Title: “Increased risk of thrombocytopenia and death in patients with bacteremia caused by high alpha toxin-producing methicillin-resistant Staphylococcus aureus”

Running Title: Alpha Toxin in S. aureus Bacteremia

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This study has not been presented elsewhere previously.

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Abstract

Background. Alpha toxin (Hla) is a major virulence factor of Staphylococcus aureus that targets platelets but clinical data on Hla pathogenesis in bacteremia (SAB) is limited.

Objective. We examined the link between in vitro Hla activity and outcome.

Methods. Study isolates obtained from 100 patients with SAB (50 survivors; 50 non-survivors) were assessed for in vitro Hla production and activity by Western immunoblotting and hemolysis assay, respectively. Relevant demographics, laboratory and clinical data were extracted from patients’ medical records to correlate Hla activity of the infecting isolates with outcome.

Results. Hla production strongly correlated with hemolytic activity (r_s=0.93) in vitro. A trend towards higher hemolytic activity was observed for MRSA compared to MSSA and with high-risk source infection. Significantly higher hemolytic activity was noted for MRSA strains isolated from patients who developed thrombocytopenia (median 52.48 vs 16.55 HU/ml in normal platelet count, p=0.012) and from non survivors (median 30.96 vs 14.87 HU/ml in survivors, p= 0.014) but hemolytic activity of MSSA strains did not differ between patient groups.

Conclusions: In vitro Hla activity of S. aureus strains obtained from patients with bacteremia may be used to predict risk for thrombocytopenia and death which supports bedside phenotyping and therapeutic targeting in the future.

Key words: Alpha toxin, virulence factors, Staphylococcus aureus bacteremia, mortality, platelets, thrombocytopenia.
Introduction.

*Staphylococcus aureus* is a leading cause of bloodstream infection, affecting an estimated 50 in 100,000 people annually with an overall mortality rate of up to 57% in adults (1). Despite receipt of antibiotic therapy, one in three patients develop persistent bacteremia, which is associated with complications, prolonged hospitalization, and increased risk of death (2). Numerous factors may contribute to the varied outcomes observed in *S. aureus* bacteremia (SAB) including heterogeneity in host immunity and variable expression of virulence factors across clinical *S. aureus* strains (1).

Alpha toxin (Hla), a water-soluble, 34 kDa monomer, is a well-characterized cytolysin that is known to play a key role in the pathogenesis of *S. aureus* infections. Upon binding of Hla to its host cell receptor, ADAM10 (A Disintegrin And Metalloproteinase domain-containing protein-10) which is widely expressed on endothelial cells, epithelial cells, and immune cells, oligomerization of the toxin occurs leading to heptameric pores in the membranes and subsequent alterations in cellular signaling and cellular lysis (3). Platelets are the most abundant, non-nucleated immune cells in circulation and have been shown to kill *S. aureus* directly and indirectly through functional enhancement of other immune cells such as macrophages (4).

Wuescher et al found reduced survival rate with increased cytokine storm and higher bacterial load in kidneys of platelet-depleted mice infected with USA300 causing bacteremia compared to wild-type (WT) mice (5). Importantly, recent studies have demonstrated that Hla targets platelets and causes platelet activation, aberrant aggregation, and injury (6, 7). Surewaard et al found more thrombocytopenia and significantly higher platelet aggregation in the livers of mice that were infected with wild-type *S. aureus* compared to an isogenic hla-deletion mutant (8).

Additionally, Hla was shown to induce platelet desialylation resulting in enhanced and premature
platelet clearance through the hepatic Ashwell-Morell receptor (AMR) (7). High Hla production was shown to increase severity and reduce survival of infection in numerous experimental models of pneumonia (9, 10), sepsis (6), peritonitis (11), and brain abscesses (12). Administration of a novel anti-alpha toxin monoclonal antibody developed to specifically target and neutralize Hla was shown to provide survival benefit in multiple animal models of infection, including pneumonia (13, 14), skin and soft-tissue infections (15, 16), and bacteremia (17). Despite the extensive research that highlights Hla as a major toxin in animal models and the considerable efforts towards developing antibodies that target and neutralize Hla in *S. aureus* infections, few studies assessed the relationship between *in vitro* Hla production by clinical isolates as a possible predictive marker for the development of thrombocytopenia and patient outcomes in SAB. Given the previously reported variation in Hla expression across *S. aureus* strains (18, 19) and its involvement in the pathogenesis of *S. aureus* infections, we hypothesized that *S. aureus* bloodstream isolates produce varying Hla levels and that high Hla-producing strains are associated with increased risk of thrombocytopenia and mortality in patients with bacteremia. Our study objectives were to: 1) measure the *in vitro* level of Hla production and hemolytic activity of *S. aureus* bloodstream isolates and 2) correlate Hla activity with platelet count and outcome of SAB.

