Comparative profiling of biofilm-production, quorum sensing system and virulence genes in human and ovine non-aureus staphylococci

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Abstract

Background: This study assessed the genetic characteristics shared by non-aureus staphylococci (NAS) responsible for human infections and those causing mastitis in dairy ewes. In a collaboration between animal and human health care professionals, we collected and identified 125 ovine and 70 human NAS isolates and compared them for biofilm production, presence of autolysins, microbial surface components recognizing adhesive matrix molecules (MSCRAMMS), pyrogenic toxins, and *agr* alleles regulating quorum-sensing systems. Ovine NAS included: *S. epidermidis* (57), *S. chromogenes* (29), *S. haemolyticus* (17), *S. simulans* (8), *S. caprae* (6), *S. warneri* (5), *S. saprophyticus*, *S. intermedius*, and *S. muscae* (1 each) while human NAS included: *S. haemolyticus* (28), *S. epidermidis* (26), *S. hominis* (4), *S. lugdunensis* (4), *S. capitis* (3), *S. warneri* (2), *S. xylosus*, *S. pasteurii*, and *S. saprophyticus* subsp. *bovis* (1 each).

Results: Based on colony characteristics on Congo Red Agar, 4 (3.2%) ovine, and 49 (70%) human isolates produced biofilm. Few *S. epidermidis* isolates harbored the *icaA/D* genes coding for the polysaccharide intercellular adhesin (PIA) and the *bhp*, *aap*, and *embp* genes coding biofilm accumulation proteins. PCR amplification of the genes coding for autolysins (*atlE* and *aae*), microbial surface components recognizing adhesive matrix molecules (MSCRAMMs, *sdrG* and *sdrF*), enterotoxins (*sea*, *seb*, *sec*, *sed*, and *see*), and the toxic shock syndrome toxin (*tsst*), revealed that 40%, 39.2%, 47.2% and 52.8% of the sheep isolates carried *atlE*, *aae*, *sdrF* and *sdrG*, respectively, against 37.1%, 42.8%, 32.8%, and 60% of human isolates. Enterotoxins and *tsst* were not detected. Fifty-nine sheep isolates (all *S. epidermidis*, 1 *S. chromogenes*, and 1 *S. haemolyticus*) and 27 human NAS (all *S. epidermidis* and 1 *S. warneri*) were positive for the accessory gene regulator (*agr*), responsible for the regulation of virulence factors: *agr-3se* (57.8%) followed by *agr-1se* (36.8%) predominated in sheep, while *agr-1se* (65.4%), followed by *agr-2se* (34.6%) predominated in humans.

Conclusions: This comparative study provided a detailed characterization of the putative virulence genes present in human and ovine NAS and indicated that the ability to form biofilms, observed mainly in human *S. epidermidis*, could be a major virulence factor facilitating colonization, infection, diffusion, and resistance.

Background

Half of the total Italian dairy sheep stock is farmed in Sardinia, an island located in the Mediterranean Sea. Sardinia has approximately 3.5 million dairy sheep, while the population is around 1.6 million inhabitants. Accordingly, a relevant part of the regional economy relies on dairy sheep farming, and the control of intra-mammary infections (IMI) is consequently of the greatest importance.

Several reports indicate that non-aureus staphylococci (NAS) are the most prevalent bacteria causing subclinical mastitis in sheep and goats [1–4], creating opportunities for the exchange of colonizing as well as pathogenic microorganisms among sheep and farmers, with their antimicrobial-resistance and

pathogenicity gene pools. Of note, NAS have emerged as predominant nosocomial agents capable of causing infection in debilitated or compromised patients and are associated with catheter-related and other indwelling medical device-related infections [5]. NAS, and in particular *S. epidermidis*, can produce a multicellular biofilm that decreases the antibiotic concentration within the colony and promotes multiplication and enhances survival of invading bacteria [6].

