., 1996). If *mecA* is transferred to *S. aureus* or CoNS inhabiting the udder, there is a possibility of public health risk especially if such gene is transferred to strains that are able to survive, invade and spread within human population (Mevius et al., 2005; Walther & Perreten, 2007). On the other side, the colonisation of MRS in human carriers could be a significant source of MRS re-spread to animals, dairy environment, and the community (Jones et al., 2002; Juhász-Kaszanyitzky et al., 2007; Kluytmans et al., 1995; Lee, 2003). This notion was supported by a Korean study where the genomes of six bovine MRSA isolates were closely related to those of human MRSA isolates (Lee, 2003). Consumption of milk and other food products obtained from these animals might be associated to the reported human infection.

4. Conclusion

The identification of *Staphylococcus* spp. isolated from milk and dairy products was facilitated by 16S rRNA GS, and all isolates were identified to the species level. Additionally, our findings elucidated the possible role of raw milk in the contribution to transmission of MRS having multidrug resistance to the surrounding environment. Therefore, continuous monitoring for the development of MR in bacteria that may have impact on animal and human health should be carried out regularly. The harbouring of MR in potentially-pathogenic bacteria in milk needs further investigations to analyse the relationship between antibiotic use patterns in farm production and emergence of resistance towards specific antimicrobials. Additionally, our findings emphasised the importance of hygienic practises within dairy farming and the crucial need for finding alternatives to antibiotics for therapeutic, prophylactic and growth promotion purposes in food-producing animals, which consequently will contribute to limiting MR spread.

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References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*, 403–410.

Anderson, K. L., Lyman, R. L., Bodeis-Jones, S. M., & White, D. G. (2006). Genetic diversity and antimicrobial susceptibility profiles among mastitis-causing *Staphylococcus aureus* isolated from bovine milk samples. *American Journal of Veterinary Research, 67*, 1185–1191.

Archer, G. L., & Niemeyer, D. M. (1994). Origin and evolution of DNA associated with resistance to methicillin in staphylococci. *Trends in Microbiology, 2*, 343–347.

Baptiste, K. E., Williams, K., Willams, N. J., Wattret, A., Clegg, P. D., Dawson, S., et al. (2005). Methicillin-resistant staphylococci in companion animals. *Emerging Infectious Diseases, 11*, 1942.

Böhme, K., Morandi, S., Cremonesi, P., Fernández-No, I. C., Barros-Velázquez, J., Castiglion, et al. (2012). Characterization of *Staphylococcus aureus* strains isolated from Italian dairy products by MALDI-TOF mass fingerprinting. *Electrophoresis, 33*, 2355–2364.

Butaye, P., Devriese, L. A., & Haesebrouck, F. (2003). Antimicrobial growth promoters used in animal feed: Effects of less well known antibiotics on gram-positive bacteria. *Clinical Microbiology Reviews, 16*, 175–188.

Capurro, A., Artursson, K., Waller, K. P., Bengtsson, B., Ericsson-Unnerstad, H., & Aspán, A. (2009). Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and tuf gene sequence-based genotyping for species-level identification of coagulase-negative staphylococci isolated from cases of bovine mastitis. *Veterinary Microbiology, 134*, 327–333.

Chambers, H. F. (1997). Methicillin resistance in staphylococci: Molecular and biochemical basis and clinical implications. *Clinical Microbiology Reviews, 10*, 781–791.

CLSI. (2008). M31–A3. In *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Approved standard* (3rd ed.). Wayne, NJ, USA: Clinical and Laboratory Standards Institute.

Couto, I., de Lencastre, H., Severina, E., Kloos, W., Webster, J. A., Hubner, R. J., et al. (1996). Ubiquitous presence of a *mecA* homologue in natural isolates of *Staphylococcus sciuri*. *Microbial Drug Resistance, 2*, 377–391.

Devriese, L., Damme, L., & Fameree, L. (1972). Methicillin (cloxacillin)-resistant *Staphylococcus aureus* strains isolated from bovine mastitis cases. *Zentralblatt für Veterinarmedizin Reihe B, 19*, 598–605.

Du, Z., Yang, R., Guo, Z., Song, Y., & Wang, J. (2002). Identification of *Staphylococcus aureus* and determination of its methicillin resistance by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Analytical Chemistry, 74*, 5487–5491.

Edwards-Jones, V., Claydon, M. A., Evason, D. J., Walker, J., Fox, A., & Gordon, D. (2000). Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. *Journal of Medical Microbiology, 49*, 295–300.

Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J., & Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *Journal of Clinical Microbiology, 38*, 1008–1015.

Erskine, R., Walker, R., Bolin, C., Bartlett, P., & White, D. (2002). Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. *Journal of Dairy Science, 85*, 1111–1118.

Felten, A., Grandry, B., Lagrange, P. H., & Casin, I. (2002). Evaluation of three techniques for detection of low-level methicillin-resistant *Staphylococcus aureus* (MRSA): A disk diffusion method with cefoxitin and moxalactam, the Vitek 2 system, and the MRSA-screen latex agglutination test. *Journal of Clinical Microbiology, 40*, 2766–2771.

Feßler, A. T., Billerbeck, C., Kadlec, K., & Schwarz, S. (2010). Identification and characterization of methicillin-resistant coagulase-negative staphylococci from bovine mastitis. *Journal of Antimicrobial Chemotherapy, 65*, 1576–1582.

Fox, L. K., Besser, T. E., & Jackson, S. M. (1996). Evaluation of a coagulase-negative variant of *Staphylococcus aureus* as a cause of intramammary infections in a herd of dairy cattle. *Journal of the American Veterinary Medical Association, 209*, 1143–1146.

Haran, K. P., Godden, S. M., Boxrud, D., Jawahir, S., Bender, J. B., & Sreevatsan, S. (2012). Prevalence and characterization of *Staphylococcus aureus*, including methicillin-resistant *Staphylococcus aureus*, isolated from bulk tank milk from Minnesota dairy farms. *Journal of Clinical Microbiology, 50*, 688–695.

Hata, E., Katsuda, K., Kobayashi, H., Uchida, I., Tanaka, K., & Eguchi, M. (2010). Genetic variation among *Staphylococcus aureus* strains from bovine milk and their relevance to methicillin-resistant isolates from humans. *Journal of Clinical Microbiology, 48*, 2130–2139.

Hiramatsu, K. (1995). Molecular evolution of MRSA. *Microbiology and Immunology, 39*, 531–543.

Hsueh, P., Teng, L., Ho, S., Hsieh, W., & Luh, K. (1996). Clinical and microbiological characteristics of *Flavobacterium indologenes* infections associated with indwelling devices. *Journal of Clinical Microbiology, 34*, 1908–1913.

Huber, H., Koller, S., Giezendanner, N., Stephan, R., & Zweifel, C. (2010). Prevalence and characteristics of meticillin-resistant *Staphylococcus aureus* in humans in contact with farm animals, in livestock, and in food of animal origin, Switzerland, 2009. *Euro Surveillance, 15*, 19542.

Huber, H., Ziegler, D., Pflüger, V., Vogel, G., Zweifel, C., & Stephan, R. (2011). Prevalence and characteristics of methicillin-resistant coagulase-negative staphylococci from livestock, chicken carcasses, bulk tank milk, minced meat, and contact persons. *BMC Veterinary Research, 7*, 1–7.

Jakobsen, R. A., Heggebø, R., Sunde, E. B., & Skjervheim, M. (2011). *Staphylococcus aureus* and *Listeria monocytogenes* in Norwegian raw milk cheese production. *Food Microbiology, 28*, 492–496.

Jones, T. F., Kellum, M. E., Porter, S. S., Bell, M., & Schaffner, W. (2002). An outbreak of community-acquired foodborne illness caused by methicillin-resistant *Staphylococcus aureus*. *Emerging Infectious Diseases, 8*, 82–84.

Juhász-Kaszanyitzky, É., Jánosi, S., Somogyi, P., Dán, Á., Van der Graaf-van Bloois, L., Van Duijkeren, E., et al. (2007). MRSA transmission between cows and humans. *Emerging Infectious Diseases, 13*, 630–632.

Kamal, R. M., Bayoumi, M. A., & Salah, F. A. (2013). MRSA detection in raw milk, some dairy products and hands of dairy workers in Egypt, a mini-survey. *Food Control, 33*, 49–53.

Kluytmans, J., van Leeuwen, W., Goessens, W., Hollis, R., Messer, S., Herwaldt, L., et al. (1995). Food-initiated outbreak of methicillin-resistant *Staphylococcus aureus* analyzed by pheno- and genotyping. *Journal of Clinical Microbiology, 33*, 1121–1128.