**Methods**

**Patient and bacterial isolate selection**

Study isolates and clinical data had been previously collected as part of a large multicenter prospective observational study of adult patients hospitalized for SAB from two affiliated medical centers in Los Angeles, USA. The study was approved by the respective IRB at each site; informed consent was waived as the study was observational in design. A total of 100 study
isolates were selected to represent equal numbers of patients who died or survived within 30 days of bacteremia onset. Survivors were those who had favorable outcomes: bacterial clearance and clinical improvement by day 4 following onset of SAB and end of therapy success while non-survivors were those whose death was deemed SAB-related in the patient’s medical record and had persistent bacteremia or lack of clinical improvement or worsening on day 4 following onset of SAB. Thrombocytopenia was defined as platelet count < 150 × 10^9/L at time of SAB onset. Source of infection was grouped based on risk of mortality as previously defined: high (> 20%; endovascular, lower respiratory tract, intrabdominal, and central nervous system foci), intermediate (10–20%; osteoarticular, soft tissue, and unknown sources), and low (< 10%; IV catheter, urinary tract infection, ear-nose-larynx, gynecologic sources, and several manipulation-related sources including digestive endoscopy, arterial catheterization, and sclerosis of esophageal varices) (20).

Patients’ medical records were retrospectively reviewed to extract relevant demographics, laboratory and clinical data (see Table 1), and managed using REDCap electronic data capture tools hosted at University of Southern California (21). Our in vitro analysis included 100 clinical isolates from those patients plus three S. aureus control strains: LAC (USA300) hla wild type, isogenic Δhla mutant lacking Hla production, and Δhla-complemented mutant strains with restored Hla production (22).

**In vitro measurement of Hla expression**

A single colony of each study isolate freshly grown on a TSA plate was inoculated in 5 ml Tryptic Soy Broth and incubated overnight at 37°C with shaking at 200 rpm. The OD_{600nm} was adjusted to 0.1 for all isolates and the bacterial suspension was incubated at 37°C for another 20 h to reach stationary growth phase (23, 24). Then, an aliquot of the culture was removed for CFU
determination by plate counting and the remainder was centrifuged at 4°C and 3,100 rpm (Eppendorf Centrifuge 5415R) for 10 min to generate cell-free supernatants, which were stored at -80°C until later analysis. All cell-free supernatants were normalized to 1x10^9 CFU and used in both Western immunoblot and hemolysis assays.

**Western immunoblot assay.** Ten µL of cell-free culture supernatants were subjected to SDS-PAGE using 4-20% Tris-Glycine extended gels. Purified Hla (Sigma-Aldrich, St. Louis, US) was used as controls diluted to in 250 ng, 125 ng, and 25ng/10 µl samples. Proteins were then transferred to a PVDF membrane and blocked with 5% BSA in TBST (Tris-buffered saline, 0.1% Tween 20, TBST) for 2 h, then incubated overnight at 4°C with the primary antibody mouse anti-SA Hla [8B7] - N-terminal (Abcam, Cambridge, United Kingdom) diluted 1:2500 in the blocking buffer. After three five-min TBST washes, the blot was incubated with goat-anti Mouse-HRP (Abcam, US) as secondary antibody diluted 1:2000 in the blocking buffer for 1 h followed by a 5 min wash in TBST. The signal was developed using TMB (3,3’,5,5’-tetramethylbenzidine) Peroxidase (HRP) Substrate Kit according to the manufacturer’s instructions (Vector kit SK4400, Vector Laboratories, Burlingame, US). Developed membranes were dried and images were acquired with a Bio-Rad ChemiDoc Touch Gel Imager (Bio-Rad, Hercules, US) and the quantitative analysis was performed using Image Lab software (6.0.1 version).

