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Pheno- and Genotyping of *Staphylococcus aureus* Isolates of Sheep Origin

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Abstract

The aim of the study was to determine the prevalence of genes encoding virulence factors in *Staphylococcus aureus* strains isolated from raw sheep milk, sheep cheese and Bryndza cheese. Genes encoding staphylococcal enterotoxin (*sea, seb, sec, sed* and *see*), toxic shock syndrome toxin-1 (*tst*), exfoliative toxins (*eta* and *etb*) and collagen-binding protein (*cna*) were detected. In a total of 79 *S. aureus* isolates all assessed toxins encoding genes were found, except for *see, eta* and *etb*. Overall, 75.9% of *S. aureus* isolates were found to be positive for one or more toxin genes. The *sec* gene was found most frequently (24.1%), followed by *tst* (22.8%), *seb* (13.9%), *sed* (10.1%) and *sea* (5.1%). The *cna* gene was detected in 55.7% of *S. aureus* isolates. Based on tandem repeats in *coa* gene, five *coa* types were observed, further divided into 16 subtypes based on their RFLP pattern. Similarly tandem repeats in *spa* gene divided *S. aureus* isolates into 7 types. In the parallel antibiotic resistance study, 69.6% isolates were resistant to at least one of the 11 tested antibiotics. The pheno- and genotyping of *S. aureus* isolates of sheep origin presented in this work update the epidemiological data in Slovakia.

Milk, cheese, sheep-bacteria, toxins, coa and spa genotyping, antimicrobial resistance

Staphylococcus aureus, the most important aetiological agent of contagious bovine and ovine mastitis, has attracted attention by its presence in the infected udder and by environmental contamination of milk during handling and processing (Scherrer et al. 2004; Jorgensen et al. 2005). Epidemiological survey is therefore important to prevent the spread of *S. aureus*.

The virulence of *S. aureus* has been postulated to depend on the expression of a wide range of cell wall-associated and secreted molecules that are believed to promote colonization of host tissues and evasion of the host immune response (Foster and Hook 1998; Dinges et al. 2000). Adherence to host tissue is the first critical step required to initiate infection and is mediated by collagen-binding protein (Cna) and adhesins of the microbial surface components, recognizing adhesive matrix molecules (MSCRAMM) (Foster and Hook 1998). *S. aureus* also produces a wide variety of extracellular toxins of which the most important are staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), and exfoliative toxins (ET). Each of these toxins has a potent effect on cells of the immune system (Dinges et al. 2000). Some SEs are potent emetics (Boerema et al. 2006). TSST-1 is unique in its ability to cross mucosal surfaces and to develop life-threatening toxic shock syndrome (Llewelyn and Cohen 2002; Proft and Fraser 2003).

Because *S. aureus* is a common pathogen for humans and animals, many studies were orientated towards staphylococcal antibiotic resistance (Lyon and Skurray 1987; Werckenthin et al. 2001; Strommenger et al. 2003; Normanno et al. 2007; Wang et al. 2008). In human medicine, antimicrobial multiresistance is frequently encountered and methicillin-resistant *S. aureus* strains (MRSA) belong to the most life threatening nosocomial bacteria. In veterinary medicine, however, MRSA and multiresistant *S. aureus* strains are only reported occasionally (Werckenthin et al. 2001; Normanno et al. 2007).

Little is known about the occurrence of virulence factors in ovine *S. aureus* strains. The

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objective of this study was pheno- and genotyping of ovine *S. aureus* strains isolated from sheep milk, sheep cheese and Bryndza cheese (typical Slovak sheep cheese) samples from Slovakia.

