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Molecular typing of *Staphylococcus aureus* isolated from Italian dairy products on the basis of coagulase gene polymorphism, multiple-locus variable-number tandem-repeat and toxin genes

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Coagulase gene restriction fragment length polymorphism (RFLP), six-locus variable-number tandem-repeat analysis patterns (MLVA) and detection of enterotoxin genes (*se*) (*sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *sel*) were used to determine the phylogenetic relationship among isolates of *Staphylococcus aureus* isolated from dairy products from different regions of Italy. A total of 25 *Staph. aureus* were subtyped into 16 coagulase genotypes by RFLP, and MLVA revealed marked genomic variability. Furthermore, 17 of the isolates harboured at least one toxin gene, with the predominance of *sea*, *sed* and *sej* among cow isolates and *sec-sel* among the goat and sheep strains. Combined RFLP, MLVA polymorphism and *se* genes were found to be useful techniques for discriminating several genetic variants in *Staph. aureus* isolates.

Keywords: *Staphylococcus aureus*, enterotoxins, coagulase, molecular typing, MLVA, RFLP.

Staphylococcus aureus is an ubiquitous bacteria commonly isolated from bulk raw milk, and from the milk of dairy cattle suffering from mastitis (Jørgensen et al. 2005). Its presence in raw milk is a major concern for the safety and quality of traditionally produced cheeses. Some *Staph. aureus* strains in the natural population can produce staphylococcal enterotoxins (SEs) (Balaban and Rasooly 2000), and these were recently reported to be responsible for food poisoning associated with reconstituted milk, in Japan (Ikeda et al. 2005), and cheese consumption, in European countries (Le Loir et al. 2003) and Brazil (do Carmo et al. 2002).

A number of typing techniques are available to help trace the source and transmission rates of *Staph. aureus* from foods and clinical specimens: restriction fragments length polymorphism analysis (RFLP), pulse-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and multiple-locus variable-number tandem-repeat (MLVA).

RFLP analysis of the coagulase (*coa*) is a useful method for typing *Staph. aureus* isolates for epidemiological study: amplified DNA fragments of different size can be further discriminated by digestion with *Alu*I. This genotyping method is an easy and useful tool that provides epidemiological information about *Staph. aureus* (Hookey et al. 1998; Moon et al. 2007).

The MLVA approach is based on the detection of the number of tandem repeats (TRs) at a specific locus in the genome of a microorganism. These can vary as a consequence of DNA polymerase enzyme slippage during replication, and these differences can be detected using PCR primers designed to anneal to the flanking regions (Keim et al. 2000). The complete TR is amplified and sized using a conventional agarose gel.

MLVA has proved very efficient, not only for *Staph. aureus* but also for numerous bacterial pathogens such as *Bacillus anthracis* and *Yersinia pestis* (Lindstedt, 2005; Merabishvili et al. 2006). It was recently applied to human *Staph. aureus* isolates and its discriminatory power was comparable to PFGE and MLST (Sabat et al. 2003; Francois et al. 2005; Malachowa et al. 2005). However, in contrast to the human isolates, data available in the literature about the spreading and typing of *Staph. aureus* isolated from bovine and from dairy products is quite limited (Vimercati et al. 2006; Gilbert et al. 2006).

In the present study, coagulase gene (*coa*) RFLP analysis, considered easy to perform and having high levels of specimen typeability and reproducibility (Chiou et al. 2000) and MLVA typing (a technique not yet used for typing *Staph. aureus* isolated from food) were used to

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Table 1. Characteristics of *Staph. aureus* isolates and distribution of *se* genes according to their origin