**Hemolysis assay.** The hemolytic activity of Hla was evaluated by measuring the hemolysis of rabbit erythrocytes (Innovative research, US). As described previously (25), 100 µl of serially diluted (1:5 up to 1:640 in PBS) culture supernatant was added to a round-bottom polystyrene 96-well plate followed by the addition of 100 µl of washed rabbit erythrocytes (1% in PBS). The plate was incubated for 1 h at 37°C and then centrifuged at room temperature, 220 rpm for 10
minutes. One hundred µl from each well was transferred to a new flat-bottom polyesterene 96-well plate and absorbance was measured at 570 nm. PBS was used as a negative control and 5000 ng purified Hla resuspended in 100 µl PBS was used as a positive control. Hla level (in hemolytic units per ml, HU/ml) was defined as the inverse of the dilution causing 50% hemolysis. All supernatants were tested in duplicates and the results were averaged.

**Data Analysis**

The correlation between Hla protein level measured by Western immunoblotting and the hemolytic activity was examined for 61 clinical isolates representing 40 MRSA; 21 MSSA; survivors and non-survivors. The relationship between the hemolytic activity of all 100 SAB isolates relative to methicillin resistance and platelet count at onset of bacteremia, source risk of infection, and 30-day mortality was analyzed.

Statistical analysis was performed using GraphPad Prism (version 9.0). Unpaired t-test, Mann-Whitney tests and Fisher’s Exact tests were used to test continuous and categorical variables where appropriate. Spearman correlation test was performed for correlation analysis. A two-tailed p-value of < 0.05 denotes statistical significance.

**Results**

**Patient characteristics and platelet trends.**

Table 1 compares the characteristics between those with and without thrombocytopenia and between survivors and non-survivors in terms of sex, age, IV drug use, and comorbid conditions that may predispose to the development of thrombocytopenia, cause and source risk of infection, and platelet count at onset. Thrombocytopenia occurred in 36 % (34/95) of our study cohort overall, with 8-times greater proportion among non-survivors than survivors (57 %, 27/47 vs
Among those who developed thrombocytopenia at onset of SAB, a greater proportion had sources of infection associated with high risk for death such as endovascular and lower respiratory tract infections (50% vs 24%, p=0.022) and had MRSA as a causative pathogen (53% vs 31%, p=0.048) when compared to those who did not, though the groups did not differ in age or comorbid conditions except for liver cirrhosis (24% vs 8%, p=0.059). On the other hand, non-survivors were older (median 62 vs. 52 years, p=0.002) with a trend towards greater proportion with active malignancy (20% vs 6%, p=0.071). Similarly, non-survivors were also more likely to have high risk source infections (44% vs 22%, p=0.032).

Of the 95 patients with available platelet counts at onset of SAB, the median platelet counts were 96.5, 241, 221, 128 x 10^9/L in the following groups respectively: thrombocytopenic, non-thrombocytopenic, survivors, and non-survivors. An analysis of platelet dynamics during the first 7 days of bacteremia showed a decline of platelet counts through day 4 with negligible recovery among non-survivors relative to survivors (median platelet count, 1 x 10^9/L): on day 1 [128 (IQR 87, 229) vs 221 (IQR 170, 315); p=0.0002], on day 4 [83 (IQR 51,159 vs 200 (IQR 161, 292); p<0.0001), and on day 7 [91 (IQR 44.7, 163) vs 237 (IQR 189, 369); p <0.0001] (Figure 1).

**High correlation of Hla protein level and hemolytic activity**

We evaluated Hla expression by first determining the correlation between protein levels and hemolytic activity measured by Western immunoblot and hemolysis assays, respectively, in a subset of 61 clinical isolates, representing survivors and non-survivors, MRSA and MSSA isolates, plus the three control strains (Figure 2). A very strong correlation was found between Hla protein level and hemolytic activity ($r_s= 0.93$, $p <0.0001$). Based on these findings and considering the labor intensity of the Western immunoblot, we performed hemolysis assays only...
on the remaining clinical isolates and all subsequent analysis was based on results obtained from
the hemolysis assay.