Materials and Methods

Bacterial strains and culture media

During the years 2004 - 2007, raw sheep milk and cheese samples were screened for staphylococcal contamination by classical microbiological examination (ISO 6888-1). Based on colony morphology (growth on Baird-Parker agar) and coagulase production 79 possible *S. aureus* isolates (49 raw sheep milk, 24 sheep cheese and 6 Bryndza cheese) were selected for further genospecies confirmation and geno-phenotyping. Reference strains used in PCR based assays were: *S. aureus* CCM 2353 (*cna* gene), *S. aureus* CCM 7056 (*eta* and *etb* genes), *S. aureus* CCM 5756 (*sea* gene), *S. aureus* CCM 5984 (*sec* gene) and *S. aureus* CCM 5972 (*see* gene) (Czech Collection of Microorganisms, Brno, Czech Republic). Working cultures of all strains were prepared by inoculation from frozen glycerol stocks into brain heart infusion (BHI) broth (Oxoid, Basingstoke, Hampshire, UK) followed by incubation at 37 °C for 16 - 18 h.

Antibiotic susceptibility testing - disc diffusion assays

Discs (Oxoid) with oxacillin (OXA), chloramphenicol (CMP), tetracycline (TET), erythromycin (ERY), gentamycin (GEN), kanamycin (KAN), streptomycin (STR), vankomycin (VAN), linkomycin (LCM), clindamycin (CLI) and spiramycin (SPI) were added onto *S. aureus* inoculated Mueller-Hinton agar plates (Oxoid) (Table 6). The zone of inhibition was determined after 24 h of incubation at 37 °C. The zone size was interpreted according to the recommendations of the CLSI criteria (CLSI, 2004).

Nucleic acid amplification - PCR: Nucleic acid amplification was performed on *S. aureus* genomic DNA isolated according to (Hein et al. 2005). PCRs were performed either in multiplex (set A, set B and set C) (Sharma et al. 2000) or as a single reaction (*mec*A, *cna*, *spa* and *coa*) (Table 1). Cycling conditions for each type of PCR were according to the authors depicted in Table 1. Amplified products were detected in 1.5% agarose gel.

DNA restriction endonuclease analysis of the PCR-amplified coagulase gene

For coagulase genotyping, a PCR-RFLP analysis of isolates was performed according to the method of Hookey et al. (1998). Ten µl of PCR product was digested with 2 U of *Hae*III (Fermentas, Vilnius, Lithuania) at 37 °C overnight. After restriction the samples were analysed by electrophoresis in 2% agarose gel.

Results and Discussion

Specific gene amplification of 16S rRNA and *S. aureus* (Martineau et al. 1998) confirmed 79 *S. aureus* isolates already selected based on the microbiological criteria like colony characterization and coagulase production.

In 79 *S. aureus* isolates all the assessed toxin genes were found, except for *see, eta* and *etb*. Overall, 75.9% *S. aureus* isolates were found to be positive for one or more toxin genes. The *sec* gene was found most frequently (24.1%), followed by *tst* (22.8%), *seb* (13.9%), *sed* (10.1%) and finally, *sea* (5.1%). The *tst* gene was the only gene present in all types of isolates (Table 2).

The presence of *sea, seb, sed, see* and *tst* genes in *S. aureus* strains associated with bovine mastitis has been described earlier (Akineden et al. 2001; Stephan et al. 2001; Scherrer et al. 2004; Zschock et al. 2004; Morandi et al. 2007). Of the staphylococcal enterotoxin encoding genes, the *sec* gene has been reported to have the highest frequency in bovine as well as ovine isolates (Scherrer et al. 2004). We obtained similar results, where out of 42 enterotoxin positive strains, 19 (45.2%) were *sec* positive.

The presence of more than one toxin genes was found in 15.2% *S. aureus* isolates. In our study, from all 18 *tst* positive isolates, 6 (33.6%) were also positive for the *sec* gene. The strains positive for *sec-tst* were isolated mostly from sheep cheese; only one with the *seb-sec-tst* gene combination was found in sheep milk isolate (Table 3).

A high co-occurrence between *sec* and *tst* has also been reported for bovine *S. aureus* strains (Akineden et al. 2001; Stephan et al. 2001; Zschock et al. 2004). Smyth et al. (2005) published a high prevalence (19.2%, 46.2% and 60.9%) of the *tst* gene in bovine, goat and sheep isolates, respectively. In the present study the *tst* gene was the only gene found in isolates from Bryndza cheese. These isolates did not show any double presence of toxin genes. Whether TSST-1 plays a role in the pathogenesis of mastitis is still unknown.

