Associations of pathogen-specific and host-specific characteristics with disease outcome in patients with Staphylococcus aureus bacteremic pneumonia

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Abstract

Objective. To understand the relationships of Staphylococcus aureus (SA) bacteremic pneumonia (SABP) outcome with patient-specific and SA-specific variables. Methods. We analysed SA bloodstream isolates and matching sera in SABP patients by sequencing SA isolates (n = 50) and measuring in vitro AT production, haemolytic activity and expression of ClfA and ClfB. Controls were sera from gram-negative bacteremia patients with or without pneumonia and uninfected subjects. Levels of IgGs, IgMs and neutralizing antibodies (NAbs) against SA antigens were quantified and analysed by one-way ANOVA. Associations of patient outcomes with patient variables, antibody levels and isolate characteristics were evaluated by univariate and multivariate logistic regression analyses. Results. SABP patients had higher levels of IgGs against eight virulence factors and anti-alpha toxin (AT) NAbs than uninfected controls. Levels of IgG against AT and IgMs against ClfA, FnbpA and SdrC were higher in clinically cured SABP patients than in clinical failures. Anti-LukAB NAb levels were elevated in all cohorts. Increased odds of cure correlated with higher haemolytic activity of SA strains, longer time between surgery and bacteremia (>30 days), longer duration of antibiotic therapy, lower acute physiology and total APACHE II scores, lack of persistent fever for >72 h and higher levels of antibodies against AT (IgG), ClfA (IgM), FnbpA (IgM) and SdrC (IgM). Discussion. Limitations included the cross-sectional observational nature of the study, small sample size and inability to measure antibody levels against all SA virulence factors. Conclusion. Our results suggest that SABP patients may benefit from immunotherapy targeting multiple SA antigens.

Keywords: antibody response, bacteremia, patient outcome, pneumonia, Staphylococcus aureus, virulence
INTRODUCTION

Staphylococcus aureus is a common bacterial pathogen that causes a multitude of life-threatening infections. In the United States alone, S. aureus infections result in more than 11 000 deaths each year, along with an estimated annual cost of $14 billion, and S. aureus pneumonia accounts for an estimated 50 000 infections per year. S. aureus bacteremic pneumonia is associated with a high mortality rate (30-day mortality, 46.9%). The increase in antibiotic resistance among S. aureus is an alarming concern. Antibiotic resistance among S. aureus infections result in more than 1000 deaths each year, along with an estimated annual cost of $14 billion, and S. aureus bacteremic pneumonia accounts for an estimated 50 000 infections per year. S. aureus bacteremic pneumonia is associated with a high mortality rate (30-day mortality, 46.9%). The increase in antibiotic resistance among S. aureus is an alarming concern. Antibiotic resistance among S. aureus infections result in more than 1000 deaths each year, along with an estimated annual cost of $14 billion, and S. aureus bacteremic pneumonia accounts for an estimated 50 000 infections per year. S. aureus bacteremic pneumonia is associated with a high mortality rate (30-day mortality, 46.9%). The increase in antibiotic resistance among S. aureus is an alarming concern.

Staphylococcus aureus possesses an arsenal of virulence factors, many of which are employed to evade and counteract the host immune system. Recent studies have correlated high preexisting serum antibody levels against several S. aureus virulence factors, including alpha toxin (AT), with a decreased risk of S. aureus infections and improved patient outcomes.

The aim of this study was to understand the relationships among patient variables, bacterial strain characteristics, antibody response and clinical outcomes by analysing S. aureus bloodstream isolates along with matching sera and clinical data in a cohort of patients with S. aureus bacteremic pneumonia. For comparators, we included cohorts of patients with gram-negative bacteremic pneumonia, patients with gram-negative bacteremia without pneumonia and uninfected control subjects who were matched to the cohort with S. aureus bacteremic pneumonia by the number of samples and patient demographic characteristics such as age, gender and race.

