Title:

Toxin profiling of *Staphylococcus aureus* strains involved in varicella superinfection

Short Title: Varicella and *S. aureus* superinfection

Key words: varicella, *S. aureus*, superinfection, toxin, France

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The most common complications of varicella are bacterial skin and soft-tissue infections, generally due to Staphylococcus aureus and group A betahaemolytic streptococci. The aim of this study was to characterize the toxin and antibiotic resistance profiles of S. aureus isolates involved in varicella complications. Between 2002 and 2007, the French Reference Centre for Staphylococci collected 58 S. aureus isolates involved in varicella superinfection. All the isolates were characterized by screening for 12 toxin genes, agr typing and mecA gene detection; some isolates were also studied by spa typing, multilocus sequence typing (MLST) and resistance profiling. A major toxin gene was detected in 53% (31/58) of the isolates (genes of exfoliative toxins A and B 17.2%, Panton-Valentine leukocidin gene 8.6%, toxic shock syndrome toxin-1 gene 27.6%). Most clinical manifestations were directly compatible with the classical activity of these toxins. Nineteen isolates (33%) were resistant to methicillin, and 12 of these isolates belonged to an emerging agr2, ST5 clone that harbors the toxic shock syndrome toxin 1 gene. These data should be considered in the management and treatment of patients with varicella complicated by S. aureus superinfection. Antibiotics that decrease toxin production, such as clindamycin, may provide benefit and their efficacy against bacterial superinfections in children with varicella should be studied.
Introduction

Varicella is an acute, generally benign childhood disease due to varicella-zoster virus. The reported frequency of serious complications is highly variable, probably owing to differences in the methods of data collection, geography, living conditions, vaccine use and hospital admission policies from one study to another. Overall, about 4 to 9% of cases of varicella are complicated (6,15), and these complications account for 71 to 80% of varicella-related hospitalizations (5,18,40). Previously healthy children account for more than 80% of patients with such complications (32). Neurological, gastrointestinal and respiratory disorders are often reported (29,32) but bacterial superinfection represents 31 to 70% of all varicella-associated events (15,18,22). Superficial skin infections account for 20 to 50% of all varicella complications and for most cases of skin scarring (18,32,40).

In previously healthy children with varicella, bacterial superinfection is facilitated by skin barrier disruption and possibly by transient virus-induced alterations of local immunity (1). *Staphylococcus aureus* and group A beta haemolytic streptococci are the two most common bacterial pathogens isolated in this setting, and both can cause invasive infections (27,40). *S. aureus* is the predominant species in recent large studies with bacteriological documentation (18,40). *S. aureus* is frequently responsible for skin and soft-tissue infections (SSTIs), sometimes involving toxin production. Staphylococcal toxins have a wide array of biological properties, including exfoliative, suppurative and superantigenic effects (9). For example, exfoliatins A and B (ETA, ETB) split the desmosomes that cement cells together. Loss of keratinocyte cell-cell adhesion can culminate in bullous impetigo or in a generalized form called staphylococcal scalded-skin syndrome (SSSS) (2). Panton Valentine leukocidin (PVL) forms pores in the outer membrane of polymorphonuclear leukocytes (24), triggering their apoptosis. PVL secretion by *S. aureus* is mainly associated with necrotic suppurative lesions such as furuncles and abscesses in skin and subcutaneous tissue, and sometimes with
deep-seated infections such as necrotizing pneumonia (48). TSST-1 can activate vast numbers of T cells, triggering inappropriate cytokine release (25). Superantigenic toxins cause a variety of illnesses, ranging from toxic shock syndrome (TSS) to staphylococcal scarlet fever and neonatal toxic shock exanthematous diseases (NTED) (11,46).

In France about 700,000 cases of varicella are diagnosed annually, of which 90% involve children under 10 years of age. A French national survey of pediatric hospitalizations for varicella in 2003-2005 showed that *S. aureus* was involved in 58% of 299 documented cases of bacterial superinfection (18). Extrapolating from this survey, it would appear that *S. aureus* superinfection occurs in about 10,000 children with varicella annually.

The aims of the present study were i) to characterize the toxin profiles of *S. aureus* isolates involved in varicella superinfection, and ii) to seek correlations between toxin expression and clinical manifestations for a better understanding of physiopathology of complications and a better care of the patients.

