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Systematic survey on the prevalence of genes coding for staphylococcal enterotoxins SEIM, SEIO, and SEIN

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Staphylococcus aureus remains a leading cause of food-poisoning with substantial impact on public health. Using a multiplex polymerase chain reaction-DNA enzyme immunoassay (PCR-DEIA), we studied the presence of genes encoding staphylococcal enterotoxin-like (SEl) superantigens *sem*, *sen*, and *seo*, associated with the enterotoxin gene cluster (*egc*), in 429 clinical *Staphylococcus aureus* isolates. 294 (68.5%) isolates tested positive for at least one of the three SEl genes. In contrast to the fixed gene combination *seg*/*sei* also located on *egc*, a substantial number of isolates (n = 108) were found to bear only one or two of the genes encoding SElM, SElN, and SElO. Regarding the origin of the *S. aureus* isolates, a significant difference (P = 0.022) was found for the possession of *seo* (61.2% of blood isolates *versus* 42.9% of nasal strains). Also *sem* (not significantly) was found more common in blood isolates (52.1% *versus* 40.5%). The survey of the newly described SEl genes *sem-seo* supports the concept that most clinical *S. aureus* isolates harbor subsets of pyrogenic toxin superantigens. The potential contribution of *seo* and *sem* to the pathogenic potential of *S. aureus* has to be further evaluated.

Keywords: Enterotoxin / Polymerase chain reaction-DNA enzyme immunoassay / Pyrogenic toxin superantigen / *Staphylococcus aureus*

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1 Introduction

Beyond providing energy and nutrients, food is a potential vehicle for pathogenic microorganisms and their toxic products [1]. While food-borne disease is being increasingly attributed to a wide spectrum of bacteria, parasites, fungi, and viruses, *Staphylococcus aureus* remained a common cause of confirmed food-poisoning with major effect on public health and economy [2]. The primary human habitat of *S. aureus* is the moist squamous epithelium of the anterior nares. *S. aureus* is recognized as a major cause of community-acquired and hospital-acquired infections. In addition to superficial lesions and systemic infections, this pathogen is responsible for toxin-mediated diseases, such as the toxic shock syndrome (TSS) and staphylococcal food-poisoning (SFP) [3]. The virulence factors causing these toxicosis are members of the family of bacterial pyrogenic toxin superantigens (PTSAgs) comprising the TSS-causing toxic shock syndrome toxins (TSST-1, $TSST_{ovine}$) and the staphylococcal enterotoxins (SEs) producing SFP [4, 5].

The PTSAgs of *S. aureus* belong to a "superfamily" of true exotoxins sharing common phylogenetic relationships, sequence homology, structure, and function [6]. Besides their specific toxic properties, they also share other biological characteristics contributing to their role in disease such as pyrogenicity, superantigenicity, and enhancing the susceptibility to endotoxin shock [7, 8]. PTSAgs are able to stimulate T-lymphocyte proliferation in a nonantigen-specific manner, to induce high fevers, to stimulate neutrophil recruitment to a site of infection resulting in local inflammation, and to enhance host susceptibility to endotoxin shock 100 000- to 1 million-fold.

The repertoire of SEs includes the five classical enterotoxins SEA-SEE [9] and several recently identified enterotoxins. For several SEs, subtypes have been characterized [10–13]. To date, the "alphabet" of SEs and their coding genes has reached the letter "U" [11, 13–22]. However, some of the novel SE homologues were shown to be nonemetic, thus,

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Abbreviations: CI, confidence interval; **DEIA**, DNA enzyme immunoassay; **MRSA**, methicillin-resistant *S. aureus*; **OR**, odds ratio; **PTSAg**, pyrogenic toxin superantigen; **SE**, staphylococcal enterotoxin; **SEl**, staphylococcal enterotoxin-like; **SFP**, staphylococcal food-poisoning

actually lacking the defining property of SEs [19, 21, 23]. According to the suggestions of a recently established International Nomenclature Committee for Staphylococcal Superantigens (INCSS), SE-like superantigens that either lack emetic activity or have not been tested should be designated "staphylococcal enterotoxin-like" (SEl) superantigens [24]. This holds also true for the three SEs studied here, thus designated as SElM, SElN, and SElO [24]. Noteworthy, SElN (up to recently named SEN) and SElO (up to recently named SEO) were originally published as SEK and SEL, but renamed in a correction note (published in *J. Immunol.* 2001, *166*, 4260) to rule out possible confusion with SEs described at the same time [16, 19]. In addition to *seg, sei,* and two pseudogenes (ψ *ent1* and ψ *ent2*), the SElM-, SElN-, and SElO-encoding genes belong to the *egc* operon, which is located on the genomic island SaPI3 [11, 20].

Various studies have suggested that the newly described SEs are commonly distributed in food-borne and clinical isolates of *S. aureus* and their role may probably underestimated [25 – 30]. Indeed, only few valid data are available to date concerning the prevalence of the newer described SEs in *S. aureus* isolates derived from clinical specimens. Systematical studies regarding their distribution among well-characterized clinical strains of *S. aureus* are generally lacking.

