Cytotoxic Virulence Predicts Mortality in Nosocomial Pneumonia Due to Methicillin-Resistant *Staphylococcus aureus*

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(See the editorial commentary by Popovich and Hayden on pages 1857–9.)

The current study identified bacterial factors that may improve management of methicillin-resistant *Staphylococcus aureus* (MRSA) nosocomial pneumonia. Isolates were obtained from 386 patients enrolled in a randomized, controlled study of antibiotic efficacy. Isolates were screened for production of virulence factors and for vancomycin susceptibility. After adjustment for host factors such as severity of illness and treatment modality, cytotoxic activity was strongly and inversely associated with mortality; however, it had no effect on clinical cure. Isolates having low cytotoxicity, which were derived largely from healthcare-associated clones, exhibited a greater prevalence of vancomycin heteroresistance, and they were recovered more often from patients who were older and frailer. Additionally, a clone with low cytotoxic activity was associated with death and poor clinical improvement. Clone specificity and attenuated virulence appear to be associated with outcome. To our knowledge, these are the first correlations between MRSA virulence and mortality in nosocomial pneumonia.

Keywords. *Staphylococcus aureus*; MRSA; nosocomial pneumonia; outcome; virulence.

Therapy of nosocomial pneumonia (NP) caused by methicillin-resistant *Staphylococcus aureus* (MRSA) is often ineffective. Mortality rates are around 20%–40%, even when treatment conditions are optimized under experimental protocols [1–3]. A rational approach for improving outcomes has sought to understand microbial factors that distinguish favorable from adverse outcomes. Recently, the role of genetically distinct strains of MRSA of community origin on the outcome of MRSA infection has attracted attention [4–7]. Community-acquired

MRSA (CA-MRSA) strains have emerged as a cause of infections inside healthcare settings [8, 9], and they have been linked with differences in virulence profile and clinically significant events, such as NP [9–12]. Explanations for conflicting outcome differences from these studies have been limited by difficulty in establishing the intrinsic virulence of a given bacterial strain. Moreover, the status of the host is critical to the outcome of MRSA infection, and particularly NP, independent of the presence or absence of virulence attributes [13–15]. Thus, the virulence or nonvirulence of a strain cannot be inferred from the outcomes of the patients from which the strain was isolated without specifying factors such as host risk and infection type. The issue is particularly relevant for subsets of NP, such as ventilator-associated pneumonia, that may have no attributable mortality [13, 16, 17].

In the present work, we sought to disentangle characteristics of the bacteria and host that predispose individuals to severe outcomes. The 381 MRSA strains studied composed all available pulmonary isolates obtained at the time of diagnosis during a large prospective clinical

trial comparing the efficacy of linezolid and vancomycin in the treatment of NP due to MRSA [18]. Pooled analysis of individual patient-level data were used to discern the impact of treatment strategies on clinical cure and mortality. The analyses took into account host-related factors and organism-related factors, namely virulence and antimicrobial resistance. Analysis included adjustment for the potentially confounding effects of clonality. We measured virulence under normalized conditions, using a combination of relative leukocytotoxic activity (in which individual leukotoxic activities are inseparable) and screening for functionality of the global virulence regulator *agr*.

METHODS

MRSA Isolates

MRSA strains were obtained during a previously described randomized, double-blinded, international, multicenter clinical trial [18]. Between October 2004 and January 2010, hospitalized patients aged ≥18 years with radiographic and clinical evidence of pneumonia were randomized to treatment with intravenous linezolid or weight-based vancomycin. The study protocol allowed for vancomycin dosage adjustments and pharmacokinetic monitoring according to the local standard of care. Extensive inclusion and exclusion criteria, as well as antimicrobial interventions and assessments, have been described previously [18]. Although invasive, quantitative methods were not required for diagnosis, reflecting common practice in the United States, most diagnoses were made by invasive methods or blood culture. Clinical outcome was assessed within 5 days after the end of treatment, defined as within 72 hours of the last dose of antimicrobial, and at the end of the study, defined as 7–30 days after the last dose.

Overall mortality was assessed at 60 days after therapy. Cultures were obtained at baseline, the end of treatment, and the end of the study. Of the 1225 patients randomized in this study, 448 were included in a modified intention-to-treat analysis of patients who received study drug and received a diagnosis of MRSA NP. Three hundred eighty-one available baseline isolates, from 160 patients with hospital- or healthcare-associated MRSA pneumonia and 221 with ventilator-associated pneumonia, were used in the analysis. Primary airway cultures, obtained by several methods, were used in all 381 cases. Five additional patients whose isolates were from blood were not initially available for determination of cytotoxic activity were included in the analysis of outcomes (n = 386) [19]. (Consistent with previous reports [20], genotypic and phenotypic characteristics of pairs of blood and respiratory isolates among the 29 other patients with bacteremic pneumonia for which both isolates were available were always identical [data not shown].) All laboratory work was performed blinded with respect to patient data.

agr Functionality

Hemolytic patterns produced by *Staphylococcus* on sheep's blood agar by cross-streaking against RN4220, which produces only β-hemolysin, were used to semiquantitatively assay *agr* functionality [21, 22]. Previous work indicates that completely nonhemolytic strains of *S. aureus* and *Staphylococcus epidermidis* harbor inactivating mutations in *agr* genes in virtually all cases [20, 23–25].

