Research in Veterinary Science 88 (2010) 427–435

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Biochemical profiles, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and multilocus variable number tandem repeat analysis (MLVA) for typing *Staphylococcus aureus* isolated from dairy products

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ARTICLE INFO

Article history:
Accepted 19 October 2009

Keywords:
Staphylococcus aureus
Biochemical profile
MLVA
RAPD
*coa*RFLP
Enterotoxin gene

ABSTRACT

The study concerns 130 *Staphylococcus aureus* strains isolated from different raw-milk dairy products (122 isolates) and human samples (eight isolates). Four different typing techniques were applied: biochemical profiles (Biolog GP), restriction fragment length polymorphism of coagulase gene (*coa*RFLP), random amplified polymorphic DNA (RAPD) and multilocus variable number tandem repeat analysis (MLVA). Moreover multiplex-PCR was used to study the distribution of genes encoding staphylococcal enterotoxins. The results of this study reveal marked genomic and phenotypic variability among the tested *S. aureus*. The considered techniques were all found useful for strain typing, but, based on discriminatory power as the key parameter of the typing system, MLVA and Biolog GP were found to be the most powerful techniques. The methods showed little concordance in terms of discerning the clusters of related strains.

1. Introduction

Staphylococcus aureus is involved in a wide variety of infections found in human beings and animals, and some strains are also responsible for human food poisoning as they produce enterotoxins in foodstuffs (Rodríguez-Calleja et al., 2006). *S. aureus* can access milk through direct excretion from udders suffering clinical and sub-clinical staphylococcal mastitis, and by environmental contamination during the milk's handling and processing (Scherrer et al., 2004; Jørgensen et al., 2005). Many biochemical and molecular methods have been used in epidemiological investigations into human, bovine and ovine staphylococcal infections such as pulsed-field gel electrophoresis (PFGE) (Prevost et al., 1991; Hennekinne et al., 2003), restriction fragment length polymorphism analysis (RFLP) (Hookey et al., 1998), random amplification of polymorphic DNA (RAPD) (Pereira et al., 2002), multilocus variable number tandem repeat analysis (MLVA) (Sabat et al., 2003; Malachowa et al., 2005; Gilbert et al., 2006; Tenover et al., 2007) and multilocus sequence typing (MLST) (Aires-de-Sousa et al., 2007). Indeed, the sensitivity and performance of polymerase chain reaction (PCR) based typing systems make them useful tools for the study of *S. aureus* of animal and human origin, and for the monitoring of the bacterium's diffusion.

The aim of the current study was to describe the genetic and phenotypic diversity of *S. aureus* isolated from raw-milk and dairy products. Three well-known and frequently used staphylococcal typing methods (RFLP, RAPD-PCR and MLVA) were used for characterization, while the fermentative profile (Biolog GP2) was evaluated to characterize phenotypic diversity (Roach et al., 2006; Di Cagno et al., 2007). Further information on *S. aureus* strains was also gained by the detection of staphylococcal enterotoxin genes (*se*) and, in particular, by studying the presence of genes encoding *sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *sel* using multiplex-PCR (mPCR).

2. Materials and methods

2.1. Bacterial strains

A group of 130 *S. aureus* isolates were selected from the CNR ISPA strain collection.

Of these, 122 were obtained from raw-milk products (milk, curd, 1–2 month old cheeses, butter and whey) from 10 different Italian regions and different animal species (81 from cow, 22 from goat, 17 from sheep and two from water buffalo) over a period of 5 years (2002–2007), while eight were collected from different clinical disease episodes in humans (Table 1). All the samples were

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doi:10.1016/j.rvsc.2009.10.005

Table 1
S. aureus ($n = 130$) used in this study and their origin.

Strains	Source	Sample	Cheese	Total
39, 59, 57, 266, 280, 286, 507, 521, 530, 587, 617, 631, 640, 642, 700, 739, 759, 810, 829, 844, 845, 868, 885, 890, 894, 919, 948, 954, 957, 983, 990, 1010, 1028, 1060, 1087, 1106, E, GP10, GP11, GP12, GP13, GP14, GP15, GP16, GP17, GP18, GP19, GP2, GP21, GP26, GP27, GP28, GP30, GP31, GP32, GP4, GP9, VS508	Cow	Raw milk		59
F, G		Curd	Soft cheese	2
RAS1, RAS3, RAS4			Raschera DOP	3
VS501, VS503, VS504, VS506			Formagèla Valseriana	4
5, 15, 92, L83, L89		Cheese	Soft cheese	5
S1G			Scimudin	1
VS507			Formagèla Valseriana	1
GP20, GP22, GP23, GP24, GP25, GP29		Butter		6
9, CA13, CA14, CA16, CA2, CA21, CA22, CA24, CA25, CA26, CA27, CA4, CA5, CA6, CA7, CA8, CA9	Goat	Raw milk		17
41, 88, CA10, CA19, V6		Cheese	Caprino cheese	5
ESP4, F3, OV10, OV13, OV16, OV17, OV18, OV24, OV3, OV30, OV38, OV43, OV8, PE1	Sheep	Raw milk		14
ESP9		Curd	Soft cheese	1
ESP11		Cheese	Soft cheese	1
ESP12		Whey	Soft cheese	1
BU1, BU2	Water Buffalo	Raw milk		2
AS, U2, U3, U7, U9, U11, U13, U17	Human			8

characterized by a content of staphylococci coagulase positive higher than 1000 cfu/g ot mL, allowing us to assume that bovine mastitic cows were the primary source of contamination of the milk and milk products. To highlight the possible differences among the isolates of different origin, the considered strain originated from different samples. The eight isolates from human disease were included to ascertain the existence of any significant differences between their characteristics and those of the strains isolated from raw-milk dairy products. The strains were maintained and propagated in Brain Heart Infusion (BHI, Oxoid, Milan, Italy) broth and incubated at 37 °C overnight.