Previously, we described the rapid and specific detection of toxigenic *S. aureus* isolates by the use of several multiplex PCR-DNA enzyme immunoassays (PCR-DEIAs) for amplification and hybridization of the classical SE genes (*sea*, *seb*, *sec*$_{1-3}$, *sed*, *see*), several recently described SE genes (*seg*, *seh*, *sei*, *sej*), and TSST-1 gene (*tst*) [25, 31]. For the study reported here, this approach was extended by developing a further multiplex PCR-DEIA targeting SElM, SElN, and SElO genes. Aims of this study were (i) to extend the existing multiplex PCR-DEIAs by a further one targeting three newly described SE genes, (ii) to investigate the prevalence of SElM-, SElN-, and SElO-encoding genes in a well-defined *S. aureus* strain collection obtained during the course of a German multicenter study, and (iii) to analyze possible differences in toxin gene equipment of these SEs comparing colonizing isolates from nasal specimens and isolates involved in severe systemic infection.

2 Materials and methods

2.1 Bacterial strains

All together, 429 *S. aureus* isolates collected during the course of a German multicenter study comprising general and intensive care units of 32 university and community hospitals [32] were tested by PCR for the prevalence of genes encoding SElM, SElN, and SElO. Only one isolate per patient was included. The *S. aureus* isolates tested comprised 219 blood isolates from patients with *S. aureus* bacteremia (with a rate of methicillin-resistant *S. aureus* (MRSA) of 9.1%, exhibiting nine genotypes). The most frequent clinically presumed causes of bacteremia were catheter-related infections (46%) followed by osteomyelitis, skin and soft tissue infections such as cutaneous abscesses, cutaneous ulcerations (27%) as well as lower respiratory tract infections (11%). In addition to the blood isolates, a total of 210 *S. aureus* nasal isolates prospectively collected during routine surveillance were also tested (MRSA rate 2.9%, exhibiting five genotypes). None of these patients who were nasally colonized and who were selected for this study subsequently developed *S. aureus* bacteremia within a 6-year-study-period [32].

2.2 DNA isolation, multiplex PCR, and DEIA procedure

Isolation of staphylococcal DNA was performed as previously described [31]. The oligonucleotide primers used to detect the SE genes *sem*, *sen*, and *seo* by a multiplex PCR procedure are listed in Table 1. The assay conditions for these genes corresponded with that of previously published multiplex PCR assays [25, 31]. For carry-over prevention, the uracil DNA glycosylase (UNG) system (Boehringer Mannheim, Germany) was used for all multiplex PCR assays. The amplification was performed in an iCycler (Bio-Rad, Munich, Germany). For hybridization of the amplified DNA generated by the multiplex PCR assays, a generic DNA enzyme immunoassay (GEN-ETI-K® DEIA, Sorin, Saluggia, Italy) was used as described elsewhere [31]. Three 5′-biotinylated oligonucleotide probes were designed to control the specificity of the multiplex PCR targeting *sem-seo* (Table 1). DNA from *S. aureus* reference strains known to harbor the *egc* operon and other toxin-producing reference strains was used as control for the PCR-DEIAs (Table 2). In addition to standard PCR controls for contamination events, *S. epidermidis* DSM 20044 served as negative control.

2.3 Statistical analysis

Regarding the possession of *sem*, *sen*, and *seo*, differences between groups were assessed using the chi square test. If necessary, the Yates correction was used. If more than one exotoxin gene in various combinations was found, multivariate analysis by logistic regression was used to establish their importance [33]. Data on possession of further PTSAg encoding genes (*sea-see*, *seg-sej*, *tst*) in the same strain collection previously published [25] were included for multivariate analysis. Epi-Info 2001 (Centers for Disease Control and Prevention, Atlanta, GA, USA) was used to perform calculations. Odds ratios (ORs) higher than 1 within a confidence interval of 95% ($CI_{0.95}$) were taken into account for

Table 1. Base sequences and gene locations for the oligonucleotide primers and oligonucleotide probes to detect enterotoxin genes *sem-seo*

Gene	Primer or probe[a)]	Oligonucleotide sequence (5′–3′)	Location within gene	Size of PCR product (bp)	Ref.
sem	mpSEM-1	CTA TTA ATC TTT GGG TTA ATG GAG AAC	1785–1811	326	[42]
	mpSEM-2	TTC AGT TTC GAC AGT TTT GTT GTC AT	2085–2110		[42]
	SEM-9B[b)]	ACT TAT TTG ATA CTG GAA CAG GAC A	2034–2058		This study
sen	mpSEN-1	ATG AGA TTG TTC TAC ATA GCT GCA AT	3813–3838	680	[42]
	mpSEN-2	AAC TCT GCT CCC ACT GAA C	4474–4492		[42]
	SEN-3B[b)]	AAA GGC TAA AGT AAC AGT ACA AGA A	4304–4328		This study
seo	mpSEO-1	AGT TTG TGT AAG AAG TCA AGT GTA GA	481–506	180	[42]
	mpSEO-2	ATC TTT AAA TTC AGC AGA TAT TCC ATC TAA C	630–660		[42]
	SEO-3B[b)]	AAA TTC TTA GAC TTC GAT TTA TTA	589–612		This study

a) Sequences and locations were derived from the published nucleotide sequences for *egc* enterotoxin gene cluster [11].
b) 5′ biotinylated.

