Available online at www.sciencedirect.com
ScienceDirect
International Journal of Food Microbiology 118 (2007) 186–193
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Characterization of *Staphylococcus aureus* strains isolated from bovine milk in Hungary

F. Peles [a,*], M. Wagner [b], L. Varga [c], I. Hein [b], P. Rieck [b], K. Gutser [b], P. Keresztúri [a], G. Kardos [d,e], I. Turcsányi [d], B. Béri [f], A. Szabó [a]

[a] *Department of Food Processing, Quality Control, and Microbiology, Center of Agricultural Sciences, University of Debrecen, Böszörményi út 138, 4032 Debrecen, Hungary*
[b] *Institute of Milk Hygiene, Milk Technology, and Food Science, Department of Veterinary Public Health and Food Science, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria*
[c] *Department of Dairy Science, Institute of Food Science, Faculty of Agricultural and Food Sciences, University of West Hungary, Lucsony u. 15-17., 9200 Mosonmagyaróvár, Hungary*
[d] *Department of Microbiology at Debrecen, Veterinary Diagnostic Directorate, Central Agricultural Office, Bornemissza u. 3-5, 4031 Debrecen, Hungary*
[e] *Department of Medical Microbiology, Medical and Health Science Center, University of Debrecen, Nagyerdei krt. 98, 4032 Debrecen, Hungary*
[f] *Department of Animal Husbandry, Center of Agricultural Sciences, University of Debrecen, Böszörményi út 138, 4032 Debrecen, Hungary*

Received 6 March 2007; received in revised form 29 June 2007; accepted 10 July 2007

Abstract

Staphylococcus aureus is a major foodborne pathogen due to its capability to produce a wide range of heat-stable enterotoxins. The primary purpose of this research was to characterize *S. aureus* isolates recovered from mammary quarter milk of mastitic cows and from bulk tank milk produced on Hungarian dairy farms of different sizes. Macrorestriction analysis of chromosomal DNA from *S. aureus* isolates was performed using the restriction enzyme *Sma*I followed by pulsed-field gel electrophoresis (PFGE). The prevalence rates of nine *S. aureus* enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*) and of the toxic shock syndrome toxin 1 gene (*tst*) were determined by multiplex polymerase chain reaction (PCR). The bulk tank milks of 14 out of 20 farms were contaminated with *S. aureus* at levels of up to 6.0×10^3 CFU/ml. Farm size had no significant effect ($P > 0.05$) on the *S. aureus* counts in bulk milk. The prevalence rates of penicillin resistance were 88.9% and 20.0% among the *S. aureus* recovered from mastitic quarter milk and bulk tank milk, respectively. After phenotypic characterization, a total of 59 *S. aureus* isolates were selected for genotyping. PFGE analysis revealed 22 distinct pulsotypes, including 14 main types and 8 subtypes, at a similarity level of 86%. Only one or two main types were observed on each of the farms tested, indicating a lack of genetic diversity among *S. aureus* isolates within farms, and there were only two pulsotypes which occurred on more than one farm. The PFGE patterns showed genetic relatedness between the *S. aureus* strains recovered from quarter milk and bulk milk on two large farms, implying that on farms having a high number of mastitic cows, *S. aureus* from infected udders may contaminate bulk milk and, subsequently, raw milk products. Sixteen (27.1%) of the *S. aureus* isolates tested by multiplex PCR were found to be positive for enterotoxin genes, with 15 of them carrying just one gene and one strain carrying two genes (*seg* and *sei*). The most commonly detected toxin genes were *seb*, *sea*, and *sec*, whereas none of our isolates possessed the *see*, *seh*, *sej*, or *tst* genes. On 75% of the dairy farms surveyed, no enterotoxigenic staphylococci were recovered from either mastitic quarter milk or bulk tank milk.
© 2007 Elsevier B.V. All rights reserved.

Keywords: *Staphylococcus aureus*; Milk; Enterotoxin gene; PFGE; PCR; Pulsotype

1. Introduction

Staphylococcus aureus is the most prevalent and economically significant pathogen causing intramammary infections in dairy ruminants (Akineden et al., 2001; Cabral et al., 2004; Katsuda et al., 2005). The organism is responsible for approximately 30% to 40% of all mastitis cases (Asperger and Zangerl, 2003). *S. aureus* can gain access to milk either by direct excretion from udders with clinical or subclinical staphylococcal mastitis or by contamination from the environment during handling and processing of raw milk (Scherrer et al., 2004;

* Corresponding author. Tel.: +36 52 508 444x88111; fax: +36 52 413 385.
E-mail address: fpeles@freemail.hu (F. Peles).

0168-1605/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2007.07.010

Jørgensen et al., 2005c). When the udder is infected, *S. aureus* is excreted in the milk with large fluctuations in counts ranging from zero to 10^8 CFU/ml (Asperger and Zangerl, 2003).

In the European Union, criteria have been established for the *S. aureus* content in raw bovine milk intended for processing without prior heat treatment. The *m* value, which separates acceptable from marginally acceptable quality, is 5.0×10^2 CFU/ml or 2.70 log_{10} CFU/ml; and the *M* value separating marginally acceptable from defective quality is 2.0×10^3 CFU/ml or 3.30 log_{10} CFU/ml (Council of the European Communities, 1992).