**Association of high hemolytic activity with thrombocytopenia and mortality**

Bloodstream *S. aureus* isolates exhibited a wide variation of Hla expression in terms of
hemolytic activity (range: 0 to 138.7 HU/ml). Overall, higher hemolytic activity was observed
for MRSA vs MSSA isolates (25.12 vs 14.76 HU/ml, \( p = 0.09 \)), isolates from patients with
thrombocytopenia vs those from patients with normal platelet count [median 28.1 HU (IQR 7.62,
61.1) vs 16.34 HU (IQR 8.8, 32.3); \( p = 0.08 \)], and from survivors vs non-survivors [median 23.1
HU (IQR 7.62, 59.2) vs 17.97 HU (IQR 5.42, 33.73); \( p = 0.252 \)], respectively (Figures 3A-B,
4A-B). When isolates were grouped based on methicillin resistance, a striking difference in
hemolytic activity was observed for MRSA isolates from patients with thrombocytopenia vs
normal platelet count [median 52.48 HU (IQR 22.19, 69.71) vs 16.55 HU (IQR 9.8, 26.82), \( p =
0.011 \)] and from patients who died vs those who survived [median 30.96 HU/ml (IQR 17, 66.50)
v215 vs 14.87 HU/ml (IQR 3.76, 29.53), \( p = 0.014 \) (Figures 3C and 4C).

On the contrary, MSSA isolates exhibited similar Hla activity regardless of platelet count status
of the infected patients [median 14.76 HU (IQR 6.4, 35.5) vs 18.9 HU (IQR 5.7, 40.6); \( p = 0.91 \]
(Figure 3C) or survival status [median 18.99 HU (IQR 5.45, 34.50) non-survivors vs 13 HU
(IQR 4.91, 39.32) survivors, \( p = 0.353 \) (Figure 4C). Notably, the mean platelet count among
MRSA-infected group was significantly lower compared to MSSA group [155.8 +/- 87.98 SD vs
219 237 +/- 125.6 SD, \( p = 0.0008 \)]. These findings support the association between Hla activity,
thrombocytopenia, and death among MRSA but not MSSA bloodstream isolates.
Association of high hemolytic activity with high-risk source of infection

We grouped the study isolates according to the source of infection relative to the associated risk of death as reported by Soriano et al (20). We found a positive trend when correlating hemolytic activity with risk source of infection [median 29.04 HU (IQR 12.08, 59.84) vs 15.06 HU (IQR 6.25, 34.74), p=0.066] comparing between isolates from high-risk source (n=33) versus intermediate-risk and low-risk sources of bacteremia (n=67), respectively, but this trend did not reach statistical significance (data not shown).

Discussion

The objective of our study was to measure the in vitro level of Hla production and hemolytic activity of S. aureus bloodstream isolates and investigate the association of S. aureus Hla activity with platelet count and outcome in patients with SAB. We examined a total of 100 S. aureus isolates that caused bacteremia chosen to represent equal number of patients who died or survived. From these isolates, in vitro Hla production and hemolytic activity were measured by Western immunoblotting and rabbit erythrocyte-based hemolysis assays using cell-free culture supernatants obtained from bacteria in stationary growth phase. We demonstrated an excellent correlation between hemolysin production in vitro and hemolytic activity. Consistent with previous reports (26, 19), our findings showed a wide variation in hemolytic activity across bloodstream isolates.

A strong association between thrombocytopenia, defined as a platelet count less than 150 x 10^9/L, and 30-day mortality has been previously shown by a retrospective study involving 1052 patients with SAB (27). In a separate study that included 49 patients with SAB, strains with high
Hla expression were associated with thrombocytopenia (platelet count < 100 × 10^9/L) from the initial blood sample and death in 4 of 9 patients (7). We extended these findings by analyzing the association of hemolytic activity of the SAB strains and platelet count measured at multiple time points during SAB as well as mortality in a larger patient cohort.