Cytotoxic Activity

Bacteriologically sterile culture filtrate preparations, obtained from early logarithmic-phase (6 hours) growth, were used for differentiating cytotoxic activity, as described elsewhere [26]. Briefly, strains were grown in 96-well, round-bottomed plates containing 150 μL of tryptic soy broth for 16 hours with shaking at 2469g at 37°C. Cultures were diluted 1:75 with fresh Roswell Park Memorial Institute plus casamino acids, and 150 μL of the diluted culture was regrown in 96-well, round-bottomed plates for 6 hours at 2469g at 37°C. Plates were centrifuged to pellet the bacteria, and culture supernatants were collected and stored at −80°C until used. To measure the cytotoxic activity within culture supernatants, we used cells from the human myeloid cell line HL-60 that were differentiated into neutrophil-like cells (PMN-HL60). PMN-HL60 cells have been extensively used to evaluate the cytotoxic activity of *S. aureus* culture supernatants and have been shown to mimic the sensitivity of primary human neutrophils [26–28]. To measure cell viability, we monitored metabolic activity using CellTiter (Promega), as described by the manufacturer. Twenty microliters of *S. aureus* supernatant was incubated with approximately 1.0×10^5 PMN-HL60 cells in a final volume of 100 μL (20% v/v supernatants) for 2 hours at 37°C, followed by 2-hour incubation with the CellTiter reagent.

Each sample was assayed in triplicate, and the experiments were repeated at least twice. Variability in the assay was observed, partially as a result of differences in growth rates among the strains, with errors skewed positively at low values and negatively at high (Supplementary Table 1 and data not shown). However, consistently high (or low) cytotoxicity as defined was a stable trait of a given clinical MRSA isolate. Although there are no biologically driven informative break points for cytotoxic activity, use of ≥90% killing of the cells within 2 hours as a marker of highly cytotoxic strains resulted in a convenient and clear-cut parameter that was analyzed dichotomously. Because a dichotomous tool ignores some of the information contained in a scoring system, we also modeled the exposure variables as a stratified score with quartiles, to allow for nonlinear associations and for ease of interpretability (compared with single-unit comparisons). The highest quartile of cytotoxicity (92% killing) closely approximated the cutoff for highly cytotoxic strains and provided a basis for why this value was chosen.

Genotyping

The relatedness of strains from respiratory tract and blood specimens from subjects, as well as variants from mixed *agr+* and *agr−* cultures, was previously determined by DNA sequence

Table 1. Clinical Data and Bacterial Characteristics Among Patients With Nosocomial Pneumonia Due to Methicillin-Resistant *Staphylococcus aureus* (MRSA), by Cytotoxicity

Table 1   continued.

