EXTENDED ABSTRACT

Antibiotic resistance assessment in *S. aureus* strains isolated from raw sheep's milk cheese

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Abstract In vitro activities of 16 antibiotics were tested against 36 *Staphylococcus aureus* (*SA*) strains isolated from raw sheep's milk cheese from six dairies. The minimum inhibitory concentration (MIC) was determined using a broth microdilution method (CLSI). All 36 isolates were analyzed for the presence of the accessory gene regulator gene, *agr* (I–IV), and genes encoding resistance to methicillin (*mecA*), erythromycin (*ermA*), penicillin (*blaZ*), and vancomycin (*vanA-B*). The isolates were also analyzed for similarities in pulsed-field gel electrophoresis (PFGE) patterns. *SA* strains showed resistance to ampicillin (36.1%), penicillin (33.3%), tetracycline (11.1%), and cloxacillin (2.8%) but were susceptible (≥94.4%) to 12 out of 16 tested antimicrobials. The overall susceptibility of the strains to oxacillin, vancomycin, and erythromycin was confirmed by the absence of the *mecA*, *vanA-B*, and *ermA* genes. The PFGE results showed that 32 strains belonged to 10 different clusters (P1–P10) while four strains were untypeable.

Keywords *S. aureus* · Sheep cheese · Antibiotic resistance · MIC · Genes

Abbreviations

SA	*Staphylococcus aureus*
MIC	minimum inhibitory concentration
AMR	antimicrobial resistance
MRSA	methicillin-resistant *Staphylococcus aureus*
PFGE	pulsed-field gel electrophoresis
CPS	coagulase-positive staphylococci

Introduction

S. aureus (*SA*) is involved in food poisoning outbreaks and systemic human diseases. Antibiotic resistance in *SA* isolated from food of animal origins has been reported. The

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European Food Safety Authority (EFSA) has identified animals as a possible reservoir of
SA antimicrobial-resistant (AMR) strains. Transmission through foods of AMR strains
involved in human outbreaks has been reported in sporadic cases (EFSA 2008). Several
mechanisms are involved in the antibiotic resistance of SA. Plasmid-encoded β-lactamase
inactivates penicillin. Methicillin and other semi-synthetic penicillins have been effective
against β-lactamase producer bacterial strains. However, the emergence of methicillin-
resistant SA (MRSA) strains have been reported. MRSA strains harbor the mecA gene,
which encodes a low-affinity penicillin-binding protein (PBP2a). Nosocomial infections
caused by MRSA can sometimes lead to death (Moreillon and Que 2004). Macrolide
resistance in SA can be caused by a target modification of a 23S rRNA methylase,
encoded by the erm genes. SA can also be resistant to vancomycin, which has been used
successfully to treat MRSA strains. Vancomycin resistance is encoded by several genes
and appears to have originated from mutations under antibiotic-selective pressure.
Antibiotic resistance is frequently acquired by exchange of mobile DNA elements during
food processing between pathogenic and non-pathogenic microorganisms. In the dairy
sheep industry, SA AMR strains can be recovered from raw milk, secretions from animals
with mastitis, and dairy products. The aim of the present study was to assess a protocol to
characterize SA isolates and their resistance to antibiotics using phenotypic and molecular
techniques. In SA strains isolated from raw sheep's milk cheese collected at early stages
of the cheese making process, minimum inhibitory concentrations (MICs) and
susceptibility to antibiotics used in human and veterinary practice were determined.
The presence of the ermA, blaZ, mecA, and vanA and B genes was also investigated. A
population study of the isolated microorganisms was carried out by pulsed-field gel
electrophoresis (PFGE).

Material and methods

SA strains were recovered from cheese made from raw sheep's milk collected 6 hours after
molding, a phase generally associated with the highest levels of SA. Twelve cheese wheels
were collected from six household plants (A-F) located on the island of Sardinia (Italy).
Each sample was representative of a different production batch, two from each plant.
Samples were analyzed according to UNI EN ISO 6888–2:2004. Thirty-six strains were
isolated from typical colonies and identified according to Scarano et al. (2005). For each
strain, the MIC was tested by the broth microdilution method (CLSI 2006a, b) for the
following antibiotics: ampicillin (AM), cefalotin (CF), cefoperazone (CFP), cloxacillin
(OB), erythromycin (E), gentamycin (GM), kanamycin (K), novobiocin (NV), ofloxacin
(OFX), oxacillin (OX), penicillin (P), rifampicin (RA), streptomicin (S), tetracycline (TE),
tobramycin (TM), and vancomycin (VA). Twelve serial dilutions of each antibiotic were
made in cation-adjusted Mueller Hinton Broth (CAMHB; Oxoid LTD Basingstoke, UK)
containing NaCl (2%), with the final concentrations of antibiotics ranging from 0.06 to
128 μg/mL (0.006-12.8 μg/mL for RA). The MIC mode, range, MIC_{50}, and MIC_{90} of the
antimicrobial agents were calculated, and antibiotic susceptibility was defined comparing
the MICs with reference values (CLSI 2006b). After DNA extraction, multiplex PCR was
used (Rosato et al. 2003; Vancraeynest et al. 2004) to detect gyrA encoding the DNA
gyrase, agr (I–IV), an accessory gene regulator, blaZ encoding a penicillinase, ermA, a gene
encoding resistance to macrolide E, mecA encoding PBP2a, and vanA-B, genes encoding
VA resistance. The templates were loaded onto an agarose gel, and electrophoresis was
performed. The population study was conducted by PFGE on SA strains according to

Prevost et al. (1992) after *Sma*I restriction digest (Invitrogen, CA, USA) of bacterial DNA. Correlations between the isolates were determined by an unweighted pair grouping by mathematical averaging (UPGMA) model with a similarity greater or equal to 80% of the Pearson correlation coefficient (Gel Compar II, Applied Math).