RESULTS

Comparison of serum IgG and NAb levels among study cohorts

Our recent studies demonstrated elevated serum levels of anti-AT immunoglobulin G (IgG) and neutralising antibodies (NAbs) in haemodialysis and surgery patients with S. aureus bacteremia in comparison with healthy control subjects. To perform a similar assessment in this study and to expand the analysis beyond AT, we measured the serum levels of IgG and IgM against additional S. aureus secreted toxins and surface proteins, such as delta toxin, clumping factor A (ClfA), ClfB, fibronectin-binding protein (FnbpA), leukocidin AB (LukAB), lipoprotein component C of ABC manganese transporter (MntC), serine-aspartate repeat-containing protein C (SdrC) and toxic shock syndrome toxin-1 (TSST-1). These antigens were previously validated as important virulence factors involved in adhesion, evasion of immune responses, cell and tissue damage, biofilm formation and inflammation. As shown in Figure 1, patients with S. aureus bacteremic pneumonia had elevated levels of IgGs against eight of nine measured antigens as compared with uninfected control subjects: AT (3.2-fold, \(P = 0.0002\)), delta toxin (2.95-fold, \(P = 0.0003\)), ClfA (2.51-fold, \(P = 0.0006\)), ClfB (1.9-fold, \(P = 0.01\)), FnbpA (2.12-fold, \(P = 0.0042\)), SdrC (1.74-fold, \(P = 0.0225\)), LukAB (3.9-fold, \(P < 0.0001\)) and MntC (7.28-fold, \(P < 0.0001\)). In contrast, serum S. aureus-specific IgG levels in patients with gram-negative bacteremia (with and without pneumonia) were similar to those in uninfected subjects. Levels of IgG against AT, as well as IgM against ClfA, FnbpA and SdrC, were higher in patients with cured S. aureus bacteremic pneumonia than in those with clinical failure.

Next, we determined serum levels of anti-AT and anti-LukAB NAbs (Figure 2). Anti-AT NAb levels were higher in patients with S. aureus bacteremic pneumonia than in uninfected subjects (2.12-fold, \(P = 0.0236\) overall, and 3.11-fold, \(P = 0.0101\) in cured patients), whereas no such differences were observed in patients with gram-negative bacteremia. Interestingly, compared with uninfected subjects, the levels of anti-LukAB NAbs were higher in patients with S. aureus bacteremic pneumonia (2.66-fold, \(P = 0.0041\)) and were elevated to an even greater extent in those with gram-negative bacteremia without pneumonia and those with gram-negative bacteremic pneumonia (4.26-fold, \(P < 0.0001\), and 5.32-fold, \(P = 0.001\), respectively). We did not observe significant differences in either anti-AT or anti-LukAB NAb levels between cured patients and those with clinical failure in any of the study cohorts. We observed a high correlation between levels of IgG and NAbs against both AT (\(r = 0.8698\); 0.7952 and 0.7911) and LukAB (\(r = 0.9044\); 0.8259 and 0.7649) in patients with S. aureus bacteremic pneumonia, gram-negative bacteremic pneumonia and gram-negative bacteremia without pneumonia, respectively. There was no correlation between...
Figure 1. Serum anti-AT IgG and IgM levels. (a) A box plot is presented for each cohort and is overlaid with values from individual subjects. The dashed line represents the assay’s lower limit of quantitation (LLOQ). Values below the LLOQ were imputed with LLOQ/2. Each sample was tested in duplicate. (b) Ratios of geometric mean level for each disease cohort relative to uninfected control group and clinical outcome for the cohort with *Staphylococcus aureus* bacteremic pneumonia relative to uninfected control group and cure versus failure, with 95% CIs and *P*-values. Fill colours ranging from purple to red represent lower to higher compared with the uninfected control group.
levels of IgG and NAbs against either AT ($r = 0.0671$) or LukAB ($r = 0.2409$) in uninfected control subjects.

**Characterisation of *S. aureus* isolates from patients with *S. aureus* bacteremic pneumonia**

To assess the relationship between *S. aureus*-specific factors and clinical outcomes, we characterised *S. aureus* bloodstream isolates from patients with *S. aureus* bacteremic pneumonia collected during the acute phase of infection. AT production was heterogeneous (96% positive by enzyme-linked immunosorbent assay [ELISA]; range, 0–70 μg mL$^{-1}$), and the majority of strains ($n = 47$) co-expressed ClfA and ClfB (data not shown). The phylogenetic relatedness, AT production, haemolytic activity and genotypic characteristics of *S. aureus* isolates in relation to *S. aureus* bacteremic pneumonia cure or failure are summarised in Figure 3. All isolates carried hld, clfA, clfB, sdrC, lukAB and mntC genes. We identified three major groups of isolates based on their position on the phylogenetic tree, multilocus sequence types (MLSTs) and presence or absence of genes of interest. The first group (Figure 3, top; $n = 23$) is predominantly composed of ST8 mecA-positive strains that carry the fnbpA gene. A majority of ST8 strains in this study (15 of 18) had elevated haemolytic activity above 100 haemolytic units (HU) per millilitre, which did not always correspond to high AT production and suggests that additional toxins other than AT contribute to