Foodborne diseases have a major public health impact. It is estimated that in the United States alone foodborne illnesses affect 6 million to 80 million people each year, causing up to 9000 deaths, and cost about 5 billion US dollars (Balaban and Rasooly, 2000). *S. aureus* is considered the third most important cause of disease in the world among the reported foodborne illnesses (Asperger and Zangerl, 2003; Normanno et al., 2005; Boerema et al., 2006). The growth of *S. aureus* in foods presents a potential public health hazard because many strains of *S. aureus* produce enterotoxins (SEs) that cause food poisoning if ingested (Akineden et al., 2001; Cenci-Goga et al., 2003; Boerema et al., 2006). Milk and dairy foods have frequently been implicated in staphylococcal food poisoning, and contaminated raw milk is often involved (De Buyser et al., 2001). SEs are a family of exoproteins forming a single chain with a molecular weight ranging from 26,000 to 29,600 Da (Balaban and Rasooly, 2000; Asperger and Zangerl, 2003; Normanno et al., 2005). Unlike the producer organism, SEs are remarkably heat resistant, showing *D*-values of 3 min to 8 min at 121 °C (Asperger and Zangerl, 2003). As a result, they may be present in foods even when viable *S. aureus* are absent (Jørgensen et al., 2005c). Traditionally, five classical antigenic SE types (SEA, SEB, SEC, SED, and SEE) were recognized. However, in recent years, the existence of new types of SEs, including enterotoxin-like (SEl) toxins (SEG, SEH, SEI, SElJ, SElK, SElL, SElM, SElN, SElO, SElP, SElQ, SElR, SElU, SElU2, and SElV) has been reported and their genes described (Letertre et al., 2003; Lina et al., 2004; Omoe et al., 2004; Jørgensen et al., 2005a; Bania et al., 2006; Boerema et al., 2006; Hata et al., 2006; Thomas et al., 2006). A distantly related protein, toxic shock syndrome toxin 1 (TSST-1), also produced by *S. aureus*, was the first toxin shown to be involved in the toxic shock syndrome of humans and animals (Akineden et al., 2001).

Over the last decade, various typing methods have been developed for the characterization of *S. aureus* isolates (Hata et al., 2006). Phenotyping methods have gradually been supplemented or replaced with genotyping methods. Numerous techniques have been described for *S. aureus* genotyping, of which pulsed-field gel electrophoresis (PFGE) is considered to be the "gold standard" because of its discriminatory power and reproducibility (Weller, 2000). The polymerase chain reaction (PCR) has been introduced as a simple technique for the detection of enterotoxigenic strains (Asperger and Zangerl, 2003; Kwon et al., 2004). Although the PCR-based approach is specific, highly sensitive, and rapid, it can only demonstrate the presence of enterotoxin genes in *S. aureus* isolates rather than the production of the SE protein (Boerema et al., 2006).

The main objectives of this study were to enumerate *S. aureus* in milk from dairy farms of different sizes and to characterize both pheno- and genotypically the *S. aureus* strains isolated from the mammary quarter milk of mastitic cows and from bulk tank milk. Genotyping was performed by PFGE analysis, and the prevalence of genes encoding various enterotoxins was determined by multiplex PCR.

2. Materials and methods

2.1. Farms

Twenty farms (7 large, 4 medium-size, and 9 small farms designated as LF1 to LF7, MF8 to MF11, and SF12 to SF20, respectively) were enrolled in the study carried out from June 2005 through August 2006. The farms were located in the eastern part of Hungary, at a distance of 15 km to 100 km from one another. Herd size varied from 4 cows to 520 cows of Holstein Friesian and/or Hungarian Red Pied breeds. The *S. aureus* counts in bulk tank milk were determined four times on each farm throughout the duration of the study. The isolates used in the molecular epidemiologic investigations were arbitrarily selected from the sampling session performed in January 2006.

2.2. Isolation and enumeration of S. aureus

From each farm at each sampling time, 50-ml samples were collected, cooled to 4 °C, and then tested for the presence of *S. aureus*. Udder quarter milk samples were taken from mastitic cows and were plated on Columbia Blood Agar (Oxoid, Basingstoke, UK) and incubated aerobically at 37 °C for 24 h. The isolation of *S. aureus* from bulk tank milk was performed according to the EN ISO 6888-1 standard procedure of the International Organization for Standardization (ISO, 1999) using Baird Parker Agar supplemented with Egg Yolk Tellurite Emulsion (Oxoid). The plates were incubated under aerobic conditions at 37 °C for 24 h to 48 h. If present, 5 egg yolk reaction-positive and 5 egg yolk reaction-negative colonies were chosen from each sample for further identification. Samples were also collected with sterile cotton swabs from the environment (e.g., teat cup liner, milk tank, exit pipe of the milk tank) of the dairy farms which had problems with elevated *S. aureus* counts. The samples were examined as described above. Randomly chosen suspect colonies were picked from each plate for further phenotypic identification.

All suspect colonies were grown aerobically in Brain Heart Infusion Broth (Oxoid) at 37 °C for 18 h to 24 h and were then also spread-plated on a second culture medium, i.e., Egg Yolk Tellurite Emulsion-supplemented Baird Parker Agar or Columbia Blood Agar for quarter milk samples or bulk tank milk (and environmental swab) samples, respectively. The isolates were identified as *S. aureus* on the basis of their colony morphology, Gram-staining, catalase reaction, tellurite reduction, lecithinase activity, hemolytic properties and by their ability to coagulate rabbit plasma (tube coagulase test) and to produce clumping factor (Staphylase test; Oxoid).

