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Antimicrobial susceptibility and genotyping of *Staphylococcus aureus* isolates collected between 1986 and 2015 from ovine mastitis

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ABSTRACT

In this research, 330 *Staphylococcus aureus* isolates, collected in Sardinia (Italy) in the period 1986–2015 from clinical ovine mastitis and used for the preparation of inactivated autogenous vaccines, were analyzed. Susceptibility to 12 antimicrobial agents was tested by disk diffusion, according to CLSI recommendations. Resistance genes were detected by PCR assays. The most of isolates (85.2%) were susceptible to all antimicrobials tested, suggesting that did not exist change of resistance over time. Two isolates were multidrug-resistant (MDR), one of them (isolate 1496) showed resistance to seven antibiotics including oxacillin and erythromycin. This MRSA harboured SCC*mec* type IV and the *erm*(C) gene. Isolates were characterized by *spa* typing and MLST. Isolates belonged to 29 *spa* types: t1773 (n = 186), t2678 (n = 53), t7754 (n = 14), t1532 (n = 5), t524 (n = 5) and t6060 (n = 4) were the most frequent *spa* types found in Sardinia. The majority of ovine isolates (t1773, t7754 and t1532) was grouped in MLST CC130 (n = 205) followed by CC133 (n = 57). MRSA 1496 was classified as t3896, ST1 and CC1, a clonal complex common in human and also reported in cattle and pig. This study suggests that the CC130/ST700/t1773 is the prevalent *S. aureus* lineage associated with ovine mastitis in Sardinia.

1. Introduction

Mastitis is the one of the most common health problems affecting dairy sheep. Sardinia, an island located in the middle of the Mediterranean, has approximately 3.5 million milking Sarda sheep, corresponding to half of the total Italian stock. The economy of this region is largely based on shepherding and, in particular, on *pecorino* cheese production. Therefore, udder health is a critical factor, and control of intra-mammary infections (IMI) is consequently of the greatest importance for the dairy farmers. A recent and large multi-center Italian study on the aetiology of bacterial mastitis in small ruminants, has confirmed that coagulase-negative staphylococci and *Staphylococcus aureus* are the main causes of subclinical and clinical IMI, respectively (Dore et al., 2016). *S. aureus* is also frequently isolated from recurrent infectious mastitis (Marogna et al., 2010, 2012).

In the control of ovine staphylococcal mastitis, antimicrobial therapy continues to play a significant role. In several studies, the antibiotic resistance of different *S. aureus* isolates from cases of ovine mastitis have been described (Mørk et al., 2005; Aires-de-Sousa et al., 2007; Lollai et al., 2008). Animal vaccination is a valid strategy for controlling diseases, as recommended by the guidelines for the prudent use of antimicrobials in veterinary medicine (2015/C299/07).

Genotyping of *S. aureus* isolated from ovine milk is an important tool in epidemiological studies of mastitis and contributes to our understanding of the pathogen's dissemination. Several molecular methods have been developed for typing *S. aureus* isolates, such as MLST, ribotyping, AFLP, PFGE and staphylococcal protein A (*spa*) typing (Porrero et al., 2012; Smith et al., 2014).

The aim of the present study was to characterise the *S. aureus* isolates collected from ovine milk samples in Sardinia (Italy) in about 30 years and used for the preparation of inactivated autogenous vaccines. We investigated their antimicrobial susceptibility and genetic diversity by means *spa* typing and MLST.

2. Materials and methods

2.1. Bacterial isolates

In this study a total of 330 *S. aureus* isolates, collected between 1986 and 2015 from ovine clinical or gangrenous mastitis in different provinces of Sardinia (Italy), were analyzed. After diagnosis of *S. aureus* mastitis, one isolate per herd was used for the preparation of inacti-

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vated autogenous vaccines, according the Italian Ministerial Decree n°287/1994. Isolates included in this study were randomly selected among our collection of ovine *S. aureus*.

2.2. DNA extraction

Genomic DNA was extracted from all 330 isolates according to Onni et al. (2011). Plasmid isolation was carried out from all TET-resistant *S. aureus* isolates using the QIAprep Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Plasmids were linearized using *Kpn*I enzyme (Roche).

2.3. In vitro antimicrobial susceptibility

Antimicrobial susceptibility testing was performed using the disk diffusion method on Mueller-Hinton agar plates in accordance with current guidelines recommended by the Clinical and Laboratory Standards Institute VET01-04 (CLSI, 2013a). The inoculum turbidity was adjusted to the 0.5 McFarland standard. The following antibiotic disks (Oxoid, Basingstore, England) were used: penicillin (PEN, 10 U.I.), streptomycin (S, 10 µg), novobiocin (NV, 30 µg), kanamycin (KAN, 30 µg), gentamicin (CN, 10 µg), erythromycin (ERY, 15 µg), trimethoprim-sulfamethoxazole (SXT, 25 µg), cephalothin (KF, 30 µg), ampicillin (AMP, 10 µg), amoxicillin-clavulanic acid (AMC, 30 µg), oxacillin (OXA, 1 µg) and tetracycline (TET, 30 µg). S. aureus ATCC 25923 were used as quality control (QC) strains. Isolates were classified as susceptible, intermediate, or resistant on the basis of the inhibition zone diameter using the breakpoint values (mm) indicated in the CLSI manual and related to *S. aureus*.

