ARTICLE

Prevalence of toxin genes in consecutive clinical isolates of *Staphylococcus aureus* and clinical impact

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Abstract Even if Panton–Valentine leukocidin (PVL), toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEB and SEC), and exfoliative toxins (ETA and ETB) may be associated with severe infections, the clinical significance of their presence in clinical isolates of *Staphylococcus aureus* remains poorly documented. In this study, we evaluated the prevalence of toxin genes and the relationship between their presence and the severity of infection. We screened for the presence of these six toxin genes among 186 consecutive *S. aureus* clinical isolates (resistant or not to methicillin) during a two-month period. We compared the toxin gene profile between strains recovered from patients presenting uncomplicated infections ($n=151$) and from patients suffering from severe infections ($n=35$). At least one toxin gene was detected in 55 (29.6%) isolates as follows: *pvl* ($n=1$), *tst*+ *sec* ($n=5$), *seb* ($n=19$), *seb*+*sec* ($n=1$), *sec* ($n=28$), and *eta* ($n=1$). The proportion of toxin-producing strains among patients with uncomplicated infections (27.8%) and patients with severe infections (37.1%) was not statistically different ($p=0.3044$), even if the severity of infection tended to be associated with the presence of *sec* ($p=0.0655$). Although the prevalence of toxin genes was relatively high herein, no statistically significant association between the severity of infection and the presence of toxin genes was observed.

Introduction

Staphylococcus aureus is a major human pathogen that causes both suppurative and toxin-mediated nosocomial and community-acquired infections worldwide [1]. Numerous toxins have been identified in *S. aureus*, including some of them that have been potentially associated with specific infections or syndromes [2].

Among these virulence factors, Panton–Valentine leukocidin (PVL) is a pore-forming toxin generally associated with complicated skin and skin structure infections, extensive cellulitis, necrotizing pneumonia, and acute osteomyelitis in children [3–5]. In particular, the leukotoxic action of PVL is responsible for the high mortality rate (up to 75%) reported in necrotizing pneumonia [4].

Several toxins have superantigenic properties, such as toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins B (SEB) and C (SEC). These superantigens directly activate T-cells through bypassing normal antigen presentation. They bind to the specific variable region of the T-cell antigen receptor-β-chain with strong affinity [2, 6]. Uncontrolled release of Th1 pro-inflammatory cytokines mediated by massive T-cell proliferation may be responsible for the symptomatology of staphylococcal toxic shock syndrome (STSS) [2]. Clinically, STSS combines rapid onset of fever, diffuse macular rash, hypotension, and multiple organ failure, with a mortality rate ranging from 4 to 22% [6, 7].

Exfoliative toxins A (ETA) and B (ETB) are epidermolytic proteases mainly responsible for staphylococcal scalded skin syndrome (SSSS), which typically occurs in neonates and infants, but can also affect predisposed adults. The syndrome is characterized by a fever and a rapid generalized desquamation without mucosal damage. The mortality rate is low in infants but can reach 67% in adults [8]. The strains producing these toxins can also cause localized forms (namely, bullous impetigo), where skin lesions are restricted to the site of infection [8].

Even if these toxins have been related to severe infections, the clinical significance of their presence in *S.*

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aureus isolates has been poorly investigated, and only among collections of non-consecutive clinical isolates [9–13]. The aim of this study was, then, to: (i) screen consecutive *S. aureus* clinical isolates (resistant or not to methicillin) for the presence of these six major toxins and (ii) assess the clinical significance of these toxin-producing strains. The proportion of virulence genes was compared between the isolates groups associated with uncomplicated infection and severe infection.

Materials and methods

Bacterial isolates

From May to June 2009, all non-duplicate *S. aureus* clinical isolates recovered from patients hospitalized at the university hospital of Caen (ca. 2,000-bed) were studied. They were identified by using conventional phenotypic methods, such as Gram staining, catalase activity, and latex agglutination test (Pastorex Staph-Plus®; Bio-Rad, Marnes-la-Coquette, France). In doubtful cases, an identification by using the ID-GP card on the Vitek 2 system (bioMérieux, Marcy-l'Etoile, France) was performed. Antimicrobial susceptibility was determined by disc diffusion on Mueller–Hinton agar according to the Antibiogram Committee of the French Society for Microbiology guidelines (http://www.sfm.asso.fr).

