Immunogenicity of Toxins during Staphylococcus aureus Infection

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Background. Toxins are important Staphylococcus aureus virulence factors, but little is known about their immunogenicity during infection. Here, additional insight is generated.

Methods. Serum samples from 206 S. aureus–infected patients and 201 hospital-admitted control subjects were analyzed for immunoglobulin (Ig) G binding to 20 toxins, using flow-cytometry based technology. Antibody levels were associated with polymerase chain reaction–defined presence of toxin genes in homologous S. aureus isolates.

Results. IgG levels directed to exfoliative toxin (ET) A, ETB, γ hemolysin B (HlgB), leukocidin (Luk) D, LukE, LukS, staphylococcal enterotoxin (SE) A, SEE, SEH, SEI, and SEI-M were higher in S. aureus–infected patients than in control subjects (P < .05). Furthermore, in the S. aureus–infected patient group, IgG levels were higher if genes encoding ETA, ETB, SEA, SEC, SEH, SEI-Q, toxic shock syndrome toxin–1 (TSST-1), or Panton-Valentine leukocidin (PVL) were present in the infectious isolate (P < .05). Levels of anti-SEA IgG increased during infections with sea-positive (median fluorescence intensity from 11,555 to 12,388; P < .05) but not sea-negative strains. In addition, anti-LukS IgG levels increased during skin and soft-tissue infections with luk-PV-positive (median fluorescence intensity from 15,231 to 15,911; P < .05) but not luk-PV-negative strains. Bacteremia was associated with sea (odds ratio, 3.4; 95% confidence interval, 1.2–10.0) and tst (odds ratio, 5.7; 95% confidence interval, 1.6–20.8). Skin and soft-tissue infections and bone and joint infections were associated with luk-PV (odds ratio, 2.5; 95% confidence interval, 1.2–5.2).

Conclusions. Many toxins are expressed in vivo and recognized by the immune system during staphylococcal infections, suggesting their involvement in S. aureus pathogenesis.

Staphylococcus aureus produces numerous virulence factors that contribute to its ability to cause infections [1, 2]. These include a variety of toxins that are known for their detrimental effects on cells of the immune system [3]. In addition, their toxinogenic activity is implicated in a broad range of S. aureus infections [4]. Staphylococcal toxins can be categorized in groups: pyrogenic toxin superantigens (PTSAgs), exfoliative toxins (ETs), leukocidins, and other toxins. The family of PTSAgs includes staphylococcal enterotoxins (SEs), SE-like toxins [5], and toxic shock syndrome toxin-1 (TSST-1). Superantigens cross-link major histocompatibility complex class II molecules on antigen-presenting cells with T cell receptors, which leads to massive T cell proliferation and cytokine release [6]. This disproportionate proinflammatory activity is implicated in the pathogenesis of food poisoning and toxic shock syndrome [3, 7]. ETs are responsible for staphylococcal scalded skin syndrome and bullous impetigo [8]. Thus far, 4 ETs are known, and 3 of these (ETA, ETB, and ETD) are linked to human infection [9]. Leukocidial toxins constitute a family of pore-forming toxins that are composed of 2 distinct components. The toxic effect depends on the synergistic action of both class S and F proteins on human neutrophils or erythrocytes. Members of the leukotoxin family are LukD, LukE, LukM, γ hemolysin (Hlg), and Panton-Valentine leukocidin (PVL) [10, 11]. PVL is associated with
necrotizing pneumonia, bone and joint infections, epidemic furunculosis, and abscesses in humans [12–15]. Toxins of the epidermal cell differentiation inhibitor family inactivate GTPases and thereby block important immune cell functions, such as chemotaxis and phagocytosis [16].

Despite the fact that toxins are important staphylococcal virulence factors and that the prevalence of S. aureus infection is continuously increasing [17], little is known about the immunoglobulin (Ig) G response directed against PTSAsgs, ETs, and leukocidins during S. aureus infection in humans. By studying the immune response, important information can be collected concerning the antigenicity and in vivo expression of the toxins. This may increase knowledge on pathogenesis of S. aureus infection and might contribute to the development of new measures against staphylococcal disease. We studied the anti-toxin humoral immune response to 20 toxins in a large number of S. aureus–infected patients and hospital-admitted control subjects. Furthermore, we associated antibody levels with the presence of toxin genes in infectious S. aureus isolates, and we related toxin gene presence to different types of staphylococcal infection.