Group means of data were compared to determine significant differences between the *S. aureus* counts of milk samples collected from different size farms. The number of CFU/ml was converted to $\log_{10}$ values, and data were analyzed with the Student's *t*-test by means of the STATISTICA data analysis software system, version 7.1 (StatSoft, Tulsa, OK). Significance was evaluated at the $P=0.05$ level. The results were compared to the microbiological criteria laid down in the EU Milk Hygiene Directive 92/46 (Council of the European Communities, 1992).

2.3. Antibiotic susceptibility testing

Antimicrobial drug susceptibility testing of the isolates was performed on Mueller–Hinton agar (Oxoid) by the disk diffusion method in accordance with Clinical Laboratory Standards Institute guidelines (CLSI, 2006). The antimicrobial agents tested included penicillin (10 U/disk), methicillin (5 μg/disk), cefoxitin (30 μg/disk), lincomycin (15 μg/disk), tetracycline (30 μg/disk), erythromycin (15 μg/disk), and sulfamethoxazole/trimethoprim (23.75/1.25 μg/disk). *S. aureus* ATCC 25923 was the control strain in every test run.

2.4. Macrorestriction analysis by PFGE

Macrorestriction analysis of chromosomal DNA from *S. aureus* isolates was performed using the restriction enzyme *Sma*I (New England BioLabs, Beverly, MA) followed by PFGE. *S. aureus* isolates were checked for purity and grown aerobically in brain heart infusion broth at 37 °C for 18 h to 24 h. The cells were harvested and resuspended in Pett IV (PIV) buffer (1 M NaCl, 25 mM Tris–HCl, pH 8.0). The suspension was mixed with equal volume of 1.2% low melting point SeaKem Gold agarose (Cambrex Bio Science, Rockland, ME). The plugs were incubated overnight at 37 °C in EC lysis buffer (6 mM Tris–HCl, 1 M NaCl, 0.1 M EDTA, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine) with 10 mg/ml RNAse, 10 mg/ml lysozyme and 5 mg/ml lysostaphin. The lysis buffer was then removed and each plug was incubated overnight at 50 °C in 1 ml of ESP buffer (0.5 M EDTA, 1% lauryl sarcosine, 1 mg/ml proteinase K). On the following day, the plugs were washed four times in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) at 54 °C for 30 min to 60 min. The plugs were then stored in TE buffer at 4 °C.

Plugs were digested with 40 U *Sma*I (New England BioLabs) according to the instructions of the manufacturer. Digested DNA was separated in a 1% SeaKem Gold agarose gel (Cambrex Bio Science) with a CHEF DR III (Bio-Rad Laboratories, Hercules, CA) pulsed-field electrophoresis system in 0.5 × Tris–borate–EDTA (1 M Tris, 0.01 M EDTA, 1 M boric acid). Running parameters were as follows: 5 s to 15 s ramping for 7 h followed by a 15 s to 60 s ramping for 19 h; 6 V/cm; 120° angle; 14 °C. Gels were stained with ethidium bromide (0.5 μg/ml) for 30 min and destained for 30 min to 60 min in fresh distilled water. The patterns were visualized using a UV transilluminator and then photographed. *Salmonella enterica* subsp. *enterica* serotype Braenderup H9812 digested with *Xba*I was used as a molecular size marker.

DNA restriction bands were analyzed by using the Molecular Analyst Fingerprinting II software package, version 3.0 (Bio-Rad). Similarity coefficients were calculated and dendrograms were constructed using the Dice coefficient and the unweighted pair group method with arithmetic averages, respectively, with an optimization value of 0.5% and a position tolerance of 1%. The cluster cutoff value was set at 86% similarity. Isolates with indistinguishable banding patterns (i.e., 100% similarity) were assigned to the same pulsotype and those with similarities ranging from 86% to 99% were designated as subtypes. Main types were designated by capital letters and subtypes by capital letters followed by Arabic numerals.

Table 1
Oligonucleotide primers for amplification of genes encoding staphylococcal enterotoxins

Gene	Primer	Primer sequence (5′ to 3′)	Amplification 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	GSEER-1	AGG TTT TTT CAC AGG TCA TCC	209	Mehrotra et al. (2000)
	GSEER-2	CTT TTT TTT CTT CGG TCA ATC		
seg	SEG-1	TGC TAT CGA CAC ACT ACA ACC	704	McLauchlin et al. (2000)
	SEG-2	CCA GAT TCA AAT GCA GAA CC		
seh	SEH-1	CGA AAG CAG AAG ATT TAC ACG	495	McLauchlin et al. (2000)
	SEH-2	GAC CTT TAC TTA TTT CGC TGT C		
sei	SEI-1	GAC AAC AAA ACT GTC GAA ACT G	630	McLauchlin et al. (2000)
	SEI-2	CCA TAT TCT TTG CCT TTA CCA G		
sej	SEJ-1	CAT CAG AAC TGT TGT TCC GCT AG	142	Monday and Bohach (1999)
	SEJ-2	CTG AAT TTT ACC ATC AAA GGT AC		
tst	GTSSTR-1	ACC CCT GTT CCC TTA TCA TC	326	Mehrotra et al. (2000)
	GTSSTR-2	TTT TCA GTA TTT GTA ACG CC		