Strains	Animal	Source	Province	*se* type
S1	Cow	Milk	Bolzano	*sec, sed, sej, sel*
S2	Cow	Milk	Bari	*sed, sej*
S3	Cow	Milk	Reggio E.	*seg*
S4	Cow	Milk	Genova	*seh*
S5	Cow	Milk	Milano	no amplification
S6	Cow	Butter	Lecco	*sea*
S7	Cow	Butter	Sondrio	*sea, sed*
S8	Cow	Milk	Sondrio	*sea*
S9	Cow	Curd	Torino	*seh*
S10	Cow	Curd	Bergamo	*sea, sed, seg, sej*
S11	Goat	Milk	Milano	*sec, sel*
S12	Goat	Milk	Varese	*sec, sel*
S13	Goat	Cheese	Varese	*sea*
S14	Goat	Milk	Sondrio	*sea, sed, sej*
S15	Goat	Milk	Milano	no amplification
S16	Goat	Milk	Varese	no amplification
S17	Goat	Milk	Bergamo	*sec, sel*
S18	Sheep	Cheese	Palermo	no amplification
S19	Sheep	Milk	Palermo	no amplification
S20	Sheep	Curd	Palermo	no amplification
S21	Sheep	Whey	Palermo	*sec, sel*
S22	Sheep	Milk	Agrigento	no amplification
S23	Sheep	Milk	Sassari	*seg, sei*
S24	Sheep	Milk	Sassari	*sec, sel*
S25	Sheep	Milk	Siena	*seg*

investigate *Staph. aureus* diversity in isolates from Italian dairy products.

Further information on *Staph. aureus* strains was found by also detecting staphylococcal enterotoxin genes (*se*).

Materials and Methods

Bacterial strains

The 25 *Staph. aureus* investigated in the present study were obtained from different dairy products coming from different regions of Italy (Table 1). To highlight the possible differences among the isolates of different origin, only one strain for each sample was considered.

In order to detect coagulase-positive *Staph. aureus* the isolates were collected on Baird Parker RPF Agar (Biolife, Milan, Italy). The *Staphylococcus* genus was identified by Gram staining, catalase activity determination and the heat stable nuclease (TNase) test using Toluidine Blu Agar (Oxoid, Milan, Italy). To determine the staphylococcal species, species-specific PCR was used according to Cremonesi et al. (2005). Strains were maintained and propagated in Brain Heart Infusion (BHI, Oxoid, Milan, Italy) broth and incubated at 37 °C overnight.

DNA extraction

DNA was extracted, as described by Cremonesi et al. (2006), using 1 ml bacterial culture, incubated in BHI broth overnight at 37 °C. Cell numbers were verified by total sample counts, following the ISO 6888 1/2:1999 procedure with Baird Parker RPF agar plate. An approximate average of 10 μg DNA was obtained from 10^8 CFU/ml.

Detection of coa gene by PCR and RFLP analysis

To detect the polymorphic region of the *coa* gene, a PCR assay was performed as described by Hookey et al. (1998). The amplifications were carried out in a thermocycler (Mastercycler ep, Eppendorf, Hamburg, Germany).

For restriction endonuclease analysis, approximately 300 ng (12 μl) of PCR products were digested at 37 °C for 2 h with 10 U of the restriction endonuclease *Alu*I (New England BioLabs, Frankfurt, Germany), according to manufacturer instructions. All the digested PCR products were analysed on 3% agarose gel (GellyPhor, Euroclone, Milan, Italy) stained with Ethidium bromide (0·05 μg/μl; Sigma Aldrich, Milan, Italy). *Staphylococcus aureus* ATCC 25923 was used as reference strain in both PCR assays as positive control. The DNA bands were visualised on UV transilluminator (Uvitec, Cambridge, UK). To determine the exact number of tandem repeat units, 25 PCR products of the *coa* gene of *Staph. aureus* were sequenced by PRIMM services (PRIMM, Milan, Italy).

The computer software (http://tandem.bu.edu/trf/trf.basic.submit.html) developed by Benson (1999), which incorporates the algorithm of the Tandem Repeat Sequence Finder, was used to count the number of repeat units whose size was 81 bp.

MLVA typing

Six variable numbers of tandem repeat (VNTR) loci *spa*, *clfA*, *clfB*, *sdr*, *sspA* and *coa* were analysed in this study.

A multiplex PCR assay was performed as described by Sabat et al. (2003), with the slight modification of Moroni et al. (2005) containing *spa*, *clfA*, *clfB*, *sdr* and *sspA* primers.

The *coa* gene was analysed according to Hookey et al. (1998) separately.