The four main biofilm formation steps are adherence/aggregation, accumulation, maturation, and detachment [7]. Specific staphylococcal autolysins, including AtlE and Aae [8, 9], mediate initial adhesion to surfaces, and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) further promote colonization and serum protein binding [10]. The best known *S. epidermidis* MSCRAMMs are the fibrinogen-binding protein SdrG [11], and the collagen/keratin-binding protein SdrF [12, 13]. The extracellular polysaccharide intercellular adhesin (PIA) mediates the accumulation phase, and in *S. epidermidis* this step is mediated by *icaABCD*-encoded proteins [6]. However, PIA-independent biofilms involving accumulation-associated protein (Aap), biofilm homologue protein (Bhp) and extracellular matrix-binding protein (Embp) [14, 15] have also been reported.

The production of various toxins can contribute to NAS virulence [16], including staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin 1 (TSST-1) [17]. Five serological types of SEs are typically known (SEA to SEE), but new types of SEs (SEG to SE1V) have also been identified and characterized [18, 19]. The *quorum-sensing* system (QS) *agr*, i.e. accessory gene regulator [20, 21] regulates biofilm formation, intercellular communication, and numerous virulence factors including toxins and autolysins. Three distinct genetic groups (types 1, 2, and 3) based on the *agr* locus polymorphism have been described in *S. epidermidis* [22], but data on the genetic polymorphisms of the *agr* locus in different species of NAS were not available in the scientific literature at the beginning of this investigation.

We compared the molecular characteristics of NAS isolated from ovine mastitis and human clinical specimens with the following aims: 1) assess the biofilm production characteristics by phenotypic and genotypic methods, 2) carry out genotypic screening for a set of MSCRAMMs, autolysins, enterotoxins and *tsst-1* genes and 3) investigate the *agr* locus and its genetic polymorphism.

Results

Ovine NAS: general findings

We analyzed a total of 125 isolates, including *S. epidermidis* (n=57), *S. chromogenes* (n=29), *S. haemolyticus* (n=17), *S. simulans* (n=8), *S. caprae* (n=6), *S. warneri* (n=5), *S. saprophyticus* (n=1), *S. intermedius* (n=1) and *S. muscae* (n=1). Table 1 reports the isolates included in the study, while Table 2 reports the primers used for PCR amplifications.

Table 3 summarizes biofilm formation results, evaluated by the phenotypic characteristics of bacterial colonies grown on Congo Red Agar (CRA) and by PCR detection of *icaA/D*, *bhp*, *aap*, *embp*, *atlE*, *aae*, *sdrF*,

sdrG and *agr*. Out of 125 isolates examined, only 4 (3.2%) were classified as strong biofilm producers because their colonies exhibited a *very black* color; the remaining 121 (96.8%) as non-biofilm producers due to *bordeaux* or *red* color. One isolate harbored both *icaA* and *icaD* genes while two had only the *icaA* gene: one of them was considered as non-biofilm-producing phenotype. On the other hand, 37 (29.6%), 22 (17.6%) and 63 (50.4%) isolates possessed the *bhp*, *aap* and *embp* genes, respectively (Table 3). Concerning autolysin genes, 50 (40%) isolates were PCR positive for *atlE* while 49 (39.2%) were positive for *aae*. Concerning adhesion (MSCRAMMS) factors, 59 (47.2%) and 66 (52.8%) isolates harbored *sdrF* and *sdrG* genes, respectively. Concerning the *agr* type, 21 (16.8%) isolates belonged to *agr*-1 whilst 33 (26.4%) to *agr*-3. None of the isolates belonged to type 2. The toxin genes analyzed did not produce amplification (data not shown).

Ovine *S. epidermidis*

S. epidermidis was the most represented ovine NAS. Out of 57 isolates, 2 (3.5%) were strong biofilm producers but only one harbored both *icaA/D* genes. On the other hand, 54 (94.7%), 30 (52.6%) and 20 (16%) isolates possessed *embp*, *bhp* and *aap* genes, respectively. Concerning autolysin genes, 48 (84.2%) were PCR-positive for *atlE* and 46 (80.7%) for *aae*. High percentages were found for MSCRAMMS genes: 87.7% for *sdrG* and 78.9% for *sdrF*. All *S. epidermidis* isolates were positive for the *agr* locus: 33 (57.8%) belonged to *agr*-3_{se} while 21 (36.8%) to *agr*-1_{se} (Table 3). Three isolates were non-typeable.

