α-Hemolysin Activity of Methicillin-Susceptible Staphylococcus aureus Predicts Ventilator-associated Pneumonia

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Abstract

Rationale: Colonization of lower airways by Staphylococcus aureus is a risk factor for the development of ventilator-associated tracheobronchitis (VAT) and ventilator-associated pneumonia (VAP). However, little is known about the virulence factors of methicillin-sensitive and -resistant S. aureus (MSSA and MRSA) that may influence host colonization and progression to VAT and VAP.

Objectives: We evaluated MRSA and MSSA endotracheal aspirates (ETA) for genotype and α-hemolysin activity in relation to the development of VAT and VAP.

Methods: Serial S. aureus ETA isolates from ventilated patients were analyzed for methicillin resistance, molecular type by Multi-Locus Sequence Typing and spa-typing, and α-hemolysin activity by semiquantitative analysis of hemolysis on sheep blood agar and quantitative measurement of cytolysis of human lung epithelial cells. The virulence of selected strains was assessed in mice by intranasal challenge.

Mechanically ventilated patients are at high risk of bacterial colonization that may progress to ventilator-associated respiratory infections, manifested as ventilator-associated tracheobronchitis (VAT) or ventilator-associated pneumonia (VAP). Both conditions contribute to prolonged ventilation and stay in the intensive care unit (ICU), increased healthcare costs, and VAP is associated with increased mortality (1–4). VAT has been proposed as an intermediate condition between simple colonization of the upper airways and VAP, although there are controversies concerning diagnostic criteria and true distinction from VAP (5). Recently, a new approach to disease management has been implemented using serial microbial analysis of endotracheal aspirates (ETAs), which is performed to identify and quantify bacteria colonizing the lower airways (4, 6). Heavy colonization defined as many (4+) and moderate (3+) growth by semiquantitative analysis of ETAs (SQ-ETA) or quantitative ETA greater than 105 cfu/ml of a respiratory pathogen is a risk factor for progression to VAT and/or VAP (4, 6). Detection of specific bacterial pathogens helps to guide earlier, targeted antibiotic treatment and antibiotic stewardship efforts (7).

Measurements and Main Results: We detected S. aureus from ETA samples in a quarter of the 231 ventilated patients analyzed; one-third of them developed VAP. VAP patients (n = 15) were mainly infected by MSSA strains (87%), whereas colonized individuals (n = 18) not progressing to disease mainly carried MRSA strains (68%). MSSA isolates from colonized or VAT patients exhibited significantly lower α-hemolysin activity than those from VAP cases; however, no such relationship was found with MRSA strains. α-Hemolysin activity of S. aureus isolates was predictive for virulence in mouse pneumonia model.

Conclusions: MSSA strains with strong blood agar hemolysis and high α-hemolysin activity are markers for VAP, but not VAT, and might be considered in differential diagnosis and initiation of therapy.

Keywords: Staphylococcus aureus; biomarker; α-hemolysin; ventilator-associated pneumonia
At a Glance Commentary

Scientific Knowledge on the Subject: Airway colonization by *Staphylococcus aureus* is a precursor for the development of methicillin-resistant *S. aureus* (MRSA) or methicillin-sensitive *S. aureus* (MSSA)-induced ventilator-associated tracheobronchitis (VAT) and/or pneumonia (VAP). However, little is known about the pathogen-associated factors of *S. aureus* that promote progression from colonization to pneumonia. Detection of *S. aureus* isolates with a high propensity to cause VAP and identification of simple, sensitive, and specific biomarkers would greatly support prophylaxis or initiation of earlier antibiotic therapy for VAT and VAP.

What This Study Adds to the Field: This study represents a comprehensive characterization of *S. aureus* isolates from endotracheal aspirates obtained by serial sampling of ventilated patients. Most of the VAP cases were caused by MSSA; in contrast, MRSA isolates were mainly recovered from colonized patients. High α-hemolysin activity of MSSA but not MRSA isolates was a marker for the progression to or the presence of VAP. The sheep blood agar hemolysis test is a simple assay that can be performed in routine microbiologic laboratories to measure α-hemolysin activity and can serve as a predictor for VAP in ventilated patients colonized with MSSA isolates. These findings may expedite initiation of earlier therapy to improve patient outcomes, such as survival, decreased ventilator days, and length of intensive care unit stay.