2.5. PCR amplification of genes encoding staphylococcal enterotoxins

The sequences of the oligonucleotide primers used for the specific amplification of nine *S. aureus* enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*) and the TSST-1 gene

(*tst*) are summarized in Table 1, along with the predicted PCR product sizes. DNA was amplified by 30 cycles of 95 °C for 60 s, 55 °C for 60 s and 72 °C for 60 s with a final extension at 72 °C for 10 min. The amplification was performed in a GeneAmp PCR System 9700 (Perkin-Elmer, Wellesley, MA) using Platinum *Taq* DNA polymerase (Invitrogen, Lofer, Austria). PCR products were resolved by agarose gel electrophoresis and visualized by UV transillumination.

3. Results and discussion

3.1. Isolation and enumeration of S. aureus

The bulk tank milks of 14 out of 20 farms were contaminated with *S. aureus* at levels of up to 6.0×10^3 CFU/ml, which was in accordance with a previous report by Stephan, Buehler & Lutz (2002), who determined that the *S. aureus* counts ranged from 1.0×10^1 CFU/ml to 3.0×10^3 CFU/ml in bulk tank milk samples in Switzerland. No staphylococci were recovered from bulk milks collected from farms LF2, LF7, MF10, MF11, SF19, and SF20. The mean *S. aureus* counts were high (>3.30 $\log_{10}$ CFU/ml or 2.0×10^3 CFU/ml) on SF15, medium (2.70 to 3.30 $\log_{10}$ CFU/ml or 5.0×10^2 to 2.0×10^3 CFU/ml) on LF3, SF12 and SF13, and low (<2.70 $\log_{10}$ CFU/ml or 5.0×10^2 CFU/ml) on the rest of the farms tested. In view of the microbiological criteria laid down in the EU Milk Hygiene Directive 92/46 (Council of the European Communities, 1992), the group (i.e., LF, MF, and SF) means of *S. aureus* counts in bulk tank milks, which ranged from 0.90 $\log_{10}$ CFU/ml to 1.74 $\log_{10}$ CFU/ml, were all below the *m* value; and no significant differences ($P > 0.05$) were observed between them (data not shown).

It is worth noting that the combined incidence rates of suspected subclinical infections and clinical mastitis cases ranged from 0% to approximately 10% on the large farms surveyed. On the small and medium-size farms involved in our study, the incidence rates of clinical or subclinical mastitis have not been recorded.

After phenotypic characterization, a total of 59 *S. aureus* isolates were selected for genotyping (Table 2). Nine isolates were from udder quarter milk and 50 isolates were from bulk tank milk. No staphylococci were recovered from the environmental swab samples.

3.2. Antibiotic susceptibility testing

All 59 *S. aureus* isolates were uniformly susceptible to methicillin, cefoxitin, lincomycin, tetracycline, erythromycin, and sulfamethoxazole/trimethoprim. Forty-one isolates were also susceptible to penicillin, whereas 18 isolates (30.5%) were

Table 2
Characterization of 59 *Staphylococcus aureus* isolates recovered from milk samples

Farm [a]	PFGE pattern	Total no. of isolates	Identification no.	Origin [b]	Phenotypic properties						Enterotoxin gene
					Tellurite reduction	Lecithinase activity	Hemolysis	Clumping factor	Coagulase test	Antibiogram pattern [c]	
LF1	A	2	9, 11	BTM	Gray	Strong	α–β	Positive	Positive	S	None
	B	2	10, 12	BTM	Black	Weak	α–β	Positive	Positive	S	*sed*
LF3	C	4	17, 18, 19, 20	BTM	Black	Strong	Weak	Positive	Positive	S	*sea*
LF4	D	5	34, 35, 37, 38, 43	BTM, UQM	Black	None	α	Positive	Positive	R (Pen)	None
	D1	2	41, 47	UQM	Black	None	α	Positive	Positive	R (Pen)	None
	E	1	48	UQM	Gray	None	α–β	Positive	Positive	S	None
LF5	D	1	30	UQM	Black	Weak	α	Positive	Positive	R (Pen)	None
	F	3	21, 26, 28	BTM, UQM	Black	Strong	Weak	Positive	Positive	R (Pen)	*seg*/*sei* (1), None (2)
	F1	2	22, 23	BTM	Gray	Strong	Weak	Positive	Positive	R (Pen)	*seb*
LF6	G	3	31, 32, 33	BTM	Gray	Strong	β	Positive	Positive	S	None
MF8	H	3	6, 7, 8	BTM	Gray	Strong	α–β	Positive	Positive	S	None
MF9	C1	3	13, 14, 15	BTM	Black	Strong	α–β (2), weak (1)	Positive	Positive	S	*sec*
	I	1	16	BTM	Gray	Strong	Weak	Positive	Positive	R (Pen)	*sec*
SF12	A2	1	65	BTM	Black	Strong	α–β	Positive	Positive	R (Pen)	None
	J	2	63, 64	BTM	Black	None	α–β	Positive	Positive	S	None
SF13	G1	3	66, 67, 68	BTM	Black	Strong	α–β	Positive	Positive	S	None
SF14	K	3	69, 70, 71	BTM	Gray	Strong	α–β	Positive	Positive	R (Pen)	*seb*
SF15	L	3	49, 50, 73	BTM	Gray	Strong	α–β	Positive	Positive	S	None
	M	1	74	BTM	Black	None	α–β	Positive	Positive	S	None
SF16	A1	4	52, 53, 75, 76	BTM	Gray	Weak	Weak	Positive	Positive	S	None
SF17	J1	3	55, 57, 78	BTM	Black	None	α–β	Positive	Positive	S	None
	N	1	79	BTM	Gray	Strong	α–β	Positive	Positive	S	None
SF18	L1	2	60, 83	BTM	Gray	Strong	Weak	Positive	Positive	S	None
	N	4	58, 59, 81, 82	BTM	Gray	Strong	α–β	Positive	Positive	S	None