In each run, a 100 bp DNA ladder (Sharpemass 100 DNA Ladder, Euroclone, Italy) was included for PCR products. All the PCR products were analysed on 3% agarose and visualized and photographed on an UV transilluminator. Strains were characterized by a MLVA profile regrouping the alleles obtained for the six primers.

Detection of se genes by multiplex PCR

Se genes, including *sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *sel* were detected by multiplex PCR assay as described by Cremonesi et al. (2005). As several studies have revealed that none of the investigated strains isolated from bovine and goat milk, and related dairy products, harbour any of the *seb*, *see* and *sek* genes, the primer pairs related to these three genes were not used when the amplification protocol

Table 2. The *coa* typing and *coa* subtypes by means of PCR and RFLP polymorphism, and number of *Staph. aureus* detected for each subtype

coa type (size, bp)	*coa* n° repeat	*coa* subtype	Number of isolates			Strains
			Cow	Goat	Sheep	
A (560±20)	5	A1	1	—	—	S3
	5	A2	1	—	—	S4
	5	A3	4	2	—	S6, S7, S8, S10, S13, S14
	5	A4	—	1	—	S15
	5	A5	—	1	—	S17
	5	A6	—	—	1	S19
B (650±20)	6	B1	—	2	2	S12, S16, S22, S24
	6	B2	—	—	1	S25
C (750±20)	7	C1	1	—	—	S1
	7	C2	1	—	—	S5
	7	C3	—	—	1	S18
	7	C4	—	—	1	S20
D (800±20)	8	D1	1	—	—	S2
	8	D2	1	—	—	S9
	8	D3	—	1	1	S11, S21
	8	D4	—	—	1	S23

for multiplex PCR assay was established. The present PCR assay included species-specific primers for 23S rRNA and TNase for DNA isolate control. The reference strains ATCC 700699 (harbouring *sea*, *sec*, *seg*, *sei* and *sel* genes), ATCC 23235 (*sed*, *seg*, *sei* and *sej*) and ATCC 19095 (*sec*, *seh*, *seg* and *sei*) were included as positive controls for the PCR assay.

Statistical analysis

The peak matrices corresponding to MLVA profiles was subjected to a cluster analysis. Binary 0/1 matrices were created based on the absence or presence of DNA bands. Pairwise distances were calculated with the SimQual option of the NTSYSpc 2.01 computer program (Applied Biostatistics Inc., USA) by employing the Jaccard coefficient for two-state data and strain clustering was performed by the UPGMA analysis. The significance of resulting UPGMA tree was checked comparing the original similarity matrix with the cophenetic similarity matrix by using Mantel test.

Results

The PCR amplification of the *coa* gene yielded a single amplification, the sizes ranging from 560±20 to 800±20 bp for the strains analysed.

All the *Staph. aureus* isolated from cow, goat and sheep dairy products were classified into four *coa* types on the basis of fragment size (A, B, C and D). The sizes of the fragments obtained by PCR were confirmed by sequence analysis, and the fragments showed different repeat numbers, ranging from 5 to 8.

Table 2 shows the number of strains of the different animals corresponding to each polymorphism and the number of repeats. All the *coa* types are found in at least two ruminant species. For the cow isolates, most are in type A (6 strains), the others being equally distributed in types C and D. For the goat products, *Staph. aureus* is present in the A, B and D *coa* types. Instead the sheep isolates were distributed in all the types, from A to D.

Furthermore, these *coa* types were subdivided into subtypes on the basis of RFLP patterns obtained by *Alu*I digestion. The *coa* subtypes were arbitrarily identified by letters followed by a numerical code (e.g. A1 and A2).

From 1 to 4 fragments were produced by *Alu*I digestion of the 25 PCR products of *coa* gene, the fragment size varying from approximately 128 to 600 bp. Isolates belonging to type A were subdivided into six restriction subtypes (denoted A1–A6), strains belonging to C and D types into four subtypes (named C1–C4 and D1–D4) and isolates belonging to B type into two subtypes (named B1 and B2) (Table 2).

The S3 strain belonging to type A did not show enzyme digestion and was defined as subtype A1.