Notably, we showed a strong association between thrombocytopenia and 30-day mortality and found that platelet count nadir occurred around day 4 from onset of bacteremia. These findings provide clear support of the current clinical practice that considers low platelet count as a poor prognostic indicator in SAB, indicative that platelet count should be followed serially particularly during the early course of bacteremia in line with previous studies that analyzed the association between mortality and multiple time points of platelet count in critically ill patients (28, 29).

Previous studies provided mechanistic insights into Hla-mediated platelet dysfunction and depletion in experimental models of S. aureus sepsis (6, 8). Therefore, we analyzed the relationship between Hla expression of the infecting S. aureus isolates and thrombocytopenia and death in patients with bacteremia. Importantly, our results confirmed the relationship between Hla expression and thrombocytopenia and cast a new light on the contribution of methicillin resistance. We found that MRSA bloodstream isolates had higher overall hemolytic activity and that high Hla-producing MRSA strains are significantly associated with thrombocytopenia and death in S. aureus bacteremia but the association between high hemolytic activity and poor outcome was not observed with MSSA strains. In line with these results, Coia et al. measured Hla production in a total of 201 isolates of MRSA and MSSA obtained from various body sites and reported significantly higher Hla production in vitro in MRSA relative to MSSA isolates (30). Our results are in partial agreement with Jacobsson et al. as they found a
positive association between Hla hemolytic activity (assessed by measuring the zones of
hemolysis in nutrient agar plates using rabbit erythrocytes) and complicated bacteremia, but not
mortality (31). On the other hand, Sharma-Kuinkel et al reported an inverse association between
SAB patients’ outcome and the in vitro Hla production (measured by Western blot and ELISA)
and hemolytic activity (measured by rabbit erythrocyte lysis assay) (25). One potential
explanation is the difference in the patient population studied since their population was limited
to postsurgical patients and those on hemodialysis whereby our study included a broader range of
patient population. Importantly, Sharma-Kuinkel et al did not analyze their results relative to
methicillin resistance which could potentially mask differences between MRSA and MSSA with
respect to Hla activity in association with patient outcome as was noted in our study.
Epidemiologic data from a recent retrospective analysis of 92,089 patients indicated that
bacteremia caused by MRSA was associated with longer hospitalization, higher rate of
readmission with bacteremia recurrence, and increased mortality compared to MSSA (32). Two
previous meta-analyses yielded similar findings when comparing mortality rates between
patients infected with MRSA and MSSA (33, 34). Our findings suggest differences in virulence
of the infecting strains and the Hla-mediated injury to platelets may offer a biologically plausible
explanation for the observed difference in mortality between MRSA and MSSA bacteremia.
It is possible that MRSA and MSSA bloodstream isolates in our cohort differ in genetic
backgrounds and that additional virulence factors may be present in MRSA strains that act
synergistically with Hla, thereby contributing to the observed difference in patient outcome.
Another potential explanation for the difference in hemolytic activity in our MRSA and MSSA
strains is the variation between MRSA vs. MSSA in the regulatory component, agr system. Agr
(the accessory gene regulator) is known to regulate the expression of many virulence genes
including Hla (9) and Cheung et al. reported that agr enhanced the regulation of methicillin resistance genes (mecA, mecR1) in community-associated MRSA (CA-MRSA) (35). Of interest, Otto et al. have previously linked the possibility of increased virulence of CA-MRSA with the increased expression of hla, partially because of the enhanced activity of Agr (36). Moreover, high Hla protein was detected among highly virulent ST93 CA-MRSA strains while agr-deficient strains displayed decreased hla expression (37). Similarly, others have shown increase in the expression of hla and agr in ST59 CA-MRSA isolates (38).

Our study on patients with SAB corroborated published literature on the deleterious effect of S. aureus Hla on platelets (e.g. platelet aberrant aggregation and desialylation) in vitro and in experimental models of sepsis (6, 7) and lend support for future investigations on measuring the virulence phenotype of the infecting strain and therapeutically targeting Hla-mediated effects on platelets to improve patient outcome. Specifically, future studies should examine the feasibility of performing phenotypic assays to measure Hla expression of the infecting S. aureus isolates that could be readily adopted into the routine workflow in clinical microbiology laboratory at the time of organism identification. As platelet count measurements are part of routine complete blood count, clinicians should closely monitor platelet count especially during the initial 4 days following onset of S. aureus bacteremia relative to Hla expression in the infected strains.