Table 2. Results of testing reference strains for staphylococcal enterotoxin genotypes *sem, sen,* and *seo* derived from agarose gel analysis of multiplex-PCR and colorimetric microtiter plate DEIA

Reference strains	Toxin genotype determined by previous work	Agarose gel analysis[a)]			Colorimetric assay[b)] (OD ratio 450/630 nm)			Reference or source
		sem	*sen*	*seo*	*sem*	*sen*	*seo*	
ATCC 13565	*sea, sed, sej*[c)]	–	–	–	0.031	0.034	0.034	ATCC
ATCC 14458	*seb*	–	–	–	0.029	0.037	0.043	ATCC
ATCC 19095	*sec, seg, seh, sei*[d)]	–	+	+	0.096	2.439	0.259	ATCC
ATCC 23235	*sed, seg, sei, sej*[e)]	+	+	+	>2.500	>2.500	2.428	ATCC
ATCC 27664	*see*	–	–	–	0.064	0.099	0.073	ATCC
FRI 569	*seh*[f)]	–	–	–	0.174	0.042	0.031	[30]
FRI 572	*seg, sei*[f)]	+	+	+	2.314	2.472	2.430	[17, 30, 43]
KN 813	*tst, seg, sei*	–	+	+	0.110	>2.500	0.309	[25, 31]
BM 10458	*eta*	–	–	–	0.031	0.056	0.038	[25, 31]
BM 10143	*etb*	+	–	+	1.448	0.059	2.403	[25, 31]
Cowan 1	*seg, sei*[g)]	–	+	+	0.152	2.415	0.316	[25, 31]
DSM 20044T	nontoxigenic[h)]	–	–	–	0.033	0.045	0.052	[25, 31]

a) –, negative; +, positive; judged by eye
b) Reactions were judged as positive if the signal reached or exceeded the cut-off value of 0.150 absorbance units above the mean value of determinations of toxin-negative reference strains.
c) Selected as prototype strain for SEA [44], however, also found to be *sed*- and *sej*-positive in a previous study [25]
d) Selected as prototype strain for SEC [45], however, also found to be *seg-, seh-,* and *sei*-positive in a previous study [25]
e) Selected as prototype strain for SED [46], however, also found to be *seg-, sei-,* and *sej*-positive in a previous study [25]
f) Strains were previously tested by PCR and DEIA as completely negative for classical exotoxin genes *sea-see*, *tst*, *eta*, and *etb* [25]
g) *Staphylococcus aureus* Cowan 1 considered nontoxigenic for classical PTSAgs and ETs, however, shown to carry enterotoxin genes *seg* ans *sei* [25]
h) Negative control strain; *Staphylococcus epidermidis* DSM 20044^T considered nontoxigenic for PTSAgs and ETs

associations. *P* values lower than 0.05 were considered to be statistically significant.

3 Results

3.1 Evaluation of the detection system

The multiplex PCR with primer pairs targeting *sem*, *sen*, and *seo* genes amplified successfully fragments from the DNA of *S. aureus* reference strains (Fig. 1, Table 2). PCR amplifications were confirmed by respective hybridization reactions in the DEIA system for positive reference strains (Table 2). The sensitivity of the multiplex PCR-EIAs reached the expected range as previously described [31]. None of the primer pairs and the respective hybridization probes reacted with the negative control, *Staphylococcus epidermidis* type strain DSM 20044^T.

S. aureus strain Cowan 1, considered nontoxigenic and shown to be negative regarding the classical PTSAg genes

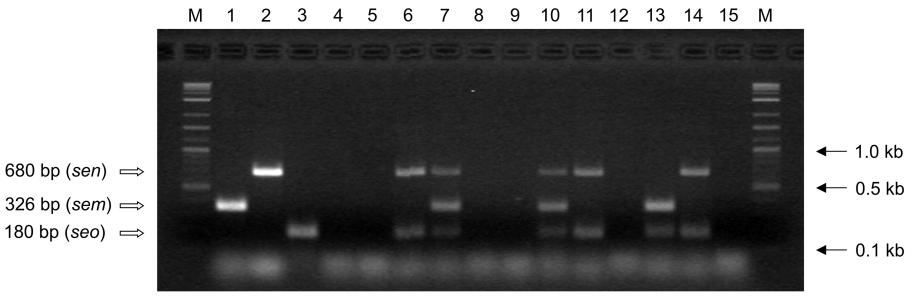

Figure 1. Agarose gel electrophoresis patterns showing PCR-amplified products of *S. aureus* (lanes 1–14) and *S. epidermidis* (lane 15) reference strains in single (lanes 1–3) and multiplex (lanes 4–15) PCR for SE genes *sem*, *sen*, and *seo*. Lanes M (left, right), DNA molecular weight marker 1 kb/100 bp DNA ladder; lanes 1–3, ATCC 23235 (*sem*-, *sen*-, *seo*-positive) using *sem*-specific primers (lane 1), *sen*-specific primers (lane 2), and *seo*-specific primers (lane 3); 4, ATCC 13565 (*sem*-, *sen*-, *seo*-negative); 5, ATCC 14458 (*sem*-, *sen*-, *seo*-negative); 6, ATCC 19095 (*sem*-negative, *sen*-, *seo*-positive); 7, ATCC 23235 (*sem*-, *sen*-, *seo*-positive); 8, ATCC 27664 (*sem*-, *sen*-, *seo*-negative); 9, FRI 569 (*sem*-, *sen*-, *seo*-negative); 10, FRI 572 (*sem*-, *sen*-, *seo*-positive); 11, KN 813 (*sem*-negative, *sen*-, *seo*-positive); 12, BM 10458 (*sem*-, *sen*-, *seo*-negative); 13, BM 10143 (*sen*-negative, *sem*-, *seo*-positive); 14, Cowan 1 (*sem*-negative, *sen*-, *seo*-positive); 15, DSM 20044 (negative control). Sizes are marked in base pairs on the left and right.