[a] LF: large farm, MF: medium-size farm, SF: small farm.
[b] UQM: udder quarter milk, BTM: bulk tank milk.
[c] S: susceptible to penicillin, methicillin, cefoxitin, lincomycin, tetracycline, erythromycin, and sulfamethoxazole/trimethoprim; R (Pen): resistant to penicillin, whereas susceptible to all other antimicrobial drugs tested.

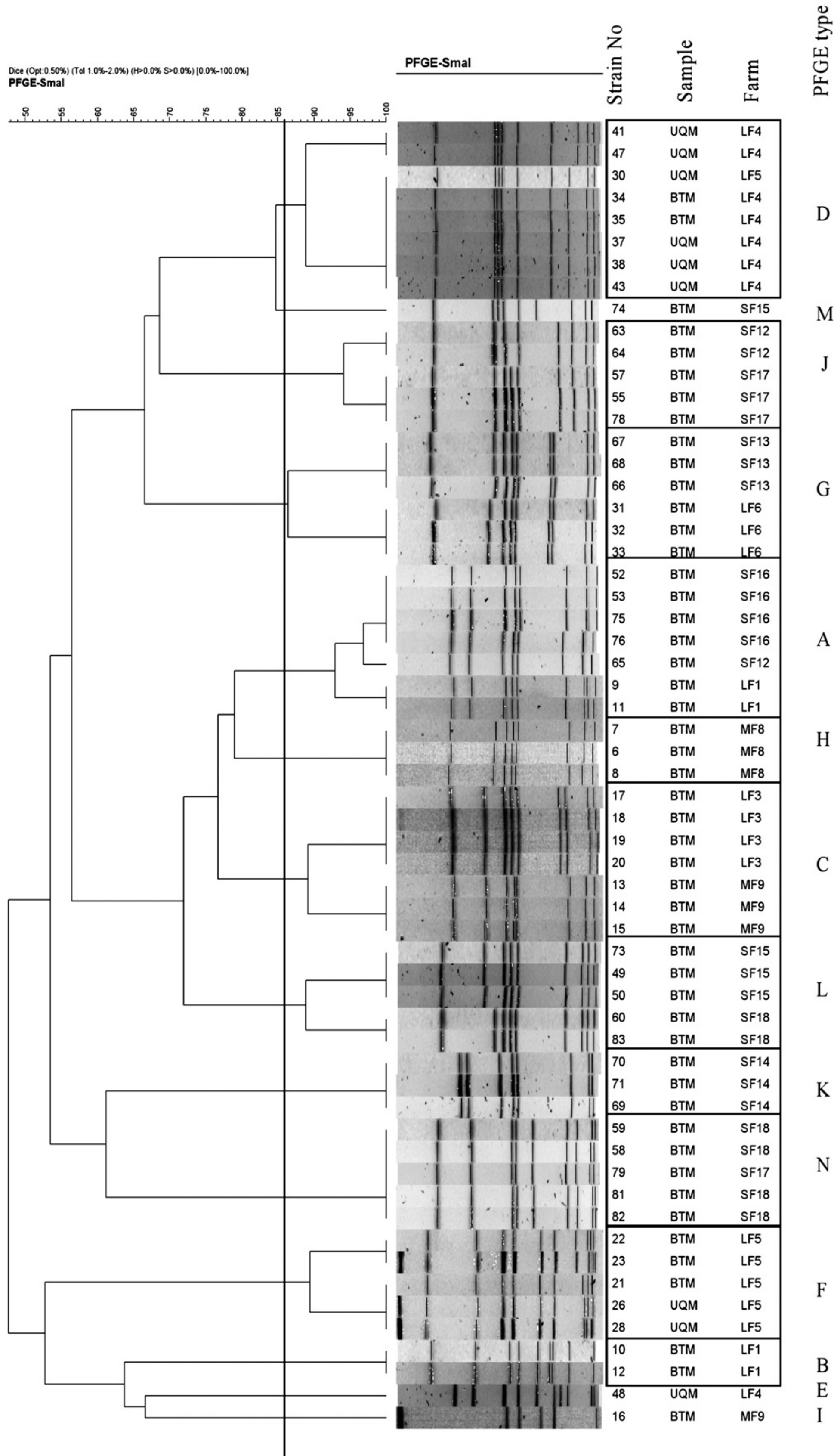

Fig. 1. Dendrogram of PFGE patterns showing the relatedness of 59 *Staphylococcus aureus* strains examined in this study. The cluster cutoff was set at 86% similarity. Columns to the right of the dendrogram show the identification number of strains, the origin of samples (UQM: udder quarter milk, BTM: bulk tank milk), the code of farms (LF: large farm, MF: medium-size farm, SF: small farm), and the PFGE types determined.

resistant to it (Table 2). The prevalence rates of penicillin resistance were 88.9% (i.e., 8 out of 9 isolates) and 20.0% (i.e., 10 out of 50 isolates) among the *S. aureus* recovered from mastitic quarter milk and bulk tank milk, respectively.