Additionally, in vivo studies confirming the modulatory potential of existing therapeutics including antibiotics with antivirulence activity (39) on Hla expression and their benefit in mitigating Hla-mediated platelet dysfunction and depletion could accelerate the translation of our findings to practice.
We acknowledge several limitations in our study. First, we measured the *in vitro* Hla production of *S. aureus* bloodstream isolates and correlated it with patient outcome; however, it remains unclear how well *in vitro* Hla production and hemolytic activity correlates with *in vivo* production during bacteremia. In murine models of pneumonia and skin and soft tissue infection, Berube et al showed a direct correlation between *in vivo* Hla production and the degree of tissue injury (40). Nonetheless, the harmful effects of Hla on platelets are supported by a clear relationship observed between high hemolytic activity of the infecting strains and thrombocytopenia during bacteremia in humans. Furthermore, it is possible that other staphylococcal cytotoxins including the beta, delta, and gamma hemolysins and the bi-component leukocidins may have contributed collectively to the observed *in vitro* hemolytic activity, as their role in the pathogenesis of *S. aureus* diseases has not been fully clarified (41, 42). Finally, we observed very strong signals of high Hla proteins in the Western immunoblot for some of the strains which suggest that the measurements may have reached or exceeded the level of saturation. Therefore, we employed the standard rabbit erythrocyte-based hemolysis assay to capture the variable expression of Hla across our bloodstream isolates. The lack of hemolysis exhibited by our *hla*-deletion strain and the recovery of hemolytic activity of the complemented mutant strain support the validity of the hemolysis assay.

In conclusion, our findings show that patients with MRSA bacteremia are more likely to be infected with high Hla-producing strains and are at higher risk for developing thrombocytopenia and death. Importantly, our study provides support for the future precision treatment of infections by phenotyping pathogen virulence and host platelet response to stratify patients who may benefit from treatment that target the Hla-platelet interface thereby improving outcome of *S. aureus* bacteremia.
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Author Contributions

Rachid Douglas-Louis and Emi Minejima contributed by collecting and providing the clinical data.

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Conflict of Interest

A.W.B. has received research funding from Merck, Inc. and MeMed Diagnostics and consulted for Merck Inc. and Ferring Pharmaceuticals. J.B.W. has a financial agreement with Aridis Pharmaceuticals related to patents owned by the University of Chicago.
References


expression among *Staphylococcus aureus* clones with hospital and community origin.