S. chromogenes, *S. haemolyticus* and minor ovine NAS

S. chromogenes (n=29) and *S. haemolyticus* (n=17) were the most prevalent species in ovine milk samples after *S. epidermidis*. Table 3 shows that almost all isolates were non-biofilm producers, except one *S. caprae* and one *S. muscae*.

Human NAS: general findings

Table 3 summarizes the distribution of virulence genes in the 70 human NAS, including *S. haemolyticus*, *S. epidermidis*, *S. lugdunensis*, *S. hominis*, *S. capitis*, *S. warneri*, *S. xylosus*, *S. pasteurii* and *S. saprophyticus subsp. bovis*.

Human *S. haemolyticus*

Out of 28 *S. haemolyticus* isolates examined, 16 (57.1%) were classified as strong biofilm producers by CRA because their colonies exhibited a *very black* color; the remaining 12 as non-biofilm producers due to *bordeaux* or *red* color. None of *S. haemolyticus* isolates harbored *icaA/D*, *bhp* and *embp*. On the contrary, three *black* (2 from blood and 1 from nasal swab) and one *bordeaux* (from glans swab) isolates possessed the *aap* gene. No amplification was observed for *atlE*, *aae*, *sdrF*, *sdrG* genes and *agr* locus (Table 3) and for all toxin genes (data not shown).

Human *S. epidermidis*

Among the 26 *S. epidermidis* isolates, 22 (84.6%) showed a *very black* color; ten (38.5%) of them (3 from catheter, 3 from blood, 2 from nasal swab, 1 from skin swab and 1 from pus) harbored both *icaA* and *icaD*. The remaining isolates were *icaA/D* negative. However, 16 (61.5%), 25 (96.1%) and 23 (88.5%) isolates possessed the *bhp*, *aap* and *embp* genes, respectively (Table 3). Out of 10 *icaA/D* positive isolates, 5 were also positive for the other biofilm genes analyzed. Among the 4 non-biofilm-producing phenotypes, all were *icaA/D* negative but 3 (2 from pus and 1 from oral swab) harbored *aap* and *embp* while 1 (from skin swab) possessed all *bhp*, *aap* and *embp* genes. Data on the prevalence of autolysins and MSCRAMMs genes by PCR are shown in Table 3. Concerning autolysin genes, all isolates were PCR positive for *aae* and almost all (25/26=96.1%) were positive for *atlE*. Regarding adhesion factors, all *S. epidermidis* isolates were positive for *sdrG* and 21/26 (80.8%) harbored *sdrF*. In four of them (2 from pus, 1 from biopsy and 1 from skin swab), a PCR product smaller than the expected size was observed. Sequence analysis of these amplicons showed the absence of an 84 bp fragment. Determination of the *agr* type was performed in all *S. epidermidis* isolates: 17 (65.4%) belonged to *agr*-1_{se} whilst 9 (34.6%) to *agr*-2_{se} (Table 4). Among the 17 *agr*-1_{se} isolates, only 3 (1 from femoral venous catheter, 1 from nasal swab and 1 from pus) carried simultaneously *icaA/D*, *bhp*, *aap*, *embp*, *atlE*, *aae*, *sdrF* and *sdrG* genes associated with biofilm formation. Among the 9 *agr*-2_{se} isolates, only 1 (from blood) possessed these genes. Regarding the pyrogenic toxin genes, amplification was not observed in any of the *S. epidermidis* isolates or in the remaining staphylococci (data not shown).

Minor human NAS

Out of the 4 *S. lugdunensis* isolates examined, 3 (2 from peritoneal fluid and 1 from vaginal swab) displayed a *very black* colour on CRA. However, none of them harbored neither *icaA/D* nor any other gene examined, except the *sdrG* gene.