**MATERIALS AND METHODS**

**Collection of serum and S. aureus strains.** S. aureus isolates and 2 serum samples were collected from 206 S. aureus–infected patients in the Mustapha Pacha hospital (Algiers, Algeria) during 2006–2007. The first serum sample was obtained 5 days (range, 0–20 days) after strain identification. The second sample was collected 14 days (range, 7–34 days) thereafter. Serum samples were stored at −80°C until use. Data on sex, age, hospital ward, and type of infection were recorded. Isolates were considered to be community acquired if a sample obtained within 48 h after admission was culture positive for S. aureus. Isolates obtained later were considered to be hospital acquired. Furthermore, serum samples were collected for control patients (n = 201). Control patients were admitted in the same period but did not have an overt S. aureus infection. Patients with an immunocompromised status (eg, human immunodeficiency virus–positive patients or patients receiving corticosteroids or other immunosuppressive therapies) were excluded. Adult patients were defined as those aged ≥18 years. All patients provided written informed consent, and the local Medical Ethics Committee of the Mustapha Pacha hospital approved the study.

**Bacterial identification and toxin gene detection.** S. aureus was identified on the basis of colony and microscopic morphology, coagulase testing with rabbit plasma (bioMérieux) and the Staphyslide agglutination test (bioMérieux). The identification was confirmed by multiplex polymerase chain reaction (PCR) amplification of the accessory gene regulator (agr) [18] and by determining theagr allelic group. The isolates were PCR-screened for genes encoding methicillin resistance (mecA); SE A, B, C, D, H, K, L, M, O, P, Q, and R (set-a-d, seh, selk-m, and selo-r); TSST-1 ( tst); ETA, ETB, and ETD ( eta, etb, and etd); PVL ( luk-PV); class F LukM leukocidin (lukM); HlgB (hlgb); and epidermal cell differentiation inhibitor (edin), as described elsewhere [18, 19].

**Recombinant toxin production.** The toxins SEC, SED, SEE, SEH, SEI, SEJ, SEI, SEIQ, SER, ETA, ETB, LukF, and LukS were produced as described elsewhere [20–22]. The S. aureus strains listed in Table 1 were used to produce the other toxins. Esch-
Figure 1. A, Toxin-specific immunoglobulin (Ig) G levels in Staphylococcus aureus–infected patients versus control patients and adults versus children. IgG levels are reflected by median fluorescence intensity (MFI) values. Black arrows indicate significant differences between the \textit{S. aureus}–infected patient and control patient group (\textit{P} < .05, by Mann Whitney \textit{U} test). Results are shown for the first serum sample. B, Differences in toxin-specific IgG level between the first and second serum sample and between different types of \textit{S. aureus} infections in adults. The first bar of each pattern represents median IgG level in the first serum sample; the second bar of the same pattern represents median IgG level in the second serum sample. ET, exfoliative toxin; HlgB, \textgamma{} hemolysin B; Luk, leukocidin; SE, staphylococcal enterotoxin; SE, staphylococcal enterotoxin-like; TSST-1, toxic shock syndrome toxin-1.

