Comparative characterisation of human and ovine non-\textit{aureus} staphylococci isolated in Sardinia (Italy) for antimicrobial susceptibility profiles and resistance genes

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Abstract

We present the comparative characterisation of 195 non-\textit{aureus} staphylococci (NAS) isolates obtained from sheep (n = 125) and humans (n = 70) in Sardinia, Italy, identified at the species level by gap gene polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis with Alul. Isolates were tested phenotypically with a disc diffusion method and genotypically by PCR, for resistance to 11 antimicrobial agents including cationic anti septic agents. Among the ovine isolates, \textit{Staphylococcus epidermidis} (n = 57), \textit{S. chromogenes} (n = 29), \textit{S. haemolyticus} (n = 17), \textit{S. simulans} (n = 8) and \textit{S. caprae} (n = 6) were the most prevalent species, while among human isolates, \textit{S. haemolyticus} (n = 28) and \textit{S. epidermidis} (n = 26) were predominant, followed by \textit{S. lugdunensis} and \textit{S. hominis} (n = 4). Of the 125 ovine isolates, 79 (63.2%) did not carry any of the resistance genes tested, while the remainder carried resistance genes for at least one antibiotic. The highest resistance rates among ovine isolates were recorded against tetracycline (20.8%), and penicillin (15.2%); none was resistant to methicillin and two exhibited multidrug resistance (MDR); one of which was positive for the antiseptic resistance \textit{smr} gene. By contrast, most human isolates (59/70, 84.3%) were resistant to \textgtr{}1 antimicrobials, and 41 (58.6%) were MDR. All 52 (74.3%) penicillin-resistant isolates possessed the \textit{blaZ} gene, and 33 of 70 (47.1%) harboured the \textit{mec} gene; of these, seven were characterised by the Staphylococcal Chromosomal Cassette (SCC\text{mec}) type IV, 6 the type V, 5 of type III and one representative each of type I and type II. The majority (57.1%) was erythromycin-resistant and 17 isolates carried only the efflux \textit{msr} gene, 11 the methylase \textit{ermC} gene and an equal number harboured both of the latter genes. Moreover, 23 (32.8%) were tetracycline-resistant and all but one possessed only the efflux \textit{tetK} gene. \textit{qacA}/B and \textit{smr} genes were detected in 27 (38.6%) and 18 (25.7%) human NAS, respectively. These results underline a marked difference in species distribution and antimicrobial resistance between ovine and human-derived NAS.

Introduction

Sardinia, an island located in the middle of the Mediterranean Sea, with a population is around 1.6 million inhabitants, has approximately 3.5 million milking Sarda sheep, corresponding to half of the total Italian national stock. A considerable part of the regional economy relies on dairy sheep farming, mainly for pecorino cheese production; as a consequence, the control of intra-mammary infections is of the greatest importance for dairy farmers. Several reports indicate that non-\textit{aureus} staphylococci (NAS) are the most prevalent bacteria recovered from sub-clinical mastitis of sheep and goats [1–4], thus creating opportunities for cross colonisation and infection among sheep and farmers, due to their antimicrobial resistance and pathogenicity gene pools. Of note, NAS have emerged as relatively frequent nosocomial agents capable of causing infection in debilitated or compromised patients as well as their association with catheter-related and other indwelling medical device-related infections [5].

In the last decade, a significant increase of antibiotic-resistant infections has been recorded among ovine NAS, especially for beta-lactams and tetracyclines, which are commonly used in veterinary practice for mastitis treatment [6, 7]. Two mechanisms confer penicillin resistance in staphylococci, the most common being production of \textbeta-lactamase, encoded by the \textit{blaZ} gene. The other mechanism is due to a penicillin-binding protein transpeptidase (PBP2a), encoded by the \textit{mec} gene [8], which is carried on a mobile chromosomal element, the Staphylococcal Chromosomal Cassette \textit{mec} (SCC\text{mec}) [9]. SCC\text{mec} types are defined by the
recombinase (ccr) gene complex and the class of the mec gene complex [10]. Recently, a novel PBP2a homologue has been described as encoded by mecC [11].

Many cationic antiseptic agents such as quaternary ammonium compounds (QACs) are widely used as surface germicides within healthcare facilities [12, 13]. Although issues of antibiotic resistance have been widely investigated, knowledge on the occurrence of antiseptic resistance genes (qacA/B, smr, qacG, qacH, qacJ) in staphylococci from dairy animals is limited [7, 14].

In most veterinary and clinical laboratories, differentiation of NAS species is based on phenotypic reactions which may be unreliable, particularly for animal isolates. Consequently, several genotypic methods are increasingly being applied for species-level identification [15]. This approach combined with genotyping by multilocus sequence typing (MLST) for the differentiation of strain populations, allows an informative analysis and insight into the evolving epidemiology of bacterial species groups in relation to their pathogenicity and antimicrobial resistance [16].

The objective of this study was to compare the molecular characteristics of NAS isolated from ovine mastitis with those from human clinical specimens, and specifically to: (1) identify NAS isolates using genotypic techniques; (2) determine their antimicrobial susceptibility profiles, and distribution of antimicrobial and antiseptic resistance genes and (3) determine the genetic relatedness of isolates within species by MLST.

Materials and methods

Isolate collection

Ovine

In total, 125 NAS isolates were collected from sheep milk samples in different provinces of Sardinia (Italy) over a period of 9 months (April–December 2017). The isolates belonged to a bank of NAS used for the preparation of inactivated autogenous vaccines, according to the Italian Ministerial Decree no. 287/1994. Basic identification of staphylococci was determined by colony morphology, Gram-stain, catalase and coagulase tests, clumping factor production (Staphylase Test, Oxoid, UK), and growth on mannitol salt agar (Becton Dickinson, Quebec, CDN).