Table 1. Characteristics of the primers used in this study

Gene	Primer	Nucleotide sequence 5´-3´	Amplicon length	PCR type	Reference
16S[1]	16S-1	CAG CTC GTG TCG TGA GAT GT	420	multiplex set A	Martineau et al. 1998; Strommenger et al. 2003
	16S-2	AAT CAT TTG TCC CAC CTT CG			
	sau1	AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG	107		
	sau2	CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA			
	Sa-U	TGT ATG TAT GGA GGT GTA AC	-	multiplex set B	Sharma et al. 2000
sea	SA-A	ATT AAC CGA AGG TTC TGT	270		
seb	SA-B	ATA GTG ACG AGT TAG GTA	165		
sec	SA-C	AAG TAC ATT TTG TAA GTT CC	102		
sec	ENT-C	AAT TGT GTT TCT TTT ATT TTC ATA A	69		
sed	SA-D	TTC GGG AAA ATC ACC CTT AA	306		
see	SA-E	GCC AAA GCT GTC TGA G	213		
tst	tst-F	ACC CCT GTT CCC TTA TCA TC	326	multiplex set C	Mehrotra et al. 2000
	tst-R	TTT TCA GTA TTT GTA ACG GC			
eta	eta-F	GCA GGT GTT GAT TTA GCA TT	93		
	eta-R	AGA TGT CCC TAT TTT TGC TG			
etb	etb-F	ACA AGC AAA AGA ATA CAG CG	226		
	etb-R	GTT TTT GGC TGC TTC TCT TG			
spa	spa-F	GAC GAT CCT TCG GTG AGC	220[2]	single PCR	Shopsin et al. 1999
	spa-R	CAG CAG TAG TGC CGT TTG C			
coa	coa-F	ATA GAG ATG CTG GTA CAG G	500[2]	single PCR	Hookey et al. 1998
	coa-R	GCT TCC GAT TGT TCG ATG C			
mecA	*mec*A-1	AAA ATC GAT GGT AAA GGT TGG C	532	single PCR	Strommenger et al. 2003
	*mec*A-2	AGT TCT GCA GTA CCG GAT TTG C			
cna	cna-F	ATG GTA CCA AGA AGA TAC G	364	single PCR	Peacock et al. 2002
	cna-R	TCT TGA TAC CAA GCT TGT G			

[1] The common "forward" primer; [2] Variable product sizes depending on the number of repeat units

Table 2. Distribution of toxin encoding genes among *S. aureus* isolates

	Toxin gene								Total
	sea	*seb*	*sec*	*sed*	*see*	*tst*	*eta*	*etb*	
Sheep milk (n = 49)	4 (8.2)	10 (20.4)	12 (24.5)	6 (12.2)	-	6 (12.2)	-	-	38 (77.6)
Sheep cheese (n = 24)	-	1 (4.2)	7 (29.2)	2 (8.3)	-	10 (41.7)	-	-	20 (83.3)
Bryndza cheese (n = 6)	-	-	-	-	-	2 (33.3)	-	-	2 (33.3)
Total (n = 79)	4 (5.1)	11 (13.9)	19 (24.1)	8 (10.1)	-	18 (22.8)	-	-	60 (75.9)

Numbers in parentheses are percentages

Table 3. Distribution of *S. aureus* isolates with different combination of toxin genes

	Toxin genes							
	sea, seb	*seb, sec*	*sec, sed*	*sec, tst*	*sed, tst*	*seb, sec, tst*	*seb, sed, tst*	*sec, sed, tst*
Sheep milk (n = 49)	1 (2.0)	2 (4.1)	-	-	1 (2.0)	1 (2.0)	1 (2.0)	-
Sheep cheese (n = 24)	-	-	1 (4.2)	4 (16.7)	-	-	-	1 (4.2)
Bryndza cheese (n = 6)	-	-	-	-	-	-	-	-
Total (n = 79)	1 (1.3)	2 (2.5)	1 (1.3)	4 (5.1)	1 (1.3)	1 (1.3)	1 (1.3)	1 (1.3)

Numbers in parentheses are percentages

TSST-1 and staphylococcal enterotoxins may act as superantigens for cells of the bovine immune system and may potentially contribute to the pathological mechanisms of bovine mastitis. Some other authors also described high co-occurrence of *sec* and *tst* genes in sheep (Orden et al. 1992; Hayakawa et al. 2000; Scherrer et al. 2004). This data suggest that the *sec* gene may be present on a pathogenicity island together with the *tst* gene (Fitzgerald et al. 2001).