![Figure 2](image_url). Serum anti-AT and anti-LukAB NAb levels. (a) A box plot is presented for each cohort and is overlaid with values from individual subjects. The dashed line represents the assay’s LLOQ. Values below the LLOQ were imputed with LLOQ/2. Each sample was tested in duplicate. (b) Ratios of geometric mean level of anti-AT and anti-LukAB NAbs for each cohort relative to the uninfected control group and clinical outcome relative to uninfected control group and cure versus failure, with 95% CIs and P-values. Fill colours ranging from purple to red represent lower to higher compared with the uninfected control group.
haemolytic activity in these strains. The second group (Figure 3, middle; \( n = 9 \)) includes a cluster of seven strains that have a Q113B stop codon in the \( hla \) gene, belong to sequence types 30 and 36 and carry the \( tst \) gene, which is consistent with previously reported findings.\(^{13,23–25}\) The third group (Figure 3, bottom; \( n = 18 \)) combines \( tst \)-negative and predominantly \( fnbpA \)-negative strains of various sequence types. One of the strains from this group (and the only strain in the collection) is null for the \( hla \) gene, and its MLST type (ST 225) is related to previously described \( hla \) gene-null isolates.\(^{26}\) We did not observe any definitive association of patient outcomes with either phylogenetic grouping or genotypic characteristics of \( S. aureus \) isolates from the cohort of patients with \( S. aureus \) bacteremic pneumonia.

We explored the relationships between certain phenotypic characteristics of these isolates, such as methicillin susceptibility, haemolytic activity and AT production, with patient outcomes. A correlation was observed between AT production and haemolytic activity (Figure 4), which was consistent with methicillin-resistant \( S. aureus \) and methicillin-susceptible \( S. aureus \). Paradoxically, higher haemolytic activity and \textit{in vitro} AT production were both associated with patient outcomes.
cure (Figure 5), which agrees with our previous observations in haemodialysis and postsurgical patients with S. aureus bacteremia.\textsuperscript{13} Both AT production and haemolytic activity were higher in methicillin-resistant than in methicillin-susceptible S. aureus isolates.

**Patient outcome analyses**

To understand associations of S. aureus- and patient-specific factors with clinical outcomes, we performed univariate analyses of patient demographics, clinical variables and S. aureus factors in relation to cure or failure (Tables 1 and 2; Figure 6a–c). Increased odds of cure were associated with longer time between surgical procedure and bacteremia (> 30 days) (odds ratio [OR], 3.93; 95% confidence interval [CI], 1.03–14.99; \(P = 0.0452\)); longer duration of antibiotic therapy (OR, 1.27; 95% CI, 1.03–1.56; \(P = 0.0259\)); lower acute physiology score (OR, 0.32; 95% CI, 0.12–0.84; \(P = 0.0215\)); total Acute Physiologic Assessment and Chronic Health Evaluation II (APACHE II) score (OR, 0.24; 95% CI, 0.09–0.67; \(P = 0.0064\)); lack of persistent fever for more than 72 h (OR, 0.26; 95% CI, 0.07–0.96; \(P = 0.0424\)); and higher levels of anti-AT IgG (OR, 2.64; 95% CI, 1.05–6.63; \(P = 0.0392\)) and IgM against ClfA (OR, 4.32; 95% CI, 1.45–12.85; \(P = 0.0086\)), FnbpA (OR, 4.65; 95% CI, 1.35–16.07; \(P = 0.0151\)) and SdrC (OR, 10.10; 95% CI, 2.04–49.92; \(P = 0.0046\)). In contrast, no significant associations were observed between odds of cure and characteristics of S. aureus isolates.

In addition to univariate analyses, we performed multivariate modelling to identify a combination of independent variables associated with clinical outcomes. Increased odds of cure were associated with higher levels of IgM against SdrC (OR, 13.95; 95% CI, 1.18–164.49; \(P = 0.0363\)) or ClfA (OR, 5.72; 95% CI, 1.16–28.24; \(P = 0.0322\)), elevated haemolytic activity of S. aureus isolates (OR, 2.94; 95% CI, 1.05–8.27; \(P = 0.0409\)) and lower total APACHE II score (OR, 0.34; 95% CI, 0.11–1.05; \(P = 0.0605\)) (Table 2; Figure 6d, e). Total APACHE II score alone was predictive of clinical outcome, and predictive performance was further improved by additional variables in this multivariate model.