Human

During the same period, 70 NAS isolates were collected from clinical specimens from 70 different patients attending clinical departments at three major hospitals in Sardinia. Isolates were anonymised without patient identifiers and thus individual consent was not required: 90% of the human NAS were recovered from hospitalised patients in intensive care unit, haematology/haematology, orthopaedics.

Species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Species identification was based on PCR amplification and PCR-RFLP of the glyceraldehyde-3-phosphate dehydrogenase gene (gap) [17]. Sequencing of the gap gene was used to identify non-specified isolates. Briefly, 15 μl of amplicons were digested in a 30 μl volume containing 10× buffer, 0.5 μl of 10 mg/ml acetylated BSA (Promega, Madison, USA) and 1 μl of 10 U/μl FastDigest AluI endonuclease (Thermo Fisher Scientific, City, Country). Samples were incubated at 37 °C for 15 min and then electrophoresed in 12% polyacrylamide gel. Fifteen reference strains were included: Staphylococcus epidermidis ATCC 14990, S. chromogenes ATCC 43764, S. xylosus ATCC 29971, S. warneri ATCC 27836, S. capitis ATCC 27840, S. hominis ATCC 27844, S. haemolyticus ATCC 29970, S. arlettae ATCC 43957, S. caprae ATCC 43958, S. caprae ATCC 35538, S. lentus ATCC 29070, S. hyicus ATCC 11249, S. epidermidis ATCC 14990 and lane 14, S. aureus ATCC 43300.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using a disc diffusion method on Mueller–Hinton agar plates conforming to the Clinical and Laboratory Standards Institute [18] with an inoculum corresponding to the 0.5 McFarland standard. The following antibacterial discs (Oxoid, Basingstoke, UK) were used: penicillin (Pn, 10 IU), tetracycline (Tε, 30 μg), streptomycin (St, 10 μg), kanamycin (Km, 30 μg), gentamicin (Gn, 10 μg), erythromycin (Er, 15 μg), trimethoprim-sulphamethoxazole (Tm, 25 μg), amoxicillin-clavulanic acid (Amc, 30 μg), cefalothin (Ki, 30 μg), cefoxitin (Kx, 30 μg, for detection of methicillin resistance) and oxacillin (Ox, 1 μg). S. aureus ATCC 25923 were used as the quality control strain. Isolates were classified as susceptible, intermediate or resistant based on inhibition zone diameters using CLSI breakpoint values (mm) for S. aureus. Multidrug resistance (MDR) was defined as resistance to at least three classes of the antimicrobials tested [19].

Detection of resistance genes and SCCmec typing

Cefoxitin/oxacillin-resistant isolates were tested for carriage of mecA gene and SCCmec type by PCR [20]. The presence of cerAB5 and of the novel cer allelotype (cerAB5) was confirmed with published primer sets [21, 22]. Genes encoding resistance to penicillin (blaZ), macrolide (msrA, ermA, ermB and ermC) and tetracycline (tetO, tetK, tetM and tetL) were also identified by PCR [23–26]. The following positive controls were included: S. aureus ATCC 33591 (blaZ), S. haemolyticus isolate 772
**Table 1. Distribution of ovine and human NAS isolates, identified by PCR-RFLP and sequencing analysis of the gap gene, according to the specimen origin**

| Source         | S. epi | S. chr | S. haemolyticus | S. simulans | S. caprae | S. warneri | S. saprophyticus | S. intermedius | S. muscae |
|----------------|--------|--------|-----------------|-------------|-----------|------------|------------------|----------------|-----------|
| **Ovine isolates** |        |        |                 |             |           |            |                  |                 |           |
| Milk           | 57     | 29     | 17              | 8           | 6         | 5          | 1                | 1              | 1         |
| **Human isolates** |        |        |                 |             |           |            |                  |                 |           |
| Nasal swab     | 6      | 5      | –               | –           | –         | 2          | –                | –              | –         |
| Blood          | 4      | 6      | –               | 1           | –         | –          | –                | –              | –         |
| Skin swab      | 2      | 3      | –               | –           | 2         | 1          | –                | –              | –         |
| Pus            | 1      | 3      | –               | –           | –         | –          | –                | 1              | –         |
| Peritoneal fluid | 2     | –      | 2               | –           | –         | –          | –                | –              | –         |
| Seminal fluid  | 4      | –      | –               | –           | –         | –          | –                | –              | –         |
| Injury         | –      | 3      | 1               | –           | –         | –          | –                | –              | –         |
| Ear swab       | 2      | –      | –               | –           | 1         | –          | –                | –              | –         |
| Oral swab      | 1      | 1      | –               | –           | –         | –          | –                | 1              | –         |
| Urine          | 2      | –      | –               | 1           | –         | –          | –                | –              | –         |
| C.V.C.         | –      | 2      | –               | –           | –         | –          | –                | –              | –         |
| Ulcer swab     | –      | 1      | –               | –           | –         | –          | –                | –              | –         |
| F.V.C.         | –      | –      | –               | –           | –         | –          | –                | –              | –         |
| Peritoneal swab| 1      | –      | –               | –           | –         | –          | –                | –              | –         |
| Vaginal swab   | –      | –      | 1               | –           | –         | –          | –                | –              | –         |
| Glans swab     | 1      | –      | –               | –           | –         | –          | –                | –              | –         |
| Pleural fluid  | –      | –      | 1               | –           | –         | –          | –                | –              | –         |
| Fluid drainage | –      | –      | –               | –           | –         | –          | –                | –              | –         |
| B.L. fluid     | 1      | –      | –               | –           | –         | 1          | –                | –              | –         |
| N.P. aspirate  | –      | –      | –               | –           | 1         | –          | –                | –              | –         |
| Bile           | 1      | –      | –               | –           | –         | –          | –                | –              | –         |
| Biopsy         | –      | 1      | –               | –           | –         | –          | –                | –              | –         |
| Prosthesis     | –      | –      | –               | 1           | –         | –          | –                | –              | –         |