2.2. Biochemical profile

The biochemical profile or "Metabolic Fingerprint" of the strains was studied using the Biolog GP2 Microplate (Biolog, Inc., Hayward, CA) according to manufacturer instructions. In order to standardize the inoculum conditions the strains were sub-cultured twice in BHI broth at 37 °C for 16 h and then cultivated on BUG agar supplemented with 5% defibrinated sheep blood at 37 °C for 24 h. All the isolates were tested twice and retested in case of discrepancies.

2.3. DNA extraction

DNA extraction, described by Cremonesi et al. (2006), was done using 1 ml of a BHI overnight culture containing approximately 1–3 $\times$ 10^9 cells.

2.4. Detection of coa by PCR and RFLP analysis

To detect the polymorphic region of the coagulase gene (*coa*), a PCR assay was performed as described by Hookey et al. (1998). 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). The experiments were replicated twice to test RFLP profile reproducibility, and the results were always the same.

2.5. RAPD-PCR

RAPD-PCR was performed with the primer AP4. The amplification conditions, as well as the electrophoresis and analysis of the amplification products, were the same as those described by Andrighetto et al. (2001), except for the amplification cycle of the AP4 primer that was modified as follows: an initial step of 95 °C for 90 s, followed by 35 cycles of 95 °C for 30 s, 36 °C for 60 s and 72 °C for 90 s. The RAPD-PCR profile grouping was done with the Gel Compar 4.1 software package (Applied Maths, Kortrjik, Belgium), using the Pearson product-moment correlation coefficient and UPGMA cluster analysis.

2.6. MLVA typing

Six tandem-repeat loci *spa*, *clfA*, *clfB*, *sdr*, *sspa* and *coa* were analysed in this study. The MLVA analysis of the *S. aureus* isolates was performed as described by Sabat et al. (2003) and Hookey et al. (1998).

2.7. 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).

2.8. Statistical analysis

The peak matrices corresponding to the Biolog GP and MLVA were subjected to cluster analysis. Binary 0/1 matrices were created based on the absence or presence of DNA bands or positive reaction. Pair-wise distances were calculated with the SimQual option of the NTSYSpc 2.01 computer program (Applied Biostatistics Inc., USA), employing the Jaccard coefficient for two-state data and strain clustering performed by UPGMA analysis. The significance of