**Patients, materials and methods**

**Patients and strain collection**

From 2002 to 2007 the French National Reference Centre for Staphylococci received 58 *S. aureus* isolates from patients with infectious complications of varicella. The isolates were submitted for toxin gene detection. A single isolate was selected per patient. For each case, demographic and clinical information was collected either passively when the French reference centre received the isolate, or actively by a retrospective search in the medical file for missing data. Clinical manifestations were subdivided into SSTIs (cellulitis, necrotizing fasciitis, abscess, bullous impetigo, and SSSS; other lesions were considered non specific) and systemic manifestations (sepsis, septic shock, staphylococcal scarlet fever, and TSS) (Table 1).
Laboratory methods

The isolates were grown on brain-heart infusion agar at 37°C overnight. Genomic DNA was extracted with a standard procedure (23). Amplification of gyrA was used to confirm the quality of each DNA extract and the absence of PCR inhibitors. The agr group (agr 1-4) was determined by PCR as previously described (23). The mecA gene (coding for methicillin resistance) was detected by PCR as described by Murakami et al. (37). Susceptibility to macrolides (erythromycin, clindamycin and pristinamycin) was determined by the agar plate disk diffusion method and interpreted with the guidelines of the Comité de l’Antibiogramme de la Société Française de Microbiologie (CA-SFM) (7).

The isolates were screened for 15 staphylococcal virulence genes (staphylococcal enterotoxin genes (sea-d, seh, sek, sel, sem, seo), toxic-shock syndrome toxin gene (tst), exfoliative toxin genes (eta, etb), PVL genes (luk-PV genes), the class F lukM leukocidin gene (lukM) and the beta-hemolysin gene (hlb)), as previously described (23). Representative isolates were spa typed as previously described (35). The repeat region of the protein A gene (spa) was amplified by PCR and sequenced. Spa types were determined with Ridom Staph Type software (Ridom GmbH, Würzburg, Germany), which automatically detects spa repeats and assigns a spa type according to Harmsen et al. (19) (http://spaserver.ridom.de). Multilocus sequence typing (MLST) was applied to representative methicillin-resistant S. aureus (MRSA) isolates, as described elsewhere (16). The allelic profiles were determined by sequencing 500-bp internal fragments of seven housekeeping genes (arcC, aroE, glpF, gmk, pta, tpi, yqiL). Nucleotide sequences were entered on the MLST home page (http://saureus.mlst.net), where seven numbers depicting the allelic profile and defining a sequence type (ST) were assigned.
Results

Between 2002 and 2007, the survey of staphylococcal infections secondary to varicella identified 58 cases (26 females and 32 males). The patients’ median age was 2 years (1 month – 22 years; mean 2.4 years). The clinical manifestations consisted of 47 SSTIs (72%) and 18 systemic disorders (28%) (Table 1). Seven patients had both local and systemic manifestations.

The diversity of agr alleles was high with 16 agr1, 25 agr2, 10 agr3 and 7 agr4 (Table 1). A major toxin gene (tst, eta/etb, luk-PV) was detected in 53% (31/58) of the isolates. Indeed, the tst and luk-PV genes were detected in 16 (28%) and 5 (9%) isolates, respectively. The eta gene was present in 10 isolates (17%), and was associated with etb in two cases. The other toxins sea, seb, sec, sed, seh, sek, sel sem and seo were found in 3, 12, 23, 13, 5, 7, 23, 37 and 37 isolates, respectively, and when isolates presenting a major toxin gene are excluded in 1, 10, 11, 2, 3, 4, 11, 15 and 15 respectively.

To identify the potential role of a toxin in the different clinical manifestations, the cases were divided into two groups (Table 1): i) the 31 cases (53%) associated with major toxins presumed to be associated with severe systemic syndromes (ETA/ETB and generalized exfoliation; PVL and necrotizing pneumonia and abscesses; and TSST-1 and toxic shock syndrome); and ii) the 27 cases associated with other toxins (such as enterotoxins) that are less clearly linked to specific clinical disorders.

Nineteen isolates (33%) harboured mecA gene and all were susceptible to clindamycin (Table 2). The molecular characterization of these MRSA isolates highlighted that most of them belonged to known community-acquired MRSA (C-MRSA) clones, harboring major toxin genes: 12 isolates belonged to Geraldine clone (agr2, ST5, spa-t002 or related, tst positive) (8), two belonged to the European ST80 clone (agr3, ST80, spa-t044, luk-PV...
positive) (47), two belonged to USA300 clone (agr1, ST8, spa-t008, luk-PV positive) (36).
The three remaining isolates clustered into two unusual sequence types (ST59 and ST88).