Patient characteristics and definitions

Demographic and clinical data were retrospectively recorded for each patient regarding age, sex, underlying conditions (including heart disease, hemodialysis, diabetes mellitus, hematological disorder, HIV infection, current malignancy, immunosuppressive therapy), site of isolation, clinical presentation, hospital ward, treatment, and clinical outcome.

Sepsis, severe sepsis, septic shock, and multiple organ dysfunction syndrome were defined according to the criteria of the American College of Chest Physicians and Society of Critical Care Medicine Conference [14], while STSS was defined as described elsewhere [6]. Hospital-acquired infections were defined according to the CDC definitions [15] (http://www.cdc.gov/ncidod/dhqp/pdf/nnis/NosInfDefinitions.pdf). All other cases were considered as community-acquired infections.

Multiplex real-time PCR

Bacterial genomic DNA was extracted by using the InstaGene Matrix® Kit (Bio-Rad) according to the manufacturer's recommendations. We developed two triplex real-time polymerase chain reaction (PCR) assays for the detection of *pvl*, *tst*, and *eta* genes (PCR A) and *etb*, *seb*, and *sec* genes (PCR B). Primers and TaqMan™ LNA probes were designed and synthesized by Sigma-Aldrich France (Table 1). PCR A and PCR B

Table 1 Primers and LNA probes used in the study

Name	Oligonucleotide sequence (direction 5′→3′)	Target gene	Nucleotide position	Tm (°C)	Size of amplicon (bp)
pvl-F	gtatggcagaaatatggatgttactca	*pvl*	771-797	65	91
pvl-R	tgcgttgtgtattctagatccttctaa		861-835	65	
pvl-probe	[6FAM]-ct[+A]ct[+A]g[+A]ag[+A]a[+C]aa[+C]aca[+C]t[+A]t-[BHQ1]		820-822	70	
tst-F	ttgcgacaactgctacagattttac	*tst*	50-74	65	73
tst-R	gatgcttttgcagttttgattatttg		122-97	65	
tst-probe	[HEX]-cc[+T]g[+T][+T]ccc[+T][+T]a[+T]ca[+T]c-[BHQ1]		76-92	71	
eta-F	tttactgtaggagctagtgcatttgttat	*eta*	49-77	64	75
eta-R	tgaaacttctgcttttgcatggt		123-101	67	
eta-probe	[ROX]-ca[+A]ga[+C]g[+A]a[+C]tg[+A]tgc[+A]a-BHQ2]		79-96	70	
etb-F	aacacgttgcaagagagaagca	*etb*	284-303	66	69
etb-R	catctctattttgagcgggtgtaa		352-329	65	
etb-probe	[6FAM]-c[+C]a[+A]a[+A]a[+C]c[+C]a[+T]cg[+A]a[+T]a-[BHQ1]		305-322	71	
seb-F	ctacacccaacgttttagcagaga	*seb*	62-85	65	70
seb-R	aatttactcgatttgtgcaactcatc		131-106	65	
seb-probe	[HEX]-t[+C]a[+A]c[+C]ag[+A]t[+C]c[+T]a[+A]a[+C]c-[BHQ1]		87-104	71	
sec-F	cgtattagcagagagccaacca	*sec*	72-93	65	63
sec-R	gtgaatttactcgctttgtgcaa		134-112	65	
sec-probe	[ROX]-ac[+C]cta[+C]g[+C]c[+A]g[+A]tga-[BHQ2]		95-110	69	

[+N], LNA monomer base; Tm, melting temperature

experiments were performed by using the QuantiFast® Multiplex PCR Kit (Qiagen, Courtaboeuf, France) in a 25-μl reaction mixture with final concentrations of 0.5 μM for each primer and 0.2 μM for each LNA probe. PCR amplifications were performed using a Rotor-Gene Q instrument (Qiagen) as follows: (i) initial denaturation step of 5 min at 95°C and (ii) 40 cycles of PCR, with one cycle consisting of 45 s at 95°C and 45 s at 60°C. The presence of amplified DNA was detected by measuring the fluorescence emitted at 470 nm (for *pvl* and *etb*), 530 nm (for *tst* and *seb*), and 585 nm (for *eta* and *sec*).