Results

Coagulase-positive staphylococci (CPS) in cheese samples ranged from 3.9 to 5.8 Log_{10} CFU/mL. Thirty-six isolates were identified as *SA* based on their biochemical and metabolic profiles and on the presence of the *gyrA* gene. All isolates produced TDNase and were coagulase positive. The isolates were characterized by PFGE clusters (P1–P10) and by the presence of *agr* and antibiotic resistance genes (Table 1). None of the tested isolates showed resistance to CF, CFP, E, GM, K, NV, OFX, RA, S, and TM. Only two isolates showed intermediate levels of resistance to K (5.6%), and one to OFX (2.8%). Four *SA* strains could not be typed by PFGE. The *SA* isolates exhibited resistance to AM (36.1%), P (33.3%), TE (11.1%), and OB (2.8%), and the MIC_{90} for these antibiotics was, respectively, 16.0, 32.0, 64.0, and 32.0 µg/mL. MIC_{90} of the isolates was below the sensitivity range of 13 out of the 16 tested antibiotics. Regarding AM and P resistance, the results of broth microdilution and molecular analysis (i.e., *blaZ* gene detection) were in agreement, showing a concordance of 94.4%. The only disagreements between results obtained with the two methods applied to an *SA blaZ+* isolate that showed resistance only to AM and one *blaZ-* isolate with resistance to AM, P, and OB.

Discussion

The *SA* strains isolated during cheese processing were resistant only to AM, P, OB, and TE, which are among the most commonly used antibiotics on dairy sheep farms. In *SA* isolated

Table 1 Genotypic characterization and antibiotic sensitivity

dairy	batch	*agr*	cluster	genes				Antibiotics						
				mecA	*blaZ*	*vanAB*	*ermA*	AM	P	OX	TE	E	VA	OB
A	L1	III	P1(4)^a-P2(2)	–	–	–	–	–	–	–	–	–	–	–
	L2	III	P1(4)^a-P2(2)	–	–	–	–	–	–	–	–	–	–	–
B	L1	IV	P8(3)	–	–	–	–	–	–	–	–	–	–	–
	L2	III	P1(2)–P7(1)	–	+(1)	–	–	+(1)	+(1)	–	–	–	–	–
C	L1	I	nt*(3)	–	–	–	–	–	–	–	+(3)	–	–	–
	L2	I(1)–II(2)	nt(1)–P9(2)	–	–	–	–	–	–	–	+(1)	–	–	–
D	L1	II	P3(6)	–	–	–	–	–	–	–	–	–	–	–
	L2	III	P3(6)	–	–	–	–	–	–	–	–	–	–	–
E	L1	III	P4(6)	–	+(3)	–	–	+(3)	+(2)	–	–	–	–	–
	L2	III	P4(6)	–	+(3)	–	–	+(3)	+(3)	–	–	–	–	–
F	L1	I(1)–II(2)	P6(1)–P10(2)	–	+(2)	–	–	+(3)	+(3)	–	–	–	–	+(1)
	L2	II	P5(1)–P10(2)	–	+(3)	–	–	+(3)	+(3)	–	–	–	–	–

* nt = nontypeable; + = resistant; - = sensitive; ^a = strain *n*

from sheep, the *agrIII* allele is the most frequently observed (De Santis et al. 2005). Fifty percent of the *SA* strains isolated from four different dairies belonged to the *agrIII* group, while 27.8% belonged to the *agrII* group and were isolated from three dairies (C, D, and F). The strains belonging to the *agrI* group (dairies C and F) and to the *agrIV* group (dairy B) showed low prevalence. A dendrogram showed a specific distribution of isolates from each dairy. Only isolates included in the P1 cluster were observed at two different dairies (A and B). Each of the isolates of the P3, P4, and P10 clusters was recovered at only one dairy (D, E, or F, respectively), but in both production batches. Our results show a good correspondence between PFGE clusters and antibiotic resistance profiles of the isolates. At two dairies (A and D), all the isolates were susceptible to the tested antibiotics. Four strains belonging to the *agrI* group (dairy C) were untypeable by PFGE and exhibited resistance to TE. In agreement with previous studies on *SA* strains isolated from raw sheep's milk (De Santis et al. 2005), the *mecA* gene was not detected in any of the tested strains. The recovery of MRSA strains from half udder milk samples is sporadic and commonly of human origin (De Santis et al. 2005). The agreement between the antibiotic resistance profiles and detection of the corresponding genes was 100% for E, OX, and VA, and 94.4% for AM and P. The determination of the antibiotic resistance profile allowed an understanding of the source of contamination and a trace back to each step of the cheese making process. This study permitted the identification of the spreading of strains with the same profile and their persistence within the cheese plant.

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