Characteristic	High Cytotoxicity[a] (n = 108)	Low Cytotoxicity (n = 273)	P Values
Genotypic characteristic			
agr group			
Overall	2.0 ± 0.2	1.3 ± 0.8	<.0001
I	83 (76.9)	68 (24.9)	<.0001
II	25 (23.1)	196 (71.8)	<.0001[b]
III	0 (0.0)	8 (2.9)	
NT	0 (0.0)	1 (0.4)	
SCCmec type			
I, II, III	20 (18.5)	243 (89.0)	<.0001
IV	88 (81.5)	30 (11.0)	
CC			<.0001
5	24 (22.2)	197 (72.2)	<.0001[c]
8	69 (63.9)	17 (6.2)	<.0001[c]
22	7 (6.5)	1 (0.4)	.0008[c]
45	0 (0.0)	13 (4.8)	.0235[c]
8/239	4 (3.7)	34 (12.5)	.008[c]
Other	4 (3.7)	11 (4.0)	
spa type 2	11 (10.2)	112 (41.0)	<.0001
pvl (positive)	39 (36.1)	9 (3.3)	<.0001
Phenotypic characteristic			
agr defective	0 (0.0)	61 (22.3)	<.0001
agr functionality[d]			
0	0 (0.0)	61 (22.3)	<.0001[e]
1	4 (3.7)	68 (24.9)	
2	104 (96.3)	144 (52.7)	
0, 1	4 (3.7)	129 (47.3)	<.0001[f]
hVISA	4 (3.7)	56 (20.5)	<.0001
Vancomycin MIC			
By the Etest			
>1	96 (88.9)	241 (88.3)	1
>1.5	22 (20.4)	99 (36.3)	.0032
By an automated method			
0.5	15 (13.9)	14 (5.1)	.0082
1	87 (80.6)	231 (84.9)	
≥2	6 (5.6)	27 (9.9)	
Linezolid MIC, by the Etest			
1	9 (8.6)	14 (5.1)	.0594
2	80 (76.2)	188 (69.1)	
4	16 (15.2)	70 (25.7)	
Demographic/clinical characteristic			
Age, y			
Overall[g]	56.6 ± 20.2	63.5 ± 17.1	.0008
>65	46 (42.6)	145 (53.1)	.0696
Weight, kg[h]	77.3 ± 21.5	78.3 ± 22.5	.6812
Male sex	76 (70.4)	167 (61.2)	.0989
Race			
Asian	11 (10.2)	48 (17.6)	.0051
Black	21 (19.4)	26 (9.5)	
White	75 (69.4)	184 (67.4)	.7168[i]
Other	1 (0.9)	15 (5.5)	
Region			
Asia	8 (7.4)	43 (15.8)	.0006
Europe	19 (17.6)	17 (6.2)	
Latin America	5 (4.6)	31 (11.4)	
Other	7 (6.5)	10 (3.7)	
United States	69 (63.9)	172 (63)	
Treatment			
Linezolid	53 (49.1)	136 (49.8)	.91
Vancomycin	55 (50.9)	137 (50.2)	
APACHE II score			
Overall[j]	17.0 ± 5.7	17.5 ± 6.3	.4248
≤20	74 (68.5)	186 (68.1)	.27[k]
>20	31 (28.7)	85 (31.1)	
Missing	3 (2.8)	2 (0.7)	
CPIS[l]	9.6 (1.9)	9.4 (2.3)	.4714
Creatinine clearance rate (MDRD)[m]	100.4 ± 48.4	91.6 ± 58.2	.1726
ICU stay at baseline	95 (88.0)	237 (86.8)	.8658
Vasopressor therapy at baseline	19 (17.6)	45 (16.5)	.8792
Corticosteriod therapy at baseline	19 (17.6)	63 (23.1)	.2702
Bacteremia	15 (13.9)	36 (13.2)	.8681
Chest radiography finding			
Bilateral	69 (63.9)	190 (69.6)	.1776
Unilateral	38 (35.2)	83 (30.4)	
Missing	1 (0.9)	0 (0.0)	
Pleural effusion	38 (35.2)	133 (48.7)	.022
Pneumonia, by type			
Hospital associated	21 (19.4)	78 (28.6)	.0034
Healthcare associated	10 (9.3)	51 (18.7)	
Ventilator associated	77 (71.3)	144 (52.7)	
Ventilation at baseline	83 (76.9)	190 (69.6)	.1674
Infecting pathogen(s)			
MRSA + anaerobe	0 (0.0)	2 (0.7)	.4757
MRSA + fungus	1 (0.9)	10 (3.7)	
MRSA + gram negative	33 (30.6)	66 (24.2)	
MRSA + gram positive	10 (9.3)	21 (7.7)	
MRSA + gram positive + gram negative	5 (4.6)	15 (5.5)	
MRSA only	59 (54.6)	159 (58.2)	
Gram-negative coverage at baseline	105 (97.2)	269 (98.5)	.4097
Anaerobic coverage at baseline	64 (59.3)	148 (54.2)	.4234
Service			
Medical	49 (45.4)	162 (59.3)	.0012
Surgical	24 (22.2)	68 (24.9)	
Trauma	35 (32.4)	43 (15.8)	

Table 1 continued.

Characteristic	High Cytotoxicity[a] (n = 108)	Low Cytotoxicity (n = 273)	P Values
Comorbidity			
Cardiac	54 (50.0)	175 (64.1)	.0146
Diabetes	29 (26.9)	121 (44.3)	.0017
Neoplastic	12 (11.1)	29 (10.6)	.8565
Renal/urinary	30 (27.8)	124 (45.4)	.0017
Pulmonary	74 (68.5)	187 (68.5)	1
Hepatobiliary	13 (12.0)	41 (15.0)	.5168
Gastrointestinal	61 (56.5)	157 (57.5)	.9087
LTCF stay at baseline	4 (3.7)	12 (4.4)	1
Outcome			
Clinical response			
At end of treatment	72 (73.5)	203 (76.6)	.5815
At end of study	51 (53.7)	130 (52.4)	.9039
Mortality			
Overall	14 (13.0)	85 (31.1)	.0002
By day 28	6 (5.6)	60 (22.0)	<.0001
By day 60	13 (12.0)	81 (29.7)	.0002

Data are no. (%) of patients or mean value ± SD.