**Results**

**Ovine NAS**

Figure 1 shows the PCR-RFLP profiles obtained for the 15 reference *Staphylococcus* strains. All, but two, of the 125 ovine isolates were assigned to seven species: *S. epidermidis* (n = 57), *S. chromogenes* (n = 29), *S. haemolyticus* (n = 17), *S. simulans* (n = 8), *S. caprae* (n = 6), *S. warneri* (n = 5) and *S. saprophyticus* (n = 1); the remaining two isolates were identified as *S. intermedius* and *S. muscae* (Table 1). Seventy-nine (63.2%) were susceptible to all antibiotics tested and the remaining 46 (36.8%) showed resistance to at least one antibiotic. The highest resistance rate was recorded against Te (n = 26; 20.8%), followed by Pn (n = 19; 15.2%), St (5.6%) and Er (4%). No resistance to Gn, Arm, Kf, Kx and Ox was detected. Only two isolates (1.6%) showed MDR. No isolate was positive for qacA/B genes but one harboured the smr antisense resistance gene.
Table 2. Antimicrobial susceptibility results, resistance genes detected and SCCmec typing of both 57 ovine and 26 human S. epidermidis isolates

| Source          | Antimicrobial resistance* | Resistance genesb | SCCmec Cmec type |
|-----------------|---------------------------|-------------------|------------------|
| **Ovine isolates** |                           |                   |                  |
| Milk            | Pn (n = 4)                | blaZ              | –                |
|                 | Te (n = 10)               | tetK              | –                |
|                 | Km (n = 1)                |                   | –                |
|                 | St (n = 1)                |                   | –                |
|                 | Pn-Te (n = 1)             | blaZ, tetK        | –                |
|                 | Pn-St (n = 2)             | tetK              | –                |
|                 | Pn-Te-Er (n = 1)          | blaZ, tetK, msrA  | –                |
|                 | Pn-Te-Km-Trs (n = 1)      | blaZ, tetK-smr    | –                |
| **Human isolates** |                           |                   |                  |
| Pus             | –                         | smr               | –                |
| Blood           | Pn                         | blaZ, smr         | –                |
| Biopsy          | Pn                         | blaZ              | –                |
| Skin swab       | Er                         | msrA, qacA/B      | –                |
| Ulcer swab      | Gn-Km                      | qacA/B            | –                |
| Oral swab       | Gn-Km                      |                   | –                |
| Pus             | Pn-Te                      | blaZ, tetK        | –                |
| Nasal swab      | Pn-Ox                      | blaZ, smr         | NT               |
| Nasal swab      | Pn-Er-Ox                   | blaZ, ermC        | Type IV          |
| C.V.C.†         | Pn-Er-Ox                   | blaZ, ermC, msrA, | Type III         |
| Injury          | Pn-Er-Ox                   |                   |                  |
| Skin swab       | Pn-Er-Trs                  | blaZ, ermC        | –                |
| Blood           | Pn-Km-Ox                   | blaZ, smr         | Type IV          |
| Injury          | Pn-Er-Gn-Km                | blaZ, msrA, qacA/B| –                |
| Blood           | Er-Gn-Km-Trs               | ermC, msrA, qacA/B| –                |
| Skin swab       | Pn-Gn-Km-Ox                | blaZ              | NT               |
| Blood           | Pn-Er-Gn-Km                | blaZ, msrA, qacA/B| –                |
| Nasal swab      | Pn-Er-Gn-Km-Ox             | blaZ, msrA, smr   | Type IV          |
| F.V.C.†         | Pn-Er-Gn-Km-Ox             | blaZ, ermC        | NT               |
| Pus             | Pn-Gn-Km-Amc-Ox            | blaZ, qacA/B      | NT               |
| Pn-Er-Amc-Ox    |                           |                   |                  |

Table 2. (Continued.)

| Source          | Antimicrobial resistance* | Resistance genesb | SCCmec Cmec type |
|-----------------|---------------------------|-------------------|------------------|
| Nasal swab      |                           |                   |                  |
| Injury          | Pn-Er-Gn-Km-Trs           | blaZ, ermC, tetK, | Type III         |
|                 |                           | tetL, smr         |                  |
| Blood           | Pn-Er-Gn-Km-Trs-Ox        | blaZ, msrA, qacA/B| Type III         |
| Nasal swab      | Pn-Er-Gn-Km-Amc-Trs-Ox    | blaZ, msrA, qacA/B| Type III         |
| C.V.C.†         | Pn-Er-Gn-Km-Amc-Trs-Ox    | blaZ, ermC,       | Type III         |
|                 |                           | smr               |                  |
| Blood           | Pn-Er-Gn-Km-Amc-Trs-Ox    | blaZ, ermC        | Type III         |

*Antibiotic abbreviations: Pn, penicillin; Er, erythromycin; Te, tetracycline; Km, kanamycin; Ox, oxacillin, Trs, trimethoprim-sulphonamethoxazole; Gn, gentamicin; Amc, amoxicillin-clavulanic acid; St, streptomycin.

†Resistance genes for Pn (blaZ), Er (ermA, ermB, ermC, msrA), Te (tetK, tetO, tetL, tetM), anti-septic agents (qacA/B, smr).