\textit{E. coli} M15 (Qiagen) and \textit{E. coli} BL21 pLys (Invitrogen) were used for plasmid amplification and genetic manipulations. Primers were designed following the identification of suitable hybridization sites in the toxin genes (Table 1). Chromosomal DNA of \textit{S. aureus} was extracted and used as a template for PCR amplification as described previously \cite{23}. The \textit{S. aureus} chromosomal DNA was digested with appropriate restriction enzymes (Promega), purified with the High Pure PCR Product Purification kit (Roche) and ligated using T4 DNA Ligase (Roche) in either the pQE-30, pQE-70 (Qiagen), or pIVEX 2.4d expression vector (Roche) digested with the restriction enzymes described in Table 1 \cite{24}. The resulting plasmids were transformed into \textit{E. coli} strain M15. For toxin expression in pIVEX 2.4d, the vector was transformed into \textit{E. coli} strain DH5\textalpha{} (Invitrogen) before transformation into \textit{E. coli} strain BL21 pLys. Protein purification. Transformed \textit{E. coli} cells growing exponentially in Luria-Bertani (LB) medium supplemented with ampicillin 100 \textmu{}g/mL were inoculated into 1 liter of fresh LB medium and incubated with continuous rotary shaking for 2–3 h at 37\textdegree{}C until \textit{OD}_{600} 0.5–0.7. Then, the expression was induced by adding isopropyl-\textbeta{}-\textgamma{}-thiogalactopyranoside to a final concentration of 1 mM for 5 h. The cultures were then centrifuged at 4000 \textit{g} for 20 min at 4\textdegree{}C. The cell pellets were stored overnight at −80\textdegree{}C. The cell pellets were thawed for 15 min on ice and resuspended in lysis buffer (Qiagen). The lysates were sonicated on ice after adding lysosome. Then, the lysates were centrifuged at 10,000 \textit{g} for 30 min at 4\textdegree{}C and the supernatants were collected. The His-tagged proteins were purified on Ni-nitrilotriacetic acid columns (Qiagen) and dialyzed against phosphate-buffered saline. Protein concentrations were determined according to the Bradford method, using bovine serum albumin as the standard. The toxins were quality controlled by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and mass spectrometry (Ultraflex MALDI-ToF; Bruker Daltonics).

Measurement of anti-toxin antibodies. Levels of IgG directed against the toxins ETA, ETB, HlgB, LukD, LukE, LukF, LukS, SEA, SEC, SE, SEE, SEG, SEH, SEI, SEI\textalpha{}, SEI\textbeta{}, SEI\gamma{}, SER, and TSST-1 were quantified simultaneously using a bead-based flow cytometry technique (xMap; Luminex Corporation). Methods were as described before \cite{25–27}. The assays were performed in independent duplicates, and the median fluorescence intensity (MFI) values, reflecting semi-quantitative antibody levels, were averaged. In each experiment, control beads (no toxin coupled) were included to determine nonspecific binding. In the event of nonspecific antibody binding, the nonspecific MFI values were subtracted from the antigen-specific results. Human pooled serum was used as a standard \cite{26}. Statistical analysis. Statistical analyses were performed with SPSS software, version 15.0 (SPSS). Kruskal-Wallis and Mann-Whitney \textit{U} tests were used to compare differences in...
The MFI values obtained for human pooled serum with female ratio was 1.1:1. Median age was 45 years (range, 0–92 years) and the male-to-female ratio of 201 control patients consisted of 143 adults and 58 children. Their median age was 40 years (range, 0–84 years). Male-to-female ratio was 1.5:1. Of the 206 patients, 142 (69%) had skin and soft tissue infection; 4 (2%) had ear, nose, or throat infection (eg, sinusitis); 2 (1%) had urinary tract infection (eg, bronchitis); 3 (1%) had ocular infection (eg, conjunctivitis or endophthalmitis); 12 (6%) had respiratory infections (eg, arthritis or osteitis); 18 (9%) had bacteremia; 22 (11%) had bone or joint infections (SSTIs; eg, furunculosis, infected skin lesions, or folliculitis); 11 (5%) had skin and soft tissue infection; tst, toxic shock syndrome toxin-1.

### RESULTS

#### Patient characteristics.

The group of 206 *S. aureus* infected patients consisted of 183 adults and 23 children. Their median age was 45 years (range, 0–92 years) and the male-to-female ratio was 1.1:1. Of the 206 patients, 142 (69%) had skin and soft tissue infections (SSTIs; eg, furunculosis, infected skin lesions, or folliculitis); 18 (9%) had bacteremia; 22 (11%) had bone or joint infections (eg, arthritis or osteitis); 12 (6%) had respiratory infection (eg, bronchitis); 3 (1%) had ocular infection (eg, conjunctivitis or endophthalmitis); 2 (1%) had urinary tract infection; 4 (2%) had ear, nose, or throat infection (eg, sinusitis); and 3 (1%) had central nervous system infection. The group of 201 control patients consisted of 143 adults and 58 children. Median age was 45 years (range, 0–92 years) and the male-to-female ratio was 1.1:1. The *χ²* test was applied for categorical variables and binary logistic regression to calculate odds ratios (ORs). To compare antibody levels in the first and second serum sample of each patient, the Wilcoxon matched pairs signed rank test was used. *P* < .05 was considered statistically significant.