*Staphylococcus aureus*, both methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA), is a frequent causative pathogen for VAT and VAP (1, 2). *S. aureus* virulence and pathogenesis have been extensively studied in animals, but the role of virulence factors in human disease is poorly understood (8, 9). Identification of relevant virulence factors and biomarkers would support more effective prevention strategies and initiation of earlier therapy of high-risk patients. One of the hallmarks of *S. aureus* pathogenesis is the production of pyrogens, of which the best characterized is α-hemolysin (Hla). Hla is highly potent in lysing bronchial and alveolar epithelial cells, as well as macrophages and lymphocytes, and is implicated in the induction of proinflammatory processes (10). Its dominant role in the pathogenesis of *S. aureus* pneumonia has been demonstrated in several different animal models (10). Recently published seroepidemiology studies suggested a correlation between higher serum levels of anti-Hla antibodies and favorable clinical outcome in the case of sepsis (11, 12). Therefore, the relationship between preexisting Hla-neutralizing antibody levels, susceptibility to VAP, and disease progression deserves future investigation. Hla has shown promise as a vaccine antigen and monoclonal antibody target in animal models of *S. aureus* disease (13–16), and is currently being evaluated in human trials of both active and passive immunization.

We compared methicillin resistance, genotypes, and Hla activity of *S. aureus* isolates from serial ETA samples from ventilated patients to assess the association between these markers and progression to VAP. Some of the results of these studies have been previously reported in the form of an abstract (17).

Methods

Patients and Samples

ETA samples and clinical data were collected from 231 ventilated patients hospitalized between May and December 2010 in two medical and one surgical ICUs at the Lahey Hospital and Medical Center (Burlington, MA). A natural history study based on data from 188 of these patients with ETA samples analyzed by quantitative and semiquantitative techniques was published earlier (4). SQ-ETA data were obtained from all 231 patients (Q-ETA for 188 patients) and was used in the current study. Heavy colonization is defined based

![Figure 1. Study patient selection. The flow chart describes the selection of patients into the study and their grouping in three patient groups. CAP = community-acquired pneumonia; ETA = endotracheal aspirates; SQ-ETA = semiquantitative ETA; VAP = ventilator-associated pneumonia; VAT = ventilator-associated tracheobronchitis.](image-url)
on semiquantitative microbiology analysis of ETA samples with many (4+) or moderate (3+) bacterial growth. In the original study the authors observed good correlation between Q-ETA criteria greater than or equal to 10^5 cfu/ml and SQ-ETA with at least moderate growth: 93% agreement and a kappa value of 0.86 (4).

The characteristics of the additional 43 patients included in this study were comparable with those 188 reported earlier (4). VAT diagnosis was based on heavy colonization, plus at least two clinical criteria (fever, leukocytosis, or purulent sputum). VAP was diagnosed as for VAT plus a new and persistent infiltrate on chest radiograph.

The research protocols were approved by the Lahey Clinic Institutional Review Board.

## Microbiologic Analyses and Molecular Typing of S. aureus Isolates

To determine the MRSA and MSSA status, strains were grown on selective agar and subjected to multiplex polymerase chain reaction (PCR) to detect mecA. S. aureus strains were genotyped by Multi-Locus Sequence Typing (MLST), spa, capsule, SCCmec, and hla typing according to standard methods (18–22). More details are provided in the online supplement.

## Blood Agar Hemolysis Test

*S. aureus* isolates were incubated for 16 hours at 37°C on Columbia agar containing 5% sheep blood (COS; bioMérieux, Marcy-l’Étoile, France) and hemolysis profiles were evaluated by visual inspection and semiquantitative assessment of cleared (complete hemolysis) zones around bacterial colonies. This test was performed with three different clones of the same isolate and results evaluated by three different individuals.

## In Vitro Cytotoxicity Assay

Cytotoxicity studies were performed using a human alveolar epithelial cell line (A549 ATCC CCL-185; LGC Standards, Teddington, UK) and bacterial culture supernatants of bacteria grown overnight in three different culture media: TSB, CCY, and RPMI + casamino acids. More details are provided in the online supplement.