Discussion

The survey of staphylococcal infections secondary to varicella highlighted that i) major toxins (PVL, TSST-1 and ETA/ETB) are highly prevalent in isolates; ii) most clinical manifestations were directly compatible with the classical activity of these toxins; and iii) prevalence of C-MRSA was surprisingly high. As the survey was based on spontaneous notifications to the French reference center, a reporting bias cannot be excluded, possibly favoring cases most evocative of toxin production, particularly severe cases, or cases of treatment failure. All these features match classical attributes of C-MRSA which could explain an overestimation of this subgroup of S. aureus isolates in the present study.

Nevertheless, the patients’ median age is concordant with that observed in epidemiological studies of varicella bacterial superinfections (6,18). Moreover, the 19 isolates from Lyon’s hospitals that corresponded to almost all isolates from patients with varicella superinfection in this geographical area were not genotypically and phenotypically different from those of the overall panel of isolates, suggesting that the bias due to the spontaneous notifications to the French reference centre for staphylococci, if existing, is not major.

The isolates harboring eta, associated or not with etb, had varied genetic backgrounds, reflected by the diversity of their agr types (Table 1). The corresponding patients’ clinical manifestations (SSSS and bullous impetigo in seven and three cases, respectively) were directly compatible with classical toxin activity (2,14,30,38).

The prevalence of PVL-positive isolates (9%) was higher than that observed in a 3-year (2000-2003) French hospital survey of PVL-producing S. aureus (0.7%) (41), as well as in a recent study indicating a prevalence of 1.4% in 2008 (unpublished data). Nevertheless,
these differences are not statistically significant and a reporting bias cannot be excluded. As classically reported, all five isolates were associated with necrotizing infections (abscesses in four cases and necrotizing fasciitis in one case) (21,31).

The most prevalent toxin gene was *tst* (28%). As expected, given the well-known superantigenic activity of TSST-1, there were six cases of scarlet fever and two cases of toxic shock syndrome (30,34). More surprisingly, 11 (69%) of the *tst*-positive isolates were recovered from patients with SSTIs. These *tst*-positive isolates were exclusively associated with non-suppurative cutaneous lesions and cellulitis, contrasting with the suppurative forms associated with PVL and the exfoliative forms associated with ETA and ETB. One of the most interesting findings is that about half the isolates recovered from cellulitis carried *tst* gene (Table 1). This suggests that *tst*-associated SSTIs are due more to an inflammatory process than to pus production or to the direct action of the toxin. Four patients had concomitant toxic manifestations (scarlet fever, n=2; TSS, n=2) and SSTIs (cellulitis, n=4). Twelve of the 16 *tst*-positive isolates belonged to an emerging MRSA clone named Geraldine (8), yet they were associated with a wide variety of clinical manifestations. This clinical heterogeneity might be explained by the complex expression of *tst*, that is influenced in vitro by environmental conditions such as pH, CO$_2$ and glucose (45) and in vivo by the patient’s immunological status (39).

The remaining 27 isolates (47%) did not harbor *tst*, *eta*/*etb* or *luk*-PV. The associated clinical manifestations were highly variable, and no clear association could be established with the toxin profiles. Combined use of *agr* typing, *spa* typing and toxin profiling failed to identify a dominant clone. Twenty-one of these isolates were associated with SSTIs, including two cases of exfoliatan-negative bullous impetigo. It is conceivable that these two strains expressed variants of ETA or ETB. One strain was associated with necrotizing fasciitis, in a patient with no evidence of concomitant group A beta-haemolytic streptococcal infection (as
also in the case of necrotizing fasciitis associated with a PVL-positive strain). The other SSTIs were not associated with specific toxins. Eight isolates were associated with systemic manifestations (concomitant with SSTIs in two cases), including five cases of scarlet fever associated with strains harboring superantigenic toxin genes (seb, n=3; sec n=2). In the last three cases, involving sepsis or septic shock, two isolates were agr1, ST045, sec-positive, and sel-o-positive; while the third was agr3, ST1, sea-positive, sec-positive, seh-positive, and sel-positive.