#### Validation and inter-assay variation of the toxin multiplex assay.

The MFI values obtained for human pooled serum with the multiplex assay (serum incubated with the differently fluorescence-colored antigen-coupled beads mixed in 1 well) were between 90% and 111% (median 96%) of the MFI values obtained with the individual assays (serum incubated with each individual color of antigen-coupled beads in separate wells). Therefore, the multiplex assay was considered reliable. Inter-assay variation was calculated from MFI values obtained for human pooled serum, which was included on each 96-wells plate. MFI values were averaged per protein. The median coefficient of variation was 11% (range, 5%–18%), which is comparable to what was found in other studies [25, 28].

#### Differences in anti-toxin IgG levels between distinct types of *S. aureus* infection.

Antibody levels to 20 *S. aureus* toxins were measured. The toxin-specific antibody levels showed extensive inter-individual variability. IgG levels directed to 11 of 20 toxins (ETA, ETB, HlgB, LukD, LukE, LukS, SEA, SEE, SEH, SEL, and SEIM) were significantly higher in adult *S. aureus*-infected patients than in adult control patients in both the first and second serum sample (*P* < .05) (Figure 1A). Adult patients with respiratory infections, as opposed to other *S. aureus* infections, had higher levels of IgG to SED (median MFI value, 2824 vs 1071; *P* < .01), SEL (5510 vs 2815; *P* < .01), and SER (461 vs 133; *P* < .05) (Figure 1B). In adult patients with SSTI, the level of anti-ETA IgG was elevated (5657 vs 2848; *P* < .05).

### Table 2. Presence of Toxin Genes in *Staphylococcus aureus* Strains Isolated from Patients with Different Types of *S. aureus* Infection

<table>
<thead>
<tr>
<th>Toxin gene</th>
<th>SSTI (n = 142)</th>
<th>Bacteremia (n = 18)</th>
<th>Bone infection (n = 22)</th>
<th>Respiratory infection (n = 12)</th>
<th>Other infection (n = 12)</th>
<th>Total (n = 206)</th>
<th>OR (95% CI)</th>
<th><em>P</em></th>
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<td>2 (17)</td>
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<td>1 (5)</td>
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<td>16 (8)</td>
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</tr>
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<td>1 (8)</td>
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<td>.009</td>
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<td>1 (1)</td>
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<td>5 (42)</td>
<td>94 (46)</td>
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<td>5 (28)</td>
<td>11 (50)</td>
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<td>3 (25)</td>
<td>94 (46)</td>
<td>2.5 (1.2–5.2)</td>
<td>.015</td>
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<td>4 (33)</td>
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<td>101 (49)</td>
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**NOTE.** Boldfaced font indicates genes that are significantly more prevalent in strains isolated from a particular type of infection. CI, confidence interval; edin, epidermal cell differentiation inhibitor; et, exfoliative toxin; hlgB, γ hemolysin; luk-PV, Panton-Valentine leukocidin; NS, not significant; OR, odds ratio; se, staphylococcal enterotoxin; sel, staphylococcal enterotoxin-like; SSTI, skin and soft-tissue infection; tst, toxic shock syndrome toxin-1.

* a ORs were calculated by use of binary logistic regression.
In the course of SSTI, respiratory and bone and joint infections, IgG levels showed no increase. In bacteremic patients, the level of IgG directed to HlgB, LukD, LukE, LukF, and LukS, and SEE seemed to increase in the course of infection but this increase was not statistically significant ($P = .05$2–.084) (Figure 1B).

In S. aureus–infected children, IgG levels directed to 6 of 20 proteins (ETA, HlgB, LukD, LukE, LukF, and LukS) were significantly higher ($P < .05$) (Figure 1A) than in children without S. aureus infections. Surprisingly, the level of anti-TSST-1 IgG was higher in young control patients (median MFI 4804 vs 8195; $P < .05$) than in patients with S. aureus infections. The prevalence of anti-TSST-1 IgG was significantly higher in children than adults ($P < .05$). No difference was shown in anti-LukD, anti-LukF, and anti-SER IgG. In the control group, IgG levels directed to all proteins were significantly higher in adults than children ($P < .001$) (Figure 1A).