Abbreviations: APACHE, Acute Physiology And Chronic Health Evaluation; CPIS, Clinical Pulmonary Infection Score; hVISA, vancomycin-heteroresistant *S. aureus;* ICU, intensive care unit; LTCF, long-term care facility; MDRD, Mediterranean diet in renal disease; MIC, minimum inhibitory concentration; NT, not typable; SCC*mec*, staphylococcal cassette chromosome *mec.*

[a] Defined as a mean of ≥90% killing of PMN-HL60 cells within 2 hours.

[b] Compared with data for types I and III combined and for NT.

[c] Compared with data for the other 4 CCs.

[d] Semiquantitative scoring of δ-hemolysin on sheep blood agar: 0, non-$ hemoltyic; 1, weakly hemolytic; 2, strongly hemolytic.

[e] Compared with data for *agr* functionality 1 and for *agr* functionality 2.

[f] Compared with data for *agr* functionality 2.

[g] Data are for 108 in the high cytotoxicity group and 273 in the low cytotoxicity group.

[h] Data are for 108 in the high cytotoxicity group and 272 in the low cytotoxicity group.

[i] Compared with data for Asian, Black, and Other combined.

[j] Data are for 105 in the high cytotoxicity group and 271 in the low cytotoxicity group.

[k] Compared with data for >20 and for Missing.

[l] Data are for 81 in the high cytotoxicity group and 185 in the low cytotoxicity group.

[m] Data are for 104 in the high cytotoxicity group and 268 in the low cytotoxicity group.

analysis of the protein A gene variable repeat region (*spa* typing), pulsed-field gel electrophoresis, and a variety of additional DNA polymorphisms including staphylococcal cassette chromosome *mec* (SCC*mec*), *agr* specificity group, and the presence or absence of the *pvl* gene [19]. Clonal complexes (CCs) were assigned on the basis of *spa* typing results, using the multilocus sequence typing mapping database (http://spa.ridom.de/mlst) or peer-reviewed reports, as described elsewhere [19]. Previously published polymerase chain reaction primers and conditions

were used to screen all study isolates for the presence of *sasX* [29].

Susceptibility Testing

Isolates were previously tested for susceptibility by broth microdilution and Etest and for heterogenous vancomycin resistance by Etest methods [19].

Statistical Analysis

Comparisons of proportions were performed by the Fisher exact test or χ^2 analysis, and comparisons of continuous variables were performed by analysis of variance or the *t* test. Outcomes were modeled using logistic regression with backward elimination and a 1000 bootstrap sample strategy for confirmation. All variables from the parent study were considered for the modeling building process [18] (Table 1). Before model building, covariate reduction techniques were used to adjust for near-zero variance, missingness, and covariate correlations, associations, and clusters. Criteria for entry into and retention within the model were *P* values of .05 and .10, respectively. No significant interactions were found.

RESULTS

Cytotoxic Virulence

Staphylococcal virulence is multifactorial, making it difficult to assess the contribution of specific virulence factors to outcomes in *S. aureus* infection. A promising approach to address virulence is to assess the role of global regulatory systems that affect a large part of the virulon, such as the quorum-sensing virulence regulator *agr* [30]. A significant fraction of clinical isolates have genotypic *agr* defects [20, 23, 24], which raises the possibility of determining the role of this system, and therefore virulence, in disease. However, *agr* functionality alone does not imply efficient expression of virulence. To more fully capture differences in virulence among isolates, we sought an additional, direct measure of virulence factor production. The leukocytotoxins, *agr*-regulated pore-forming toxins (bicomponent leukocidins and alpha-hemolysin) [31, 32], and membrane-damaging cytolytic peptides [33] that are found in virtually all staphylococcal isolates are attractive candidates for a wide-ranging virulence factor whose presence can effectively distinguish low- and high-virulence strains.