Table 3. Results of testing 429 clinical *S. aureus* isolates for SE genes encoding SEIM, SEIN, and SEIO by multiplex-PCR

Result of PCR testing	No. and percentage of isolates						*P* values[a]
	Blood ($n = 219$)		Anterior nares ($n = 210$)		Total ($n = 429$)		
	n	%	*n*	%	*n*	%	
sem-positive	114	52.1	85	40.5	199	46.4	0.170
sen-positive	144	65.8	143	68.1	287	66.9	0.328
seo-positive	134	61.2	90	42.9	224	52.2	**0.022**

a) The *P* values were assessed by multivariate analysis using logistic regression. The boldface value indicates significant differences between blood and nasal isolates. Analysis of *P* values regarding the possession of *sem*, *sen*, and *seo* necessitated involvement of PTSAgs gene data studied earlier [25], thus, the updated *P* values are as follows: *sea*, 0.598; *seb*, 0.387; *sec*, 0.06; *sed/sej*, 0.008; *see*, 0.937; *seg/sei*, 0.225; *seh*, 0.368; and *tst*, 0.191.

[31] was tested previously PCR-positive for the *seg/sei* gene combination [25]. Here, these strains were shown to possess also the genes encoding the enterotoxins SEIN and SEIO, but not SEIM (Table 2). Noteworthy, several reference strains considered as prototype strains for single classical SEs (ATCC catalogue, 19th edition) tested PCR-positive for two or three of the newly described SEs (Table 2): ATCC 19095 (prototype strain for SEC) yielded amplification products with primers specific for *sen* and *seo*, as did ATCC 23235 (prototype strain for SED) with primers specific for *sem*, *sen* and *seo*. This was confirmed by respective hybridization procedures (Table 2).

3.2 Detection of SEIM-SEIO encoding genes

Data on the prevalence of SEIM-SEIO genes obtained by testing 429 clinical *S. aureus* isolates by multiplex-PCR are shown in Table 3. Overall, 294/429 (68.5%) isolates were positive for at least one of the three SE genes tested. Most of these PCR-positive isolates ($n = 186/294$; 63.3%) harbored all three SE genes, however, a substantial number ($n = 108$) of isolates were found to carry only one or two of these SE genes (Fig. 2). Whereas the detection of the toxin genotypes $sem^+/sen^-/seo^-$ ($n = 1$) and $sem^-/sen^-/seo^+$ ($n = 1$) was extremely seldom, the possession of *sen* without concomitant possession of *sem* and *seo*, was found in 62/294 (21.1%) isolates. The most frequent combination of two genes detected was *sen* plus *seo* ($n = 32/294$; 10.9%). Other possible combinations were only rarely observed. Ten (blood isolates, $n = 6$; nasal isolates, $n = 4$) of the 23 MRSA isolates included tested *sem-seo* PCR-negative. Nine isolates (all from blood) tested positive for all three SE genes investigated. The remaining MRSA isolates harbored *sen* alone ($n = 3$) or, in one isolate, *sen* plus *seo*.

Encompassing the data of a previously performed study analyzing the genes encoding the classical PTSAgs (*sea-see*, *tst*) as well as the first of the newly described SEs (*seg-sej*) [25], the rate of those isolates possessing at least one of the PTSAg encoding genes reached 80.0% ($n = 343/429$). Thirty-one (7.2%) isolates (blood isolates, $n = 19$; nasal isolates, $n = 12$) were found to be only *sem*-, *sen*- and/or *seo*-positive, but negative for any other PTSAg gene. Con-

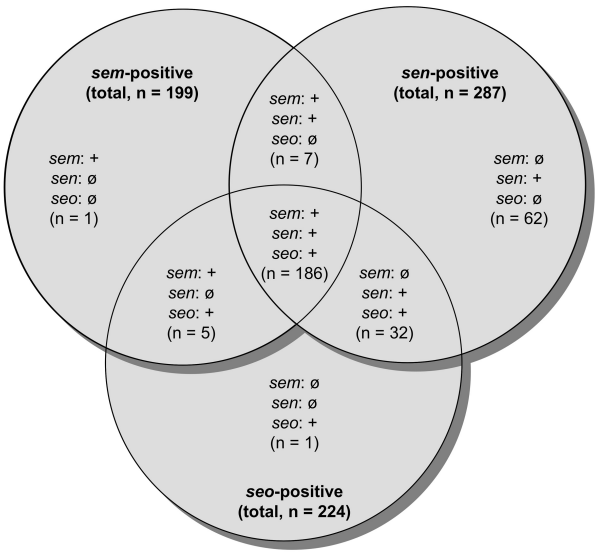

Figure 2. Schematic representation (not scaled) of the number of *S. aureus* isolates exhibiting different toxin genotypes resulting from various possession of genes encoding SEIM, SEIN, and SEIO.

sequently, *sem*, *sen* or *seo* were not necessarily found, if an isolate harbored the *seg*/*sei* combination known to be further components of the *egc* operon.