## Murine Pneumonia Model

Virulence studies were performed by intranasal challenge of anesthetized BALB/cJrj mice (Janvier Labs, Saint-Berthevin Cedex, France) with three different challenge doses of clinical isolates. Survival of animals was monitored for 7 days after challenge. More details are provided in the online supplement.

## Data Analyses and Statistics

For spa typing and MLST, published software packages were applied. Spa types were analyzed with the Ridom Staph Type software (Ridom GmbH, Würzburg, Germany). We acknowledge the use of the *S. aureus* MLST database, which is located at Imperial College London and is funded by the Wellcome Trust.

Five types of statistical methods were applied: Fisher exact two-tailed probability test using the Prism 6 software (GraphPad, La Jolla, CA) and the one-way analysis of variance, two-sample *t* test, Kruskal-Wallis test, and two-sample Wilcoxon rank-sum test using the SPSS V19.0 software (IBM, Armonk, NY). The method used for each dataset is described in the corresponding text and the figure legends.

## Results

## Collection of S. aureus Isolates from ETA Samples of Ventilated Patients

*S. aureus* was isolated from 56 of the 231 study patients (24%), and 45 of them infected only with *S. aureus* were selected.

### Table 1. Baseline Characteristics of the Study Patients

<table>
<thead>
<tr>
<th>Baseline Variables*</th>
<th>VAP (n = 15)</th>
<th>VAT (n = 12)</th>
<th>Colonized (n = 18)</th>
<th>P Value</th>
<th>MICU (n = 31)</th>
<th>SICU (n = 14)</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>60.1 ± 12.7</td>
<td>62.8 ± 14.0</td>
<td>67.7 ± 13.5</td>
<td>0.2875‡</td>
<td>61.4 ± 2.7</td>
<td>69.4 ± 2.6</td>
<td>0.0751</td>
</tr>
<tr>
<td>Male, %</td>
<td>46.7</td>
<td>58.3</td>
<td>66.7</td>
<td>0.5291†</td>
<td>83.9</td>
<td>50.0</td>
<td>0.0169</td>
</tr>
<tr>
<td>BMI</td>
<td>26.8 ± 6.9</td>
<td>36.8 ± 17.9</td>
<td>30.9 ± 5.8</td>
<td>0.0805‡</td>
<td>32.1 ± 2.3</td>
<td>28.9 ± 2.0</td>
<td>0.3966</td>
</tr>
<tr>
<td>Charlson Comorbidity Index</td>
<td>0.9 ± 1.0</td>
<td>1.4 ± 1.0</td>
<td>2.1 ± 2.0</td>
<td>0.1039‡</td>
<td>1.6 ± 0.3</td>
<td>1.4 ± 0.5</td>
<td>0.722</td>
</tr>
<tr>
<td>APACHE II</td>
<td>18.4 ± 4.1</td>
<td>18.5 ± 5.7</td>
<td>20.4 ± 6.8</td>
<td>0.5535‡</td>
<td>20.5 ± 1.1</td>
<td>16.7 ± 1.2</td>
<td>0.0377</td>
</tr>
<tr>
<td>GCS</td>
<td>13.3 ± 0.4</td>
<td>12.6 ± 2.9</td>
<td>12.9 ± 2.8</td>
<td>0.9401†</td>
<td>12.0 ± 1.1</td>
<td>14.0 ± 0.3</td>
<td>0.1275</td>
</tr>
<tr>
<td>GCS-intubated</td>
<td>7.2 ± 2.1</td>
<td>6.9 ± 1.6</td>
<td>7.4 ± 1.3</td>
<td>0.8463†</td>
<td>7.3 ± 0.4</td>
<td>6.7 ± 0.7</td>
<td>0.4375</td>
</tr>
<tr>
<td>Mortality in hospital, %</td>
<td>20.0</td>
<td>41.2</td>
<td>22.9</td>
<td>0.4035†</td>
<td>25.8</td>
<td>28.6</td>
<td>0.8503</td>
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<tr>
<td>Antibiotics during ICU stay, %</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>NS</td>
<td>100.0</td>
<td>100.0</td>
<td>NS</td>
</tr>
<tr>
<td>SICU, %</td>
<td>53.3</td>
<td>16.7</td>
<td>22.2</td>
<td>0.0393§</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Emergency surgery trauma, %</td>
<td>46.7</td>
<td>25.0</td>
<td>22.2</td>
<td>0.2914‡</td>
<td>9.7</td>
<td>78.6</td>
<td>&lt;0.0001</td>
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<tr>
<td>Acute renal failure, %</td>
<td>13.3</td>
<td>8.3</td>
<td>33.3</td>
<td>0.1885‡</td>
<td>25.8</td>
<td>7.1</td>
<td>0.1541</td>
</tr>
<tr>
<td>Chronic organ insufficiency, %</td>
<td>6.7</td>
<td>8.3</td>
<td>22.2</td>
<td>0.3725†</td>
<td>16.1</td>
<td>7.1</td>
<td>0.4232</td>
</tr>
<tr>
<td>Duration of hospitalization, d</td>
<td>26.2 ± 10.2</td>
<td>20.9 ± 9.7</td>
<td>16.2 ± 6.8</td>
<td>0.1989/0.0027/0.1426†</td>
<td>19.1 ± 1.6</td>
<td>24.6 ± 3.1</td>
<td>0.0800</td>
</tr>
<tr>
<td>Duration of ICU stay, d</td>
<td>24.2 ± 9.7</td>
<td>15.3 ± 6.7</td>
<td>10.3 ± 5.2</td>
<td>0.0146/0.0001/0.0374‡</td>
<td>13.6 ± 1.4</td>
<td>22.2 ± 3.0</td>
<td>0.0037</td>
</tr>
<tr>
<td>Ventilation, d</td>
<td>14.0 ± 4.7</td>
<td>11.1 ± 3.8</td>
<td>6.8 ± 2.5</td>
<td>0.1089/0.0001/0.0131†</td>
<td>9.6 ± 0.9</td>
<td>11.9 ± 1.3</td>
<td>0.1491</td>
</tr>
</tbody>
</table>

