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Short communication: Characterization of *Staphylococcus aureus* isolated along the raw milk cheese production process in artisan dairies in Italy

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ABSTRACT

Staphylococcus aureus is a common cause of foodborne intoxications. Several staphylococcal food poisoning outbreaks have been linked to consumption of raw milk cheeses and artisanal cheese production. However, information on *Staph. aureus* isolated from artisanal raw milk cheeses and small-scale dairy production environments is very limited. Therefore, we aimed to characterize *Staph. aureus* isolated along the artisanal raw milk production chain by determining (1) the population structure, and (2) the presence/absence of enterotoxin genes, *mecA/C*, and *pvl*. We collected 276 samples from different production stages (raw milk, whey, curd, brine, drying worktops, and cheese) at 36 artisan dairies in Italy. A total of 102 samples from 25 dairies tested positive for *Staph. aureus*, with 80% positive samples among the tested artisan cheeses. All isolates were further characterized by *spa* typing and PCR screening for staphylococcal enterotoxin genes, the *mecA/mecC* genes characteristic for methicillin-resistant *Staph. aureus*, and the *pvl* gene encoding Panton-Valentine leukocidin. The 102 isolates represented 15 different *spa* types and were assigned to 32 different *Staph. aureus* strains. The *spa* type most frequently detected was t2953 (30%), which is associated with genotype B strains causing high within-herd levels of bovine mastitis. In addition, 3 novel *spa* types (t13269, t13277, and t13278) were identified. Although none of the strains harbored *mecA/mecC* or *pvl*, 55% of the isolates exhibited at least one enterotoxin gene. Many strains were present in samples from multiple dairies from different regions and years, highlighting the spread of *Staph. aureus* in small-scale cheese production plants. Our findings demonstrate that enterotoxigenic *Staph. aureus* and in particular t2953 (genotype B) isolates commonly occur in artisanal dairies and raw milk cheeses in Italy. It is particularly alarming that 80% of the artisan cheeses sampled in our study were positive for *Staph. aureus*. These findings stress the need for effective measures preventing staphylococcal food poisoning by limiting *Staph. aureus* growth and enterotoxin formation along the production chain and in the final product.

Key words: *spa* typing, enterotoxin, staphylococcal food poisoning, methicillin-resistant *Staphylococcus aureus*

Short Communication

Staphylococcus aureus is frequently isolated from raw milk intended for cheese production, dairy processing equipment, and environments, as well as food handlers. Its presence in raw milk represents a source for introduction of *Staph. aureus* into the dairy product supply chain (D'amico and Donnelly, 2011). Ingestion of staphylococcal enterotoxins produced by *Staph. aureus* in food can result in staphylococcal food poisoning, and several outbreaks linked to consumption of raw milk cheeses and artisanal cheeses have been reported (Johler et al., 2015a,b). Enterotoxigenic strains originating from small-scale cheese productions may also carry a wide variety of important resistance and virulence determinants including *mecA/mecC*, conferring methicillin resistance or *pvl* coding for the β-pore-forming toxin Panton-Valentine leukocidin (Herrera et al., 2016).

Information on *Staph. aureus* from artisanal raw milk cheeses and dairy production environments is scarce. Although a large amount of the cow milk produced in Italy is used in the manufacture of typical raw milk cheeses, few data on the occurrence and genomic characteristics of *Staph. aureus* from these sources are available. A previous study screening raw milk and raw milk cheeses in the Turin area reported that 39% of samples were positive for *Staph. aureus* and that 21% of the isolates carried enterotoxin genes (Bianchi et al., 2014). Another study performed in the Apulia and Basilicata region detected methicillin-resistant *Staph. aureus* in

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2.5% of bulk tank milk samples tested (Parisi et al., 2016).

Phenotypic or genotypic typing methods can be used to discriminate different *Staph. aureus* isolates, for host attribution or to determine the relatedness of different isolates. Sequencing and analysis of the polymorphic X region of the protein A gene (*spa* typing) was shown to yield excellent discriminatory power (Koreen et al., 2004).

The aim of this study was to characterize isolates of *Staph. aureus* from raw milk cheese productions of small-scale artisan cheese manufactures in Italy by determining *spa* types and the presence/absence of enterotoxin genes, *mecA/C*, and *pvl*.