Many authors studied the production of exfoliative toxins among *S. aureus* isolates from bovine mastitis and have reported a rare prevalence of these toxins (Hayakawa et al. 2000; Akineden et al. 2001; Larsen et al. 2002; Endo et al. 2003). The present study confirms that these genes are rare in *S. aureus* isolates from sheep; *eta* and *etb* genes were not found in any of our isolates.

In this study we found a high prevalence of the collagen binding protein (*cna*) gene (55.7%). The gene was found most frequently in isolates from Bryndza cheese (83.3%), followed by sheep milk (55.1%) and sheep cheese (50%). The collagen binding protein mediates bacterial adherence to collagen substrates and collagenous tissue and can therefore be a virulence factor of *S. aureus* (Peacock et al. 2002; Zong et al. 2005). The *cna* gene is usually not present in *S. aureus* strains (Elasri et al. 2002).

Production of coagulase encoding by the *coa* gene is an important phenotypic feature used worldwide to identify *S. aureus* (Sutra and Poutrel 1994; Panizzi et al. 2004). In the present study five different amplicons of the *coa* gene with sizes ranging from 500-820 bp were found: type A (500 bp with 4 tandem repeats; 4TR), type B (580 bp, 5TR), type C (660 bp, 6TR), type D (740 bp, 7TR) and type E (820 bp, 8TR). Only type D (7TRs) was present in all isolates. 6TR and 7TR together account for the majority of the isolates (63%) (Table 4).

The use of PCR-RFLP for analysis of the *coa* gene allowed a more detailed characterization of the *S. aureus* isolates. In this study the highest polymorphism was recorded in type C, with 4 or 5 subtypes in sheep milk and sheep cheese isolates, respectively, and in type D with 4, 2 or 1 subtype in sheep milk, sheep cheese and Bryndza cheese isolates, respectively. Some *coa* types or subtypes were recorded only in *S. aureus* isolates from sheep cheese (B, E and C5-C7) or sheep milk (C3, C4, D3 and D4) (Table 4).

Katsuda et al. (2005) and other authors (Lange et al. 1999; Schlegelová et al. 2003) described a number of tandem repeats in the *coa* gene ranging from 3 to 9 and bovine

Table 4. Polymorphism in *coa* gene of *S. aureus* isolates

PCR product (bp)	Type and no. of TR[a] / subtype[b]	Sheep milk (n = 49)	Sheep cheese (n = 24)	Bryndza cheese (n = 6)	Total (n = 79)
500	A / 4TR	10 (20.4)	2 (8.3)	–	12 (15.2)
	A1	1 (2.0)	1 (4.2)	–	2 (2.5)
	A2	9 (18.4)	1 (4.2)	–	10 (12.7)
580	B / 5TR	–	1 (4.2)	–	1 (1.3)
	B1	–	1 (4.2)	–	1 (1.3)
660	C / 6 TR	19 (38.8)	7 (29.2)	–	26 (32.1)
	C1	6 (12.2)	2 (8.3)	–	8 (10.1)
	C2	5 (10.2)	1 (4.2)	–	6 (7.6)
	C3	3 (6.1)	–	–	3 (3.8)
	C4	5 (10.2)	–	–	5 (6.3)
	C5	–	1 (4.2)	–	1 (1.3)
	C6	–	1 (4.2)	–	1 (1.3)
	C7	–	2 (8.3)	–	2 (2.5)
740	D / 7TR	20 (40.8)	11 (45.8)	6 (100)	37 (44.3)
	D1	13 (26.5)	10 (41.6)	6 (100)	29 (36.7)
	D2	3 (6.1)	1 (4.2)	–	4 (5.1)
	D3	1 (2.0)	–	–	1 (1.3)
	D4	3 (6.1)	–	–	3 (3.8)
820	E / 8TR	–	3 (12.5)	–	3 (3.8)
	E1	–	2 (8.3)	–	2 (2.5)
	E2	–	1 (4.2)	–	1 (1.3)