S. epidermidis

S. epidermidis was the most represented species among ovine isolates. Of the 57 isolates, 22 (38.6%) were resistant to one or more antimicrobials with Te- and Pn-resistance in 26.3% and 14% of all isolates, respectively. Two were MDR: one was resistant to three different classes (Pn, Er and Te) and one to four different classes (Pn, Te, Km and Trs) (Table 2). All Pn-resistant S. epidermidis had the blaZ gene while all Te-resistant isolates harboured only tetK. The Er-resistant isolate carried msrA, and the isolate resistant to four different antibiotics was positive for smr (Table 2).

An MLST profile was assigned to 54 of the 57 isolates with the most prevalent being ST225 (n = 15), ST6 (n = 11) and ST100 (n = 5) (Table 3). Eight novel allelic profiles were submitted and designated as ST971, ST974, ST975, ST976, ST977, ST978, ST979 and ST980 in the MLST database (http://sepidermidis.mlst.net/). The two MDR isolates were ST81 and ST979, respectively.

S. chromogenes, S. haemolyticus and minor ovine NAS

S. chromogenes (n = 29) and S. haemolyticus (n = 17) were the next most prevalent species isolated from ovine milk samples. Only one of the 17 S. haemolyticus isolates was resistant to Te (tetK gene) (Table 4). Among S. chromogenes, 11 isolates were resistant to a single antibiotic and four to two agents. The highest resistance was found against Pn (n = 7), followed by Te (n = 6) and Er (n = 3). All Pn-resistant isolates had the blaZ gene while, among the six S. chromogenes isolates resistant to Te, five were positive for tetK and one for tetM. Of the three Er-resistant isolates, one had both ermB and ermC, one only ermC, and the other, ermB (Table 5).

Regarding the remaining ovine isolates, two of four S. simulans were resistant to Te (tetK or tetM gene), one to Er (ermC gene) and one to St. Two of the three S. caprae were resistant to both Pn and Te (blaZ and tetK genes) and one to Pn alone (blaZ). The single Pn-resistant isolate of S. warneri contained the blaZ gene (Table 5).
Sixty-four NAS isolates of human origin were identified as: *S. haemolyticus* (*n* = 28), *S. epidermidis* (*n* = 26), *S. hominis* (*n* = 4), *S. capitis* (*n* = 3), *S. warneri* (*n* = 2) and *S. xylosus* (*n* = 1). Six additional isolates were identified by DNA sequencing as *S. lugdunensis* (*n* = 4), *S. pasteuri* (*n* = 1) and *S. saprophyticus* subsp. *bovis* (*n* = 1) (Table 1). Only 10 (14.3%) of the 70 isolates of NAS were susceptible to all antimicrobials tested, with the following distribution: *S. lugdunensis* (*n* = 4), *S. capitis* (*n* = 2), *S. haemolyticus* (*n* = 2) and one isolate each of *S. epidermidis* and *S. warneri*. The remainder were resistant to one (21.6%), two (13.4%) and three or more (65%) classes of antibiotics.

### S. haemolyticus

All, but two, of 28 isolates of this species were resistant to one or more antimicrobials, with high rates to Pn (*n* = 25, 89.3%), Km (*n* = 23, 82.1%), Er (*n* = 22, 78.6%), Ox/Kx (*n* = 18, 64.3%) and Te (*n* = 15, 53.6%; the great majority being MDR) (Table 4). All 18 Ox-resistant *S. haemolyticus* isolates were mecA positive with SCCmec type V represented by six and SCCmec type IV in three. A single isolate of SCCmec type VII with recombination genes ccrA5 and ccrB5 was also PCR-positive for the ccrAB5T ALLotype. Eight isolates were non-typeable for SCCmec (Table 4). All Pn-resistant isolates harboured the blaZ gene. Among the 22 Er-resistant isolates, five possessed only the ermC gene, eight only the msrA gene, eight carried simultaneously ermC and msrA genes, and one was positive for both ermA and msrA genes (Table 4). All 15 Te-resistant isolates had the tetK gene. Antiseptic-resistance genes: qacA/B alone, or smr alone, were detected in 42.8% (*n* = 12) and in 21.8% (*n* = 6) of the *S. haemolyticus* isolates, respectively. An additional three isolates possessed both qacA/B and smr genes.

### S. epidermidis

All, but one, of the 26 *S. epidermidis* isolates were resistant to ≥1 antibiotic (Table 2) with 21 (80.7%) resistant to Pn, 15 (57.7%) each to the macrolide Er and aminoglycosides, 14 (53.8%) to Ox/Kx and 4 (15.4%) to Te. Half of the species isolates were MDR. The 14 Ox/Kx-resistant isolates (SCCmec type II = 1, SCCmec type IV = 4 and SCCmec type III = 5). Four isolates were non-typeable for SCCmec and were also PCR-negative for ccrAB5 and ccrAB5T. All Pn-resistant isolates were positive for the blaZ gene; six of 15 Er-resistant isolates were positive only for the msrA gene, five the ermC gene and four both ermC and msrA genes. The four Te-resistant isolates carried the tetK gene, one of them also had the tetL gene. Nine (34.6%) isolates possessed the qacA/B gene alone and the same number only the smr gene. Three isolates from blood, skin swab and seminal fluid carried both genes. Interestingly, the smr gene was detected in the antimicrobial-susceptible *S. epidermidis* isolated from pus.

MLST analysis showed that ST59 and ST5 were each represented by six isolates and ST80 by five. ST981 proved to be a novel type.

### Minor human NAS

Three of the four *S. hominis* isolates were MDR and one of them, isolated from urine, was including Er (ermC gene) and Te (tetK gene); one of them, isolated from urine, was SCCmec type I. Of note, each of the four *S. hominis* isolates was positive for the blaZ gene and two also harboured the qacA/B gene (Table 5). *S. pasteuri* and *S. capitis* isolates were resistant to Er (both ermC and msrA genes) and Pn (blaZ gene), respectively. *S. saprophyticus* subsp. *bovis*, resistant to Te, possessed the tetK gene. In the *S. warneri* isolate, collected from an ear swab and susceptible to all antibiotics tested, the qacA/B gene was found (Table 5).