A total of 16 different RFLP patterns (*coa* subtype) were identified, and 13 of these subtypes contained one single isolate. An analysis of the strain distribution showed that only one *coa* subtype (A3) contained isolates from both cow and goat, and only two *coa* subtype (B1 and D3) contained *Staph. aureus* from goat and sheep together. No *coa* subtype was observed to contain isolates from all ruminant species.

The polymorphic degree of the different tandem repeats was evaluated with a set of 25 *Staph. aureus* isolates.

The strains were subtyped on the basis of PCR amplicon fragment size. Amplification by PCR gave a single

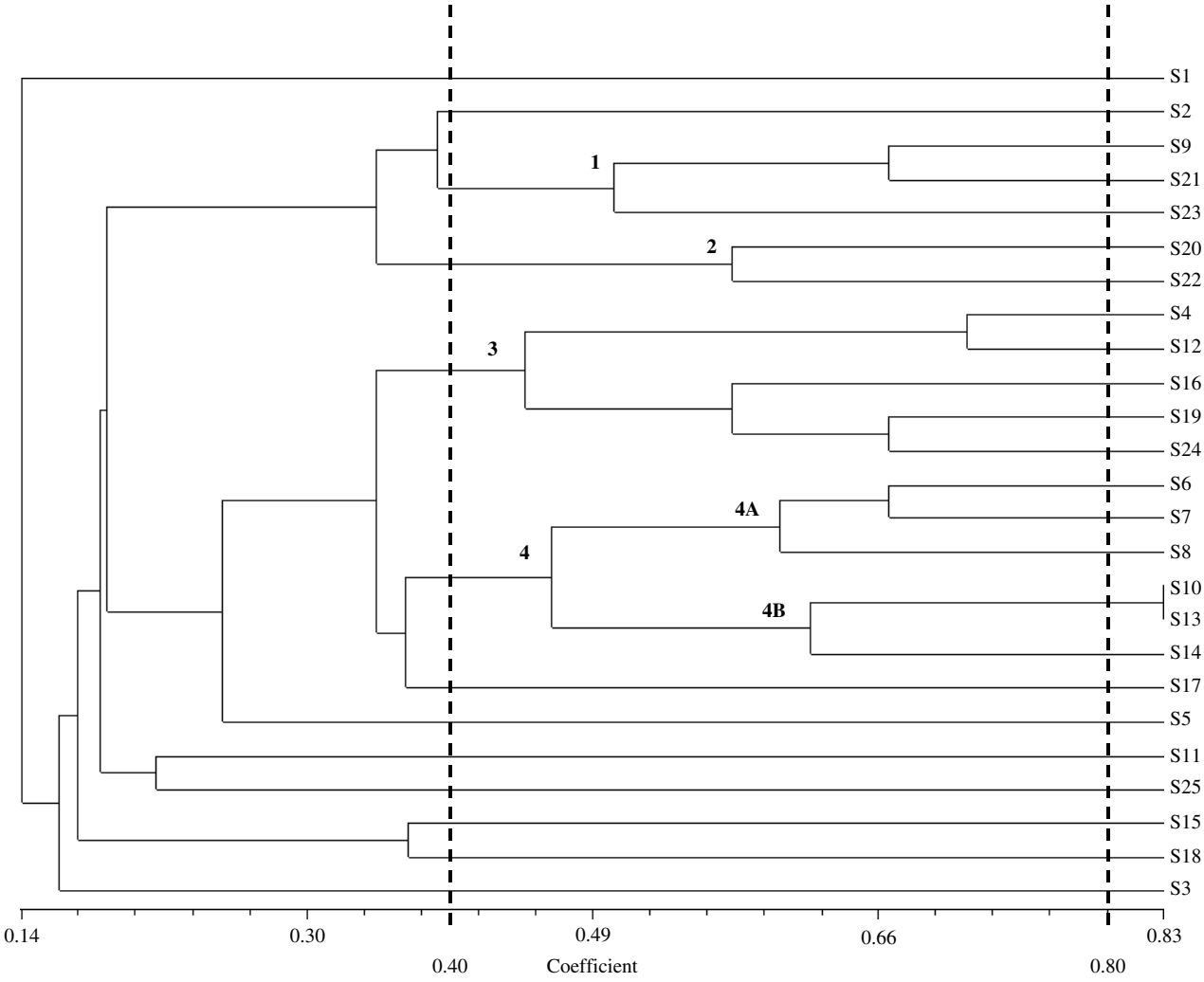

Fig. 1. UPGMA dendrogram derived from MLVA analysis showing the percentages of genetic similarity among 25 *Staph. aureus* isolates.

amplicon for each gene studied (*spa*, *clfA*, *clfB*, *sdr*, *sspA* and *coa*), except for one goat strain (S12) where the *sdr* gene showed 2 fragments (500 and 600 bp approximately).