Comparison of the cytotoxic activity of 381 individual isolates revealed that a majority (n = 273) were not highly cytotoxic (Table 1 and Supplementary Table 1). When cytotoxic activities were linked to the genetic background of the strain, we found that for about 90% of the isolates the 3 major MRSA CCs (CC5, CC8, and CC8/239) displayed a characteristic level (Figure 1A). CC8 strains were consistently the most cytotoxic. In contrast, CC5 variants had no specific pattern of cytotoxicity, and CC8/239 strains were on average weakly cytotoxic. Not
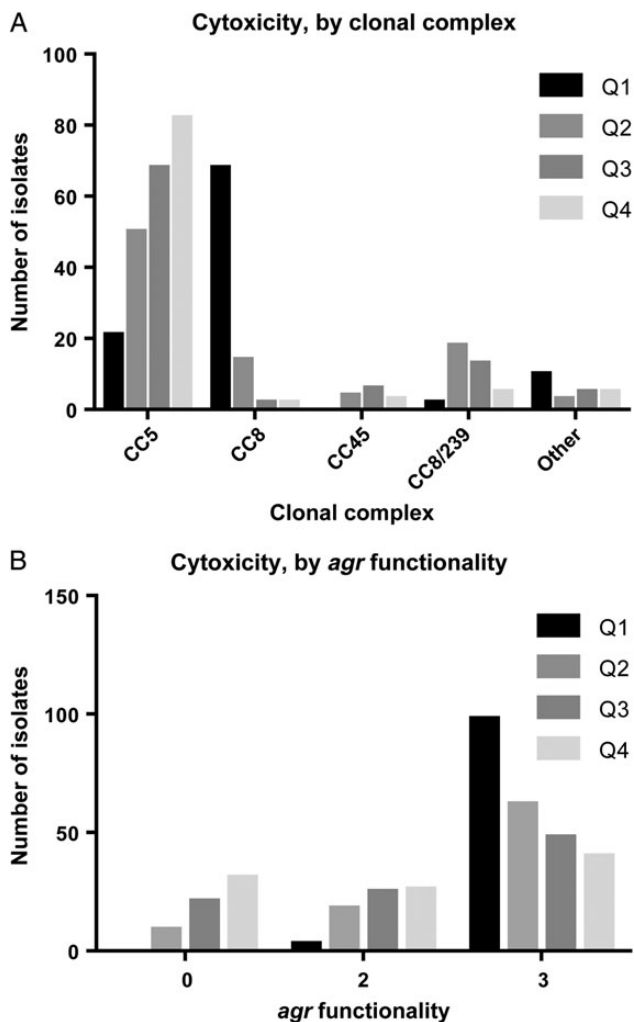
Figure 1. Cytotoxic activity, by major clonal complex (CC; *A*) and *agr* functionality (*B*). CCs are considered major if there are ≥10 strains with the same CC. Cell viability of PMN-HL60 cells with 20 μL of supernatant for 2 hours, measured with the metabolic dye CellTiter. δ-hemolysin semi-quantitative scoring on sheep blood agar: 0, nonhemoltyic; 1, weakly hemolytic; 2, strongly hemolytic. Q, quartile range of cytotoxicity, from the upper quartile (Q1) to the lower quartile (Q4).

surprisingly, the relative level of *agr* expression by distinct MRSA strains correlated with cytotoxic properties ($P < .0001$; Table 1 and Figure 1*B*). However, cytotoxic profiles varied remarkably within each CC and even within the same *agr* functional group.

Antimicrobial Susceptibility and Cytotoxicity

Reduced susceptibility to vancomycin has been linked to clinical failure of vancomycin treatment [34] and *agr* dysfunction [35, 36]. It may also influence *S. aureus* pathogenicity independent of *agr* function [37, 38]. Overall, the vancomycin minimum inhibitory concentrations (MICs) for study isolates were comparable to those of recent series (Table 1 and Supplementary

Table 1). Six of 60 vancomycin-heteroresistant *S. aureus* (hVISA) isolates had a standard Etest MIC of ≤1.0, 27 had an MIC of 1.5, and 27 had had an MIC of ≥ 2 μg/mL. Thus, we observed poor concordance between vancomycin heteroresistance and high MICs. However, an elevated vancomycin MIC (≥1.5 μg/mL) was associated with low cytotoxic activity (36% vs 20%; $P = .0032$; Table 1) and CC ($P = .0022$; not shown) but not with *agr* functionality (31% vs 31%; $P = 1.0$; data not shown). The MICs of linezolid were below the resistance break point (≤4 μg/mL) for all isolates. More than 90% of the linezolid MICs were identical within 1 dilution (2.0–4.0); thus, the usefulness of linezolid susceptibility as a marker for outcome was limited.

CA-MRSA Genes

MRSA strains prevalent in the community differ from health-care-associated strains in that they carry a different SCC*mec* (SCC*mec*IV), are resistant to fewer antibiotic classes (frequently only β-lactams and erythromycin), and often have genes encoding toxins such as Panton-Valentine leukocidin [39]. Many different strains of CA-MRSA now have a worldwide distribution. In contrast, MRSA strains carrying SCC*mec* types I, II, and III and their derivatives are usually restricted to hospital-acquired MRSA (HA-MRSA) clones that are not found widely within the healthy population. Strains belonging to SCC*mec*IV demonstrated high cytotoxic activity (82% vs 11%; $P < .0001$; Table 1) and high susceptibility to vancomycin (79% vs 63%; $P = .0029$; data not shown). In contrast, many HA-MRSA strains produced very weak cytotoxic activity, below levels expected to correlate with mortality in animals. Indeed, a number of strains (22/381 [6%] HA-MRSA) produced no detectable cytotoxic activity. Nevertheless, HA-MRSA strains were still the predominant isolate from patients with NP.