Overall, a multitude of PTSAg gene combinations with *sem*, *sen*, and/or *seo* was observed including isolates additionally harboring *sea*, *seb*, *sec*, *sed*/*sej*, *seg*/*sei*, *seh*, and/or *tst*, however, no combination with *see* was detected. Separately itemized for *sem*, *sen*, or *seo*, these results are listed in Table 4. The most frequent combination with *sem*, *sen*, and/or *seo* was found for the fixed gene combination *seg*/*sei* (overall concomitant possession, $n = 236$ of the total of 429 (55.0%) isolates, and $n = 236$ of 294 (80.3%) *sem*-, *sen*-, and/or *seo*-positive isolates, respectively). Overall, the co-possession of *sem*, *sen*, and/or *seo* with other PTSAgs was as follows: *sea* ($n = 41$), *seb* ($n = 22$), *sec* ($n = 40$), *sed*/*sej* ($n = 21$), *seh* ($n = 14$), and *tst* ($n = 79$). Thus, combination of *sem*, *sen*, and/or *seo* with one ($n = 16$), two ($n = 100$), three ($n = 95$), four ($n = 47$), and five ($n = 5$) other PTSAg genes were observed.

3.3 Comparing blood and nasal isolates

The association between the detection of SE genes and the origin of isolates was studied by multivariate analysis (Table 3). No significant difference ($P = 0.977$) was found concerning the overall possession of genes encoding SEIM, SEIN, and/or SEIO between blood isolates ($n = 143/210$; 68.1% PCR-positive) and those isolates derived from nasal swabs ($n = 151/219$; 68.9% PCR-positive). In contrast, analysis of subgroups showed that *sem* and *seo* PCR-positive isolates (independently from concomitant possession of other SE genes) were more present in blood isolates. However, only differences in possession of *seo* reached significance (*seo*, OR = 1.4; $CI_{0.95}$ (1.04; 1.89); $P = 0.022$) by multivariate analysis using logistic regression. The likelihood ratio chi square test showed a value of 5.26 with 2 degrees of freedom.

Isolates tested positive only for *sen*, but not for *sem* and *seo* genes (toxin genotype $sem^-/sen^+/seo^-$ ($n = 62$)), were significantly ($P = <0.00001$, OR = 4.3; $CI_{0.95}$ (2.22; 8.57)) less common in isolates from blood ($n = 14$) than in nasal carriage isolates ($n = 48$). *sem*- and/or *seo*-positive isolates tested *sen*-negative (toxin genotypes: $sem^+/sen^-/seo^-$ ($n = 1$), $sem^-/sen^-/seo^+$ ($n = 1$), and $sem^+/sen^-/seo^+$ ($n = 5$))

Table 4. Concomitant possession of PTSAg-encoding genes itemized for *sem*, *sen*, or *seo*

PTSAg gene	No.[a] and percentage[b] of isolates								
	Blood ($n = 219$)			Anterior nares ($n = 210$)			Total ($n = 429$)		
	sem ($n = 114$)	*sen* ($n = 144$)	*seo* ($n = 134$)	*sem* ($n = 85$)	*sen* ($n = 143$)	*seo* ($n = 90$)	*sem* ($n = 199$)	*sen* ($n = 287$)	*seo* ($n = 224$)
sea	14 (12.3%)	21 (14.6%)	19 (14.2%)	3 (3.5%)	18 (12.6%)	6 (6.7%)	17 (8.5%)	39 (13.6%)	25 (11.2%)
seb	6 (5.3%)	6 (4.2%)	7 (5.2%)	13 (15.3%)	15 (10.5%)	11 (12.2%)	19 (9.5%)	21 (7.3%)	18 (8.0%)
sec	15 (13.2%)	15 (10.4%)	15 (11.2%)	22 (25.9%)	25 (17.5%)	23 (25.6%)	37 (18.6%)	40 (13.9%)	38 (17.0%)
sed/sej	16 (14.0%)	17 (11.8%)	16 (11.9%)	3 (3.5%)	4 (2.8%)	3 (3.3%)	19 (9.5%)	21 (7.3%)	19 (8.5%)
see	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
seg/sei	88 (77.2%)	115 (79.9%)	105 (78.4%)	68 (80.0%)	117 (81.8%)	71 (78.9%)	156 (78.4%)	232 (80.8%)	176 (78.6%)
seh	2 (1.8%)	3 (2.1%)	3 (2.2%)	3 (3.5%)	10 (7.0%)	6 (6.7%)	5 (2.5%)	13 (4.5%)	9 (4.0%)
tst	9 (7.9%)	36 (25.0%)	27 (20.1%)	2 (2.4%)	42 (29.4%)	8 (8.9%)	11 (5.5%)	78 (27.2%)	35 (15.6%)

a) Multiple possession of PTSAg encoding genes may occur.
b) Percentage given in relation to *sem*-, *sen*-, and *seo*-positive isolates, respectively

were detected only in blood isolates. These associations of *sem* and/or *seo* with blood isolates were significant (P = <0.00035, OR = 26.1; $CI_{0.95}$ (2.9; 614.5)). Furthermore, the concomitant possession of all three SE genes tested (*sem*$^+$/*sen*$^+$/*seo*$^+$) was significantly (P = 0.025, OR = 0.6; $CI_{0.95}$ (0.43; 0.96)) more common in blood isolates (n = 107) than in nasal isolates (n = 80). However, including the two other SEs encoded by the *egc* operon, *i.e.*, *seg* and *sei* (*sem*$^+$/*sen*$^+$/*seo*$^+$/*seg*$^+$/*sei*$^+$), no significant difference between both groups of staphylococcal isolates was observed (blood isolates, n = 86; nasal isolates, n = 64; P = 0.056).