**DISCUSSION**

Our results demonstrated associations of clinical cure in patients with S. aureus bacteremic pneumonia with increased haemolytic activity of the infecting strains and various patient characteristics (longer time between surgery and onset of bacteremia, lower APACHE II scores, shorter persistent fever, longer duration of antibiotic therapy). Higher levels of IgG against AT and IgM against ClfA, FnbpA and SdrC were observed in cured patients than in patients with clinical failure. We hypothesise that elevated levels of antibodies specific to these antigens may correlate with protection against S. aureus bacteremic pneumonia.

In this study, irrespective of any specific S. aureus clonal lineage or clade, higher haemolytic activity and in vitro AT production were both associated with patient cure, in agreement with our prior findings in haemodialysis and postsurgical patients with S. aureus bacteremia.\textsuperscript{13} We previously hypothesised that the paradoxical correlation between AT production and patient survival in S. aureus bacteremia resulted from a stronger overall immune response against S. aureus because of AT proinflammatory properties.\textsuperscript{13} In agreement with this hypothesis, our results showed higher levels of IgG against a variety of S. aureus secreted toxins and surface proteins in patients with S. aureus bacteremic pneumonia than in uninfected control subjects and patients with gram-negative bacteremia. Reduced virulence of AT-hyperproducing S. aureus strains has been observed in a rabbit model of experimental endocarditis, and such a paradoxical
association between high AT production and decreased virulence may result from higher AT-mediated release of cationic platelet microbicidal proteins.27

Staphylococcal surface proteins such as ClfA, FnbpA and SdrC have been shown to facilitate attachment of S. aureus to host tissue cells and initiation of infection.16,28–30 We observed direct associations between elevated serum levels of IgM against ClfA, FnbpA and SdrC and increased odds of cure, which may suggest the importance of targeting these adhesins early at the onset of infection for favorable clinical outcomes in patients with S. aureus bacteremia pneumonia.

LukAB (LukGH) is a bicomponent leukotoxin that is essential for S. aureus escape from within polymorphonuclear leucocytes (PMNLs) and S. aureus-mediated killing of PMNLs.31,32 Both S. aureus components and lipopolysaccharide of gram-negative bacteria induce high sensitivity of human PMNLs to LukAB.33 We hypothesise that the elevated serum levels of anti-LukAB NAbs that were observed in this study in patients with S. aureus bacteremic pneumonia and in those with gram-negative bacteremia may be important in protecting PMNLs from LukAB-mediated killing.

We observed a high correlation between levels of IgG and NAbs against both AT and LukAB in patients with S. aureus bacteremic pneumonia, confirming our previous findings in other patient populations with S. aureus bacteremia.13,14 We observed a similar correlation in patients with gram-negative bacteremic pneumonia and gram-negative bacteremia without pneumonia, indicating that this phenomenon is not specific to S. aureus infection and includes gram-negative infection groups. Interestingly, no such correlation was observed in uninfected subjects, suggesting that high correlation between levels of IgG and NAbs against both AT and LukAB may be an attribute of an acute infection.

This study had several limitations. All samples were collected at a single hospital, and the sample size was relatively small. The samples were collected over a 13-year period, and study results might have been affected by changes in antibiotic treatment or other aspects of clinical care during this time. The customised multiplex assay used in this study could accommodate a maximum of only nine antigens, and therefore, we were not able to measure antibody levels against all of the numerous S. aureus virulence factors. Because of the cross-sectional observational nature of the study, we could measure antibody levels only in the acute stage of disease and were unable to correlate antibody levels before S. aureus exposure or infection with clinical outcome. Nevertheless, the antibody levels in the uninfected control subjects were also measured as part of this study, providing a range of the preexisting antibody levels. The antibody levels in S. aureus-infected patients were generally higher than those in uninfected subjects (i.e. preexisting antibody levels), suggesting that elevated antibody levels resulted from the acute infection.