PCR amplification produced 5 or 6 bands, approximately between 100 to 1200 bp. Indeed, 14 strains showed 5 bands, there being co-migration of the amplicons of the tested genes: 7 isolates showed the co-migration of *clfA–clfB*, 2 of *clfB–sdr*, 2 of *sdr–coa* and 2 of *clfA–coa;* on one single strain there was the co-migration of the bands of the *clfB* and *sspA* genes.

The considerable genomic variability in *Staph. aureus* strains is reflected by MLVA analysis. In fact MLVA produced 24 different DNA banding patterns among the 25 *Staph. aureus* isolates (Fig. 1).

Using the arbitrarily chosen cut off of 80%, only one cluster comprising 2 strains (S10 and S13) was distinguished. Only by applying a 40% similarity cut-off was it possible to achieve a grouping of four clusters, designated by the numbers 1 to 4.

No link was found among the animal and enterotoxin groups. Cluster 3 contains 3 isolates belonging to the RFLP subtype B1, and 2 strains (S4 and S19) that belong to different RFLP subtypes (A2 and A6); moreover S22, differently from the other 3 strains of RFLP B1 subtype, belongs to MLVA cluster 2.

Of the 25 examined isolates of *Staph. aureus*, 18 (72%) were found to be positive for one or more toxin genes, and, on the basis of combined enterotoxin genes, a total of 10 *se* combination types were observed. Table 1 shows the distribution of toxin genes according to the origin of the strains. The more commonly detected *se* genotypes were *sea*, *sec*, *sed* and *sel*. Seven isolates possessed only one kind of *se* gene (*sea*, *seg* and *seh*) and 11 *Staph. aureus* harboured more than one toxin gene. *Sej* were usually identified in combination with *sed* in cow strains (except for strain S7), while *sec* and *sel* in combination were predominately found in goat and sheep *Staph. aureus*. *Seh* was detected only in cow strains (isolates named S4 and

S9) and *sei* was present only in one isolate coming from sheep (strain S23).

Discussion

The results of the present study suggest a certain degree of heterogeneity among the *Staph. aureus* strains of different Italian dairy products.

RFLP is considered a simple and accurate method for typing *Staph. aureus* isolates: according to da Silva et al. (2006) this technique is suitable for epidemiological investigations of *Staph. aureus* as it is very easy to execute and interpret. Indeed, the four identified PCR types and the 16 *Alu*I RFLP patterns suggest the presence of different *Staph. aureus* genotypes.

The MLVA results confirmed the genetic diversity found using *coa* gene polymorphism. MLVA provided a higher degree of discriminatory power over the single RFLP technique: RFLP identified 16 individual subtypes while MLVA revealed 24 different profiles. Hence the MLVA typing method presented in this paper represents a robust and easy approach to characterize *Staph. aureus* isolates.

There was also considerable diversity in the *se* gene frequencies in the cow and small ruminant, as suggested by Smyth et al. (2005). The findings that some *se* positive and some *se* negative strains generate identical or similar RFLP and MLVA patterns suggest that the genetic profile associated with toxin production is not in any way correlated with RFLP and MLVA characterization, therefore such findings could be a further element to study biodiversity.

Our finding show strains with RFLP profiles that are the same but that belong to different MLVA clusters, supporting the hypothesis that the combination of RFLP, MLVA and *se* genes increases discrimination, compared to using the individual methods alone.

To the best of our knowledge, the present study is the first in Italy to use the MLVA technique to study the polymorphism of *Staph. aureus* isolated from different dairy products. Even though only a restricted number of isolates was investigated, it was confirmed that *Staph. aureus* typing can benefit from the analysis of *coa* genes and MLVA.

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