[a] Tandem repeats
[b] Subtypes determined by PCR-RFLP
Numbers in parentheses are percentages

S. aureus strains mostly showing five tandem repeats. Scherrer et al. (2004) studied polymorphisms in the *coa* gene of *S. aureus* isolates from sheep and goat milk. They report an 80.6% prevalence of eight tandem repeats in sheep milk samples. In sheep isolates we only found one with 5TR. This could indicate a connection between mastitis and 5TR in the *coa* gene of *S. aureus* isolates, as our isolates were not specifically from animals with mastitis.

The detection of variation in the X region of the *spa* gene is also used for the epidemiological study of *S. aureus*. Amplicons of the *spa* gene with different sizes ranging from 224 - 392 bp were observed in 82.4% *S. aureus* isolates (Table 5). Based on the size of the corresponding amplicons, 4 to 11 highly polymorphic tandem repeats (TR, 24 bp long) (Frenay et al. 1994; Shopsin et al. 1999) were supposed to be present in this X region. In the present study only two *spa* types (5TR and 6TR) were found in all isolates. Some *spa*

Table 5. Polymorphism in numbers of tandem repeats in X region of *spa* gene of *S. aureus* isolates

	Amplicon size (bp) / Number of tandem repeats in X region of *spa* gene							
	224 / 4TR	248 / 5TR	272 / 6TR	296 / 7TR	344 / 9TR	368 / 10TR	392 / 11TR	Total
Sheep milk (n = 49)	10 (20.4)	5 (10.2)	2 (4.1)	6 (12.2)	–	9 (18.4)	6 (12.2)	38 (77.5)
Sheep cheese (n = 24)	–	8 (33.3)	5 (20.8)	–	1 (4.2)	7 (29.2)	–	21 (87.5)
Bryndza cheese (n = 6)	–	1 (16.7)	5 (83.3)	–	–	–	–	6 (100)
Total (n = 79)	10 (12.7)	14 (17.7)	12 (15.2)	6 (7.6)	1 (1.3)	16 (20.3)	6 (7.6)	65 (82.4)

Numbers in parentheses are percentages

types were observed only in isolates from sheep milk (4TR, 7TR and 11TR) or only from sheep cheese (9TR) (Table 5).

Several authors studied polymorphisms in the X region of the *spa* gene of bovine *S. aureus*. Lange et al. (1999) recorded the presence of 5TR to 12TR (except 10TR) with a predominance of 6TR. Stephan et al. (2001) recorded the presence of 2TR, 6TR, 10TR and 11TR with a predominance of 6TR and Kalorey et al. (2007) recorded the presence of 4TR, 5TR, and 8TR with a predominance for 4TR. To the best of our knowledge, this publication describes the variation of the X-region of the *spa* gene in isolates from sheep milk and sheep cheese for the first time.

Over the last few decades, there has been an enormous increase and emergence of *S. aureus* strains resistant to the antibiotic methicillin (MRSA strains). MRSA is known to be one of the most prevalent nosocomial pathogens throughout the world and to be capable of causing a wide range of hospital-linked infections (Mehrotra et al. 2000). In the present study, methicillin resistance was not present in any of the tested *S. aureus* isolates. Pengov and Ceru (2003) also recorded a low prevalence of resistance to penicillin and ampicillin in ovine isolates. This can be explained by the reduction of the use of b-lactam antibiotics for treatment of sheep; the presence of a different population of *S. aureus* genotypes in the ovine mammary gland is likely to be partly responsible for this as well.