Despite its limitations, the current study offered several advantages, including well-matched study cohorts; availability of matched serum samples, S. aureus isolates and clinical data; and minimal
Table 1. Clinical measurements and outcomes in patients with S. aureus bacteremic pneumonia

| Variable                                      | Cure (n = 26) | Failure (n = 24) | P     |
|-----------------------------------------------|---------------|-----------------|-------|
| Age, years (mean ± SD)                        | 57.0 ± 14.5   | 63.5 ± 9.6      | 0.0760|
| Gender, n (%)                                 |               |                 |       |
| Female                                        | 12 (57.1)     | 9 (42.9)        | 0.5362|
| Male                                          | 14 (48.3)     | 15 (51.7)       |       |
| Race, n (%)                                   |               |                 |       |
| Black/African American                         | 3 (30.0)      | 7 (70.0)        | 0.3117|
| White                                         | 22 (57.9)     | 16 (42.1)       |       |
| Other                                         | 1 (50.0)      | 1 (50.0)        |       |
| Length of stay, days (mean ± SD)              | 30.1 ± 39.0   | 37.4 ± 42.8     | 0.5265|
| Days with symptoms of infection, mean ± SD    | 5.0 ± 9.0     | 2.4 ± 4.0       | 0.2573|
| Route of infection, n (%)                     |               |                 |       |
| Hospital acquired                             | 9 (40.9)      | 13 (59.1)       | 0.2538|
| Community acquired, healthcare associated     | 12 (66.7)     | 6 (33.3)        |       |
| Community acquired, non-healthcare associated | 4 (44.4)      | 5 (55.6)        |       |
| Surgery performed in 30 days before bacteremia, n (%) | 22 (61.1) | 14 (38.9) | 0.0452|
| No                                            |               |                 |       |
| Yes                                           | 4 (28.6)      | 10 (71.4)       |       |
| Haemodialysis dependent, n (%)                |               |                 |       |
| No                                            | 25 (53.2)     | 22 (46.8)       | 0.5144|
| Yes                                           | 1 (33.3)      | 2 (66.7)        |       |
| Diabetic, n (%)                               |               |                 |       |
| No                                            | 15 (48.4)     | 16 (51.6)       | 0.5144|
| Yes                                           | 11 (57.9)     | 8 (42.1)        |       |
| APACHE II score, mean ± SD                    | 3.0 ± 2.3     | 3.8 ± 1.6       | 0.1521|
| Age score                                      |               |                 |       |
| Chronic health                                | 4.0 ± 1.6     | 4.6 ± 1.0       | 0.1143|
| Acute physiology                              | 9.6 ± 6.2     | 14.2 ± 6.5      | 0.0215|
| Total                                         | 16.6 ± 6.7    | 22.6 ± 6.4      | 0.0064|
| Corticosteroid use, n (%)                     |               |                 |       |
| No                                            | 15 (46.9)     | 17 (53.1)       | 0.3356|
| Yes                                           | 11 (61.1)     | 7 (38.9)        |       |
| Transplant recipient, n (%)                   |               |                 |       |
| No                                            | 22 (51.2)     | 21 (48.8)       | 0.7693|
| Yes                                           | 4 (57.1)      | 3 (42.9)        |       |
| Patient being treated for neoplasm/cancer, n (%) | 21 (53.8) | 18 (46.2) | 0.6238|
| No                                            |               |                 |       |
| Yes                                           | 5 (45.5)      | 6 (54.5)        |       |
| Fever resolved within 72 h, n (%)             |               |                 |       |
| No                                            | 7 (38.9)      | 11 (61.1)       | 0.0424|
| Yes                                           | 17 (70.8)     | 7 (29.2)        |       |
| NA                                            | 2 (25.0)      | 6 (75.0)        |       |
| Main antibiotic regimen, n (%)                |               |                 |       |
| β-lactamase-resistant penicillin              | 4 (80.0)      | 1 (20.0)        | 0.6581|
| Cefazolin/first-generation cephalosporin      | 3 (100.0)     | 0 (0.0)         |       |
| Ceftriaxone                                   | 1 (100.0)     | 0 (0.0)         |       |
| Daptomycin                                    | 1 (100.0)     | 0 (0.0)         |       |
| Linezolid                                     | 3 (75.0)      | 1 (25.0)        |       |

Table 1. Continued.

| Variable                                      | Cure (n = 26) | Failure (n = 24) | P     |
|-----------------------------------------------|---------------|-----------------|-------|
| Vancomycin                                    | 13 (40.6)     | 19 (59.4)       |       |
| Other                                         | 1 (25.0)      | 3 (75.0)        |       |
| Duration of antibiotic therapy, days (mean ± SD) | 3.64 ± 23.0 | 19.2 ± 24.9 | 0.0259|

SD, standard deviation. Clinical measurements were summarised as number and percentage for categorical variables; in median, minimum and maximum for ordinal variables; and as mean ± standard deviation for continuous variables. *P*-values for the association between clinical measurements and patient outcome were calculated from logistic regression.