Table 1. Comparison of patient characteristics grouped by development of thrombocytopenia and 30-day survival.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Thrombocytopenia (n=34)</th>
<th>Non-thrombocytopenia (n=61)</th>
<th>P value</th>
<th>Non-survivors (n=50)</th>
<th>Survivors (n=50)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male)</td>
<td>21 (61%)</td>
<td>42 (68.8%)</td>
<td>0.504</td>
<td>31 (62%)</td>
<td>35 (70%)</td>
<td>0.53</td>
</tr>
<tr>
<td>Age, y, mean (SD)</td>
<td>58.91 (15.24)</td>
<td>56.84 (18.28)</td>
<td>0.575</td>
<td>62.66 (14.74)</td>
<td>52.60 (17.86)</td>
<td>0.002*</td>
</tr>
<tr>
<td>IV drug use</td>
<td>3 (8.8%)</td>
<td>7 (11.4%)</td>
<td>&gt;0.99</td>
<td>6 (12%)</td>
<td>5 (10%)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Alcohol use</td>
<td>5 (14.7%)</td>
<td>12 (19.6%)</td>
<td>0.591</td>
<td>3 (6%)</td>
<td>14 (28%)</td>
<td>0.006*</td>
</tr>
<tr>
<td>Active malignancy</td>
<td>5 (14.7%)</td>
<td>6 (9.8%)</td>
<td>0.498</td>
<td>10 (20%)</td>
<td>3 (6%)</td>
<td>0.071</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>8 (23.5%)</td>
<td>5 (8.2%)</td>
<td>0.059</td>
<td>7 (14%)</td>
<td>6 (12%)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Renal disease</td>
<td>12 (35.3%)</td>
<td>21 (34.4%)</td>
<td>&gt;0.99</td>
<td>17 (34%)</td>
<td>17 (34%)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>High risk mortality *</td>
<td>17 (50%)</td>
<td>15 (24.5%)</td>
<td>0.022*</td>
<td>22 (44%)</td>
<td>11 (22%)</td>
<td>0.032*</td>
</tr>
<tr>
<td>Intermediate risk mortality b</td>
<td>10 (29.4%)</td>
<td>20 (32.7%)</td>
<td>0.649</td>
<td>17 (34%)</td>
<td>17 (34%)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Low risk mortality c</td>
<td>7 (20.5%)</td>
<td>26 (42.6%)</td>
<td>0.042*</td>
<td>11 (22%)</td>
<td>22 (44%)</td>
<td>0.032*</td>
</tr>
<tr>
<td>MRSA as causative Pathogen</td>
<td>18(53%)</td>
<td>19 (31%)</td>
<td>0.048*</td>
<td>25 (50%)</td>
<td>15 (30%)</td>
<td>0.065</td>
</tr>
<tr>
<td>Platelet count at onset (Median, IQR)</td>
<td>96.5 (56, 123)</td>
<td>241 (201, 319)</td>
<td>&lt;0.0001*</td>
<td>128 (87, 229)</td>
<td>221 (170, 315)</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Death</td>
<td>27 (79%)</td>
<td>20 (32.8%)</td>
<td>&lt;0.0001*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: *endovascular sources, lower respiratory tract, IA, and CNS foci; b osteoarticular sources, soft tissue sources, and unknown sources; c IV catheter, UTI, ear-nose-larynx, gynecologic sources, and several manipulation-related sources including digestive endoscopy, arterial catheterization, and sclerosis of esophageal varices.
*denotes statistical significance as determined by Student t-test or Fisher’s exact test where appropriate.

**Figure 1.** Association between platelets dynamics during SAB. Strong positive association between serial measurements of platelets (plt) counts during first 7 days of bacteremia and survival. *p* values as determined by Mann-Whitney tests.
Figure 2. Correlation of Western immunoblot and hemolysis assays. (A) Representative image of hemolysis assay with supernatants that were tested in duplicates, collected from control strains (1. isogenic Δhla mutant lacking Hla production, 2. LAC (USA300) hla wild type and 3. Δhla-complemented mutant strains with restored Hla production) and clinical isolates (4. HH92, 5. HH37, and 6. LAC82) in a 96 well plate. (B) Western immunoblot for (a) known amounts of purified Hla, (b) control strains and (c) representative SAB clinical isolates. (C) Correlation between Hla protein concentration and hemolytic activity, p values as determined by Spearman correlation test.
Figure 3. (A) Distribution of hemolytic activity across SAB isolates grouped by platelet status. Control strains: LAC (USA300) hla wild type, isogenic Δhla mutant lacking Hla production, and Δhla-complemented mutant strains with restored Hla production. (B) Comparison of S. aureus hemolytic activity from patients with normal platelet count and thrombocytopenia (including both MSSA and MRSA). (C) Comparison of S. aureus hemolytic activity from patients with normal platelet count and thrombocytopenia grouped by MSSA or MRSA. plt: platelet count. p values as determined by Mann-Whitney test.
**Figure 4.** (A) Distribution of alpha hemolysin hemolytic activity across SAB isolates grouped by survival status. Control strains: LAC (USA300) *hla* wild type, isogenic Δ*hla* mutant lacking Hla production, and Δ*hla*-complemented mutant strains with restored Hla production. (B) Comparison of *S. aureus* hemolytic activity from survivors and non-survivors (including both MSSA and MRSA). (C) Comparison of *S. aureus* bloodstream isolates hemolytic activity from survivors and non-survivors grouped by MSSA or MRSA. p values as determined by Mann-Whitney test.