Definition of abbreviations: APACHE = Acute Physiology and Chronic Health Evaluation; BMI = body mass index; GCS = Glasgow Coma Scale; GCS-intubated = GCS of patients intubated prior scoring; MICU = medical intensive care unit; NA = not applicable; NS = not significant; SICU = surgical intensive care unit; VAP = ventilator-associated pneumonia; VAT = ventilator-associated tracheobronchitis.

*Mean ± SD shown for age, BMI, Charlson Comorbidity Index, APACHE II, GCS, GCS-intubated, hospitalization days, ICU days, and ventilation days categories.

‡Two-sample *t* test (VAP vs. VAT/VAP vs. colonized/VAT vs. colonized).

*One-way analysis of variance of mean of three groups.

†Fisher exact two-tailed probability test.
Distribution of MRSA and MSSA Strains in the Different Patient Groups
MRSA and MSSA were found in 23 and 26 samples of 45 patients, respectively (three patients carried both MRSA and MSSA) (Figure 2). A total of 75% of isolates from the surgical ICU were MSSA (12 of 16), whereas more MRSA than MSSA were detected among the medical ICU isolates (19 of 33) \( (P = 0.0164, \text{ Fisher exact two-tailed probability test}) \). Nasal swabs taken at admission to ICUs followed by weekly sampling were approximately 75% predictive for the presence of MRSA or MSSA in subsequently collected ETA samples (Figure 2).

Distribution of MRSA and MSSA strains in the different patient groups was significantly different. Although 67% of colonized patients (12 of 18) carried MRSA, 87% (13 of 15) of VAP patients were infected with MSSA \( (P = 0.0062, \text{ two-sample t test}) \). Only four ETA samples from VAP patients contained MRSA, two of these occurred together with MSSA. MRSA and MSSA strains were equally represented in VAP patients who did not progress to VAT.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Collection of serial samples from ventilated patients. Data are shown in three groups according to the diagnosis of patients: ventilator-associated pneumonia (VAP), ventilator-associated tracheobronchitis (VAT), and colonized. Length of ventilation is given as time-line (days) and time of VAT and VAP diagnosis is indicated by green and red diamonds, respectively. Methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) positive endotracheal aspirates (ETA) samples are marked with closed and open circles, respectively. Strength of...
VAP (7 and 6 out of 12, respectively, Figure 2).