**Prevalence of genes encoding S. aureus toxins.** The prevalence of genes encoding S. aureus toxins in the 206 infectious isolates is shown in Table 2. Of 206 isolates, 182 (88%) harbored $\geq$1 toxin gene. The most prevalent genes were edin, luk-PV, and etd, which were detected in 49%, 46%, and 46% of isolates, respectively. Genes encoding SED, SER, and LukM were found in none of the isolates. Bacteremia was associated with a higher prevalence of the sea and tst gene. Isolates recovered from 6 (33%) of 18 bacteremic patients versus isolates from 24 (13%) of 188 other patients were sea positive. Isolates from 4 (22%) of 18 bacteremic patients versus isolates from 9 (5%) of 188 other patients were tst positive ($P < .05$ and $P < .01$, respectively) (Table 2). SSTI and bone and joint infections were associated with a higher prevalence of luk-PV ($P < .05$). Isolates recovered from 82 (50%) of 164 patients with SSTI or bone infection versus 12 (29%) of 42 other patients were luk-PV positive ($P < .05$) (Table 2).

Out of all infectious S. aureus isolates, 112 (54%) were methicillin susceptible (MSSA), and 94 (46%) were methicillin resistant (MRSA). The prevalence of sea, seb, sec, seh, sell, selm, selq, tst, and hlgb was higher among MSSA stains; etd, luk-PV, and edin were more prevalent among MRSA strains ($P < .05$) (Table 3). Within the group of MSSA or MRSA infections, the prevalence of toxin genes was not significantly different between hospital-acquired and community-acquired infections (Table 3).

**Association of anti-toxin IgG levels with presence of toxin genes in infectious S. aureus isolates.** Data on both gene presence and anti-staphylococcal antibody levels were available for 13 combinations (ETA, ETB, SEA, SEC, SED, SEH, SELM, SELQ, SER, TSST-1, HlgB, LukS, and LukF). There were no sed- and ser-positive isolates. Antibody levels were elevated if the gene was present in 8 (eta, etb, sea, sec, seh, selq, tst, and luk-PV) of 11 combinations ($P < .05$) (Figure 2). Furthermore, in patients with sea-positive S. aureus infection, the anti-SEA

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### Table 3. Difference in Presence of Toxin Genes between Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Methicillin-Susceptible *S. aureus* (MSSA) Isolates

<table>
<thead>
<tr>
<th>Toxin gene</th>
<th>MSSA ($n = 112$)</th>
<th>MRSA ($n = 94$)</th>
<th>HA-MSSA ($n = 66$)</th>
<th>CA-MSSA ($n = 48$)</th>
<th>HA-MRSA ($n = 61$)</th>
<th>CA-MRSA ($n = 33$)</th>
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</tr>
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<td>0 (0)$^b$</td>
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</tr>
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<td>47 (77)</td>
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<td>edin</td>
<td>21 (19)</td>
<td>80 (85)$^b$</td>
<td>13 (20)</td>
<td>8 (17)</td>
<td>50 (82)</td>
<td>30 (91)</td>
</tr>
<tr>
<td>hlgb</td>
<td>12 (11)</td>
<td>0 (0)$^a$</td>
<td>8 (12)</td>
<td>4 (9)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

**NOTE.** CA, community acquired; HA, hospital acquired.

- $^a$ $P$ values <.05 (by the $\chi^2$ test) were considered to be statistically significant.
- $^b$ $P < .01$.
- $^c$ $P < .05$. 

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*Immunogenicity of S. aureus Toxins • CID 2010:50 (1 January) • 65*
IgG titers increased significantly in the course of infection (median MFI, from 11,555 to 12,388; P < .05). In addition, in patients with a SSTI caused by a luk-PV-positive strain, the level of anti-LukS (not anti-LukF) IgG increased significantly (median MFI, from 15,231 to 15,911; P < .05). No increase in IgG level was seen in sea- and luk-PV-negative S. aureus infections.