PFGE

Fingerprinting by pulsed-field gel electrophoresis (PFGE) was performed on toxin gene-positive strains as previously described [16]. *Sma*I-macrorestricted patterns were obtained by using a contour-clamped homogeneous electric field DR-III apparatus (Bio-Rad) as follows: ramped pulse times of 5 s and 35 s at 6 V/cm for 20 h at 14°C. Fingerprint® II software (Bio-Rad) was used to analyze PFGE patterns. The calculation of similarity matrices and dendrograms were obtained with the unweighted pair group method with arithmetic averages (UPGMA). Strains were considered to be clonally-related if the Dice coefficients were greater than 85%.

Comparative analysis

In order to evaluate the role of toxins in the severity of infections, patients were classified into two groups: (i) uncomplicated infections (UI) defined as sepsis and (ii) severe infections (SI) defined as severe sepsis, septic shock, multiple organ dysfunction syndrome, or STSS. These two groups were compared regarding demographic and clinical characteristics, as well as toxin gene prevalence.

Statistical analysis

Statistical analysis was performed using the GraphPad Software (http://www.graphpad.com/quickcalcs). The χ^2 test or Fisher's exact test was used, as appropriate, to compare categorical variables, whereas Student's *t*-test was used to compare continuous variables. Differences between groups were considered to be statistically significant for *p*-values <0.05.

Results

Clinical data

During the two-month period, 186 strains were recovered from 186 patients, including 75 isolates obtained from patients

Table 2 Demographic and clinical characteristics of 186 patients with *Staphylococcus aureus* infections

Characteristic	Number (%) of *S. aureus* isolates			
	Uncomplicated infections (*n*=151)	Severe infections (*n*=35)	Total infections (*n*=186)	*p*-value
Mean age (range), years	52.7 (0–97)	61.4 (0–83)	54.4 (0–97)	0.0700
M/F sex ratio	1.52	1.92	1.6	0.7005
Hospital ward				**<0.0001**
Intensive care	23 (15.2)	16 (45.7)	39 (21.0)	
Medicine	60 (39.7)	12 (34.3)	72 (38.7)	
Surgery	68 (45)	7 (20.0)	75 (40.3)	
Type of infections				**<0.0001**
Superficial	74 (49)	1 (2.9)	75 (40.3)	
Invasive	77 (51)	34 (97.1)	111 (59.7)	
Prior hospitalization (<3 months)	48 (31.8)	16 (45.7)	64 (34.4)	0.1660
Nosocomial infection	78 (51.7)	21 (60.0)	99 (53.2)	0.4531
Underlying condition(s)	72 (47.7)	22 (62.9)	94 (50.5)	0.1336
Prior surgery	32 (21.2)	8 (22.9)	40 (21.5)	0.8217
MRSA infection	37 (24.5)	11 (31.4)	48 (25.8)	0.3982
Antimicrobial therapy	102 (67.5)	32 (91.4)	134 (72.0)	**0.0034**
Surgical treatment	42 (27.8)	4 (11.4)	46 (24.7)	0.0503
28-day mortality	10 (6.6)	11 (34.1)	21 (11.3)	**0.0002**

MRSA, methicillin-resistant *S. aureus*

Table 3 Polymerase chain reaction (PCR) detection of *S. aureus* toxin genes and comparison of toxin profiles between uncomplicated and severe infections