These data strongly suggest that MSSA strains were more likely to cause VAP than MRSA strains in this study population.

**Determination of S. aureus Genotypes**

We further characterized the isolates based on MLST and spa typing. At least one strain from each patient was included in the analysis. When multiple samples were collected, the first and last available isolates were tested, and even more when different sheep blood agar hemolysis patterns or both MSSA and MRSA were detected in individual patients. MLST and spa typing revealed that all 26 MSSA strains belonged to a different clonal type, whereas the 23 MRSA strains were represented by five sequence types. The most dominant clonal lineage was the ST5-II-t002, a well-known hospital-associated MRSA also represented by the USA100 PFGE standard strains. This was detected in 37% of patients and was responsible for 71% of MRSA infections. The ST5-II-t002 clone was isolated throughout the 7 months of the study. Three MRSA clonal types occurred in two different patients without temporal association (see Table E1 in the online supplement).

**Sheep Blood Agar Hemolysis Patterns**

On culturing the S. aureus isolates on sheep blood agar, we detected a broad range of hemolytic activities (Figure 3A). Most of the isolates displayed a hemolysis pattern characteristic for Hla causing β-hemolysis and a clear halo on sheep blood agar plates. A few isolates showed hemolysis with a turbid halo, which is typical for α-hemolysin (Hlb) production of S. aureus. The presence of the functional hlb gene in these isolates was confirmed by PCR (data not shown).

We detected comparable proportion of high and low hemolytic S. aureus isolates among MRSA and MSSA (4+ or 3+: 12 of 23 and 12 of 26, respectively). However, VAP patients significantly more likely carried highly hemolytic (3+ or 4+) MSSA strains than non-VAP patients (10 out of 13 vs. 2 out of 11, respectively; \( P = 0.0123 \), Fisher exact two-tailed probability test). Such correlation between hemolysis strength and VAP occurrence was not found with MRSA strains (Figure 3B).

VAP patients who were infected with MSSA strains exhibiting moderate, low, or no blood agar hemolysis were taken off the ventilator earlier than those infected with highly hemolytic strains (Figure 2). Isolates from serial samples of the individual patients displayed similar blood agar hemolysis profile, except when more than one molecular type was identified by MLST or spa typing (data not shown).

Based on these data, MSSA strains strongly hemolytic on sheep blood agar are much more likely to cause VAP than weakly hemolytic isolates.

**α-Hemolysin Activity of Isolates**

To correlate the hemolytic profiles on sheep blood agar with Hla activity, we determined the strength of cytolysis of serially diluted overnight culture supernatants from S. aureus isolates using a human lung alveolar epithelial cell line (A549). The specificity of this assay for Hla was demonstrated by the lack of cell lysis by a hla gene-deletion mutant S. aureus strain (TCH1516, USA300) and in the presence of

*Figure 3.* Hemolytic phenotypes of Staphylococcus aureus isolates on sheep blood agar plates. (A) S. aureus isolates were plated on sheep blood agar plates and categorized as strong to weak or no β-hemolysis (from 4+ to –) (characteristic for α-hemolysin activity), according to the diameter of cleared halo around the colonies based on semiquantitative evaluation. Turbid halos surrounding the colonies were accounted for β-hemolysin (Hlb) expression. (B) Distribution of S. aureus strains with different blood agar hemolysis patterns among the different patient groups is indicated. (Left) Methicillin-sensitive S. aureus; (right) methicillin-resistant S. aureus. The \( P \) value is given for ventilator-associated pneumonia (VAP) versus non-VAP patient groups calculated with the Fisher exact two-tailed probability test. Col = colonized; n.s. = not significant; VAT = ventilator-associated tracheobronchitis.
a Hla-neutralizing monoclonal antibody (see Figure E1).