Modeling of Mortality

To define potential predictors of mortality, we performed univariate and multivariate logistic regression analyses. Five additional patients whose isolates were from blood specimens and were not available for determination of cytotoxic activity were included in the analysis of outcomes because the MRSA genotypes were previously determined (n = 386) [19]. All variables examined, including baseline patient data from the original studies, are listed in Table 1 and Supplementary Table 1. Results of the multivariate analysis and a comparison with the univariate results are provided in Table 2.

In the univariate analysis, low Acute Physiology and Chronic Health Evaluation (APACHE) II score and 11 patient comorbidities were statistically significantly associated with mortality ($P < .05$). Among baseline bacterial characteristics, strains having low cytotoxic activity and a non-SCC*mec*IV phenotype were uniquely and paradoxically associated with lethality. Only 14 of the 108 patients (13%) infected with highly cytotoxic strains died, whereas 85 of 273 (31%) infected with low cytotoxic strains died

Table 2. Risk Factors Associated With Mortality Among Patients With Nosocomial Pneumonia Due to Methicillin-Resistant *Staphylococcus aureus*

Factor	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	P Values	OR (95% CI)	P Values
Genotypic characteristic				
CC8	0.80 (.46–1.41)	.4442	2.97 (1.20–7.36)	.0183
CC8/239	2.59 (1.33–5.07)	.0053	3.36 (1.29–8.77)	.0131
SCCmec (type IV vs other)	0.60 (.35–1.01)	.0544	. . .	
Cytotoxicity				
Low (>10 vs ≤10)[a]	3.04 (1.64–5.63)	.0004	4.36 (1.77–10.76)	.0014
Per 10-unit increase	1.10 (1.03–1.17)	.0043	. . .	
Quartile[b]		.0108		
Q2 vs Q1	2.50 (1.23–5.10)		. . .	
Q3 vs Q1	2.44 (1.21–4.95)		. . .	
Q4 vs Q1	3.13 (1.57–6.23)		. . .	
Median (>45 vs ≤45)	1.72 (1.08–2.74)	.0215	. . .	
Demographic/clinical characteristic				
Age				
Per 5-unit increase	1.20 (1.11–1.30)	<.0001	1.19 (1.08–1.31)	.0003
>65 y	2.89 (1.78–4.69)	<.0001	. . .	
APACHE II score				
Per 5-unit increase	1.71 (1.40–2.10)	<.0001	1.70 (1.34–2.16)	<.0001
>20 vs ≤20	3.44 (2.13–5.56)	<.0001	. . .	
Creatinine clearance rate (MDRD)				
Per 20-unit increase	0.87 (.79–.95)	.0037	. . .	
30–80 vs >80	2.78 (1.68–4.61)	<.0001	. . .	
<30 vs >80	3.28 (1.55–6.92)		. . .	
Bacteremia	2.06 (1.14–3.72)	.0172	. . .	
Chest radiography finding (bilateral vs unilateral)	1.72 (1.02–2.89)	.0413	. . .	
Vasopressor therapy at baseline	2.30 (1.32–4.01)	.0033	. . .	
Service		.0328		
Surgical vs medical	0.74 (.43–1.29)		. . .	
Trauma vs medical	0.41 (.21–.81)		. . .	
Nonmedical vs medical	0.58 (.36–.93)	.0239	. . .	
Region		.0133		
Asia vs US	0.65 (.31–1.37)		0.62 (.25–1.52)	.0027
Europe vs US	0.46 (.17–1.24)		0.55 (.18–1.72)	
Latin America vs US	2.56 (1.25–5.22)		4.55 (1.72–12.07)	
Other vs US	1.56 (.55–4.39)		3.79 (.97–14.75)	
Comorbidity				
Cardiac	1.97 (1.20–3.21)	.0068	. . .	
Diabetes	1.64 (1.04–2.59)	.0345	. . .	
Neoplastic	1.96 (1.00–3.84)	.0506	. . .	
Renal/urinary	1.88 (1.19–2.97)	.0073	. . .	
Gastrointestinal	2.19 (1.35–3.56)	.0016	2.51 (1.36–4.61)	.0031

Data are only for factors that were statistically significant in univariate or multivariate analysis. The full set of factors included is listed in Table 1.

Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation; CI, confidence interval; CPIS, Clinical Pulmonary Infection Score; MDRD, Mediterranean diet in renal disease; OR, odds ratio; SCCmec, staphylococcal cassette chromosome *mec*.

[a] Defined as a mean of <90% killing of PMN-HL60 cells within 2 hours.

[b] Defined as the percentage of viable cells after intoxication of PMN-HL60. Q1, <9%; Q2, ≥9% to <45%; Q3, ≥45% to <78%; Q4, ≥78%.

($P = .0002$). Correlation of mortality with low cytotoxic activity was observed across geography, genotypes, and type of pneumonia (ventilator-associated pneumonia or hospital-associated pneumonia; Table 3). Additionally, CC8/239, a clone with low cytotoxic activity, was associated with mortality (odds ratio [OR], 2.6; $P = .0053$).