Negative results were also obtained for all *S. hominis* isolates, except for a PCR positivity for *aap* and *sdrG* genes. Among the 3 *S. capitis* isolates, 1 (from skin swab) exhibited *black* color whereas 2 were phenotypically *red* but, despite this, all isolates were PCR-positive for *icaA* and *sdrG* genes. Table 4 reports the PCR results for *S. warneri*, *S. xylosus*, *S. pasteurii*, and *S. saprophyticus* subsp. *bovis*. Of note, one *bordeaux* *S. warneri* isolate (from fluid drainage) was PCR negative for *icaA/D* and *bhp* genes but positive for *aap*, *embp*, *atlE*, *aae*, *sdrG/F* and *agr* genes. However, we were not able to type the *agr* locus.

Discussion

This work results from an integrated collaboration between animal and human health care professionals for understanding if non-*aureus* staphylococci (NAS) responsible for human diseases share some genetic characteristics with those circulating in sheep. A total of 195 NAS isolates, 125 from ovine mastitis and 70 from human clinical specimens, were analyzed for biofilm production and presence of autolysins, MSCRAMMS and pyrogenic toxins. We also typed *agr* alleles by PCR because many virulence determinants involved in staphylococcal infections, including autolysins, adhesins and toxins are regulated by *quorum sensing* system [16]. In sheep, the main NAS detected were *S. epidermidis* followed

by *S. chromogenes* and *S. haemolyticus*. In humans, we found primarily *S. haemolyticus* and *S. epidermidis*, followed by *S. lugdunensis*, *S. hominis*, *S. capitis*, *S. warneri*, *S. xylosus*, *S. pasteurii* and *S. saprophyticus* subsp. *bovis*. *S. haemolyticus* has been associated with septicemia in neonates and skin infections; *S. epidermidis* is the main pathogen isolated in catheter-associated bloodstream infections (BSI); *S. lugdunensis* can cause acute endocarditis; *S. hominis* and *S. capitis* may induce BSI in neonates; *S. warneri* is associated with device-related bone and joint infections, while *S. pasteurii*, *S. xylosus* and *S. saprophyticus* subsp. *bovis* are not associated to a particular clinical infection, and their appearance as nosocomial pathogens could be related to previous contact with animals, mainly pig, cattle, sheep and goats [23]; *S. epidermidis* represents the most frequently isolated species from ovine mastitis and human clinical specimens [1, 23, 24].

Overall, 53 NAS were able to form biofilm onto CRA plates, according to the Arciola criteria [25]; however, the percentage of strong biofilm producers in sheep isolates was much lower than for human isolates: 3.2% (4/125) versus 70% (49/70). Only in 11 *S. epidermidis* isolates (10 human and 1 ovine), we found a correlation between black color development and *ica* operon presence. Some authors proposed to use this correlation as a pathogenesis marker to distinguish invasive from commensal isolates [26, 27]. However, we and others [15, 28, 29] demonstrated that PCR positivity for *icaA/icaD* genes can also be found in non-biofilm producers. Since the correlation between biofilm production and positivity for *ica*, *bhp*, *aap* and *embp* genes is not well known; we suggest considering all isolates that possess such genes as potentially invasive. In this work, only 1 *S. epidermidis* with these characteristics was isolated from ovine mastitis while the other 5 derived from femoral venous catheter, blood, nasal swab, skin swab and pus. Nasal and skin swabs were collected from patients in the intensive care unit. Noteworthy, high positivity (80.7 to 100 percent) for the genes encoding the bifunctional adhesins/autolysins AtlE and Aae was found in both animal and human *S. epidermidis* isolates [3, 5]. In addition to bacteriolytic activity, AtlE and Aae act as adhesins by binding noncovalently to vitronectin and by causing the release of extracellular DNA (eDNA), a critical adherence/aggregation factor in biofilm formation [30]. The presence of the *atlE* and *aae* genes was accompanied by a high prevalence of *embp*, *sdrG* and *sdrF*, encoding proteins that mediate adherence to substrates containing fibronectin, fibrinogen and collagen [4, 31]. This observation confirms a pivotal role of AtlE, Aae, Embp, and SdrG in the *S. epidermidis* pathogenesis.