A possession of *sem*, *sen*, and *seo* simultaneously with the fixed gene comination *sed*/*sej* was significantly different between blood (n = 17) and nasal (n = 4) isolates (P = 0.01, OR = 0.2; $CI_{0.95}$ (0.06; 0.75)). The presence of *sed*/*sej* [25] remained signficantly (P = 0.008) more common for blood isolates even so the possession of *sem*, *sen*, and/or *seo* genes was included into the multivariate analysis of all PTSAg genes analyzed so far (Table 3).

4 Discussion

Among the predominant bacteria involved in food-borne diseases, *S. aureus* is a leading cause of gastroenteritis resulting from consumption of SE contaminated or spoiled food. Classically, SEs were discovered in studies on *S. aureus* strains implicated in outbreaks of food-borne diseases. In addition, it has been shown that a substantial number of animal-derived *S. intermedius* isolates harbor the potential for enterotoxin production and that this species has been clearly involved in SFP outbreaks [34, 35]. In contrast, the occurrence of SE genes is at least very rare in human-associated coagulase-negative staphylococci [36]. The number of known SEs has increased sharply over the last few years mainly due to *S. aureus* genome sequencing projects as well as due to the discovery and further analyses of a enterotoxin gene cluster (*egc*) suggested as an enterotoxin gene nursery [11, 13, 20, 37]. These new SEs were first discovered on the basis of sequence similarities with classical SEs and further confirmed by respective structural analogies and biochemical characteristics.

The classical SEs were named because of their role in food-poisoning, but their role in staphylococcal virulence is multifaceted. While the significance of the SEs in food-borne diseases have been shown in numerous studies, their impact in systemic infections as well as in several allergic and autoimmune diseases is still a matter of debate, in particular regarding the newly described SEs. The SEs are able to stimulate T-lymphocyte proliferation in a nonantigen-specific manner by recognizing the T cell antigen receptor and interacting directly with the class II major histocompatibility complex products on the surface of antigen-presenting cells [6]. Thus, by bypassing intracellular processing, they are the most potent activators of T lymphocytes stimulating at nano- to picogram concentrations up to 30% of T cells, hence they are termed "superantigens" [38]. Superantigenicity and emetic activity have been demonstrated to result from distinct regions of the SE molecule [39].

In this study, it was shown that *sem* (46.4%), *sen* (66.9%), and *seo* (52.2%) belong to the most frequent PTSAg-encoding genes in the *S. aureus* collection tested comparable with the previously described prevalence of the *seg*/*sei* gene combination detected in 55% of *S. aureus* strains [25]. Including all PTSAg genes analyzed (*sea-see*, *sed-sej*, *sem-seo*, *tst*), the overall rate of toxin gene-positive isolates reached 80.0%. The survey of the newly described SE genes *sem-seo* supports the concept that most clinical *S. aureus* isolates harbor subsets of PTSAg genes [25]. This is in contrast to former opinions that nearly all strains of *S. aureus* produce enzymes and hemolysins that contribute to their pathogenicity but only some strains produce PTSAgs [9, 40].

Comparing *S. aureus* isolates derived from blood *versus* carriage isolates, no significant differences were found concerning the overall possession of SElM, SElN, and/or SElO-encoding genes tested. However, analyzing the prevalence of single genes in both study populations, *sem* tended to be and *seo* was significantly more common in blood isolates. In contrast, *sen* was found to be uniformly distributed, irrespective of the origin of the isolates. Also analyzing subsets of different gene combinations of *sem*, *sen*, and/or *seo*, a significant association of *sem* and *seo* with blood isolates was found. In contrast, the exclusive possession of *sen*, was significantly more common in nasal isolates. Thus, it might be speculated that the possession of *sem* and/or *seo* facilitates *S. aureus* infections. However, further studies including isolates of different sources also are needed to confirm a contribution of these newer SE genes to the pathogenicity of *S. aureus*.

In contrast to *seg* and *sej* observed previously as strictly linked [25], the possession of *sem*, *sen*, and *seo* was found to be more variable suggesting a more heterogeneous composition of the *egc* cluster than assumed so far and/or a more frequent allelic variation affecting, *e.g.*, the primer binding sites. These findings are in agreement with the paper of Letertre *et al.* [13], which reports on a new putative enterotoxin SElU (originally described as SEU). The encoding gene (*seu*) was demonstrated to be the result of a DNA insertion into one of the *egc* pseudogene sequences (ψ *ent1*) leading to a putative open reading frame. Moreover, the authors described a variant $SElU_{382F}$ with only 95% identity with the other SElU. In addition, a further *egc* located SE, *i.e.*, SEG, was shown to exist in several variants. Jarraud *et al.* [11] described a SEG_{L29P} variant and

Abe *et al.* [12] characterized a variant SEG designated SEGv. Furthermore, a possible incorporation of parts of the *egc* operon into plasmids could be discussed as known for *seb* and sec_1, found to be on pathogenicity islands, but also occasionally on plasmids. Previously, the gene combination *sed*/*sej* located on a penicillinase plasmid (pIB485) was found to be significantly more associated with blood isolates than with nasal isolates. It was shown that *sej* contributed independently to the virulence of *S. aureus* [25, 41].