Table 3. Relative Risk of Mortality Among Patients With Nosocomial Pneumonia Due to Methicillin-Resistant *Staphylococcus aureus* (MRSA), by Isolate and Patient Characteristic

Covariate	Low Cytotoxic Activity (n = 273)	High Cytotoxic Activity[a] (n = 108)	RR (95% CI)
	Patients Who Died/Patients Evaluated (%)		
Genotypic characteristic			
agr group			
I	27/68 (39.7)	13/83 (15.7)	2.54 (1.42–4.52)
II			
No (groups I, III)	27/76 (35.5)	13/83 (15.7)	2.27 (1.27–4.07)
Yes	58/196 (29.6)	1/25 (4.0)	7.40 (1.07–51.09)
III	0/8 (0.0)	0/0 (NA)	. . .
NT	0/1 (0.0)	0/0 (NA)	. . .
SCC*mec* type			
I, II, III	75/243 (30.9)	1/20 (5.0)	6.17 (.91–42.09)
IV	10/30 (33.3)	13/88 (14.8)	2.26 (1.11–4.60)
Clonal complex			
5			
No	27/76 (35.5)	13/84 (15.5)	2.30 (1.28–4.12)
Yes	58/197 (29.4)	1/24 (4.2)	7.07 (1.02–48.72)
8			
No	77/256 (30.1)	3/39 (7.7)	3.91 (1.30–11.78)
Yes	8/17 (47.1)	11/69 (15.9)	2.95 (1.41–6.19)
22			
No	85/272 (31.3)	14/101 (13.9)	2.25 (1.34–3.78)
Yes	0/1 (0.0)	0/7 (0.0)	. . .
45	2/13 (15.4)	0/0 (NA)	. . .
No	83/260 (31.9)	14/108 (13.0)	2.46 (1.46–4.14)
Yes	2/13 (15.4)	0/0 (NA)	. . .
8/239	17/34 (50.0)	1/4 (25.0)	2.00 (.35–11.29)
No	68/239 (28.5)	13/104 (12.5)	2.28 (1.32–3.93)
Yes	17/34 (50.0)	1/4 (25.0)	2.00 (.35–11.29)
Other	0/11 (0.0)	1/4 (25.0)	. . .
spa type 2			
2	35/112 (31.3)	0/11 (0.0)	. . .
Other	50/161 (31.1)	14/97 (14.4)	2.15 (1.26–3.68)
pvl			
No	81/264 (30.7)	7/69 (10.1)	3.02 (1.46–6.25)
Yes	4/9 (44.4)	7/39 (17.9)	2.48 (.92–6.68)
Phenotypic characteristic			
agr functionality[b]			
0	13/61 (21.3)	0/0 (NA)	. . .
1	26/68 (38.2)	1/4 (25.0)	1.53 (.27–8.58)
2	46/144 (31.9)	13/104 (12.5)	2.56 (1.46–4.48)
0, 1	39/129 (30.2)	1/4 (25.0)	1.21 (.22–6.74)
1, 2	72/212 (34.0)	14/108 (13.0)	2.62 (1.55–4.42)
Vancomycin susceptibility			
Heteroresistant	16/56 (28.6)	0/4 (0.0)	. . .
Susceptible	69/217 (31.8)	14/104 (13.5)	2.36 (1.40–3.99)
Vancomycin MIC, µg/mL			
By the Etest			
≤1			
Yes	7/32 (21.9)	0/12 (0.0)	. . .
No	78/241 (32.4)	14/96 (14.6)	2.22 (1.32–3.72)

Table 3 continued.