Because it is known that the composition of growth media greatly influences toxin expression, S. aureus isolates (two individually picked clones) were grown in three different culture media: TSB, CCY, or RPMI supplemented with casamino acids. We found that blood agar hemolysis strength was overall in agreement with cytolytic activity, and CCY culture supernatants showed the best resolution between the different hemolysis category groups (Figure 4). Hla activity of isolates that expressed Hlb became quantifiable (masked by the turbid hemolysis pattern on blood agar).

Similarly to blood agar hemolysis, a great variation in Hla activity measured in the A549 viability assays was observed among the S. aureus isolates. The serial samples of individual patients, often collected more than a week apart, were remarkably similar to each other in their cytolytic pattern (Figure 5A). When patients were infected with two different S. aureus clonal types, these were recognizably different from each other (e.g., patients P22, P39, and P198). The ST5-II-002 isolates behaved differently when isolated from different patients and displayed a broad range of cytotoxicity from no or low to high activity (Figure 5B). We also observed different Hla activities with other clonal types when isolated from different patients (examples shown in Figure 5C).

By comparing the cytotoxicity of MRSA and MSSA strains (the first available isolate from each patient), we found a comparable distribution and median value (Figure 6), which is in good agreement with the semiquantitative blood agar hemolysis data. However, culture supernatants of MSSA strains obtained from VAP patients were overall more cytotoxic than those from colonized or VAT patients based on group median values. This trend was consistent with all three growth media tested (Figures 6A–6C), but did not reach statistical significance. Such trend was not observed with MRSA strains.

**Virulence Testing in Murine Pneumonia Model**

We investigated whether MRSA and MSSA strains, both with high and low hemolytic activity, were associated with different virulence profiles in murine pneumonia. The three strains characterized with high Hla activity were associated with 100% lethality at the highest dose tested, whereas most animals survived at the same dose of isolates characterized with low or no detectable in vitro Hla activity (Table 2). Two lower bacterial challenge doses also differentiated well between strains with low and high Hla activity.

These results confirmed the major role of Hla in the murine S. aureus pneumonia
pathogenesis; however, the survival outcomes did not reflect MRSA or MSSA background or disease outcome (colonized vs. VAP).

Discussion

In this study, we analyzed a unique collection of \textit{S. aureus} isolates obtained by serial sampling of ventilated patients and correlated our data with the diagnosis of patients. We found that one-third of the patients heavily colonized with \textit{S. aureus} developed VAP and most of them carried MSSA strains. VAP and MSSA strains were much more likely to be detected in the surgical ICU, whereas VAT and colonized patients and MRSA strains were more likely to be found in the medical ICU. Higher prevalence of VAP in general and MSSA-induced VAP in particular in surgical/truma versus medical ICU patients have also been reported by others (23, 24). The significantly lower prevalence of MRSA versus MSSA in VAP patients and the inverse prevalence in colonized patients suggests that MRSA strains are associated with lower pneumonia-causing potential, supporting the notion that antibiotic resistance is associated with reduced virulence (25).

Although MSSA strains were genetically very diverse, 87% of MRSA isolates were ST5-II-1002 and ST5-II-1242. These molecular types were reported to belong to the USA100 PFGE type, the dominant hospital-associated MRSA in North America until recently (26–30). The isolates characterized in this study were collected in 2010 in North America (Burlington, MA). Since then, USA300 CA-MRSA strains replaced a significant portion of USA100 infections in US hospitals (31).

The most important finding of this study is the significantly higher blood agar hemolytic activity of MSSA strains isolated from VAP patients compared with those from VAT or colonized patients. Such correlation was not observed for MRSA isolates. By using a quantitative cytolytic assay with human airway cells, specifically measuring Hla activity, we detected a good correlation with the blood agar hemolysis profile. There was a tendency for higher Hla activity of MSSA associated with VAP compared with VAT and asymptomatic

Figure 5. Cytotoxic activities of \textit{Staphylococcus aureus} isolates greatly differ, but are very similar in serial samples from a given patient. A549 cells were incubated with culture supernatants of \textit{S. aureus} isolates grown in three different media as indicated and cytotoxicity measured as described in the Methods. Mean values ± SEM obtained with independent biologic replicates of the same isolates are shown. (A) Serial samples from individual patients with the genotypes indicated; (B) ST5-II-1002 isolates; (C) ST8-1334 isolates.
colonization (without reaching statistical significance with this low sample size).