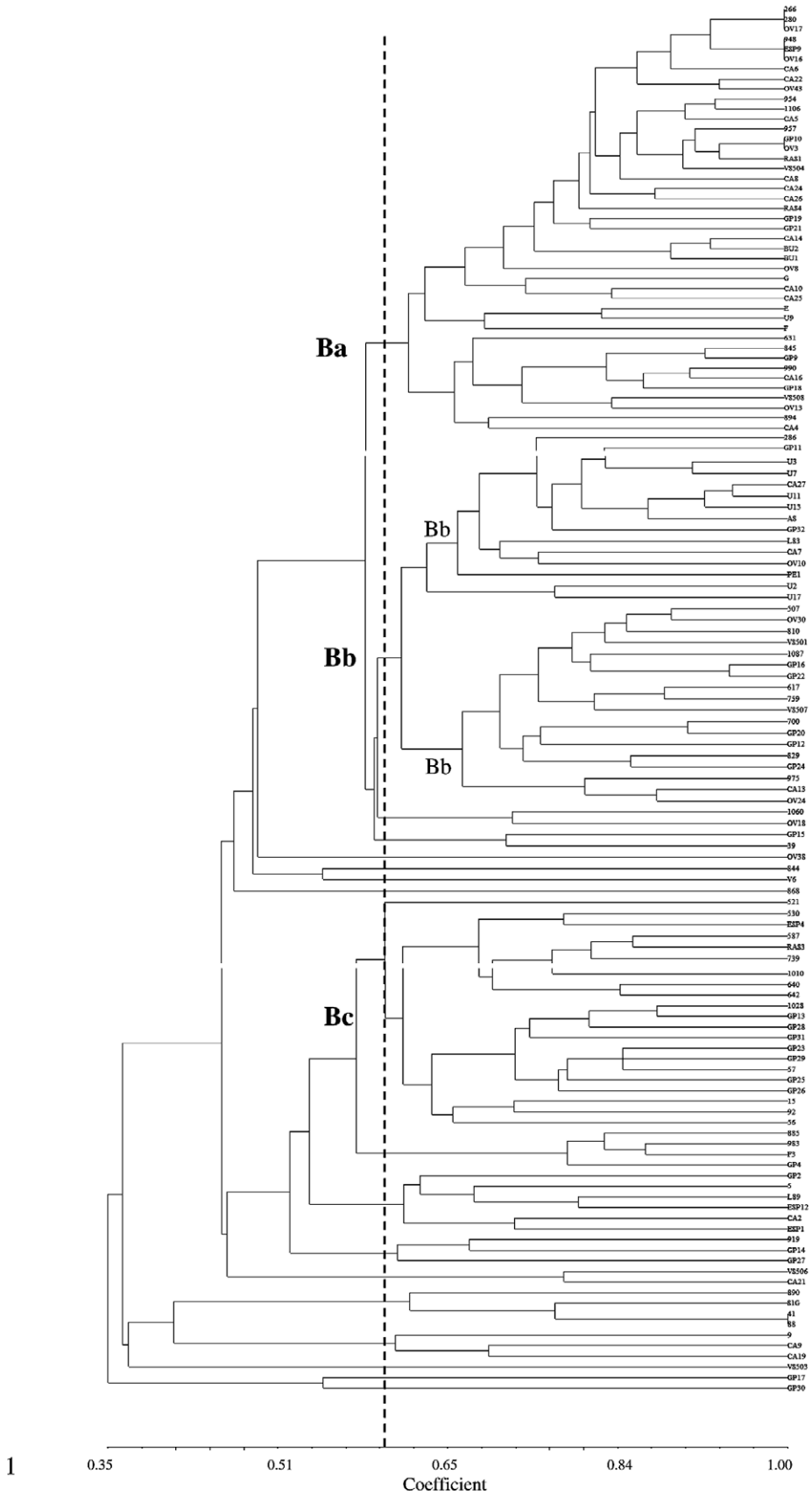

Fig. 1. Dendrogram showing the relatedness of the different Biolog GP2 profiles of *S. aureus* isolates. Similarity analysis was performed from a combined binary matrix based on the presence or absence of positive reaction using the Jaccard coefficient for two-state data, and strain clustering performed by the unweighted pair group method with arithmetic mean (UPGMA) analysis. A 60% similarity was arbitrarily chosen as a discriminating threshold to define the homogenous clusters (Ba, Bb, Bc) and sub-clusters (Bb1, Bb2).

the resulting UPGMA tree was checked by comparing, using the Mantel test, the original similarity matrix with the cophenetic similarity matrix (Mantel, 1967).

2.9. Discriminatory power

The discriminatory power of the used methods can be calculated on the basis of Simpson's index of diversity (*D*). Ideally, the index, based on the testing of a large number of epidemiologically unrelated isolates, should be equal to 1.0. (Hunter, 1990).

3. Results

3.1. Metabolic fingerprinting – Biolog GP2

Based on the Biolog GP2 test, different profiles were found for the strains. All the strains fermented dextrin, *N*-acetyl-D-glucosemine, α-D-glucose, L-lactic acid, 2′-deoxy adenosine, inosine, thymidine, pyruvic acid methyl ester while α-cyclodrestrin, β-cyclodextrin, inulin, amygdalin, L-arabinose, arbutin, D-arabitol, D-arabitol, L-fucose, D-galactose, D-galacturonic acid, m-inositol, mannan, D-melibiose, α-methyl-D-galactoside, β-methyl-D-galactoside, α-methyl-D-mannoside, L-rhamnose, salicin, sedoheptulosan, stachyose, D-tagatose, xylitol, D-xylose, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxybutyric acid, lactamide, D-malic acid, propionic acid, L-alaninamide, L-asparagine, L-pyroglutamic acid, putrescine were not used by the 130 *S. aureus* strains.

The 130 *S. aureus* isolates produced 124 Biolog patterns (Fig. 1). Three distinct clusters, designated Ba, Bb and Bc, were identified with 60% similarity. The first cluster (Ba) contained 43 *S. aureus* isolates (22 strains isolated from cow, 11 from goat, seven from sheep, two from water buffalo and one from human samples). The second cluster (Bb) had 33 strains (19 from cow, three from goat, four from sheep and seven from human samples). This Bb cluster grouped seven of the eight human isolates (87%). The 33 strains fell into two sub-clusters designated Bb1 and Bb2 with a similarity level of 63%. The third cluster (Bc) grouped many *S. aureus* isolated from cow dairy products (18 of the 19 strains) and one from sheep samples.

On considering a genetic similarity threshold of 60%, 35 strains were not within any cluster.

The comparison between the cophenetic similarity matrix and the original similarity matrix done with the Mantel's test gave a product-moment correlation of 0.77.

3.2. coa gene typing

The PCR amplification of the *coa* gene yielded a single amplification product with a size ranging from 420 ± 20 to 800 ± 20 bp. The products of sizes 560, 800 and 650 bp were the most frequent, and accounted for 50%, 23% and 15% of the isolates respectively.

Restriction enzyme digestion (by *Alu*I) of the PCR products generated 31 different *coa*RFLP patterns or clusters. The number of fragments produced upon *Alu*I digestion varied from 1 to 4, and their sizes from 90 ± 20 and 560 ± 20 bp. Considering the clusters grouping more than three isolates, (Table 2) the RFLP pattern A1 dominated, containing, in fact, 44 strains (39 from cow, three from goat, one from sheep and one from human samples). Moreover, the RFLP typing results showed that one cluster grouped three strains, six clusters two isolates, and 15 only single strains.