A major finding in this study is the high frequency of meca, the gene conferring methicillin resistance: 19 (33%) of the 58 S. aureus isolates were meca-positive. In Europe, MRSA account for less than 5% of paediatric S. aureus isolates in the community setting (20,44), and for less than 11% of isolates from SSTI patients in France (10). Sixteen of the 19 MRSA isolates belonged to known C-MRSA clones. The main C-MRSA clone was the Geraldine clone (n=12, 63%). It has been described as an emerging clone in France, initially associated with both toxic shock syndrome and suppurative infections, mainly in children with a median age of 3 years (13). In a recent French multicenter study, this clone accounted for 6.3% of invasive MRSA isolates (8). Its high prevalence in the present study suggests broad geographical dissemination and frequent colonization of children. The other C-MRSA clones were sporadic. Two isolates belonged to the European PVL-positive ST80-IV clone (agr3, spa-t044) (Table 2) that is the most prevalent C-MRSA clones in Europe (47). Two other MRSA isolates shared characteristics of the American epidemic PVL-positive C-MRSA clone USA300 (agr1, ST8, spa-t008) (36,47). One isolate belonged to the ST88 eta-positive agr3 clone that has been reported frequently in Japan but rarely in Europe (28,49). The two remaining isolates were seb-positive and sek-positive, ST59, agr1 and spa-t216.

The present study described S. aureus strains involved in varicella superinfections. Varicella characteristically produces disseminated vesicular lesions at varying degrees of
maturity and is associated with severe pruritus. This leads to itching and subsequent excoriations, skin barrier disruption, exposition of tissue containing extracellular matrix underlying the cutaneous tissue and endothelium as well as plasma clots. However an extensive substrate repertoire exists among *S. aureus* adhesins (typically known as MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family such as fibronectin-binding protein A (FnBPA), collagen-binding protein (Can), fibrinogen-binding protein (ClfA, ClfB) or Sdr proteins), with many being able to bind these exposed ligands. The initial step of adherence is a crucial factor before invasion, toxin expression and/or dissemination of *S. aureus*. Staphylococcal syndromes complicating varicella infections are not strictly different from other staphylococcal infection syndromes. One of the main interests of our study is to highlight that severe forms of varicella superinfections due to *S. aureus* are mostly related to MRSA strains. Moreover, the clinical manifestations of these cases of staphylococcal superinfection in varicella patients were largely compatible with direct effects of toxins (ETA/ETB, PVL, and TSST-1), suggesting that toxin inhibition could be clinically beneficial. Various antimicrobial agents showed activity on staphylococcal and streptococcal toxin production in vitro (3,12,43). In particular, clindamycin and linezolid have inhibitory actions on protein synthesis including superantigen production. Clindamycin could appear to be an interesting therapeutic option in this setting, as i) it shows good pharmacodynamic behaviour in cutaneous tissue; ii) it had bacteriostatic activity on all 19 MRSA isolates studied here (Table 2); and iii) paediatricians have extensive experience with this antibiotic (17). Currently, there is scant published clinical evidence showing the addition of an inhibiting toxin-production antibiotic is beneficial in staphylococcal infections (42) but no comparative study using clindamycin (or linezolid) in this setting. In France where prevalence of clindamycin-resistant group A betahaemolytic streptococci is low (4,33), further studies are needed to determine the efficacy of protein synthesis inhibiting antibiotics.
either alone or in conjunction with the usual beta-lactam regimens used in varicella superinfection, especially when the clinical manifestations are evocative of toxin activity (26). In conclusion, this first toxin profiles study of *S. aureus* isolates involved in varicella superinfections shows that major toxins (TSST-1, ETA, ETB and PVL) are present in about half the cases and *mecA* gene in one-third of cases. These data must be taken into account for the management of patients and the interest of toxin-targeting treatment, such as clindamycin, would deserve to be evoked and studied for such bacterial superinfections in children with varicella.
TABLE 1. Characterization of *S. aureus* isolates associated with varicella superinfection