Gene	Number (%) of *S. aureus* isolates			
	Uncomplicated infections (*n*=151)	Severe infections (*n*=35)	Total infections (*n*=186)	*p*-value
pvl	1 (0.7)	0	1 (0.5)	–
tst and *sec*	3 (2.0)	2 (5.7)	5 (2.7)	0.2373
seb	17 (11.2)	2 (5.7)	19 (10.2)	0. 5355
seb and *sec*	1 (0.7)	0	1 (0.5)	–
sec	19 (12.5)	9 (25.7)	28 (15.1)	0.0655
eta	1 (0.7)	0	1 (0.5)	–
etb	0	0	0	–
Total	42 (27.8)	13 (37.1)	55 (29.6)	0.3044

with superficial infections (68 skin and soft tissue infections, six ear, nose, throat [ENT] infections, and one genital infection) and 111 from patients with invasive infections (33 respiratory tract infections, 30 bacteremia, 21 urinary tract infections, 17 bone or joint infections, and 10 deep abscesses). Among the 186 patients enrolled in the study, 151 suffered from uncomplicated infections and 35 suffered from severe infections (18 severe sepsis, 15 septic shock, two multiple organ dysfunction syndromes). No patients met the diagnostic criteria for STSS. Most infections were hospital-acquired (99/186; 53.2%), while the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) infection was 25.8%. Forty surgical site infections (21.5%) were reported.

The demographic and clinical characteristics of the patients are shown in Table 2. There were no significant differences between UI and SI groups regarding age, sex, underlying diseases, prior hospitalization, prior surgery, nosocomial infection, MRSA infection, and surgical therapy (Table 2). By contrast, there was a significant difference for hospitalization ward, type of infection (superficial or invasive), and antimicrobial therapy (Table 2). Unsurprisingly, the 28-day crude mortality was significantly higher among SI patients than among UI patients (34.1% vs. 6.6%; *p*=0.0002).

Prevalence of toxin genes

Of the 186 isolates, 55 (29.6%) harbored at least one toxin gene (Table 3), comprising 13 of 35 strains (37.1%) recovered from SI patients and 42 of 151 strains (27.8%) recovered from UI patients. The most prevalent gene was *sec* (15.1%), followed by *seb* (10.2%). Five (2.7%) strains were positive for both *tst* and *sec* genes, while *seb* and *sec* genes were detected in a single isolate. Finally, the *eta* gene was detected in one strain, whereas no strain was positive for the *etb* gene. Noteworthy, one or more toxin genes were detected in 46 (33.3%) of 138 methicillin-susceptible *S. aureus* (MSSA) and in 9 (18.7%) of 48 MRSA.