3.3. RAPD-PCR

The genomic variability in the *S. aureus* strains is also reflected by RAPD-PCR analysis using the primer AP4 (Fig. 2). The reproducibility value of the RAPD-PCR assay, calculated from two repetitions of independent amplification of staphylococcal type strains, was higher than 90%. At 80% similarity six distinct clusters (I, II, III, IV, V and VI) were detected. Cluster I grouped 10 *S. aureus* strains, five isolated from goats and three from sheep dairy products and two cow isolates. Cluster II grouped many *S. aureus* isolated from cow dairy products (23 of the 24 strains) and one from human samples. The 24 strains, except for two isolates, showed a high similarity coefficient (>95%). Cluster III contained 29 isolates: 25 isolated from cow, three from goat and one from human samples. Considering a similarity level of 85%, cluster III can be divided into two sub-clusters, IIIa and IIIb. The strains in the IIIb sub-cluster showed a high similarity coefficient (>95%). In cluster IV goat and sheep isolates predominated. Also note that eight of the nine strains had a high similarity coefficient (>95%). Cluster V contained six *S. aureus* isolates (four came from goat dairy products, one from cow and one from humans) showing a similarity level of 95%. Cluster VI contained 19 isolates, and could be divided into two sub-clusters VIa and VIb. Considering a threshold of 80% genetic similarity, 33 strains do not fall into any cluster.

3.4. MLVA typing

PCR amplification gave a single amplicon for each studied gene (*spa*, *clfA*, *clfB*, *sdr*, *sspa* and *coa*), except for 15 strains (eight from cow, four goat, one from sheep and two from human isolates) where the *sdr* gene showed two fragments. Seven isolates showed the co-migration of *clfA*–*clfB*, two of *clfB*–*sdr*, two of *sdr*–*coa* and two of *clfA*–*coa*; on one single strain there was the co-migration of the bands of the *clfB* and *sspA* genes. PCR amplification produced 4–7 bands, approximately between 100 and 1200 bp. Indeed, 18 strains showed four bands, 61 strains five bands, 47 strains six bands and four strains seven bands.

Table 2
Coagulase gene (*coa*) subtyping by means of polymerase chain reaction and restriction fragment length polymorphism (RFLP), and number of isolates detected for each subtype.

coa Type (size, bp)	*coa* Subtype	Number of strains	Dairy origin				
			Bovine	Goat	Sheep	Water buffalo	Human
A (560 ± 20)	A1	44	39	3	1		1
	A2	5	3	1		1	
	A3	4	4				
	A4	6	2	3			1
B (650 ± 20)	B	10		5	4		1
C (750 ± 20)	C	5		2	3		
D (800 ± 20)	D1	6	1	1	4		
	D2	16	14	1			1
	D3	4	3			1	