DISCUSSION

For the majority of toxins, anti-staphylococcal IgG levels were higher in adults than children and IgG levels were higher in S. aureus–infected patients than in hospital-admitted control subjects (Figure 1A and 1B). This suggests that the anti-staphylococcal humoral immune state of an individual develops over the years and probably depends on the history of confrontations with S. aureus. IgG levels in serum of patients were higher if the toxin gene was present in their infectious isolates in 8 of 11 combinations (Figure 2). This indicates that eta, etb, sea, sec, seh, selq, tst, and luk-PV are actively expressed and stimulate the humoral immune system during S. aureus infections. For PVL and SEA, there was additional support for expression in vivo during S. aureus infections, as was found earlier by Croze et al [29] for PVL. The level of anti-SEA IgG increased in a time period of 2 weeks during infections caused by sea-positive but not sea-negative strains. The level of anti-SEA IgG did not increase, although earlier studies observed cross-reactivity with anti-SEA antibodies [36]. Additionally, the level of anti-LukS antibodies increased in the course of two weeks in patients suffering from SSTI caused by luk-PV-positive but not luk-PV-negative strains (P < .05). In contrast to LukS, IgG levels to LukF were not higher if isolates were luk-PV positive, and no increase anti-LukF IgG was detected in patients with a luk-PV-positive SSTI. In mice, a dominant anti-LukS IgG2a and 2b response developed after immunizing mice subcutaneously with both components. Furthermore, intranasally-vaccinated mice generated anti-LukS IgA, but not anti-LukF IgA [30]. Therefore, it seems that LukS is the dominant antigenic protein subunit, in mice as well as in humans.

For S. aureus infections that were caused by strains other than strains positive for sea or luk-PV, the level of toxin-specific IgG showed no increase in the course of infection. This suggests that, in the case of SSTI, respiratory and bone and joint infections, these infections did not elicit a strong systemic humoral immune response. Alternatively, differences in antibody levels between the 2 samples were nonsignificant because of the high premorbid anti-toxin IgG levels. High preexisting IgG levels might be the result of the high incidence of S. aureus infections in this particular population. Subsequently, this would suggest that these antibodies do not protect against these types of staphylococcal infection. Preformed anti-toxin IgG might also explain the high anti-SED and anti-SER IgG levels that were found in patients with a respiratory infection, even though the causative S. aureus isolates were sed and ser negative.

Bacteremia was associated with a high prevalence of sea and tst, SSTI and bone and joint infections with a high prevalence of luk-PV, in agreement with earlier studies [13, 31–33]. The most prevalent genes in the 206 clinical S. aureus isolates of
Algerian patients were edin (49%), luk-PV (46%), and etd (46%) (Table 2). These genes were more prevalent among MRSA than among MSSA strains (P < .05) (Table 3). Likely, this is due to the frequent occurrence of the luk-PV-, etd-, and edin-positive MRSA-ST80 clone that is predominant in Algeria [34, 35].

We measured IgG binding to toxins, but we do not have data on neutralizing capacity and cross-reactivity of these antibodies. In earlier studies, neutralizing capacity for anti-TSST-1, SEA, SEB, SEC, and SEE antibodies was observed [6]. Cross-reactivity was shown against anti-SEA and anti-SEE antibodies and between anti-SEB and anti-SEC antibodies [36, 37]. For other enterotoxins, antibody titers specific for heterologous toxins were 10-fold lower than those directed against the toxin used for immunization, which argues against a strong cross-reactivity [6, 38]. Knowledge on cross-reactivity and functionality of the anti-toxin antibodies should be increased, though. Furthermore, we do not know the S. aureus carrier state of the patients. Because nasal carriers of S. aureus have an increased risk of infection and nearly 80% of the infections are endogenous, their level of IgG might be influenced by colonization and/or previous infections with their colonizing strain [39, 40]. Therefore, including nasal swab cultures in future studies is important.

In conclusion, during S. aureus infection, the toxins ETA, ETB, SEA, SEC, SEH, SElQ, TSST-1, and Luk-PV are actively expressed and recognized by the humoral immune system. S. aureus bacteremia is associated with a high prevalence of sea and tst whereas SSTI and bone and joint infections are associated with presence of luk-PV. Significant increases in anti-SEA IgG and anti-Luk-PV IgG levels are observed during all types of infection and SSTI, respectively, suggesting their involvement in staphylococcal pathogenesis.

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