In the present study all staphylococcal isolates were susceptible to oxacillin, tetracycline and gentamycin. A low level of resistance to chloramphenicol, kanamycin and streptomycin was recorded in isolates from sheep milk only. Resistance to vankomycin was recorded only in one isolate from sheep cheese. A high prevalence of resistance to linkomycin, spiramycin and to clindamycin was recorded (Table 6). The prevalence of resistance was higher in isolates from sheep milk than in isolates from sheep cheese and from Bryndza cheese. We also found multiresistant *S. aureus* isolates. Multiresistance to CMP-ERY-KAN-STR-LCM-SPI, ERY-VAN-LCM-CLI-SPI or LCM-CLI-SPI was recorded in one staphylococcal isolate from sheep milk, sheep cheese and Bryndza cheese, respectively. Resistance to LCM-SPI was recorded in 10 isolates; resistance to LCM-CLI was recorded in 4 isolates.

Table 6. Distribution of antibiotic resistance among of *S. aureus* isolates

	Antibiotics; Disc content										
	OXA	TET	CMP	GEN	KAN	STR	VAN	LCM	CLI	SPI	ERY
	5 µg	30 µg	30 µg	10 µg	30 µg	10 µg	30 µg	10 µg	10 µg	20 µg	15 µg
Sheep milk (n = 49)	–	–	2 (4.1)	–	1 (2.0)	1 (2.0)	–	20 (40.8)	2 (4.1)	6 (12.2)	1 (2.0)
Sheep cheese (n = 24)	–	–	–	–	–	–	1 (4.2)	8 (33.3)	2 (8.3)	3 (12.5)	1 (4.2)
Bryndza cheese (n = 6)	–	–	–	–	–	–	–	3 (50.0)	2 (33.3)	2 (33.3)	–
Total (n = 79)	–	–	2 (2.5)	–	1 (1.3)	1 (1.3)	1 (1.3)	31 (39.2)	6 (7.6)	11 (13.9)	2 (2.5)

OXA, oxacillin; TET, tetracycline; CMP, chloramphenicol; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; VAN, vankomycin; LCM, lincomycin; CLI, clindamycin; SPI, spiramycin; ERY, erythromycin
Numbers in parentheses are percentages

The reason for a high prevalence of resistance to linkomycin, spiramycin and clindamycin in our study is probably the use of these antibiotics for treatment of sheep mastitis. Wang at al. (2008) also reported a considerably higher resistance to lincosamides (linkomycin and clindamycin) or macrolides (erythromycin and spiramycin) in *S. aureus* strains isolated from bovine mastitis.

To our knowledge, this is the first study providing comprehensive characterisation data of *S. aureus* strains originating from sheep milk and sheep cheese samples in Slovakia.

Feno- a genotypizácia ovčích izolátov *Staphylococcus aureus*

Cieľom tejto práce bolo stanoviť prevalenciu génov kódujúcich faktory virulencie v izolátoch *Staphylococcus aureus* získaných zo surového ovčieho mlieka, ovčieho syra a bryndze. Boli sledované gény kódujúce stafylokokové enterotoxíny (*sea, seb, sec, sed* a *see*), toxín syndrómu toxického šoku-1 (*tst*), exfoliatívne toxíny (*eta* a *etb*) a proteín viažuci kolagén (*cna*). Vo všetkých 79 *S. aureus* izolátoch boli nájdene vyššie spomenuté gény s výnimkou *see, eta* a *etb* génov. Celkovo bolo 75,9 % *S. aureus* izolátov pozitívnych pre jeden a viac toxínových génov. Najčastejšie bol nájdený *sec* gén (24,1 %), potom *tst* (22,8 %), *seb* (13,9 %), *sed* (10,1 %) a *sea* (5,1 %) gény. 55,7 % *S. aureus* izolátov malo *cna* gén. Na základe analýzy tandemových repetícií v *coa* géne bolo nájdených 5 *coa* typov, ktoré boli na základe RFLP analýzy rozdelené do 16 podtypov. Podobne analýza počtu tandemových repetícií v *spa* géne rozdelila izoláty *S. aureus* na 7 typov. V súbežnej štúdii antibiotikorezistencie bolo zistené, že 69,6 % izolátov bolo rezistentných aspoň na jedno z 11 testovaných antibiotík. Feno- a genotypizácia ovčích izolátov *S. aureus* prezentovaná v tejto práci aktualizuje epidemiologické údaje na Slovensku.

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