By using a cut-off value of 85% for the Dice coefficient, 16 and 10 different PFGE clusters were identified in the group of *pvl*-, *tst*- and *sec*-, *seb*-, *seb*- and *sec*-, and *eta*-positive strains (*n*=27) and in the group of *sec*-positive strains (*n*=28), respectively (Fig. 1). Noteworthy, three of the five *tst*-positive isolates were MRSA, while the single *pvl*-positive isolate was also MRSA (Fig. 1a). Concerning the 28 *sec*-positive strains, 13 (46.4%) clustered in a major group (Fig. 1b). Although they exhibited identical antimicrobial resistance profiles, they were recovered at different dates from patients hospitalized in different hospital wards (Fig. 1b).
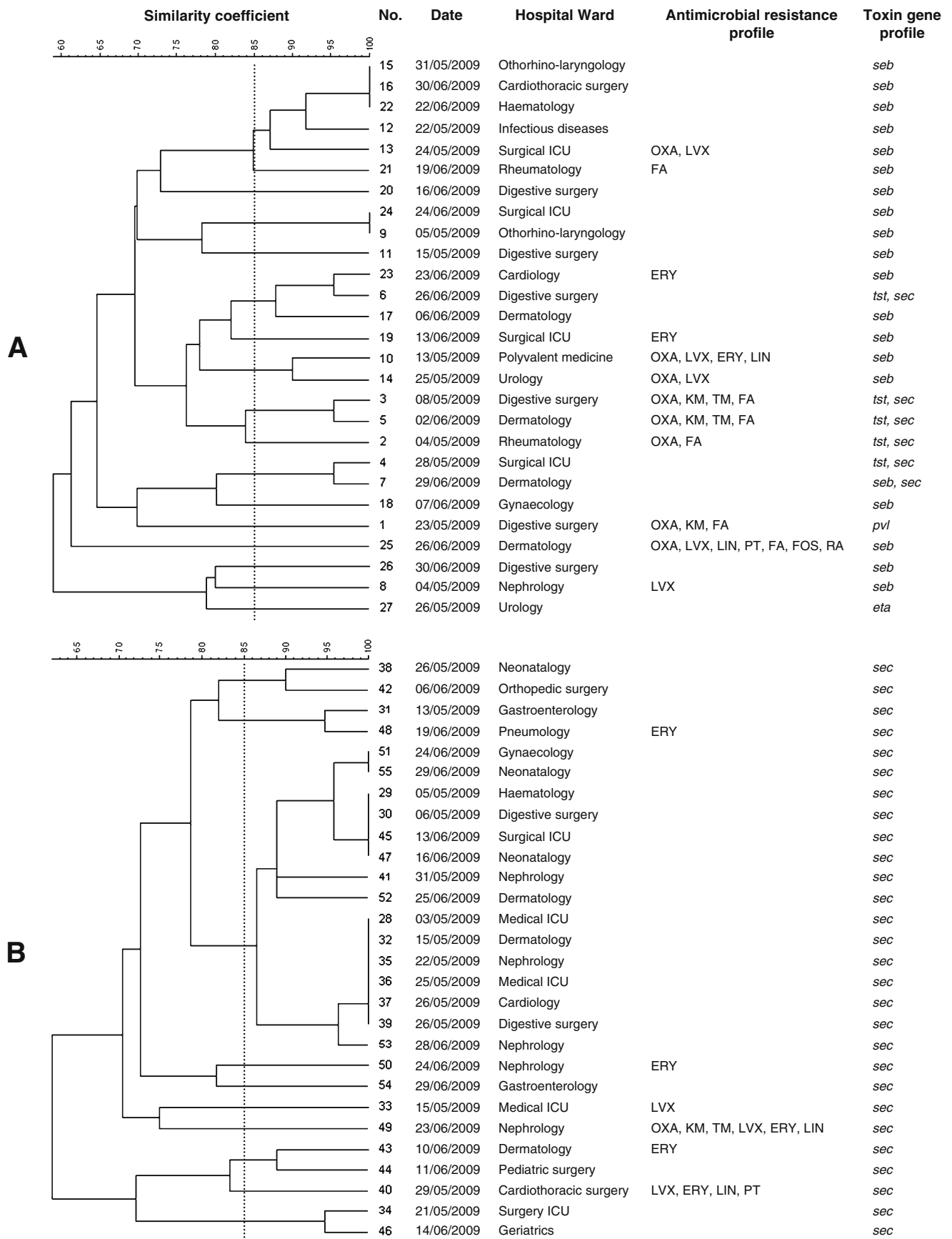
Clinical significance of toxin-producing strains

The overall prevalence of toxin-producing strains among UI patients (27.8%) and SI patients (37.1%) was not statistically different (*p*=0.3044) (Table 3). In addition, none of the detected genes was more prevalent in one of the two groups. Nonetheless, SI tended to be associated with a higher prevalence of the *sec* gene (25.7% vs. 12.5%; *p*=0.0655).