### Discussion

This study is the result of an integrated collaboration between veterinary and human health care professionals to determine whether NAS recovered from human infections share some
Table 4. Antimicrobial susceptibility results, resistance genes detected and SCCmec typing of the 17 ovine and 28 human S. haemolyticus isolates

| Source                    | Antimicrobial resistance | Resistance genes | SCCmec type |
|---------------------------|--------------------------|------------------|-------------|
| **Ovine isolates**        |                          |                  |             |
| Milk                      | Te                       | tetK             |             |
| **Human isolates**        |                          |                  |             |
| Peritoneal fluid          | –                        |                  |             |
| Ear swab                  | –                        | qacA/B           |             |
| Seminal fluid             | Pn                       | blaZ             |             |
| Seminal fluid             | Pn-Er-Te                 | blaZ, ermC, tetK, qacA/B |             |
| Pus                       | Pn-Er-Km-Te              | blaZ, msrA, tetK, qacA/B |             |
| Nasal swab                | Pn-Er-Km-Te              | blaZ, ermC, tetK, smr |             |
| Urine                     | Pn-Km-Te-Ox              | blaZ, tetK, smr  | Type IV     |
| Nasal swab                | Pn-Km-Trs-Ox             | blaZ             | Type V      |
| Peritoneal swab           | Er-Gn-Km-Te-Trs          | ermC, tetK, smr  |             |
| Blood                     | Pn-Er-Km-Te-Trs          | blaZ, ermC, tetK, smr |             |
| Seminal fluid             | Pn-Gn-Km-Te-Ox           | blaZ, tetK, qacA/B, smr | Type IV    |
| B.L. fluid*               | Pn-Er-Gn-Km-St-Te        | blaZ, msrA, tetK, qacA/B |             |
| Blood                     | Pn-Kf-Er-Gn-Km-Te        | blaZ, ermC, msrA, tetK, smr |             |
| Ear swab                  | Pn-Er-Gn-Km-Trs-Ox       | blaZ, msrA       | Type V      |
| Blood                     | Pn-Kf-Er-St-Amc-Ox       | blaZ, ermA, msrA | Type IV     |
| Seminal fluid             | Pn-Er-Gn-Km-Te-Amc-Ox    | blaZ, ermC, tetK, qacA/B | Type V      |
| Nasal swab                | Pn-Kf-Er-Gn-Km-Te-Trs-Ox | blaZ, ermC, msrA, tetK, | Type V      |
| Skin swab                 | Pn-Kf-Er-Gn-Km-Te-Amc-Ox | blaZ, msrA, tetK, qacA/B | NT          |
| Nasal swab                | Pn-Kf-Er-Gn-Km-Te-Amc-Ox | blaZ, msrA, tetK, qacA/B | NT          |
| Oral swab                 | Pn-Kf-Er-Gn-Km-Te-Amc-Ox | blaZ, msrA, tetK, qacA/B | NT          |
| Bile                      | Pn-Kf-Er-Gn-Km-St-Amc-Ox | blaZ, ermC, msrA, qacA/B | NT          |
| Skin swab                 | Pn-Kf-Er-Gn-Km-St-Amc-Ox | blaZ, ermC, msrA, qacA/B | NT          |
| Nasal swab                | Pn-Kf-Er-Gn-Km-Amc-Trs-Ox| blaZ, msrA, qacA/B | NT          |
| Nasal swab                | Pn-Kf-Er-Gn-Km-Amc-Trs-Ox| blaZ, ermC, msrA, qacA/B | Type V      |
| Glans swab                | Pn-Kf-Er-Gn-Km-Amc-Trs-Ox| blaZ, ermC, msrA, qacA/B | Type VII    |
| Blood                     | Pn-Kf-Er-Gn-Km-St-Amc-Trs-Ox | blaZ, ermC, msrA, qacA/B | NT          |
| Peritoneal fluid          | Pn-Kf-Er-Gn-Km-St-Amc-Trs-Ox | blaZ, ermC, msrA, qacA/B | NT          |
| Urine                     | Pn-Kf-Er-Gn-Km-St-Amc-Trs-Ox | blaZ, msrA, tetK | Type V      |

Antibiotic abbreviations: Pn, penicillin; Er, erythromycin; Te, tetracycline; Km, kanamycin; Ox, oxacillin; Trs, trimethoprim-sulphamethoxazole; Gn, gentamicin; St, streptomycin; Kf, cephalothin; Amc, amoxicillin-clavulanic acid.

Resistance genes for Pn (blaZ), Er (ermA, ermB, ermC, msrA), Te (tetK, tetO, tetL, tetM), antiseptic agents (qacA/B, smr).

NT, isolate non typeable for SCCmec.

Specimen abbreviations: B.L. fluid, bronchoalveolar lavage fluid.

genetic characteristics with those circulating in sheep. In the current study, 125 NAS from ovine mastitis and 70 from human clinical specimens were assigned to species level by PCR-RFLP of the gap gene. Among the ovine isolates, S. epidermidis was the most common followed by S. chromogenes and S. haemolyticus, while among human isolates S. haemolyticus and S. epidermidis were predominant, followed by S. lugdunensis and S. hominis.

S. epidermidis is widely recognised to be the most prevalent NAS recovered from ovine mastitis and human clinical specimens [1, 7, 29]. This study confirms our previous findings that ovine isolates of this species is the major reservoir of antimicrobial resistance genes, in particular for tetracycline and penicillin [6, 7]. However, the frequency of MDR in sheep isolates was considerably lower than that for human isolates (1.6% vs. 58.6%). Among the latter group, MDR was associated more frequently with S. haemolyticus (35.7%) than S. epidermidis (18.6%). This finding is in agreement with previous reports of resistance to a wide range of antimicrobials among S. haemolyticus from...
human clinical specimens, and also supports the view that this species may constitute an important reservoir for the transfer of resistance genes to other Staphylococcus species [30, 31].