In summary, our study is the first systematic search for the recently described SElM-, SElN-, and SElO-encoding genes in a large number of well-characterized clinical *S. aureus* isolates. The studied genes were shown to belong to the most frequent PTSAg genes in *S. aureus* isolates originating from hospitalized patients. Although described as part of the *egc* operon, the possession of *sem*, *sen*, and *seo* was not strictly linked and a multitude of combinations among themselves and with other PTSAg genes were observed. However, *seo* (significantly) and *sem* (tending) were more common in blood isolates than in nasal carriage strains. The potential contribution of *seo* and/or *sem* to the pathogenic potential of *S. aureus* has to be further investigated. Nevertheless, 80% of all *S. aureus* isolates studied harbored at least one of the 13 PTSAg genes analyzed, thus, toxinogenic and/or superantigenic capacities seem to be a habitual feature of this pathogen.

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5 References

[1] Hedberg, C. W., MacDonald, K. L., Osterholm, M. T., Changing epidemiology of food-borne disease: a Minnesota perspective. *Clin. Infect. Dis.* 1994, *18*, 671–680.

[2] Zhang, S., Stewart, G. C., Staphylococcal enterotoxins. In: Honeyman, A. L., Friedman, H., Bendinelli, M. (Eds.). *Staphylococcus aureus infection and disease*. Kluwer Academic/Plenum Publishers, New York 2001, pp. 117–136.

[3] Lowy, F. D., *Staphylococcus aureus* infections, *N. Engl. J. Med.* 1998, *339*, 520–532.

[4] Bohach, G. A., Fast, D. J., Nelson, R. D., Schlievert, P. M., Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. *Crit. Rev. Microbiol.* 1990, *17*, 251–272.

[5] Balaban, N., Rasooly, A., Staphylococcal enterotoxins, *Int. J. Food Microbiol.* 2000, *61*, 1–10.

[6] Llewelyn, M., Cohen, J., Superantigens: microbial agents that corrupt immunity. *Lancet Infect. Dis.* 2002, *2*, 156–162.

[7] Kotb, M., Bacterial pyrogenic exotoxins as superantigens. *Clin. Microbiol. Rev.* 1995, *8*, 411–426.

[8] Schlievert, P. M., The role of superantigens in human disease. *Curr. Opin. Infect. Dis.* 1995, *8*, 170–174.

[9] Dinges, M. M., Orwin, P. M., Schlievert, P. M., Exotoxins of *Staphylococcus aureus. Clin. Microbiol. Rev.* 2000, *13*, 16–34.

[10] Marr, J. C., Lyon, J. D., Roberson, J. R., Lupher, M., *et al.*, Characterization of novel type C staphylococcal enterotoxins: biological and evolutionary implications. *Infect. Immun.* 1993, *61*, 4254–4262.

[11] Jarraud, S., Peyrat, M. A., Lim, A., Tristan, A., *et al.*, *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus. J. Immunol.* 2001, *166*, 669–677.

[12] Abe, J., Ito, Y., Onimaru, M., Kohsaka, T., Takeda, T., Characterization and distribution of a new enterotoxin-related superantigen produced by *Staphylococcus aureus. Microbiol. Immunol.* 2000, *44*, 79–88.

[13] Letertre, C., Perelle, S., Dilasser, F., Fach, P., Identification of a new putative enterotoxin SEU encoded by the egc cluster of *Staphylococcus aureus*. *J. Appl. Microbiol.* 2003, *95*, 38–43.

[14] Ren, K., Bannan, J. D., Pancholi, V., Cheung, A. L., *et al.*, Characterization and biological properties of a new staphylococcal exotoxin. *J. Exp. Med.* 1994, *180*, 1675–1683.

[15] Yarwood, J. M., McCormick, J. K., Paustian, M. L., Orwin, P. M., *et al.*, Characterization and expression analysis of Staphylococcus aureus pathogenicity island 3: implications for the evolution of staphylococcal pathogenicity islands. *J. Biol. Chem.* 2002, *277*, 13138–13147.

[16] Fitzgerald, J. R., Monday, S. R., Foster, T. J., Bohach, G. A., *et al.*, Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J. Bacteriol.* 2001, *183*, 63–70.

[17] Munson, S. H., Tremaine, M. T., Betley, M. J., Welch, R. A., Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. *Infect. Immun.* 1998, *66*, 3337–3348.

[18] Zhang, S., Iandolo, J. J., Stewart, G. C., The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (*sej*). *FEMS Microbiol. Lett.* 1998, *168*, 227–233.

[19] Orwin, P. M., Leung, D. Y., Donahue, H. L., Novick, R. P., Schlievert, P. M., Biochemical and biological properties of staphylococcal enterotoxin K. *Infect. Immun.* 2001, *69*, 360–366.

[20] Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., *et al.*, Whole genome sequencing of meticillin-resistant *Staphylococcus aureus. Lancet* 2001, *357*, 1225–1240.

[21] Orwin, P. M., Leung, D. Y., Tripp, T. J., Bohach, G. A., *et al.*, Characterization of a novel staphylococcal enterotoxin-like superantigen, a member of the group V subfamily of pyrogenic toxins. *Biochemistry* 2002, *41*, 14033–14040.