Discussion

To the best of our knowledge, this is the first study that has evaluated the prevalence of several major toxins on a collection of consecutive (methicillin-resistant or -susceptible) *S. aureus* clinical isolates. To do that, we

Fig. 1 Unweighted pair group method with arithmetic averages ▶ (UPGMA) dendrogram of pulsed-field gel electrophoresis (PFGE) results based on the Dice distance matrix of *pvl*-, *tst*- and *sec*-, *seb*-, *seb*- and *sec*-, and *eta*-positive (**a**) and *sec*-positive (**b**) *Staphylococcus aureus* clinical isolates. The 85% cut-off value of the Dice coefficient is shown by a vertical dotted line. Date of isolation, hospital ward, and antimicrobial resistance and toxin gene profiles are also indicated. OXA, oxacillin; KM, kanamycin; TM, tobramycin; LVX, levofloxacin; ERY, erythromycin; LIN, lincomycin; PT, pristinamycin; FA, fusidic acid; FOS, fosfomycin; RA, rifampicin

Similarity coefficient

No.	Date	Hospital Ward	Antimicrobial resistance profile	Toxin gene profile
15	31/05/2009	Othorhino-laryngology		seb
16	30/06/2009	Cardiothoracic surgery		seb
22	22/06/2009	Haematology		seb
12	22/05/2009	Infectious diseases		seb
13	24/05/2009	Surgical ICU	OXA, LVX	seb
21	19/06/2009	Rheumatology	FA	seb
20	16/06/2009	Digestive surgery		seb
24	24/06/2009	Surgical ICU		seb
9	05/05/2009	Othorhino-laryngology		seb
11	15/05/2009	Digestive surgery		seb
23	23/06/2009	Cardiology	ERY	seb
6	26/06/2009	Digestive surgery		tst, sec
17	06/06/2009	Dermatology		seb
19	13/06/2009	Surgical ICU	ERY	seb
10	13/05/2009	Polyvalent medicine	OXA, LVX, ERY, LIN	seb
14	25/05/2009	Urology	OXA, LVX	seb
3	08/05/2009	Digestive surgery	OXA, KM, TM, FA	tst, sec
5	02/06/2009	Dermatology	OXA, KM, TM, FA	tst, sec
2	04/05/2009	Rheumatology	OXA, FA	tst, sec
4	28/05/2009	Surgical ICU		tst, sec
7	29/06/2009	Dermatology		seb, sec
18	07/06/2009	Gynaecology		seb
1	23/05/2009	Digestive surgery	OXA, KM, FA	pvl
25	26/06/2009	Dermatology	OXA, LVX, LIN, PT, FA, FOS, RA	seb
26	30/06/2009	Digestive surgery		seb
8	04/05/2009	Nephrology	LVX	seb
27	26/05/2009	Urology		eta

A

No.	Date	Hospital Ward	Antimicrobial resistance profile	Toxin gene profile
38	26/05/2009	Neonatalogy		sec
42	06/06/2009	Orthopedic surgery		sec
31	13/05/2009	Gastroenterology		sec
48	19/06/2009	Pneumology	ERY	sec
51	24/06/2009	Gynaecology		sec
55	29/06/2009	Neonatalogy		sec
29	05/05/2009	Haematology		sec
30	06/05/2009	Digestive surgery		sec
45	13/06/2009	Surgical ICU		sec
47	16/06/2009	Neonatalogy		sec
41	31/05/2009	Nephrology		sec
52	25/06/2009	Dermatology		sec
28	03/05/2009	Medical ICU		sec
32	15/05/2009	Dermatology		sec
35	22/05/2009	Nephrology		sec
36	25/05/2009	Medical ICU		sec
37	26/05/2009	Cardiology		sec
39	26/05/2009	Digestive surgery		sec
53	28/06/2009	Nephrology		sec
50	24/06/2009	Nephrology	ERY	sec
54	29/06/2009	Gastroenterology		sec
33	15/05/2009	Medical ICU	LVX	sec
49	23/06/2009	Nephrology	OXA, KM, TM, LVX, ERY, LIN	sec
43	10/06/2009	Dermatology	ERY	sec
44	11/06/2009	Pediatric surgery		sec
40	29/05/2009	Cardiothoracic surgery	LVX, ERY, LIN, PT	sec
34	21/05/2009	Surgery ICU		sec
46	14/06/2009	Geriatrics		sec