<table>
<thead>
<tr>
<th>Toxin genes</th>
<th>Number (%)</th>
<th>eta and/or etb&lt;sup&gt;a&lt;/sup&gt;</th>
<th>luk-PV&lt;sup&gt;b&lt;/sup&gt;</th>
<th>tst&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Others</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>10 (17.2)</td>
<td>5 (8.6)</td>
<td>16 (27.6)</td>
<td>27 (46.5)</td>
<td></td>
</tr>
<tr>
<td>Methicillin resistant (mecA +)</td>
<td>19 (33)</td>
<td>1 (5.3)</td>
<td>4 (21.1)</td>
<td>12 (63.2)</td>
<td>2 (10.5)</td>
<td></td>
</tr>
<tr>
<td>agr group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>agr1</td>
<td>16</td>
<td>-</td>
<td>2 (100)</td>
<td>1 (50)</td>
<td>13 (81.2)</td>
<td></td>
</tr>
<tr>
<td>agr2</td>
<td>25</td>
<td>4 (16)</td>
<td>-</td>
<td>12 (48)</td>
<td>9 (36)</td>
<td></td>
</tr>
<tr>
<td>agr3</td>
<td>10</td>
<td>1 (10)</td>
<td>2 (20)</td>
<td>3 (30)</td>
<td>4 (40)</td>
<td></td>
</tr>
<tr>
<td>agr4</td>
<td>7</td>
<td>5 (71.4)</td>
<td>-</td>
<td>1 (14.3)</td>
<td>1 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Skin and soft tissues infections</td>
<td>47 (72)</td>
<td>10 (21.3)</td>
<td>5 (10.6)</td>
<td>11 (23.4)</td>
<td>21 (44.6)</td>
<td></td>
</tr>
<tr>
<td>Non-specific cutaneous lesions</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>2 (22.2)</td>
<td>7 (77.8)</td>
<td></td>
</tr>
<tr>
<td>Cellulitis</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>9 (52.9)</td>
<td>8 (47.1)</td>
<td></td>
</tr>
<tr>
<td>Necrotizing fasciitis</td>
<td>2</td>
<td>-</td>
<td>1 (100)</td>
<td>- (0)</td>
<td>1 (50)</td>
<td></td>
</tr>
<tr>
<td>Abcess</td>
<td>6</td>
<td>-</td>
<td>4 (100)</td>
<td>- (0)</td>
<td>2 (50)</td>
<td></td>
</tr>
<tr>
<td>Bullous impetigo</td>
<td>6</td>
<td>3 (50)</td>
<td>-</td>
<td>- (0)</td>
<td>3 (50)</td>
<td></td>
</tr>
<tr>
<td>Scalded skin syndrome (SSSS)</td>
<td>7</td>
<td>7 (100)</td>
<td>-</td>
<td>- (0)</td>
<td>- (0)</td>
<td></td>
</tr>
<tr>
<td>Systemic manifestations</td>
<td>18 (28)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>10 (55.6)</td>
<td>8 (44.4)</td>
<td></td>
</tr>
<tr>
<td>Sepsis</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1 (100)</td>
<td>1 (50)</td>
<td></td>
</tr>
<tr>
<td>Septic shock</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>1 (66.7)</td>
<td>2 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Scarlet fever</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>6 (54.5)</td>
<td>5 (45.5)</td>
<td></td>
</tr>
<tr>
<td>Toxic shock syndrome (TSS)</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2 (100)</td>
<td>- (0)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> eta and etb: the genes coding for exfoliatin toxin A and exfoliatin toxin B

<sup>b</sup> luk-PV: the gene coding for Panton Valentine leukocidin

<sup>c</sup> tst: the gene coding for toxic shock syndrome toxin-1
<table>
<thead>
<tr>
<th>Clone designation</th>
<th>n</th>
<th>ST</th>
<th>Spa type</th>
<th>agr group</th>
<th>Toxin genes</th>
<th>Susceptibility to clindamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geraldine Clone</td>
<td>12</td>
<td>5</td>
<td>t002</td>
<td>2</td>
<td>tst, sec, sed, sei, sem, seo</td>
<td>12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>European ST80 clone</td>
<td>2</td>
<td>80</td>
<td>t044</td>
<td>3</td>
<td>luk-PV</td>
<td>2</td>
</tr>
<tr>
<td>USA 300 clone</td>
<td>2</td>
<td>8</td>
<td>t008</td>
<td>1</td>
<td>luk-PV, sek</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Undesignated</td>
<td>2</td>
<td>59</td>
<td>t216</td>
<td>1</td>
<td>seb, sek</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>88</td>
<td>t186</td>
<td>3</td>
<td>eta</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> 4 isolates resistant to erythromycin, with induction between erythromycin and lincomycin

<sup>b</sup> 1 isolate resistant to erythromycin, with no induction between erythromycin and lincomycin
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