S. aureus has developed penicillin resistance and multidrug resistance worldwide, although reported prevalence rates indicate that wide variations exist regionally (De Oliveira et al., 2000; Waage et al., 2002; Normanno et al., 2007). Depending on the origin of the sample, the prevalence of penicillin resistant *S. aureus* strains ranges from less than 10% to more than 50% (Stephan et al., 2001; Pitkälä et al., 2004; Jørgensen et al., 2005a,c; Anderson et al., 2006). In Hungary, the β-lactams have long been one of the most widely used antimicrobial agents for treatment of bovine mastitis. As a result, penicillin resistance is a frequent occurrence among *S. aureus* strains. In a study conducted by Kaszanyitzky-Juhász (2003), 96%, 55%, and 45% of the *S. aureus* isolates recovered from humans, bovine mastitis, and foods, respectively, tested positive for β-lactamase. These data underline the need for a policy on the judicious use of antimicrobials in Hungarian dairy production.

3.3. Macrorestriction analysis by PFGE

PFGE analysis showed 22 distinct pulsotypes, including 14 main types and 8 subtypes, at a similarity level of 86% (Fig. 1 and Table 2). The *S. aureus* strains originating from quarter milk belonged to 3 main types (D, E, and F) and 1 subtype (D1). Isolates from bulk tank milk were divided into 13 main types (A to D and F to N) and 7 subtypes (A1, A2, C1, F1, G1, J1, and L1). On each of the farms tested, only one or two main types were observed, indicating a lack of genetic diversity among *S. aureus* isolates within farms. There were only two pulsotypes (D and N) which occurred on more than one farm. The PFGE patterns revealed genetic relationship between the strains recovered from udder quarter milk and bulk tank milk on both farms (i.e., LF4 and LF5) where *S. aureus* isolates of quarter milk origin were available. A plausible explanation for this finding is that on farms having a high number of cows with subclinical or clinical mastitis, as was the case on LF4 and LF5, *S. aureus* from infected udders may contaminate bulk milk and, as a result, raw milk products (Stephan et al., 2002; Asperger and Zangerl, 2003; Jørgensen et al., 2005a). The results in Table 2 also indicate that the penicillin resistant isolates belonged to 7 pulsotypes, including main types D, F, I, and K and subtypes A2, D1, and F1.

3.4. PCR amplification of genes encoding staphylococcal enterotoxins

As shown in Table 2, 16 (27.1%) of the 59 *S. aureus* isolates tested by multiplex PCR were positive for SE genes. Fifteen of them carried just one gene and one strain carried two genes (*seg* and *sei*). These occurrence rates are considerably lower than those obtained by other authors (Stephan et al., 2002; Scherrer et al., 2004; Jørgensen et al., 2005a,b; Katsuda et al., 2005; Boerema et al., 2006; Hata et al., 2006; Srinivasan et al., 2006), who reported that 52.5% to 93.6% of the *S. aureus* strains isolated from mastitic or bulk milk samples harbored at least one of the enterotoxin genes studied. Both the *sea* and *sec* genes were present in four isolates (on LF3 and MF9, respectively), the *seb* gene in five (on LF5 and SF14), the *sed* gene in two (on LF1), and the *seg*/*sei* genes in one isolate (on LF5). The fact that *seb* was the most commonly detected SE genotype in our study is in agreement with the findings of Boerema et al. (2006), who determined the enterotoxigenic status and molecular genotype of 90 *S. aureus* isolates. The only strain (no. 26) that harbored newly described SE genes (*seg* and *sei*) originated from mastitic quarter milk, whereas classical SE genes (*sea* to *sed*) were detected solely in strains isolated from bulk tank milk (Fig. 1 and Table 2). The majority of *seg* and *sei* distribution studies show that these genes are always detected together in *S. aureus* because they coexist on a common genetic element, a so-called enterotoxin gene cluster (Jarraud et al., 2001; Rosec and Gigaud, 2002; Stephan et al., 2002; Scherrer et al., 2004; Katsuda et al., 2005; Bania et al., 2006; Boerema et al., 2006; Hata et al., 2006; Kérouanton et al., 2007). It should also be noted that none of our isolates possessed the *see*, *seh*, *sej*, or *tst* genes, and on 15 out of 20 farms tested, no enterotoxigenic staphylococci were recovered from either mastitic quarter milk or bulk tank milk (Table 2).

4. Conclusions

S. aureus, the most prevalent pathogen causing mastitis in dairy cows, is regularly found in bulk tank milk because the principal source of microbial contamination of raw milk is the infected udder. For this reason, *S. aureus* counts in bulk milk are related to the mastitis situation of the herd and may range from less than 10 to several thousands CFU/ml. *S. aureus* was recovered from the majority (55%) of bulk milk samples examined in this research. The spread of antibiotic resistant bacteria with milk and dairy foods is another major concern. In the present study, nearly 70% of the *S. aureus* isolates were penicillin sensitive, however, all of the isolates harboring *seb*, the most commonly detected SE genotype, proved to be resistant to penicillin. Moreover, even though staphylococci carrying any of the SE genes tested were recovered from neither mastitic quarter milk nor bulk tank milk on 75% of the dairy farms surveyed, the sporadic presence of potentially SE producing strains in raw milk and raw milk products may pose a health risk to consumers of these commodities. Further research is needed to determine the prevalence of enterotoxin production among *S. aureus* strains in the region studied.