Kobayashi, N., Taniguchi, K., Kojima, K., Urasawa, S., Uehara, N., Omizu, Y., et al. (1996). Genomic diversity of mec-regulator genes in methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Epidemiology and Infection, 117*, 289–295.

Kobayashi, N., Taniguchi, K., & Urasawa, S. (1998). Analysis of diversity of mutations in the *mecI* gene and *mecA* promoter/operator region of methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrobial Agents and Chemotherapy, 42*, 717–720.

Kumar, S., Nei, M., Dudley, J., & Tamura, K. (2008). MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics, 9*, 299–306.

Kumar, R., Yadav, B., & Singh, R. (2011). Antibiotic resistance and pathogenicity factors in *Staphylococcus aureus* isolated from mastitic Sahiwal cattle. *Journal of Biosciences, 36*, 175–188.

Lee, J. H. (2003). Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Applied and Environmental Microbiology, 69*, 6489–6494.

Lee, J. H. (2006). Occurrence of methicillin-resistant *Staphylococcus aureus* strains from cattle and chicken, and analyses of their mecA, mecR1 and mecI genes. *Veterinary Microbiology, 114*, 155–159.

Lee, J. H., Jeong, J. M., Park, Y. H., Choi, S. S., Kim, Y. H., Chae, J. S., et al. (2004). Evaluation of the methicillin-resistant *Staphylococcus aureus* (MRSA)-Screen latex agglutination test for detection of MRSA of animal origin. *Journal of Clinical Microbiology, 42*, 2780–2782.

Leonard, F., & Markey, B. (2008). Meticillin-resistant *Staphylococcus aureus* in animals: A review. *The Veterinary Journal, 175*, 27–36.

Luthje, P., & Schwarz, S. (2006). Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. *Journal of Antimicrobial Chemotherapy, 57*, 966–969.

McCabe, K. M., Zhang, Y., Huang, B., Wagar, E. A., & McCabe, E. R. (1999). Bacterial species identification after DNA amplification with a universal primer pair. *Molecular Genetics and Metabolism, 66*, 205–211.

Mevius, D., Sampimon, O., & Sol, J. (2005). Antimicrobial resistance in mastitis organisms as a public health threat. In H. Hogeveen (Ed.), *Mastitis in dairy production. Current knowledge and future solutions* (pp. 102–109). Wageningen, The Netherlands: Wageningen Academic Publishers.

Monecke, S., Kuhnert, P., Hotzel, H., Slickers, P., & Ehricht, R. (2007). Microarray based study on virulence-associated genes and resistance determinants of *Staphylococcus aureus* isolates from cattle. *Veterinary Microbiology, 125*, 128–140.

Moon, J., Lee, A., Kang, H., Lee, E., Kim, M., Paik, Y., et al. (2007). Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. *Journal of Dairy Science, 90*, 1176–1185.

Normanno, G., Corrente, M., La Salandra, G., Dambrosio, A., Quaglia, N., Parisi, A., et al. (2007). Methicillin-resistant *Staphylococcus aureus* (MRSA) in foods of animal origin product in Italy. *International Journal of Food Microbiology, 117*, 219–222.

O'Mahony, R., Abbott, Y., Leonard, F., Markey, B., Quinn, P., Pollock, P., et al. (2005). Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from animals and veterinary personnel in Ireland. *Veterinary Microbiology, 109*, 285–296.

Peles, F., Wagner, M., Varga, L., Hein, I., Rieck, P., Gutser, K., et al. (2007). Characterization of *Staphylococcus aureus* strains isolated from bovine milk in Hungary. *International Journal of Food Microbiology, 118*, 186–193.

Pereira, V., Lopes, C., Castro, A., Silva, J., Gibbs, P., & Teixeira, P. (2009). Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal. *Food Microbiology, 26*, 278–282.

Prèrea, M. F., Barona, O., Cohen Bacriea, S., & Fayetb, O. (2006). Genotype MRSA, a new genetic test for the rapid identification of staphylococci and detection of *mecA* gene. *Pathologie Biologie, 54*, 502–505.

Quintela-Baluja, M., Böhme, K., Fernández-No, I., Morandi, S., Alnakip, M., Caamaño, S., et al. (2013). Characterisation of different food-isolated *Enterococcus* strains by MALDI-TOF mass fingerprinting. *Electrophoresis, 34*, 2240–2250.

Rolinson, G. (1961). "Celbenin"-resistant staphylococci. *British Medical Journal, 1*, 125.