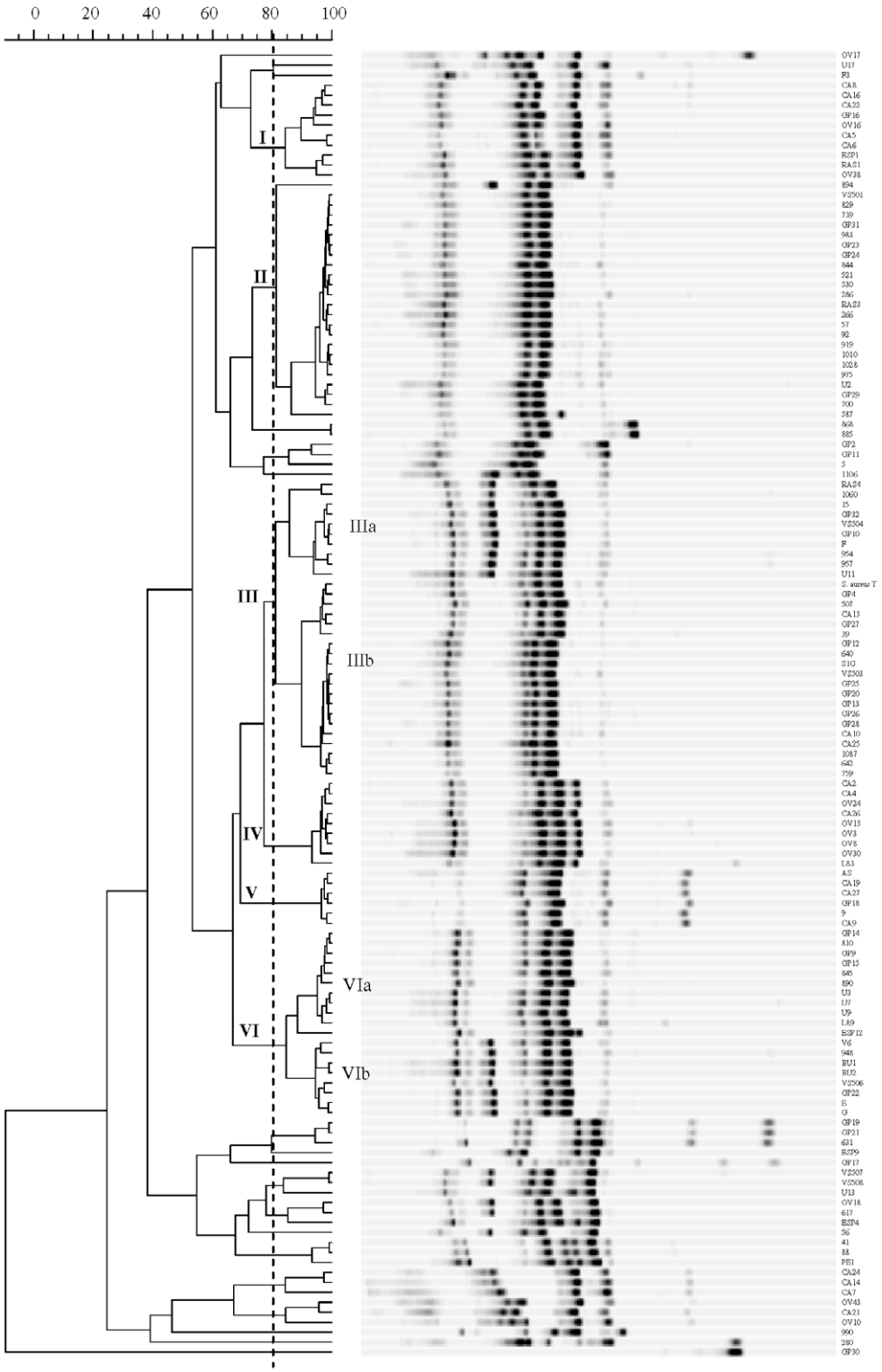

Fig. 2. Dendrogram derived from the random amplification of polymorphic DNA (RAPD-PCR) profiles generated with primer AP4. The RAPD-PCR profile grouping was done with the Gel Compar 4.1 software package using the Pearson product-moment correlation coefficient and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis. A 80% similarity was arbitrarily chosen as a discriminating threshold to define the homogenous clusters (I–VI) and sub-clusters (IIIa, IIIb, VIa, VIb).

In order to study reproducibility, two strains of *S. aureus* were inserted blindly into the study five times, along with all the other isolates, and the same results were obtained.

The genomic variability in the *S. aureus* strains is considerable, and this is reflected in the MLVA analysis that revealed that the 130 *S. aureus* isolates produced 124 different DNA banding patterns. Only applying a 30% similarity cut-off, and considering the clusters that grouped more than five isolates, was it possible to achieve a grouping of seven clusters including 82 strains (ML1, ML2, ML3, ML4, ML5 ML6 and ML7) (Fig. 3). The subtypes obtained did not group the isolates univocally on the basis of different animal species. Comparison of the cophenetic similarity matrix and

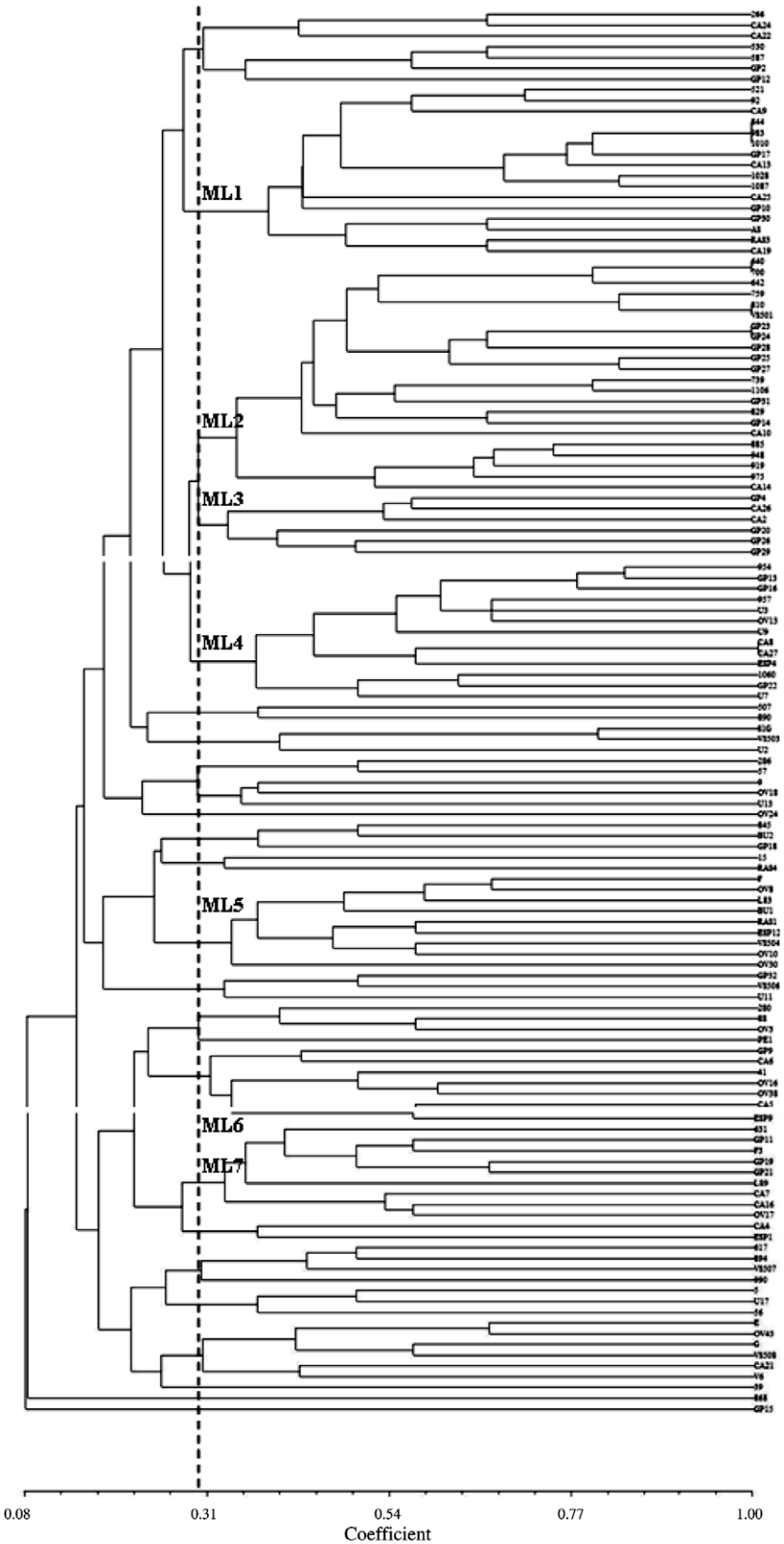

Fig. 3. Dendrogram showing the relatedness of the different multilocus variable number tandem repeat analysis (MLVA) profiles generated from the derived from studied genes (*spa*, *clfA*, *clfB*, *sdr*, *sspa* and *coa*) of *S. aureus*. Similarity analysis was performed from a combined binary matrix based on the absence or presence of positive reaction using the Jaccard coefficient for two-state data and strain clustering performed by unweighted pair group method with arithmetic mean (UPGMA) analysis. A 30% similarity was arbitrarily chosen as a discriminating threshold to define the homogenous clusters (ML1–ML7).