**METHODS**

**Subject selection and ethics statement**

The Duke University Institutional Review Board approved this investigation. Eligible patients met the following criteria: (1) adults hospitalised at Duke University Medical Center with monomicrobial S. aureus bacteremia; (2) no neutropenia (absolute neutrophil count of > 100 neutrophils μL⁻¹); (3) availability of clinical data, bloodstream S. aureus isolate and acute-phase sera in the S. aureus Bacteremia Group biorepository; and (4) availability of S. aureus bacteremic pneumonia samples collected from 2002 to 2015. After informed consent was obtained, blood was drawn from the hospitalised patients within 1–3 days after the first S. aureus-positive blood culture. Blood was allowed to clot by incubating for 30–60 min at room temperature, and serum was then separated by centrifugation at 1000–2000× g for 10 min and then divided into aliquots, which were stored at –80°C. A single serum aliquot per patient was used for serological assays. A total of 50 samples (both isolates and sera) from patients with S. aureus bacteremic pneumonia were included in the study, along with sera from patients with gram-negative bacteremic pneumonia (n = 50), patients with gram-negative bacteremia without pneumonia (n = 50) and uninfected control subjects (n = 50) who were matched with S. aureus bacteremic pneumonia patients by the number of samples and demographic characteristics such as age, gender and race.
Clinical outcomes and definitions

Clinical outcomes were defined as either ‘Cure’ or ‘Failure’ by site investigators as described previously. 13 ‘Cure’ was defined as occurring when a patient was alive with no evidence of recurrent *S. aureus* infection at 12 weeks after the initial positive blood culture. ‘Failure’ was defined as occurring when a patient died for any reason or experienced culture-confirmed recurrent *S. aureus* infection within 12 weeks of the initial positive blood culture.

*S. aureus* bacteremic pneumonia’ was defined as bacteremia with an initial source of *S. aureus* pneumonia, as diagnosed by the physician providing care to the patient.

‘Gram-negative bacteremic pneumonia’ was defined as bacteremia with an initial source of pneumonia caused by gram-negative bacteria, as diagnosed by the physician providing care to the patient. In patients with gram-negative bacteremia without pneumonia, the initial sources

Table 2. Results of statistical analyses

| Effect/variable | OR       | 95% CI       | P     |
|-----------------|----------|--------------|-------|
| **Univariate model** |          |              |       |
| **Clinical** | | | | |
| Age (units = 10 years) | 0.64 | 0.40–1.05 | 0.0760 |
| Gender (female vs male) | 1.43 | 0.46–4.42 | 0.5362 |
| Race (black/African American vs white) | 0.31 | 0.07–1.39 | 0.1272 |
| Chronic Health Points score (units = 5) | 0.15 | 0.01–1.58 | 0.1143 |
| Acute Physiology score (units = 10) | 0.32 | 0.12–0.84 | 0.0215 |
| Total APACHE II score (units = 10) | 0.24 | 0.09–0.67 | 0.0064 |
| Did fever go away ≤ 72 h? (No vs Yes) | 0.26 | 0.07–0.96 | 0.0424 |
| Was surgery performed ≤ 30 days? (No vs Yes) | 3.93 | 1.03–14.99 | 0.0452 |
| Duration of antibiotic treatment (units = 7 days) | 1.27 | 1.03–1.56 | 0.0259 |
| **Serology** | | | | |
| NAb_AT | 1.90 | 0.87–4.15 | 0.1085 |
| NAb_LukAB | 1.97 | 0.91–4.26 | 0.0857 |
| IgG_AT | 2.64 | 1.05–6.63 | 0.0392 |
| IgG_ClfA | 2.28 | 0.88–5.93 | 0.0899 |
| IgG_ClfB | 2.20 | 0.78–6.23 | 0.1386 |
| IgG_DT | 1.86 | 0.77–4.50 | 0.1658 |
| IgG_FnbA | 1.79 | 0.63–5.08 | 0.2751 |
| IgG_LukAB | 2.47 | 0.95–6.42 | 0.0637 |
| IgG_MntC | 1.17 | 0.60–2.31 | 0.6420 |
| IgG_SdrC | 0.74 | 0.25–2.24 | 0.5977 |
| IgG_TST1 | 1.29 | 0.77–2.16 | 0.3284 |
| IgM_AT | 1.37 | 0.31–5.99 | 0.6785 |
| IgM_ClfA | 4.32 | 1.45–12.85 | 0.0086 |
| IgM_ClfB | 2.17 | 0.82–5.73 | 0.1175 |
| IgM_DT | 1.53 | 0.64–3.65 | 0.3378 |
| IgM_FnbA | 4.65 | 1.35–16.07 | 0.0151 |
| IgM_LukAB | 1.12 | 0.44–2.81 | 0.8159 |
| IgM_MntC | 1.60 | 0.65–3.96 | 0.3044 |
| IgM_SdrC | 10.10 | 2.04–49.92 | 0.0046 |
| IgM_TST1 | 1.31 | 0.55–3.13 | 0.5467 |
| **Staphylococcus aureus isolate** | | | | |
| Haemolytic activity (10-fold increase) | 1.75 | 0.92–3.33 | 0.0903 |
| AT ELISA (10-fold increase) | 2.02 | 0.73–5.57 | 0.1731 |
| MSSA vs MRSA | 1.78 | 0.55–5.77 | 0.3356 |
| fnbA (negative vs positive) | 0.48 | 0.15–1.55 | 0.2195 |
| hla (negative vs positive) | 0.50 | 0.10–2.35 | 0.3763 |
| tst (negative vs positive) | 1.45 | 0.34–6.18 | 0.6175 |
| **Multivariate model** | | | | |
| Total APACHE II (score increase by 10) | 0.34 | 0.11–1.05 | 0.0605 |
| Haemolytic activity (10-fold increase) | 2.94 | 1.05–8.27 | 0.0409 |
| IgM SdrC (10-fold increase) | 13.95 | 1.18–164.49 | 0.0363 |
| IgM C1fA (10-fold increase) | 5.72 | 1.16–28.24 | 0.0322 |

MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*.

*Serology OR corresponds to a 10-fold increase in antibody levels.*
of bacteremia were endovascular, skin, soft tissue, joint or bone, gastrointestinal, genitourinary or unknown excluding pneumonia. The uninfected control subjects were hospitalised patients who had no infection at the time of screening and enrolment and no history of S. aureus or gram-negative infections.

**Staphylococcus aureus isolates**

Staphylococcus aureus bloodstream isolates were identified from patients who provided written informed consent and were linked to the clinical details via a unique study number. For patients with multiple positive blood cultures, the initial isolate was used for all characterisations.

**Genotypic characterisation of S. aureus isolates**

Whole-genome sequencing of S. aureus isolates was performed via MiSeq 2 × 250 runs (Illumina, San Diego, CA) with a targeted depth of 150-fold. S. aureus genomic DNA was purified from bacterial cultures via bead beating, followed by extraction with a PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, MA). Sequencing libraries were prepared by Covaris mechanical shearing, followed by a NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA). MLSTs were assigned to each genome according to the S. aureus MLST database (available from PubMLST at http://www.pubmlst.org) by mapping to known alleles, using SRST2. The sequence type was calculated by using the best-scoring alleles. Read sets were screened for virulence genes with a direct-read mapping approach implemented in SRST2, using a 90% coverage cut-off. Sequences were assembled de novo with SPAdes 3.11.1. Phylogenetic trees were generated with kSNP3 and were visualised and annotated with ITOL v3.

**Serological assays**

Serum levels of IgG against nine S. aureus antigens were measured with a customised multiplex assay (Meso Scale Discovery; Meso Scale Diagnostics, Rockville, MD) according to the manufacturer’s instructions. Sulfo tag-labelled HyTest 2A11 anti-human IgG (2 μg mL⁻¹) or anti-human/nonhuman primate IgM (1 μg mL⁻¹) was used for detection. Anti-AT and anti-LukAB NAb titres were measured with a red blood cell haemolytic assay and an assay with human monocytic cell line HL-60 (American Type Culture Collection, Manassas, VA), respectively, as described previously.

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**Figure 6.** Associations of Staphylococcus aureus-specific and patient-specific factors with clinical outcome in patients with S. aureus bacteremic pneumonia. The OR of cure and associated 95% CI from univariate logistic regression analysis are shown for (a) clinical characteristics, (b) serum antibody levels and (c) S. aureus isolate characteristics. The ORs for antibody levels, AT production and haemolytic activity correspond to a 10-fold increase. As shown in Figure 3, hld, clfA, clfB, sdrC, lukAB and mntC genes were present in all isolates and therefore were not included in the univariate analysis. (d) OR and associated 95% CI from the multivariate logistic model with independent predictors. (e) Receiver operating characteristics curve of the multivariate logistic model, using leave-one-out cross-validation. The associated area under the curve (AUC) is shown.
Preparation of *S. aureus* supernatants

For each *S. aureus* isolate, a single colony from a tryptic soy agar plate was inoculated into 3 mL of tryptic soy broth and grown overnight with 250-rpm shaking at 37°C. The overnight culture was diluted 1:100 in 10 mL of tryptic soy broth, incubated with shaking for an additional 16 h and centrifuged at 3700 × g, yielding a culture supernatant that was used to quantify AT production and haemolytic activity.