Acknowledgments

The authors gratefully acknowledge the collaboration and cooperation of the 20 farms involved in this study. This research was supported in part by grants from the Austro-Hungarian Action Fund (460-7/2005) and the Institute of Milk Hygiene, Milk Technology, and Food Science of the University of Veterinary Medicine at Vienna.

References

Akineden, Ö., Annemüller, C., Hassan, A.A., Lämmler, C., Wolter, W., Zschöck, M., 2001. Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. Clinical and Diagnostic Laboratory Immunology 8, 959–964.

Anderson, K.L., Lyman, R.L., Bodeis-Jones, S.M., White, D.G., 2006. Genetic diversity and antimicrobial susceptibility profiles among mastitis-causing *Staphylococcus aureus* isolated from bovine milk samples. American Journal of Veterinary Research 67, 1185–1191.

Asperger, H., Zangerl, P., 2003. *Staphylococcus aureus*. In: Roginski, H., Fuquay, J.W., Fox, P.F. (Eds), Encyclopedia of Dairy Sciences, vol. 4. Academic Press and Elsevier Science, Amsterdam, Boston, London, New York, Oxford, Paris, San Diego, San Francisco, Singapore, Sydney, Tokyo, pp. 2563–2569.

Balaban, N., Rasooly, A., 2000. Staphylococcal enterotoxins. International Journal of Food Microbiology 61, 1–10.

Bania, J., Dabrowska, A., Bystron, J., Korzekwa, K., Chrzanowska, J., Molenda, J., 2006. Distribution of newly described enterotoxin-like genes in *Staphylococcus aureus* from food. International Journal of Food Microbiology 108, 36–41.

Boerema, J.A., Clemens, R., Brightwell, G., 2006. Evaluation of molecular methods to determine enterotoxigenic status and molecular genotype of bovine, ovine, human and food isolates of *Staphylococcus aureus*. International Journal of Food Microbiology 107, 192–201.

Cabral, K.G., Lämmler, C., Zschöck, M., Langoni, H., De Sá, M.E.P., Victória, C., Da Silva, A.V., 2004. Pheno and genotyping of *Staphylococcus aureus*, isolated from bovine milk samples from São Paulo State, Brazil. Canadian Journal of Microbiology 50, 901–909.

Cenci-Goga, B.T., Karama, M., Rossitto, P.V., Morgante, R.A., Cullor, J.S., 2003. Enterotoxin production by *Staphylococcus aureus* isolated from mastitic cows. Journal of Food Protection 66, 1693–1696.

Clinical Laboratory Standards Institute (CLSI), 2006. Performance standards for antimicrobial susceptibility testing. 16th informational supplement (M100-S16). CLSI, Wayne, PA.

Council of the European Communities, 1992. Council Directive 92/46/EEC of 16 June 1992 laying down the health rules for the production and placing on the market of raw milk, heat-treated milk and milk-based products. Official Journal of the European Communities. L 268, 1–32.

De Buyser, M.L., Dufour, B., Maire, M., Lafarge, V., 2001. Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. International Journal of Food Microbiology 67, 1–17.

De Oliveira, A.P., Watts, J.L., Salmon, S.A., Aarestrup, F.M., 2000. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and the United States. Journal of Dairy Science 83, 855–862.

Hata, E., Katsuda, K., Kobayashi, H., Ogawa, T., Endo, T., Eguchi, M., 2006. Characteristics and epidemiologic genotyping of *Staphylococcus aureus* isolates from bovine mastitic milk in Hokkaido, Japan. The Journal of Veterinary Medical Science 68, 165–170.

International Organization for Standardization (ISO), 1999. EN ISO 6888-1 Microbiology of food and animal feeding stuffs: horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species). Part 1: Technique using Baird–Parker agar medium. ISO, Geneva.

Jarraud, S., Peyrat, M.A., Lim, A., Tristan, A., Bes, M., Mougel, C., Etienne, J., Vandenesch, F., Bonneville, M., Lina, G., 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. The Journal of Immunology 166, 669–677.

Jørgensen, H.J., Mørk, T., Caugant, D.A., Kearns, A., Rørvik, L.M., 2005a. Genetic variation among *Staphylococcus aureus* strains from Norwegian bulk milk. Applied and Environmental Microbiology 71, 8352–8361.

Jørgensen, H.J., Mørk, T., Høgåsen, H.R., Rørvik, L.M., 2005b. Enterotoxigenic *Staphylococcus aureus* in bulk milk in Norway. Journal of Applied Microbiology 99, 158–166.

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

Kaszanyitzky-Juhász, É., 2003. Az antibiotikum-rezisztencia mechanizmusok ismeretének gyakorlati jelentősége: a *Staphylococcus*-ok rezisztenciája (The Practical Significance of Antibiotic Resistance Mechanisms: Staphylococcal Resistance). PhD Dissertation, Szent István University, Gödöllő-Budapest, Hungary.