Table 3
Distribution of enterotoxin genes (*sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *sel*) detected by multiplex-PCR assay in *S. aureus* strains ($n = 130$) according to the origin of the isolates.

Number of strains	Origin of isolates					*se* genes							
	Cow	Goat	Sheep	Water buffalo	Human	*sea*	*sec*	*sed*	*seg*	*seh*	*sei*	*sej*	*sel*
17	11	2	–	–	4	+	–	–	–	–	–	–	–
2	–	–	–	–	2	+	+	–	–	+	–	–	+
6	6	–	–	–	–	+	–	+	–	–	–	–	–
1	1	–	–	–	–	+	–	+	+	–	+	+	–
1	1	–	–	–	–	+	–	+	+	–	–	+	–
14	13	1	–	–	–	+	–	+	–	–	–	+	–
1	1	–	–	–	–	+	–	–	+	–	–	–	–
1	–	1	–	–	–	+	–	–	+	+	+	–	–
1	–	1	–	–	–	+	–	–	+	–	+	–	–
1	–	–	–	–	1	+	–	–	–	+	–	–	–
1	1	–	–	–	–	–	+	+	–	–	–	+	+
14	–	7	7	–	–	–	+	–	–	–	–	–	+
3	3	–	–	–	–	–	–	+	–	–	–	–	–
13	13	–	–	–	–	–	–	+	–	–	–	+	–
1	1	–	–	–	–	–	–	+	+	–	–	+	–
1	–	–	1	–	–	–	–	–	+	–	–	–	–
3	1	–	2	–	–	–	–	–	+	–	+	–	–
3	3	–	–	–	–	–	–	–	–	+	–	–	–
1	1	–	–	–	–	–	–	–	–	–	+	–	–
45	24	10	8	2	1	–	–	–	–	–	–	–	–
130	**80**	**22**	**18**	**2**	**8**	**45**	**17**	**40**	**10**	**7**	**7**	**31**	**17**

the original similarity matrix, done using Mantel's test, gave a product-moment correlation of 0.64.

3.5. Detection of sea, sec, sed, seg, seh, sei, sej and sel

Of the 130 *S. aureus* isolates examined, 85 (65%) were found to be positive for one or more toxin genes and demonstrated marked genotype variability, so much so that we subdivided them into 19 groups according to gene presence (Table 3). Twenty strains possessed only one type of toxin gene (17 *sea*, three *sed*, one *seg*, three *seh* and one *sei*), while the remaining 60 harboured more than one toxin gene. The enterotoxins *sea* and *sed*, found in 45 and 40 strains respectively, were the most frequent among the toxins looked for. In general, there was considerable variation in the *se* gene types among the isolates of different origin. Enterotoxins *sea*, *sed* and *sej* were found more frequently in cow isolates while the enterotoxins *sec* and *sel* predominated in goat and sheep strains.

3.6. Distribution of enterotoxin genes among isolates according to the different typing techniques

The distribution of the enterotoxin genes among the clusters derived from the different typing techniques used in this study is reported in Table 4. The results show that the subtypes obtained with the different typing methods group together strains harbouring different *se* genes, indeed enterotoxin genes appear to be widely distributed among the clusters.

4. Discussion

The results of the study reveal marked genetic and phenotypic variability among the tested *S. aureus*, highlighted by three well-known and frequently used staphylococcal typing techniques (RFLP, RAPD-PCR and MLVA) and a metabolic fingerprint method (Biolog GP2). The different isolation years and origins were found to have no noticeable correlation with the pattern strains. However some minor variability was observed when the same techniques were used on the strains isolated from clinical human cases (Sabat et al., 2003 Malachowa et al., 2005; Neela et al., 2005; Tenover et al., 2007). In this study of 130 *S. aureus* strains, typed using four different methods, we were able to determine the discriminatory power of each technique by calculating the *D* index. Note that the MLVA ($D = 0.99$) and Biolog GP ($D = 0.99$) techniques yielded the most different patterns. Furthermore, RAPD ($D = 0.94$) and RFLP of the coagulase gene ($D = 0.86$) showed a good Simpson's index. Our results differ from those of Vautor et al. (2005) who, using a small number of isolates ($n = 26$), claimed that RAPD ($D = 0.35$) and *coa*RFLP ($D = 0.53$) were not sufficiently discriminatory for use as the sole typing method for *S. aureus*. However Biolog GP is an expensive, time-consuming and labour-intensive technique requiring particular equipment, while the big advantage of the RAPD, RFLP and MLVA approaches is their short duration (about 7 h), compared to Biolog GP which needs three days for a complete analysis. These rapid PCR-based methods are excellent for a fast and inexpensive typing of *S. aureus*, and could serve as an initial screening of isolates for public health and epidemiological studies. Indeed, the evident discriminatory power of MLVA makes it a useful and reliable starting point for a short term epidemiological investigation of *S. aureus*. None of the genetic techniques showed differences between the strains of human origin and those isolated from dairy products. However our results did show that the metabolic fingerprinting (Biolog GP2) grouped seven of the eight human *S. aureus* strains in the Bb2 sub-cluster; in fact these isolates showed similar biochemical profiles. In addition, as reported by other authors (Araki et al., 2002; Naffa et al., 2006), the results of our research showed that there was no correlation among Biolog GP, RFLP, RAPD-PCR and the MLVA clusters and toxin gene distribution. Of the 130 strains, only 16 (12%) were grouped in the same clusters by the different techniques, an index of the great genetic variability of the studied strains. All the methods were able to group the isolates, though not univocally, on the basis of some of the considered characteristics (different animal species and enterotoxin codifying genes). Note also that all the considered techniques were found useful for strain typing, though the methods were not highly concordant in terms of discerning clusters of related, and probably related, strains. Some strains that were indistinguishable by one method remained quite unrelated by the others, but it must be remembered that they were characterized by different DNA polymorphism explorations, which could explain the discrepancies among the molecular techniques used. Thus,