**AT ELISA**

AT production was measured by ELISA as described previously. 26, 38 Ninety-six-well Maxisorp plates (Thermo Fisher Scientific) were coated overnight with anti-AT monoclonal antibody LC1029 (0.1 µg mL⁻¹) in 0.2 M carbonate/bicarbonate buffer. Plates were then washed with phosphate-buffered saline (PBS)-Tween (PBS-T), blocked for 1 h at room temperature with PBS plus 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and then incubated after three washes in PBS-T for 1 h at room temperature, using serial dilutions of supernatants in PBS or purified AT as a reference standard. After washes in PBS-T, rabbit polyclonal anti-AT IgG was added at 2 µg mL⁻¹ in PBS for 1 h at room temperature. After washes in PBS-T, rabbit IgG was detected by addition of goat anti-rabbit IgG horseradish peroxidase conjugate (Jackson Laboratories, Bar Harbor, ME) diluted at 1:10 000 in PBS. After 1 h of incubation at room temperature, plates were washed in PBS-T and IgG binding was detected by adding 100 µL of 3,3′,5′-tetramethylbenzidine substrate (KPL, Gaithersburg, MD) per well, followed by 100 µL of 0.2 M H₂SO₄. Optical density at 450 nm was measured with a spectrophotometer ( Molecular Devices, San Jose, CA).

**Haemolytic activity assay**

Haemolytic activity was measured by haemolytic assay with rabbit red blood cells as described previously. 26, 38 A total of 50 µL of washed red blood cells (Pel-Freez Biologicals, Rogers, AR) was incubated with 50 µL of serially diluted supernatant for 2 h at 37°C in U-bottomed, 96-well plates (VWR, Radnor, PA). Plates were centrifuged for 3 min at 930 × g, and 50 µL of supernatant was transferred to a 96-well, flat-bottomed plate. One hundred percent haemolysis was obtained by incubating red blood cells with 0.1% sodium dodecyl sulphate. Optical density at 450 nm was measured with a spectrophotometer ( Molecular Devices), and haemolytic activity, expressed as HU per millilitre, was calculated as inverse of the dilution resulting in 50% haemolysis.

**Cytofluorimetry**

Expression of ClfA and ClfB in *S. aureus* isolates was measured by cytofluorimetry as described previously. 26, 38 *S. aureus* isolates were grown to mid-log phase, washed with fluorescence-activated cell sorting (FACS) buffer (0.1% Tween 20, 0.1% bovine serum albumin in 1 × PBS) and incubated with irrelevant human IgG1 R34738 for 60 min at 4°C to block nonspecific binding of primary antibodies by protein A. Cells were then washed with FACS buffer and incubated with purified anti-ClfA or anti-ClfB polyclonal rabbit IgG for 30 min at 4°C, followed by washing with FACS buffer. Finally, the cells were incubated for 30 min at 4°C with goat anti-rabbit Alexa Fluor 647 conjugate (Abcam, Cambridge, MA). Plates were then washed three times with FACS buffer, and cells were resuspended in 200 µL of FACS buffer. Data were acquired with a FACS Canto II analyzer (Becton Dickinson, Franklin Lakes, NJ). A total of 30,000 events were collected for each sample. The acquired data were analysed with FlowJo software (FlowJo, Ashland, OR).

**Statistical analyses**

One-way analysis of variance with heterogeneous variance was applied for comparison of antibody levels in disease cohorts with the uninfected control cohort. Logistic regression was used to evaluate the univariate association of patient outcomes with clinical variables, antibody levels and characteristics of *S. aureus* isolates. For variables that significantly correlated with patient outcomes in the univariate assessment, a multivariate logistic regression model was applied to identify independent variables to predict patient outcomes. The predictive performance of the multivariate logistic regression model was assessed through a receiver operating characteristic curve and associated area under the curve. The receiver operating characteristic curve was constructed from the probability value of each patient predicted from the multivariate logistic regression model estimated with the remaining N–1 patients, that is, leave-one-out cross-validation, to assess the predictive performance of the model. Spearman correlation coefficient was used to evaluate correlations.

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