Hla is one of the best characterized virulence factors in *S. aureus* pneumonia pathogenesis in animals (10). However, very little evidence exists that Hla contributes to pneumonia in clinical settings. Gene prevalence studies are not supportive to assess the role of Hla, because the *hla* gene is present in all *S. aureus* isolates sequenced to date (>300 genomes) and is also present in all isolates of this study (based on PCR analysis, data not shown). We are aware of only one study that correlated the extent of hemolysis on sheep blood agar to disease severity in the case of peritonitis (32).

It is known from the literature that Hla expression is influenced by the composition of the culture medium and growth conditions. The presence of red blood cells is known to up-regulate virulence factor expression (33); therefore, culturing on sheep blood agar as done for assessing the hemolysis profiles in a semiquantitative manner is a highly relevant condition. It was rather surprising to detect a very broad range of Hla activities from isolates representing the same clonal lineage but isolated from different patients. We could even detect different responses of isolates in terms of Hla expression to the different culture media. Based on analysis of serially collected samples, Hla activity remained unchanged during colonization and over the course of disease within individual patients. This suggests that strains have a "personal history" and undergo changes while colonizing individuals. Hla expression is under complex regulation (10). Mutations in global regulators of toxin expression, such as the *agr* system and Sar, are described in MRSA strains (34–36). Genetic alterations affecting *hla* itself are also implicated in altered activity in both directions (37, 38). Further genetic studies are needed to uncover the molecular basis of the high variability in Hla expression of clonally related *S. aureus* strains.

It is highly relevant for the ICU patient population, being extensively treated with multiple antiinfectives, that several antibiotics, even at subinhibitory concentrations, have been shown to increase toxin production of *S. aureus* strains (39, 40). In several studies, improved therapeutic efficacy of linezolid over vancomycin was observed; however, this was not associated with a more rapid microbial clearance suggesting that mechanisms other than direct antibacterial activity are involved (41, 42). It was shown in a therapeutic rabbit pneumonia model that the use of linezolid was associated with lower toxin production by *S. aureus* and lower cytokine levels compared with treatment with vancomycin (43).

On testing *S. aureus* isolates in murine pneumonia model, we observed good

**Figure 6.** Cytotoxicity profiles of strains involved in colonization (col), ventilator-associated tracheobronchitis (VAT), and ventilator-associated pneumonia (VAP). A549 cells were incubated with culture supernatants of *Staphylococcus aureus* isolates grown in (A) TSB, (B) CCY, and (C) RPMI supplemented with casamino acids. Cytotoxicity indices were determined as described in the *Methods* and are shown for methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) isolates in different groups of patients as indicated. Each patient is represented by the first available isolate, or by two isolates in case two different molecular types were identified. MSSA and MRSA isolates are indicated by closed or open circles, respectively; and multiple isolates from the same patient by squares. Horizontal lines indicate group median values.

...
shown to be species (human) specific virulence mechanisms. Moreover, several to overestimation of toxin-mediated (physiologic bacterial challenge doses) model in ventilated pigs using more mechanical ventilation). Therefore, alternative (heavy colonization, underlying diseases, and major risk factors associated with VAP (certain leukotoxins) and mice do not have the indicated as percentage of live versus total number of animals used (n = 10 mice per isolate and challenge dose).

Percent Survival after Intranasal Challenge (Day 7)

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Hemolysis Pattern</th>
<th>9 × 10^6 cfu</th>
<th>3 × 10^6 cfu</th>
<th>1 × 10^6 cfu</th>
<th>Molecular Type</th>
</tr>
</thead>
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<tr>
<td>P125.1 (col)</td>
<td>1+</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>ST5-II-t002</td>
</tr>
<tr>
<td>P159.1 (col)</td>
<td>–</td>
<td>30</td>
<td>100</td>
<td>100</td>
<td>ST5-II-t002</td>
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<tr>
<td>P28.1 (VAP)</td>
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<tr>
<td>P178.1 (col)</td>
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<td>76.7</td>
<td>100</td>
<td>100</td>
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<tr>
<td>P44.1 (col)</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>P151.1 (VAP)</td>
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<td>0</td>
<td>50</td>
<td>100</td>
<td>STT2-t148</td>
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Average survival