The *agr* locus is a regulatory system that responds to host and environmental stimuli and controls the production of many virulence factors [21]. In *S. epidermidis*, three distinct *agr* groups have been recognized [22]. Li et al. [32] have linked genetic polymorphism of the *agr* locus to pathogenicity; group-1_{se} was associated with pathogenicity, while healthy people mainly carried group-2_{se}. In our human *S. epidermidis* isolates, *agr*-1_{se} was predominant (n=17), followed by *agr*-2_{se} (n=9). It is interesting to notice that almost all (n=9) isolates possessing *ica* genes belonged to *agr*-1_{se}. This may suggest a correlation of these virulence genes with a specific *agr* locus. However, other 8 *icaA*⁻/*D*⁻ isolates were present in the group-1_{se}. The feature shared by all 17 isolates belonging to this group was the PCR positivity for the *atlE*, *aae* and *sdrG* genes. On the other hand, among the 9 isolates grouped in the *agr*-2_{se}, 1 (from blood) was *icaA*⁺/*D*⁺, while the remaining ones were *ica*-negative. The common denominator of these 9 isolates were

the PCR positivity for the *aap*, *aae*, *sdrG* and *embp* genes. These findings suggest that the relationship between *agr* groups and pathogenicity of *S. epidermidis* will require further investigation. As observed in our previous study [28], *agr-3_{se}* (n=33) was predominant among ovine *S. epidermidis* isolates followed by *agr-1_{se}* (n=21). These results may suggest a possible transmission of *S. epidermidis* isolates from the milkers to the ewes.

Unlike *S. haemolyticus* from ovine mastitis, several human isolates exhibited a positive result in the qualitative test CRA but did not harbor *icaA/D*. According to Fredheim et al. [33], *S. haemolyticus* mainly produces a PIA-independent biofilm. However, in the present study, we did not detect the *bhp* and *embp* genes in any of the investigated isolates by PCR. Only 4 human *S. haemolyticus* (3 *black* and 1 *bordeaux* isolates) possessed the *aap* gene coding a protein that mediates biofilm formation in strains lacking the *ica* genes [11]. Our data suggest that *ica*, *bhp*, and *embp* genes do not contribute significantly to *S. haemolyticus* biofilms' protein components.

In *S. aureus* and in many other bacteria, toxins are critical contributors to aggressive virulence, even though *S. epidermidis* is not generally accepted as an enterotoxin producer [34, 35]. Based on our findings, the primary enterotoxin genes (*sea*, *seb*, *sec*, *sed* and *see*) and the *tsst-1* gene were absent in all ovine and human NAS analyzed. On the contrary, Pedroso et al. [16] and Da Cunha et al. [36] detected high percentage of *sea* and *sec* genes in coagulase-negative staphylococci collected from hospitals of Brazil; also, Giormezis et al. [35] found a higher number of isolates positive for *tsst* among NAS collected from hospitals in Greece.

Conclusion

In conclusion, our study demonstrated the presence of intercellular adhesion genes (*icaAB*) and other genes related to biofilm formation only in *S. epidermidis*. At the same time, *S. caprae* and *S. saprophyticus* (ovine NAS) and *S. capitis* and *S. xylosus* (human NAS) harbored only *icaA*. The remaining isolates carried few virulence determinants. The biofilm formation ability observed mainly in human isolates, especially *S. epidermidis*, could be a significant virulence factor facilitating colonization, infection, diffusion, and resistance.

Methods

Isolate collection

Ovine isolates: A total of 125 NAS isolates were collected from sheep milk samples in different provinces of Sardinia (Italy) over a period of 9 months (April-December 2017) (Table 1). Isolates were identified as: *S. epidermidis* (n=57), *S. chromogenes* (n=29), *S. haemolyticus* (n=17), *S. simulans* (n=8), *S. caprae* (n=6), *S. warneri* (n=5), *S. saprophyticus* (n=1), *S. intermedius* (n=1), and *S. muscae* (n=1), by means of PCR-RFLP [24].