Katsuda, K., Hata, E., Kobayashi, H., Kohmoto, M., Kawashima, K., Tsunemitsu, H., Eguchi, M., 2005. Molecular typing of *Staphylococcus aureus* isolated from bovine mastitic milk on the basis of toxin genes and coagulase gene polymorphisms. Veterinary Microbiology 105, 301–305.

Kérouanton, A., Hennekinne, J.A., Letertre, C., Petit, L., Chesneau, O., Brisabois, A., De Buyser, M.L., 2007. Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. International Journal of Food Microbiology 115, 369–375.

Kwon, N.H., Kim, S.H., Park, K.T., Bae, W.K., Kim, J.Y., Lim, J.Y., Ahn, J.S., Lyoo, K.S., Kim, J.M., Jung, W.K., Noh, K.M., Bohach, G.A., Park, Y.H., 2004. Application of extended single-reaction multiplex polymerase chain reaction for toxin typing of *Staphylococcus aureus* isolates in South Korea. International Journal of Food Microbiology 97, 137–145.

Letertre, C., Perelle, S., Dilasser, F., Fach, P., 2003. Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. Journal of Applied Microbiology 95, 38–43.

Lina, G., Bohach, G.A., Nair, S.P., Hiramatsu, K., Jouvin-Marche, E., Mariuzza, R., 2004. Standard nomenclature for the superantigens expressed by *Staphylococcus*. The Journal of Infectious Diseases 189, 2334–2336.

McLauchlin, J., Narayanan, G.L., Mithani, V., O'Neill, G., 2000. The detection of enterotoxins and toxic shock syndrome toxin genes in *Staphylococcus aureus* by polymerase chain reaction. Journal of Food Protection 63, 479–488.

Mehrotra, M., Wang, G., Johnson, W.M., 2000. Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. Journal of Clinical Microbiology 38, 1032–1035.

Monday, S.R., Bohach, G.A., 1999. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. Journal of Clinical Microbiology 37, 3411–3414.

Normanno, G., Firinu, A., Virgilio, S., Mula, G., Dambrosio, A., Poggiu, A., Decastelli, L., Mioni, R., Scuota, S., Bolzoni, G., Di Giannatale, E., Salinetti, A.P., La Salandra, G., Bartoli, M., Zuccon, F., Pirino, T., Sias, S., Parisi, A., Quaglia, N.C., Celano, G.V., 2005. Coagulase-positive staphylococci and *Staphylococcus aureus* in food products marketed in Italy. International Journal of Food Microbiology 98, 73–79.

Normanno, G., La Salandra, G., Dambrosio, A., Quaglia, N.C., Corrente, M., Parisi, A., Santagada, G., Firinu, A., Crisetti, E., Celano, G.V., 2007. Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. International Journal of Food Microbiology 115, 290–296.

Omoe, K., Imanishi, K., Hu, D.L., Kato, H., Takahashi-Omoe, H., Nakane, A., Uchiyama, T., Shinagawa, K., 2004. Biological properties of staphylococcal enterotoxin-like toxin type R. Infection and Immunity 72, 3664–3667.

Pitkälä, A., Haveri, M., Pyörälä, S., Myllys, V., Honkanen-Buzalski, T., 2004. Bovine mastitis in Finland 2001—prevalence, distribution of bacteria, and antimicrobial resistance. Journal of Dairy Science 87, 2433–2441.

Rosec, J.P., Gigaud, O., 2002. Staphylococcal enterotoxin genes of classical and new types detected by PCR in France. International Journal of Food Microbiology 77, 61–70.

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. Veterinary Microbiology 101, 101–107.

Srinivasan, V., Sawant, A.A., Gillespie, B.E., Headrick, S.J., Ceasaris, L., Oliver, S.P., 2006. Prevalence of enterotoxin and toxic shock syndrome toxin genes in *Staphylococcus aureus* isolated from milk of cows with mastitis. Foodborne Pathogens and Disease 3, 274–283.

Stephan, R., Annemüller, C., Hassan, A.A., Lämmler, Ch., 2001. Characterization of enterotoxigenic *Staphylococcus aureus* strains isolated from bovine mastitis in north-east Switzerland. Veterinary Microbiology 78, 373–382.

Stephan, R., Buehler, K., Lutz, C., 2002. Prevalence of genes encoding enterotoxins, exfoliative toxins and toxic shock syndrome toxin 1 in *Staphylococcus aureus* strains isolated from bulk-tank milk samples in Switzerland. Milchwissenschaft 57, 502–504.

Thomas, D.Y., Jarraud, S., Lemercier, B., Cozon, G., Echasserieau, K., Etienne, J., Gougeon, M.L., Lina, G., Vandenesch, F., 2006. Staphylococcal enterotoxin-like toxins U2 and V, two new staphylococcal superantigens arising from recombination within the enterotoxin gene cluster. Infection and Immunity 74, 4724–4734.

Waage, S., Bjorland, J., Caugant, D.A., Oppegaard, H., Tollersrud, T., Mørk, T., Aarestrup, F.M., 2002. Spread of *Staphylococcus aureus* resistant to penicillin and tetracycline within and between dairy herds. Epidemiology and Infection 129, 193–202.

Weller, T.M.A., 2000. Methicillin-resistant *Staphylococcus aureus* typing methods: which should be the international standard? The Journal of Hospital Infection 44, 160–172.