Table 4
Distribution of enterotoxin genes (*sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *sel*) detected by multiplex-PCR assay in *S. aureus* strains (n = 130) among Biolog GP2, restriction fragment length polymorphism (RFLP) of coagulase gene (*coa*), and, random amplification of polymorphic DNA (RAPD-PCR) and multilocus variable number tandem repeat analysis MLVA clusters. n.a.: no *se* genes amplifications.

Bioog GP		*coa*-RFLP		RAPD-PCR		MLVA	
$n°$	*se* genes	$n°$	*se* genes	$n°$	*se* genes	$n°$	*se* genes
Ba							
5	*a*	**A1**	9	*a*	**I**	1	*a*	**ML1**	2	*a*
1	*ad*		6	*ad*		2	*cl*		6	*adj*
1	*adgij*		13	*adj*		1	*h*		2	*d*
1	*ai*		1	*adgij*		6	n.a.		1	*dj*
8	*cl*		3	*d*					1	*dgj*
3	*dj*		9	*dj*	**II**	5	*a*		4	n.a.
1	*gi*		1	*dgj*		3	*ad*			
1	*h*		2	n.a.		6	*adj*	**ML2**	3	*a*
22	n.a.					1	*adgj*		2	*ad*
		A2	1	*a*		2	*d*		6	*adj*
Bb1	3	*a*		1	*cl*		5	*dj*		1	*adgj*
	2	*aclh*		1	*dj*		1	*dgj*		1	*d*
	1	*ah*		1	*h*		1	n.a.		6	*dj*
	2	*cl*		1	n.a.					3	n.a.
	1	*g*				**IIIa**	1	*a*			
	1	*gi*	**A3**	1	*a*		2	*dj*	**ML3**	1	*a*
	1	*h*		3	n.a.		7	n.a.		2	*ad*
	4	n.a.								2	*cl*
			A4	1	*a*	**IIIb**	5	*a*		1	*dj*
Bb2	3	*a*		1	*adj*		3	*ad*			
	1	*ad*		4	n.a.		7	*adj*	**ML4**	4	*a*
	4	*adj*					3	*dj*		1	*achl*
	1	*adgj*	**B**	1	*a h*		1	n.a.		1	*adj*
	1	*d*		6	*c l*					2	*cl*
	4	*dj*		3	n.a.	**IV**	7	*cl*		2	*dj*
	2	*cl*					1	*gi*		3	n.a.
	2	n.a.	**C**	5	n.a.		1	n.a.			
									ML5	3	*cl*
Bc	2	*a*	**D1**	4	*cl*	**V**	1	*a*		1	*gi*
	3	*ad*		1	*gi*		5	n.a.		1	*h*
	6	*adj*		1	n.a.					4	n.a.
	1	*d*				**VIa**	3	*a*			
	1	*dgj*	**D2**	1	*a*		1	*adgij*	**ML6**	1	*ag*
	2	*dj*		2	*dj*		1	*ag*		1	*agi*
	4	n.a.		13	n.a.		1	*ah*		5	n.a.
							1	*cl*			
			D3	1	*gi*		2	*dj*	**ML7**	1	*a*
				3	n.a.		1	*gi*		3	*cl*
							1	*i*		1	*h*
										4	n.a.
						VIb	1	*dj*			
							7	n.a.			

the combined use of molecular techniques, biochemical profiles and enterotoxin gene patterns can lead to a more effective strain differentiation than using the individual methods alone.

References

Aires-de-Sousa, M., Parente, C.E.S.R., Vieira-da-Motta, O., Bonna, I.C.F., Silva, D.A., de Lencastre, H., 2007. Characterization of *Staphylococcus aureus* isolated from buffalo, bovine, ovine and caprine milk samples collected in Rio de Janeiro state, Brazil. Appl. Environ. Microbiol. 73, 3845–3849.

Andrighetto, C., Knijff, E., Lombardi, A., Torriani, S., Vancanneyt, M., Kesters, K., Swings, J., Dellaglio, F., 2001. Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. J. Dairy Res. 68, 303–316.

Araki, M., Kariyama, R., Monden, K., Tsugawa, M., Kumon, H., 2002. Molecular epidemiological studies of *Staphylococcus aureus* in urinary tract infections. J. Infect. Chemother. 8, 168–174.