Human isolates: During the same period, 70 NAS isolates were collected from different clinical specimens at the microbiology laboratories of three major Sardinia hospitals. Isolates and their origin are summarized in Table 1; 90% of the human NAS were recovered from hospitalized patients in intensive care unit, hematology, and orthopedics. The 70 isolates were identified by PCR-RFLP as *S. haemolyticus* (n=28), *S. epidermidis* (n=26), *S. lugdunensis* (n=4), *S. hominis* (n=4), *S. capitis* (n=3), *S. warneri* (n=2), *S. xylosus* (n=1), *S. pasteurii* (n=1), and *S. saprophyticus subsp. bovis* (n=1) [24].

Statements of owner consent or patient consent were not required in this case since personal or sensitive data never accompanied samples. All Isolates were anonymized regarding the originating animal, flock, or patient, and were processed for phenotypic and molecular analyses without any original information linked to them.

Phenotyping evaluation of biofilm production

Phenotypic evaluation of biofilm production was assessed on CRA [37]. On this medium, slime-producing strains form *very black*, *black* and *almost black* colonies, while non-producing strains develop *bordeaux*, *red* and *very red* colonies [25]. CRA plates were prepared and incubated as described by Abbondio et al. [28]. We used the *S. epidermidis* reference strain (RS) RP62A (ATCC 35984) as a positive control and ATCC 12228 as a negative control.

Detection of biofilm, autolysins, MSCRAMMs and pyrogenic toxins genes

Genomic DNA was extracted from all 195 NAS isolates and RS according to Onni et al. [38]. Primer sets for detecting of genes related to biofilm production, autolysins, MSCRAMMs and pyrogenic toxins are detailed in Table 2. PCR tests were carried out in a GeneAmp9700 DNA thermal cycler (Applied Biosystems, now Thermo Fisher Scientific, Waltham, MA, USA). The following RS were used as positive controls: *S. epidermidis* ATCC 35984 (*ica*), *S. epidermidis* ATCC 35984 (*atlE*, *aae*, *sdrF*, *sdrG*), *S. aureus* ATCC 13565 (*sea*), *S. aureus* ATCC 14458 (*seb*), *S. aureus* ATCC19095 (*sec*), *S. aureus* ATCC 23235 (*sed*), *S. aureus* ATCC 27664 (*see*) and *S. aureus* ATCC 33586 (*tsst-1*).

Typing of agr alleles

A 200 bp conserved region of the *agr* operon was amplified as described previously [16]. For isolate typing, we targeted *agr-1_{se}* to *agr-3_{se}* sequences [39]. As controls, we used *S. epidermidis* ATCC 35984 (*agr-1_{se}*), *S. epidermidis* isolate 1037 (*agr-2_{se}*) and *S. epidermidis* isolate 43027 (*agr-3_{se}*). Table 2 reports primer sets and related references.

Abbreviations

CRA, Congo Red Agar; MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; NAS, non-aureus staphylococci; QS, quorum-sensing system.

Declarations

Ethics approval and consent to participate

Does not apply.

Consent for publication

Not applicable

Availability of data and material

The data used in this study is under the regulation of the Istituto Zooprofilattico Sperimentale della Sardegna. It will be available from the corresponding author.

Competing interests

The authors declare that they have no conflicts of interest associated with this study.

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Authors' contributions

EA: carried out isolation and identification of ovine NAS; performed the experiments; analyzed the data. CML: carried out isolation and identification of ovine NAS; performed the experiments; SA, SS, and MS: carried out isolation and identification of human NAS; MFA: interpreted the data; drafted and revised the manuscript; ST: Conceived the study, analyzed, and interpreted the data; drafted the manuscript; supervised the project. All authors read, edited, and approved the final version of the manuscript.

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Tables

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