All isolates used in this study were collected in Italy between 2010 and 2012. We sampled and re-sampled 36 dairies producing artisanal raw milk cheeses from bovine milk in Piedmont and Aosta Valley. Samples were taken from raw milk (n = 36), whey (n = 36), curd (n = 36), brine (n = 36), drying worktops (n = 36), and cheese (n = 96). Information on sample type, dairy, and year is included in Supplemental Table S1 (https://doi.org/10.3168/jds.2017-13815). Food samples were processed according to the ISO 6888–2 (ISO, 1999) method for the detection and enumeration of coagulase-positive staphylococci. For each sample, 5 colonies displaying a phenotype characteristic for coagulase-positive staphylococci were streaked on blood agar plates and identified as *Staph. aureus* using the VITEK system GP card (bioMérieux, Marcy l'Etoile, France). Environmental swab samples were placed in 10 mL of Mueller-Hinton broth (Oxoid, Basingstoke, Hampshire, UK) supplemented with 6.5% NaCl (Sigma-Aldrich, Arklow, Ireland), vortexed, and incubated for 24 h at 37°C. After incubation, an aliquot of 100 μL was pour plated in rabbit plasma fibrinogen Baird Parker agar plates (Oxoid) and incubated at 37°C. Species confirmation for colonies displaying a *Staph. aureus* characteristic phenotype after 24 or 48 h was performed by VITEK system GP card (bioMérieux).

Genomic DNA was extracted from *Staph. aureus* strains using InstaGene Matrix (Bio-Rad, Milan, Italy). Each colony was suspended in 100 μL of matrix buffer, incubated at 56°C for 60 min, followed by incubation at 95°C for 45 min. The suspensions were centrifuged at 20,000 × *g* for 5 min and supernatants were used for PCR screening of all isolates for enterotoxin genes, *pvl*, *mecA*, and *mecC*. Enterotoxin genes were detected using 2 multiplex PCR assays (see Table 1) according to the protocols of the European Union Reference Laboratory for Coagulase-Positive Staphylococci (Kérouanton et al., 2007). The following reference strains were included as positive controls: FRIS6 (*sea*, *seb*), FRI137 (*seg*, *seh*, *sei*), FRI326 (*see*), FRI361 (*sec*, *sed*, *ser*), and HMPL280 (*seg*, *sei*, *selj*, *selp*). The PCR screening for *mecA/mecC* and *pvl* was performed as previously described (Stegger et al., 2012). We determined *spa* types as previously described (Johler et al., 2011) and depicted results by constructing a minimum spanning tree (BioNumerics software ver. 7.6, Applied Maths Inc., Austin, TX). Iso-

Table 1. Primers used for the detection of *Staphylococcus aureus* enterotoxin genes by a multiplex PCR approach[1]

Gene	Primer	Primer sequence (5′–3′)	Product size (bp)	Reference
sea	GSEAR-1	GGT TAT CAA TGT GCG GGT GG	102	Mehrotra et al., 2000
	GSEAR-2	CGG CAC TTT TTT CTC TTC GG		
seb	GSEBR-1	GTA TGG TGG TGT AAC TGA GC	164	Mehrotra et al., 2000
	GSEBR-2	CCA AAT AGT GAC GAG TTA GG		
sec	GSECR-1	AGA TGA AGT AGT TGA TGT GTA TGG	451	Mehrotra et al., 2000
	GSECR-2	CAC ACT TTT AGA ATC AAC CG		
sed	GSEDR-1	CCA ATA ATA GGA GAA AAT AAA AG	278	Mehrotra et al., 2000
	GSEDR-2	ATT GGT ATT TTT TTT CGT TC		
see	SA-U	TGT ATG TAT GGA GGT GTA AC	213	Sharma et al., 2000
	SA-E rev	GCC AAA GCT GTC TGA G		
ser	SER 1	AGA TGT GTT TGG AAT ACC CTA T	123	Chiang et al., 2008
	SER 2	CTA TCA GCT GTG GAG TGC AT		
seg	SEG-F	GTT AGA GGA GGT TTT ATG	198	Bania et al., 2006
	SEG-R	TTC CTT CAA CAG GTG GAG A		
seh	SEH-F	CAA CTG CTG ATT TAG CTC AG	173	Bania et al., 2006
	SEH-R	CCC AAA CAT TAG CAC CA		
sei	SEI-F	GGC CAC TTT ATC AGG ACA	328	Bania et al., 2006
	SEI-R	AAC TTA CAG GCA GTC CA		
selj	SEJ-F	GTT CTG GTG GTA AAC CA	131	Bania et al., 2006
	SEJ-R	GCG GAA CAA CAG TTC TGA		
selp	SEP-F	TCA AAA GAC ACC GCC AA	396	Bania et al., 2006
	SEP-R	ATT GTC CTT GAG CAC CA		

[1]Annealing temperatures used were 55°C for the first multiplex PCR (*sea-see*, *ser*), and 52°C for the second multiplex PCR (*seg-selj*, *selp*).

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