[22] Omoe, K., Hu, D. L., Takahashi-Omoe, H., Nakane, A., Shinagawa, K., Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. *Infect. Immun.* 2003, *71*, 6088–6094.

[23] Orwin, P. M., Fitzgerald, J. R., Leung, D. Y., Gutierrez, J. A., *et al.*, Characterization of Staphylococcus aureus enterotoxin L. *Infect. Immun.* 2003, *71*, 2916–2919.

[24] Lina, G., Bohach, G. A., Nair, S. P., Hiramatsu, K., *et al.*, Standard nomenclature for the superantigens expressed by *Staphylococcus. J. Infect. Dis.* 2004, *189*, 2334–2336.

[25] Becker, K., Friedrich, A. W., Lubritz, G., Weilert, M., *et al.*, Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. *J. Clin. Microbiol.* 2003, *41*, 1434 – 1439.

[26] Jarraud, S., Cozon, G., Vandenesch, F., Bes, M., *et al.*, Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *J. Clin. Microbiol.* 1999, *37*, 2446 – 2449.

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

[28] Omoe, K., Ishikawa, M., Shimoda, Y., Hu, D. L., *et al.*, Detection of *seg*, *seh*, and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolates harboring *seg*, *seh*, or *sei* genes. *J. Clin. Microbiol.* 2002, *40*, 857 – 862.

[29] Rosec, J. P., Gigaud, O., Staphylococcal enterotoxin genes of classical and new types detected by PCR in France. *Int. J. Food Microbiol.* 2002, *77*, 61 – 70.

[30] Su, Y.-C., Wong, A. C. L., Identification and purification of a new staphylococcal enterotoxin H. *Appl. Environ. Microbiol.* 1995, *61*, 1438 – 1443.

[31] Becker, K., Roth, R., Peters, G., Rapid and specific detection of toxigenic *Staphylococcus aureus*: use of two multiplex PCR enzyme immunoassays for amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene. *J. Clin. Microbiol.* 1998, *36*, 2548 – 2553.

[32] von Eiff, C., Becker, K., Machka, K., Stammer, H., *et al.*, Nasal carriage as a source of *Staphylococcus aureus* bacteremia. *N. Engl. J. Med.* 2001, *344*, 11 – 16.

[33] Dicker, R. C., Analyzing and interpreting data. In: Gregg, M. B., Dicker, R. C., Goodman, R. A. (Eds.), *Field Epidemiology*. Oxford University Press, New York 1996, pp. 92 – 131.

[34] Becker, K., Keller, B., von Eiff, C., Brück, M., *et al.*, Enterotoxigenic potential of *Staphylococcus intermedius. Appl. Environ. Microbiol.* 2001, *67*, 5551 – 5557.

[35] Khambaty, F. M., Bennett, R. W., Shah, D.B., Application of pulsed-field gel electrophoresis to the epidemiological characterization of *Staphylococcus intermedius* implicated in a food-related outbreak. *Epidemiol. Infect.* 1994, *113*, 75 – 81.

[36] Becker, K., Haverkämper, G., von Eiff, C., Roth, R., Peters, G., Survey of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene in non-*Staphylococcus aureus* species. *Eur. J. Clin. Microbiol. Infect. Dis.* 2001, *20*, 407 – 409.

[37] Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., *et al.*, Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* 2002, *359*, 1819 – 1827.

[38] Alouf, J. E., Muller-Alouf, H., Staphylococcal and streptococcal superantigens: molecular, biological and clinical aspects. *Int. J. Med. Microbiol.* 2003, *292*, 429 – 440.

[39] Stelma, G. N., Jr., Bergdoll, M. S., Inactivation of staphylococcal enterotoxin A by chemical modification. *Biochem. Biophys. Res. Commun.* 1982, *105*, 121 – 126.

[40] Bohach, G. A., Foster, T. J., *Staphylococcus aureus* exotoxins. In: Fischetti, V. A., Novick, R. P., Ferretti, J. J., Portnoy, D. A., Rood, J. I. (Eds.), *Gram-positive pathogens*. ASM Press, Washington, DC 2000, pp. 367 – 378.

[41] Peacock, S. J., Moore, C. E., Justice, A., Kantzanou, M., *et al.*, Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus. Infect. Immun.* 2002, *70*, 4987 – 4996.

[42] Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., *et al.*, Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. *Infect. Immun.* 2002, *70*, 631 – 641.

[43] Monday, S. R., Bohach, G. A., Genes encoding staphylococcal enterotoxins G and I are linked and separated by DNA related to other staphylococcal enterotoxins. *J. Nat. Toxins* 2001, *10*, 1 – 8.

[44] Casman, E. P., Bergdoll, M. S., Robinson, J., Designation of staphylococcal enterotoxins. *J. Bacteriol.* 1963, *85*, 715 – 716.

[45] Bergdoll, M. S., Borja, C. R., Avena, R. M., Identification of a new enterotoxin as enterotoxin C. *J. Bacteriol.* 1965, *90*, 1481 – 1485.

[46] Casman, E. P., Bennett, R. W., Dorsey, A. E., Issa, J. A., Identification of a fourth staphylococcal enterotoxin, enterotoxin D. *Biochemistry* 1967, *94*, 1875 – 1882.