<table>
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<th>Isolate ID</th>
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<th>9 × 10^6 cfu</th>
<th>3 × 10^6 cfu</th>
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<tr>
<td>P178.1 (col)</td>
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<td>0</td>
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<tr>
<td>P151.1 (VAP)</td>
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<td>0</td>
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</table>

Average survival

Table 2. α-Hemolysin–mediated In Vitro Cytotoxicity Predicts In Vivo Virulence in Mice

definition of abbreviations: col = colonized; VAP = ventilator-associated pneumonia.

Four methicillin-resistant Staphylococcus aureus and two methicillin-sensitive S. aureus isolates selected based on their α-hemolysin activity and sheep blood agar hemolysis profile were used for intranasal challenge of mice at three different bacterial challenge doses (cfu given per mouse). Survival is indicated as percentage of live versus total number of animals used (n = 10 mice per isolate and challenge dose).

correlation between Hla activity and virulence (assessed by lethality). However, we did not detect higher virulence of MSSA VAP isolates compared with MRSA colonizing strains, suggesting that murine models do not reflect all important aspects of disease-causing potential in humans. This is most likely caused by the dominant role of Hla in murine pneumonia model documented in numerous studies (reviewed in 10). Because mice are not natural hosts for S. aureus, a high inoculum is needed to induce lethal infection (∼10^8 cfu), leading to overestimation of toxin-mediated virulence mechanisms. Moreover, several virulence factors of S. aureus have been shown to be species (human) specific (e.g., certain leukotoxins) and mice do not have the major risk factors associated with VAP (heavy colonization, underlying diseases, and mechanical ventilation). Therefore, alternative animal models that are more representative for the human setting, such as the pneumonia model in ventilated pigs using more physiologic bacterial challenge doses (∼10^6 cfu), should also be considered for assessing virulence of S. aureus strains (44).

In addition, well-designed clinical research studies performed in different geographic regions are of utmost importance to understand the difference between MSSA and MRSA pathogenesis and the host-pathogen interactions. Changes in innate host immunity, such as complement activity and cytokine expression, are also reported to be associated with or predictive for VAP and/or VAP and can have a contribution in this clinical setting (45, 46).

In conclusion, our data suggest that airway colonization with MSSA strains with high Hla activity is a predictor of progression to VAP. Measuring Hla activity in cell-based assays is too laborious and clinically not applicable. Therefore, we propose to assess the hemolytic activity of MSSA isolates from ETA samples of ventilated patients on blood agar plates. Although this test is semiquantitative and therefore to a certain extent subjective, it is very simple and performed routinely in hospital microbiology laboratories. Molecular diagnostic approaches, such as quantification of hla mRNA with reverse transcriptase PCR or measuring the amount of Hla with antibody-based methods, are worth being explored. Based on our data, the results of such analyses can support differential diagnosis (VAP vs. VAT vs. colonization) and be useful for identifying patients for earlier antibiotic therapy or prophylaxis with Hla-neutralizing monoclonal antibodies.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank team members Gabor Nagy for critical review of the manuscript; Marisa Caccamo, Zehra Visram, Karin Gross, and Marian Fürsatz for technical help; Yuxiu Lei (Lahey Clinic Medical Center, Burlington, MA) for support in clinical data collection; Valéria Szajjartó (Aranseat), Robin Ruthazer (Biostatistics Research Center, Institute for Clinical Research and Health Policy Studies, Tufts Medical Center, Boston, MA), and Abderrahim Oulhaj (College of Medicine and Health Sciences, Al-Ain, United Arab Emirates) for help with statistical analyses; Knut Ohlsen (University of Wuerzburg, Germany) for providing the pKFT Staphylococcus aureus strain; and Fuminori Kato (Hiroshima University, Japan) for sharing the pKFT shuttle-vector.

References


Stulik, Malafa, Hudcova, et al.: Highly Hemolytic MSSA in ETA Predicts VAP

ORIGINAL ARTICLE