Cremonesi, P., Luzzana, M., Brasca, M., Morandi, S., Lodi, R., Vimercati, C., Agnellini, D., Caramenti, G., Moroni, P., Castiglioni, B., 2005. Development of a multiplex PCR assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. Mol. Cell. Probe. 19, 299–305.

Cremonesi, P., Castiglioni, B., Malferrari, G., Biunno, I., Vimercati, C., Moroni, P., Morandi, S., Luzzana, M., 2006. Improved method for rapid DNA extraction of mastitis pathogens directly from milk. J. Dairy Sci. 89, 163–169.

Di Cagno, R., De Angelis, M., Gallo, G., Settanni, L., Berloco, M.G., Siragus, S., Parente, E., Corsetti, A., Gobbetti, M., 2007. Genotypic and phenotypic diversity of *Lactobacillus rossiae* strains isolated from sourdough. J. Appl. Microbiol. 103, 821–825.

Gilbert, F.B., Fromageau, A., Gelineau, L., Poutrel, B., 2006. Differentiation of bovine *Staphylococcus aureus* isolates by use of polymorphic tandem repeat typing. Vet. Microbiol. 117, 297–303.

Hennekinne, J.A., Kerouanton, A., Brisabois, A., De Buyser, M.L., 2003. Discrimination of *Staphylococcus aureus* biotypes by pulsed-field gel electrophoresis of DNA macro-restriction fragments. J. Appl. Microbiol. 94, 321–329.

Hookey, J.V., Richardson, J.F., Cookson, B.D., 1998. Molecular typing of *Staphylococcus aureus* based on PCR restriction fragments length polymorphism and DNA sequence analysis of the coagulase gene. J. Clin. Microbiol. 36, 1083–1089.

Hunter, P.R., 1990. Reproducibility and indices of discriminatory power of microbial typing methods. J. Clin. Microbiol. 28, 1903–1905.

Jørgensen, H.J., Mørk, T., Rørvik, L.M., 2005. The occurrence of *Staphylococcus aureus* on a farm with a small-scale production of raw milk cheese. J. Dairy Sci. 88, 3810–3817.

Malachowa, N., Sabat, A., Gniadkowski, M., Kryszton-Russjan, J., Empel, J., Miedzobrodzki, J., Kosowska-Shick, K., Appelbaum, P.C., Hryniewicz, W., 2005. Comparison of multiple-locus variable-number tandem-repeat analysis with pulse-filed gel electrophoresis, *spa* typing, and multilocus sequence typing for clonal characterization of *Staphylococcus aureus* isolates. J. Clin. Microbiol. 43, 3095–3100.

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27, 209–220.

Naffa, R.G., Bdour, S.M., Migdadi, H.M., Shehabi, A.A., 2006. Enterotoxicity and genetic variation among clinical *Staphylococcus aureus* isolates in Jordan. J. Med. Microbiol. 55, 183–187.

Neela, V., Mariana, N.S., Radu, S., Zamberi, S., Raha, A.R., Rosli, R., 2005. Use of RAPD to investigate the epidemiology of *Staphylococcus aureus* infection in Malaysian hospitals. World J. Microbiol. Biotechnol. 21, 245–251.

Pereira, M.S.V., Leal, N.C., Leal, T.C.A., Sobreira, M., de Almeida, A.M.P., Siqueira-Junior, J.P., Campos-Takaki, G.M., 2002. Typing of human and bovine *Staphylococcus aureus* by RAPD-PCR and ribotyping-PCR. Lett. Appl. Microbiol. 35, 32–36.

Prevost, G., Pottecher, B., Dahlet, M., Bientz, M., Mantz, J.M., Pimont, Y., 1991. Pulsed-field gel electrophoresis as new epidemiologic tool for monitoring methicillin-resistant *Staphylococcus aureus* in an intensive care unit. J. Hosp. Inf. 17, 255–269.

Roach, J.C.M., Levett, P.N., Lavoie, M.C., 2006. Identification of *Streptococcus iniae* by commercial bacterial identification systems. J. Microbiol. Meth. 67, 20–26.

Rodríguez-Calleja, J.M., García-López, I., Santos, J.A., Otero, A., García-López, M.L., 2006. Molecular and phenotypic typing of *Staphylococcus aureus* isolates from rabbit meat. Res. Microbiol. 157, 496–502.

Sabat, A., Krzyszton-Russjan, J., Strzalka, W., Filipek, R., Kosowska, K., Hryniewicz, W., Travis, J., Potempa, J., 2003. New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates. J. Clin. Microbiol. 41, 1801–1804.

Scherrer, D., Corti, S., Muehlherr, J.E., Zweifel, C., Stephan, R., 2004. Phenotypic and genotypic characteristics of *Staphylococcus aureus* isolates from raw bulk-tank milk samples of goats and sheep. Vet. Microbiol. 101, 101–107.

Tenover, F.C., Vaughn, R.R., McDougal, L.K., Fosheim, G.E., McGowan, J.E., 2007. Multiple-locus variable-number tandem-repeat assay analysis of methicillin-resistant *Staphylococcus aureus* strains. J. Clin. Microbiol. 45, 2215–2219.

Vautor, E., Jay, C., Chevalier, N., Visomblin, N., Vernet, G., Pepin, M., 2005. Characterization of 26 isolates of *Staphylococcus aureus*, predominantly from dairy sheep, using four different techniques of molecular epidemiology. J. Vet. Diagn. Invest. 17, 363–368.