None of the ovine NAS was methicillin resistant in contrast to 53.8% of human isolates. The mecA gene is a constituent of the mobile genetic element SCCmec, which acts as a vehicle for horizontal transfer of antibiotic resistance genes [32]. Among the oxacillin-resistant S. epidermidis, we found type III to be the predominant SCCmec, followed by type IV, the latter being common in human isolates of the species [32, 33], while SCCmec type III is more widely distributed among NAS species; the mechanism responsible for this is not understood. The identification of SCCmec type V as the most prevalent in S. haemolyticus is consistent with previous reports of the high frequency of class C2 mec-ccrC complexes (type V) in S. haemolyticus isolated from outpatients living in Algeria, Mali, Moldova, Cambodia and China [32, 33]. By contrast, S. haemolyticus collected in South Brazil and India mainly harboured SCCmec type I [34, 35]. We identified ccrA5–ccrB5 recombinase genes in one of the S. haemolyticus isolates from a human glans specimen. This gene complex has only been reported in S. pseudintermedius from animals [21], and to the best of our knowledge, this is the first report describing the detection of SCCmec type VII in S. haemolyticus from human clinical specimens. The ccrABSTEP allotype was described by Pi et al. [22] in S. haemolyticus isolates resistant to methicillin and harbouring arginine catabolic mobile element (ACME) cluster genes. This group suggested that ccrABSTEP had a similar function to the known ccr allotype, that is, to catalyse the integration and excision of SCCmec and ACME. Our inability to establish the SCCmec type for eight of the S. haemolyticus studied here could be attributed to the presence of novel ccr allotypes.

The plasmid-located tetK gene was the most prevalent determinant encoding tetracycline resistance, indicating that the resistance mechanism is mainly mediated by the tetracycline efflux

### Table 5. Antimicrobial susceptibility results, resistance genes detected and SCCmec typing of S. chromogenes, S. simulans, S. warneri and S. caprae collected from milk and of 10 minor human NAS isolates

| Source       | Species                       | Antimicrobial resistance | Resistance genes | SCCmec type | Cmec type |
|--------------|-------------------------------|--------------------------|------------------|-------------|-----------|
| **Ovine isolates** |                               |                          |                  |             |           |
| Milk         | S. chromogenes (n = 3)        | Te                       | tetK             |             |           |
|              | S. chromogenes (n = 6)        | Pn                       | blaZ             |             |           |
|              | S. chromogenes                | Er                       | ermB             |             |           |
|              | S. chromogenes                | Er-St                    | ermC             |             |           |
|              | S. chromogenes                | Pn-Te                    | blaZ, tetM       |             |           |
|              | S. chromogenes                | Pn-Te                    | blaZ, tetK       |             |           |
|              | S. chromogenes                | St-Te                    | tetK             |             |           |
|              | S. simulans                   | Er                       | ermC             |             |           |
|              | S. simulans                   | Te                       | tetM             |             |           |
|              | S. simulans                   | Te                       | tetK             |             |           |
|              | S. simulans                   | St                       | –                |             |           |
|              | S. caprae                     | Pn                       | blaZ             |             |           |
|              | S. caprae (n = 2)             | Pn-Te                    | blaZ, tetK       |             |           |
|              | S. warneri                    | Pn                       | blaZ             |             |           |
|              | S. warneri                    | Pn                       | blaZ             |             |           |
| **Human isolates** |                               |                          |                  |             |           |
| Pus          | S. pasteuris                  | Er                       | ermC, msrA       |             |           |
|              | N.P. aspiratec                | S. capitis               | blaZ             |             |           |
| Oral swab    | S. saprophyticus              | Te                       | tetK             |             |           |
| Ear swab     | S. warneri                   | –                        | qacA/B           |             |           |
| Biopsy       | S. warneri                   | Pn-Amc                   | blaZ             |             |           |
| Prosthesis   | S. hominis                   | Pn-Te                    | blaZ, tetK       |             |           |
| Blood        | S. hominis                   | Pn-Er-Km-Te-Trs          | blaZ, ermC tetK, qacA/B |             |           |
| Pleural fluid| S. hominis                   | Pn-Er-Gn-Km-Te-Trs       | blaZ, ermC, tetK, qacA/B |             |           |
| Urine        | S. hominis                   | Pn-Er-Gn-Km-Te-Trs-Ox    | blaZ, ermC tetK  |             | Type I    |

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aAntibiotic abbreviations: Pn, penicillin; Er, erythromycin; Te, tetracycline; Km, kanamycin; Ox, oxacillin, Trs, trimethoprim-sulphamethoxazole; Gm, gentamicin; Amc, amoxicillin-clavulanic acid; St, streptomycin.

bResistance genes for Pn (blaZ), Er (ermA, ermB, ermC, msrK), Te (ermK, tetO, tetL, tetM), antiseptic agents (qacA/B, smr).

cSpecimen abbreviations: N.P. aspirate, nasopharyngeal aspirate.
pump [36]. In *S. aureus*, a strong association between tetK gene and SCCmec type V has been reported by Larsen et al. [37] but we did not observe any such correlation in our NAS isolates, although the tetK gene was amplified in oxacillin-negative isolates. The detection of msrA gene encoding an ATP-dependent efflux pump among the erythromycin-resistant NAS is consistent with other reports from France [38] and Tunisia [39].