B

optimized a multiplex real-time PCR assay that appeared to be a useful tool for the detection of *S. aureus* toxin genes during epidemiological surveys, as previously reported [17, 18]. Overall, 29.6% of all tested isolates were positive for one or more toxin genes, this prevalence being similar to those reported in previous studies [10, 11, 13, 19]. More specifically, only a small proportion of strains were positive for *pvl* (0.5%) and *eta* (0.5%) genes and none for the *etb* gene. Such low prevalence has also been reported in other studies [11, 13, 20]. The single *pvl*-positive strain harbored a typical antimicrobial resistance pattern (resistance to methicillin, kanamycin, and fusidic acid, and susceptibility to tobramycin, gentamicin, and fluoroquinolones), suggesting that it could belong to the MRSA sequence type 80 (ST80) European clone that has emerged and spread in Europe since the late 1990s [21]. We found a lower proportion of *tst* gene (2.8%) in comparison with other studies (8.7 to 30% of infections) [10, 11, 13, 19], but in Peacock et al.'s study, this prevalence decreased after adjustment for effects of clonality [13]. Among the five *tst*-positive strains (also *sec*-positive), three isolates had a particular antimicrobial resistance phenotype (phenotype 1: resistance to methicillin, kanamycin, tobramycin, and fusidic acid, and susceptibility to gentamicin and fluoro-quinolones; phenotype 2: resistance to methicillin and fusidic acid, and susceptibility to kanamycin, tobramycin, gentamicin, and fluoroquinolones) assumed to belong to the MRSA ST5 Geraldine clone, which was recently reported in France [22]. The analysis of PFGE patterns showed the polyclonality of circulating strains in our hospital during the period of the study. Only one major cluster of *sec*-positive isolates (46.4%) was identified, suggesting that these strains were epidemiologically related. However, no outbreak was identified during the two-month study period.

In this study, we demonstrated no statistically significant association between the severity of infection and the presence of the six detected toxin genes, even if the *sec* gene tended to be more common in SI patients than in UI patients (25.7% vs. 12.5%; *p*=0.065). This tendency has not yet been reported [10, 11, 13, 19] and remains to be confirmed.

Our study presented several limitations: (i) this study was a single-center investigation, (ii) clinical data have been retrospectively collected, and (iii) the SI patients group (*n*=35) was relatively small compared to the UI patients group. Furthermore, the molecular analysis of strains does not ascertain the in vivo production of toxins.

Other toxins have been shown to play a role in severity. For instance, Ferry et al. demonstrated in *S. aureus* bacteremia a link between the presence of the *sea* gene and septic shock, whereas the presence of the *egc* genes was negatively correlated with the severity of infection [10]. In addition, several virulence factors could act in

combination for enhancing the clinical severity of infections [13]. Even if only one *pvl*-positive strain was identified in this study, the role of PVL as a major virulence determinant in severe infections remains controversial [9, 23–28]. Finally, the interaction between the host immune system (including genetic predispositions) and the pathogen may play a major part in *S. aureus* toxin-mediated infections' pathogenesis. Not all colonized or infected patients with a toxin-producing strain of *S. aureus* go on to develop STSS. A lack of detectable antibodies to STSS-associated superantigens seems to be a major risk for the development of STSS [2, 6]. These individuals may be predisposed to repeated episodes of STSS. In addition, toll-like receptor type 2 (TLR-2) polymorphism may place individuals at a higher risk of Gram-positive septic shock, as well as certain HLA haplotypes can also impact on the clinical susceptibility to toxic effects of superantigens [29].

In conclusion, we did not observe a statistically significant relation between the severity of the infection and the presence of toxin gene(s). However, it would be interesting to complete these first results by a multicenter investigation with a larger population. So far, the detection of toxin genes in *S. aureus* has been considered to be of interest for the diagnosis of toxin-mediated diseases leading to an early antitoxinic therapy, such as clindamycin, linezolid, and/or intravenous immunoglobulins. Although these molecules have been shown to be effective for reducing exotoxin production in vitro [30–33], the clinical benefits should be more thoroughly evaluated.

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