Sampimon, O., Lam, T., Mevius, D., Schukken, Y., & Zadoks, R. (2011). Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine milk samples. *Veterinary Microbiology, 150*, 173–179.

Sampimon, O., Zadoks, R. N., De Vliegher, S., Supré, K., Haesebrouck, F., Barkema, et al. (2009). Performance of API Staph ID 32 and Staph-Zym for identification of coagulase-negative staphylococci isolated from bovine milk samples. *Veterinary Microbiology, 136*, 300–305.

Sawant, A., Gillespie, B., & Oliver, S. (2009). Antimicrobial susceptibility of coagulase-negative Staphylococcus species isolated from bovine milk. *Veterinary Microbiology, 134*, 73–81.

Shah, M. M., Iihara, H., Noda, M., Song, S. X., Nhung, P. H., Ohkusu, K., et al. (2007). dnaJ gene sequence-based assay for species identification and phylogenetic grouping in the genus *Staphylococcus*. *International Journal of Systematic and Evolutionary Microbiology, 57*, 25–30.

Spohr, M., Rau, J., Friedrich, A., Klittich, G., Fetsch, A., Guerra, B., et al. (2011). Methicillin-resistant *Staphylococcus aureus* (MRSA) in three dairy herds in southwest Germany. *Zoonoses and Public Health, 58*, 252–261.

Suzuki, E., Hiramatsu, K., & Yokota, T. (1992). Survey of methicillin-resistant clinical strains of coagulase-negative staphylococci for mecA gene distribution. *Antimicrobial Agents and Chemotherapy, 36*, 429–434.

Suzuki, E., Kuwahara-Arai, K., Richardson, J. F., & Hiramatsu, K. (1993). Distribution of mec regulator genes in methicillin-resistant *Staphylococcus* clinical strains. *Antimicrobial Agents and Chemotherapy, 37*, 1219–1226.

Taponen, S., & Pyörälä, S. (2009). Coagulase-negative staphylococci as cause of bovine mastitis-Not so different from *Staphylococcus aureus*? *Veterinary Microbiology, 134*, 29–36.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*, 4876–4882.

Thorberg, B., Kühn, I., Aarestrup, F. M., Brändström, B., Jonsson, P., & Danielsson-Tham, M. (2006). Pheno-and genotyping of *Staphylococcus epidermidis* isolated from bovine milk and human skin. *Veterinary Microbiology, 115*, 163–172.

Tsubakishita, S., Kuwahara-Arai, K., Sasaki, T., & Hiramatsu, K. (2010). Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. *Antimicrobial Agents and Chemotherapy, 54*, 4352–4359.

Turutoglu, H., Ercelik, S., & Ozturk, D. (2006). Antibiotic resistance of *Staphylococcus aureus* and coagulase-negative staphylococci isolated from bovine mastitis. *Bulletin of the Veterinary Institute in Pulawy, 50*, 41–45.

Ubukata, K., Nonoguchi, R., Song, M. D., Matsuhashi, M., & Konno, M. (1990). Homology of *mecA* gene in methicillin-resistant *Staphylococcus haemolyticus* and *Staphylococcus simulans* to that of *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy, 34*, 170–172.

Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermans, K., & Butaye, P. (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. *Veterinary Microbiology, 144*, 166–171.

Virgin, J., Van Slyke, T., Lombard, J., & Zadoks, R. (2009). Short communication: Methicillin-resistant *Staphylococcus aureus* detection in US bulk tank milk. *Journal of Dairy Science, 92*, 4988–4991.

Walther, C., & Perreten, V. (2007). Methicillin-resistant *Staphylococcus epidermidis* in organic milk production. *Journal of Dairy Science, 90*, 5351–5362.

Weller, T. M. (1999). The distribution of *mecA, mecR1* and *mecI* and sequence analysis of *mecI* and the *mec* promoter region in staphylococci expressing resistance to methicillin. *Journal of Antimicrobial Chemotherapy, 43*, 15–22.

Wulf, M., Sørum, M., Van Nes, A., Skov, R., Melchers, W., Klaassen, C., et al. (2008). Prevalence of methicillin-resistant *Staphylococcus aureus* among veterinarians: An international study. *Clinical Microbiology and Infection, 14*, 29–34.

Zhang, Y., Agidi, S., & LeJeune, J. (2009). Diversity of staphylococcal cassette chromosome in coagulase-negative staphylococci from animal sources. *Journal of Applied Microbiology, 107*, 1375–1383.