The inappropriate use of disinfectants can lead to a concentration gradient in the environment, which could select for isolates with reduced susceptibility to these agents. Here, we found a substantial difference between animal and human NAS as QAC resistance was identified in only a single ovine isolate compared to 42 (60%) of 70 human isolates. The ovine *S. epidermidis* strain harboured the *smr* gene whereas in human isolates, *qacA/B* genes were more prevalent (37.1%) than the *smr* gene (25.7%); both genes were similarly distributed in *S. haemolyticus* and *S. epidermidis* of human origin (was 70% and 69%, respectively). The predominance of *qacA/B* genes compared to the *smr* gene was also observed in NAS from Hong Kong [40] and Turkey [13]. Furthermore, it is noteworthy that almost all MDR-*S. haemolyticus* isolates from nasal, skin and oral swabs of patients in intensive care unit were PCR-positive for *qacA/B* genes.

NAS may therefore play a clinically significant role as reservoirs of resistance genes, especially *S. epidermidis* in sheep and *S. haemolyticus* in humans. The carriage of such genes in human NAS and the prevalence of MDR isolates were markedly higher compared with those of ovine origin. Similarly, no methicillin-resistant strains were found in the latter group which may reflect a more appropriate use of antimicrobials in humans as compared with dairy ruminants. Moreover, these findings may suggest that human NAS represent reservoirs of resistance genes which are transmissible to sheep isolates especially if they constitute part of the commensal skin microbiota of animal care or farm workers. On the other hand, the role of sheep NAS as reservoirs of resistance genes for human NAS seems reasonably lower. Nevertheless, this will require further studies, as well as periodic surveillance measures to monitor the spread of antimicrobial genes and maintain control.

Concerning the presence of sequence types in the two species, MLST genotyping of *S. epidermidis* revealed that ST225, ST6 and ST100 predominated in sheep, compared with T59, ST5 and ST80 in humans. Reports of sequence typing studies on animal-derived *S. epidermidis* are limited and currently none has analysed ovine milk isolates. Among human *S. epidermidis*, ST2 has usually been reported to be the most prevalent in humans [16, 41], but only one representative isolate of this ST was identified in our collection. Similarly, to our knowledge, only two studies have so far reported ST5 to be more common than ST2 [42, 43], which might be indicative of a different distribution in Sardinia from other geographical areas. Finally, it is noteworthy that one *S. epidermidis* ovine isolate was typed as ST5, which might suggest a possible human to animal transmission. This highlights the risk for strain and gene passage in this direction and underscores the need for periodic surveillance measures.

In conclusion, we believe that this is the first study to investigate and compare the occurrence of antimicrobial/antiseptic resistance and associated genetic determinants in human and ovine NAS in Italy. Our results document the great importance of NAS as reservoirs of resistance genes, in particular *S. epidermidis* for sheep and *S. haemolyticus* for humans. As many of the resistance determinants are located on transmissible plasmids, we propose that periodic surveillance might provide important information relevant to the control of animal and human infections, and thus help to limit the spread of MDR bacteria from humans to animals.

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**Data availability statement.** The authors confirm that the data supporting the findings of this study are available within the paper.

**References**

1. Marogna G et al. (2010) Clinical findings in sheep farms affected by recurrent bacterial mastitis. *Small Ruminant Research* 88, 119–125.
2. Onni T et al. (2012) Identification of coagulase-negative staphylococci isolated from caprine milk samples by PCR-RFLP of groEL gene. *Small Ruminant Research* 104, 185–190.
3. Vanderhaeghen W et al. (2015) Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants. *Veterinary Journal* 203, 44–51.
4. Martins KB et al. (2017) Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical mastitis. *Journal of Dairy Science* 100, 2184–2195.
5. von Eiff C et al. (2002) Pathogenesis of infections due to coagulase-negative staphylococci. *Lancet Infectious Diseases* 2, 677–685.
6. Onni T et al. (2011) Antimicrobial susceptibilities and population structure of *Staphylococcus epidermidis* associated with ovine mastitis. *Veterinary Microbiology* 148, 45–50.
7. Turchi B et al. (2020) Coagulase-negative staphylococci from ovine milk: genotypic and phenotypic characterization of susceptibility to antibiotics, disinfectants and biofilm production. *Small Ruminant Research* 183, 106030.
8. Matsushashi M et al. (1986) Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to beta-lactam antibiotics in *Staphylococcus aureus*. *Journal of Bacteriology* 167, 975–980.
9. Saber H et al. (2017) A review of staphylococcal cassette chromosome mec (SCCmec) types in coagulase-negative staphylococci (CoNS) species. *Malaysian Journal of Medical Sciences* 24, 7–18.
10. Katayama Y et al. (2001) Genetic organization of the chromosome region surrounding mecA in clinical staphylococcal strains: role of IS431-mediated mec deletion in expression of resistance in mecA-carrying, low-level methicillin-resistant *Staphylococcus haemolyticus*. *Antimicrobial Agents & Chemotherapy* 45, 1955–1963.
11. Ballhausen B et al. (2014) The mecA homolog mecC confers resistance against beta-lactams in *Staphylococcus aureus*. *Journal of Bacteriology* 196, 2132–2137.
12. Teixeira CF et al. (2010) Widespread distribution of *qacA/B* gene among coagulase-negative *Staphylococcus* spp. in Rio de Janeiro, Brazil. *Journal of Hospital Infection* 75, 333–344.
13. Ignak S et al. (2017) Frequency of antisepctic resistance genes in clinical staphylococci and enterococci isolates in Turkey. *Antimicrobial Resistance & Infection Control* 6, 88–95.
14. Bjorland J et al. (2005) Widespread distribution of disinfectant resistance genes among staphylococci of bovine and caprine origin in Norway. *Journal of Clinical Microbiology* 43, 4363–4368.
15. Heikens A et al. (2005) Comparison of genotypic and phenotypic methods for species-level identification of clinical isolates of coagulase-negative staphylococci. *Journal of Clinical Microbiology* 43, 2286–2290.
16. Miraglia M et al. (2008) Comparison of molecular typing methods for characterization of *Staphylococcus epidermidis*: proposal for clone definition. *Journal of Clinical Microbiology* 46, 118–129.
PCR-restriction fragment length polymorphism of Staphylococcus spp. by PCR-restriction fragment length polymorphism of gap gene. Journal of Clinical Microbiology 39, 3693–3695.