Multidrug-resistant (MDR) isolate was defined as non-susceptibility to at least one agent in three or more antimicrobial categories.

2.4. Detection of antimicrobial resistance genes and SCCmec typing

The presence of the *mec*A gene was studied by PCR in oxacillin resistant phenotype isolates. The SCC*mec* analysis was carried out by identification of *ccr* complex (*ccr*AB1, *ccr*AB2, *ccr*AB3, *ccr*AB4 and *ccr*C) and the *mec* complex (class A, B and C) by conventional PCRs (Kondo et al., 2007).

The ribosomal methylases encoded by *erm*A, *erm*B and *erm*C which confer resistance to erythromycin were studied by PCR in resistant isolates (Jensen et al., 1999). In addition, specific PCRs were performed for detecting the presence of *tet*K, *tet*M, *tet*L and *tet*O determinants, responsible of tetracycline resistance (Aarestrup et al., 2000; Ullah et al., 2012).

2.5. Genotyping

Amplification of the polymorphic x region of the *spa* region was performed using primers spa-1113f and spa-1514r (Moodley et al., 2006). The *spa* types were determined using the Ridom spaServer software (http://spaserver.ridom.de/). PCR amplifications were performed as described elsewhere (Moodley 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. Alleles at the seven loci, *arc*C, *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 (http://saureus.mlst.net/). Sequence types (STs) were assigned to clonal complexes (CCs) using the eBURST algorithm (Feil et al., 2004).

Table 1
Antimicrobial resistance profiles of *S. aureus* isolates used in this study.

Resistance to [a]:	Year (no of isolates)
CN	**1986** (1)
S	**1998** (1), **2000** (1), **2011** (2)
AMP/PEN	**1991** (1), **1998** (1), **2000** (1), **2009** (1), **2014** (1), **2015** (3)
TET	**1987** (1), **1994**(1), **1995** (2), **1998** (1), **1999** (2), **2000** (1), **2006** (1), **2011** (1), **2012** (1), **2015** (2)
AMP/PEN-TET	**2015** (1)
S-TET	**1990** (1)
AMP/PEN-AMC	**2007** (1), **2013** (1)
AMP/PEN-S-TET	**1999** (1)
AMP/PEN-KF-ERY-KAN-S-AMC-OXA	**2012** (1)
Intermediate to:	
S	**2005** (1), **2011** (2), **2013** (1), **2014** (7), **2015** (6)

[a] Antibiotic abbreviations: CN (gentamicin), S (streptomycin), AMP (ampicillin), PEN (penicillin), TET (tetracycline), AMC (amoxicillin/clavulanic acid), KF (cephalothin), ERY (erythromycin), KAN (kanamycin), OXA (oxacillin).

3. Results

3.1. Antimicrobial resistance

Susceptibility testing was performed on all 330 isolates. The majority of isolates (281/330 corresponding to 85.2%) were susceptible to all antibiotics tested, 17 isolates (5.1%) were classified as intermediate whereas 32 (9.7%) as resistant to one or more antibiotic. The distribution of antimicrobial-resistant isolates in relation to the year of isolation is shown in Table 1. The resistance to TET was the most common finding (16/32), followed to resistance to AMP/PEN (13/32) and S (7/32). Only two isolates were multidrug-resistant (MDR); one of them showed resistance to seven antibiotics including the oxacillin. The oxacillin-resistant *S. aureus* isolate 1496 (collected in 2012) was positive for *mec*A and carried SCC*mec* type IV (2B). In addition, this isolate harboured the *erm*(C) gene. Of 16 *S. aureus* isolates that were resistant to tetracycline, *tet*(K) was identified in 15 (94%). One of them, isolate MDR 4438 of 1999, also carried the *tet*(O) gene. The *tet*(M) gene was amplified only in the isolate 2412 of 2015. None of the isolates harboured the *tet*(L) gene. From plasmid profiling, it was observed that all *tet*(K)-positive isolates shared a plasmid with an estimated size of 4.3 kb, after *Kpn*I digestion. While the tetracycline resistance did not seem related to time variable; on the contrary, a high percentage of streptomycin −intermediate isolates (13/17 corresponding to 76.4%) were collected in 2014 and 2015 (Table 1).

3.2. Genotyping

Twenty-nine different *spa* types were identified among the ovine *S.aureus* isolates collected in the period 1986–2015 (Table 2). t1773 (186/330 = 56.3%) and t2678 (53/330 = 16.1%) are the most frequent *spa* types, found during the whole period of investigation. In contrast, other *spa* types: t7754 (14/330 = 4.2%), t1403 (9/330 = 2.7%), t524 (5/330 = 1.5%), t1532 (5/330 = 1.5%) and t6060 (4/330 = 1.2%) were correlated to specific time periods. Two *spa* types were found in 3 and 2 isolates, respectively; whereas the remaining 20 types were represented by a single isolate. The isolate MRSA 1496, collected in 2012, belonged to t3896. MLST analysis of the main *spa*-types allowed to identify two new STs, ST3730 (t1403) and ST3731 (t524). MLST data are summarized in Table 3. Interestingly, using MLST data, eBURST algorithm assigned most of the identified *spa* types to two Clonal Complex (CC), t1773, t7754 and t7754 to CC130 whereas t2678, t3731 and t6060 to CC133. The three isolates of *spa* t524 did not share any of the studied MLST alleles and were typed as ST521 (n = 2) and ST3731 (n = 1).