CLSI—Clinical and Laboratory Standards Institute (2018) Performance Standards for Antimicrobial Susceptibility Testing. M100, 28th Edn, Wayne, PA: Clinical and Laboratory Standards Institute.

Magiorakos AP et al. (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical Microbiology & Infection 18, 268–281.

Kondo Y et al. (2007) Combination of multiplex PCRIs for staphylococcal cassette chromosome mec type assignment: rapid identification system for mec, ccr, and major differences in junkyard regions. Antimicrobial Agents & Chemotherapy 51, 264–274.

Descloux S et al. (2008) Characterization of new staphylococcal cassette chromosome mec type in humans and animal models. Journal of Clinical Microbiology 46, 1818–1823.

Pi B et al. (2009) Distribution of the ACME-arcA gene among methicillin-resistant Staphylococcus haemolyticus and identification of a novel ccr allele in ACME-arcA-positive isolates. Journal of Medical Microbiology 58, 731–736.

Olsen JE et al. (2006) Diversity and evolution of blaZ from Staphylococcus aureus and coagulase-negative staphylococci. Journal of Antimicrobial Chemotherapy 57, 450–460.

Ojo KK et al. (2006) Staphylococcus efflux msr(A) gene characterized in Streptococcus, Enterococcus, Corynebacterium, and Pseudomonas isolates. Antimicrobial Agents & Chemotherapy 50, 1089–1091.

Jensen LB et al. (1999) Presence of erm gene classes in Gram-positive bacteria of animal and human origin in Denmark. FEMS Microbiology Letters 170, 151–158.

Ullah F et al. (2012) Investigation of the genetic basis of tetracycline resistance in Staphylococcus aureus from Pakistan. Tropical Journal of Pharmaceutical Sciences 11, 925–931.

Noguchi N et al. (2005) Susceptibilities to antiseptic agents and distribution of antiseptic-resistance genes qacA/B and Smr of methicillin-resistant Staphylococcus aureus isolated in Asia during 1998–1999. Journal of Medical Microbiology 54, 557–565.

Thomas J et al. (2007) Improved multilocus sequence typing scheme for Staphylococcus epidermidis. Journal of Clinical Microbiology 45, 616–619.

Becker K et al. (2014) Coagulase-negative staphylococci. Clinical Microbiology Reviews 27, 870–926.

Nunes APF et al. (2005) Genomic characterization of oxacillin-resistant Staphylococcus epidermidis and Staphylococcus haemolyticus isolated from Brazilian medical centers. Journal of Hospital Infection 59, 19–26.

Szcuka E et al. (2016) Diversity of staphylococcal cassette chromosome mec elements in nosocomial multi-resistant Staphylococcus haemolyticus isolates. Journal of Applied Genetics 57, 543–547.

Zong Z et al. (2011) Diversity of SCCmec elements in methicillin-resistant coagulase-negative staphylococci clinical isolates. PLoS One 6, e20191.

Ruppé E et al. (2009) Diversity of staphylococcal cassette chromosome mec structures in methicillin-resistant Staphylococcus epidermidis and Staphylococcus haemolyticus strains among outpatients from four countries. Antimicrobial Agents & Chemotherapy 53, 442–449.

Mombach AB et al. (2007) Distribution of staphylococcal cassette chromosome mec (SCCmec) types I, II, III and IV in coagulase-negative staphylococci from patients attending a tertiary hospital in southern Brazil. Journal of Medical Microbiology 56, 1328–1333.

Ghosh A et al. (2017) Staphylococcal cassette chromosome mec (SCCmec) typing of clinical isolates of coagulase-negative staphylococci (CoNS) from a tertiary care hospital in New Delhi, India. Indian Journal of Medical Research 143, 365–370.

Roberts MC (1996) Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiology Reviews 19, 1–24.

Larsen J et al. (2016) Co-presence of tet(K) and tet(M) in livestock-associated methicillin-resistant Staphylococcus aureus clonal complex 398 is associated with increased fitness during exposure to sublethal concentrations of tetracycline. Antimicrobial Agents & Chemotherapy 60, 4401–4403.

Lina G et al. (1999) Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. Antimicrobial Agents & Chemotherapy 43, 1062–1066.

Zmantar T et al. (2011) Detection of macrolide and disinfectant resistance genes in clinical Staphylococcus aureus and coagulase-negative staphylococci. BMR Research Notes 4, 453–462.

Zhang M et al. (2011) Prevalence of antiseptic-resistance genes in Staphylococcus aureus and coagulase-negative staphylococci colonising nurses and the general population in Hong Kong. Antimicrobial Agents & Chemotherapy 60, 2209–2221.

Widerstrom M et al. (2009) Clonality among multidrug-resistant hospital-associated Staphylococcus epidermidis in Northern Europe. Scandinavian Journal of Infectious Diseases 41, 642–649.

Mendes RE et al. (2012) Molecular epidemiology of Staphylococcus epidermidis clinical isolates from U.S. Hospitals. Antimicrobial Agents & Chemotherapy 56, 4656–4661.

Ahlstrand E et al. (2014) Long-term molecular epidemiology of Staphylococcus epidermidis blood culture isolates from patients with hematological malignancies. PLoS ONE 9, e99045.