| Covariate | Patients Who Died/Patients Evaluated (%) | | RR (95% CI) |
	Low Cytotoxic Activity (n = 273)	High Cytotoxic Activity[a] (n = 108)	
≤1.5			
Yes	63/174 (36.2)	10/86 (11.6)	3.11 (1.68–5.76)
No	22/99 (22.2)	4/22 (18.2)	1.22 (.47–3.19)
By an automated method			
0.5	1/14 (7.1)	0/15 (0.0)	. . .
1	75/231 (32.5)	12/87 (13.8)	2.35 (1.35–4.11)
≥2	9/27 (33.3)	2/6 (33.3)	1.00 (.29–3.49)
Linezolid MIC, by the Etest			
1	4/14 (28.6)	2/9 (22.2)	1.29 (.29–5.63)
2	62/188 (33.0)	9/80 (11.3)	2.93 (1.53–5.61)
4	19/70 (27.1)	3/16 (18.8)	1.45 (.49–4.30)
Demographic/clinical characteristic			
Age, y			
≤65	26/128 (20.3)	5/62 (8.1)	2.52 (1.02–6.24)
>65	59/145 (40.7)	9/46 (19.6)	2.08 (1.12–3.86)
Sex			
Female	36/106 (34.0)	4/32 (12.5)	2.72 (1.05–7.06)
Male	49/167 (29.3)	10/76 (13.2)	2.23 (1.20–4.16)
Race			
Asian	10/48 (20.8)	1/11 (9.1)	2.29 (.33–16.08)
Black	9/26 (34.6)	3/21 (14.3)	2.42 (.75–7.83)
White			
No	22/89 (24.7)	4/33 (12.1)	2.04 (.76–5.48)
Yes	63/184 (34.2)	10/75 (13.3)	2.57 (1.39–4.73)
Other	3/15 (20.0)	0/1 (0.0)	. . .
Region			
Asia	9/43 (20.9)	1/8 (12.5)	1.67 (.24–11.46)
Europe	3/17 (17.6)	2/19 (10.5)	1.68 (.32–8.86)
Latin America	16/31 (51.6)	1/5 (20.0)	2.58 (.43–15.39)
Other	5/10 (50.0)	1/7 (14.3)	3.50 (.51–23.81)
United States			
Yes	52/172 (30.2)	9/69 (13.0)	2.32 (1.21–4.44)
No	33/101 (32.7)	5/39 (12.8)	2.55 (1.07–6.05)
Treatment			
Linezolid	44/136 (32.4)	8/53 (15.1)	2.14 (1.08–4.24)
Vancomycin	41/137 (29.9)	6/55 (10.9)	2.74 (1.24–6.09)
APACHE score			
≤20	42/186 (22.6)	6/74 (8.1)	2.78 (1.24–6.27)
>20	42/85 (49.4)	8/31 (25.8)	1.91 (1.02–3.61)
Missing	1/2 (50.0)	0/3 (0.0)	
ICU stay at baseline			
No	9/36 (25.0)	1/13 (7.7)	3.25 (.45–23.22)
Yes	76/237 (32.1)	13/95 (13.7)	2.34 (1.37–4.01)
Vasopressor therapy at baseline			
No	64/228 (28.1)	9/89 (10.1)	2.78 (1.44–5.33)
Yes	21/45 (46.7)	5/19 (26.3)	1.77 (.79–4.00)
Corticosteriod therapy at baseline			
No	61/210 (29.0)	10/89 (11.2)	2.59 (1.39–4.81)
Yes	24/63 (38.1)	4/19 (21.1)	1.81 (.72–4.57)

Table 3 continued.

Covariate	Low Cytotoxic Activity (n = 273)	High Cytotoxic Activity[a] (n = 108)	RR (95% CI)
	Patients Who Died/Patients Evaluated (%)		
Bacteremia			
No	68/237 (28.7)	11/93 (11.8)	2.43 (1.34–4.38)
Yes	17/36 (47.2)	3/15 (20.0)	2.36 (.81–6.88)
Chest radiography finding			
Bilateral	64/190 (33.7)	11/69 (15.9)	2.11 (1.19–3.76)
Unilateral	21/83 (25.3)	3/38 (7.9)	3.20 (1.02–10.09)
Missing	0/0 (NA)	0/1 (0.0)	
Pleural effusion			
No	48/140 (34.3)	9/70 (12.9)	2.67 (1.39–5.12)
Yes	37/133 (27.8)	5/38 (13.2)	2.11 (.89–5.00)
Pneumonia, by type			
Hospital associated	17/78 (21.8%)	2/21 (9.5%)	2.29 (.57–9.13)
Healthcare associated	17/51 (33.3%)	1/10 (10.0%)	3.33 (.50–22.27)
Ventilator associated	51/144 (35.4%)	11/77 (14.3%)	2.48 (1.37–4.47)
Ventilation at baseline			
No	20/83 (24.1)	1/25 (4.0)	6.02 (.85–42.68)
Yes	65/190 (34.2)	13/83 (15.7)	2.18 (1.28–3.74)
Infecting pathogen(s)			
MRSA + anaerobe	0/2 (0.0)	0/0 (NA)	. . .
MRSA + fungus	5/10 (50.0)	0/1 (0.0)	. . .
MRSA + gram negative	20/66 (30.3)	4/33 (12.1)	2.50 (.93–6.72)
MRSA + gram positive	6/21 (28.6)	0/10 (0.0)	. . .
MRSA + gram positive + gram negative	5/15 (33.3)	1/5 (20.0)	1.67 (.25–11.07)
MRSA only	49/159 (30.8)	9/59 (15.3)	2.02 (1.06–3.85)
MRSA + other	36/114 (31.6)	5/49 (10.2)	3.09 (1.29–7.41)
Anaerobic coverage at baseline			
No	35/125 (28.0)	4/44 (9.1)	3.08 (1.16–8.17)
Yes	50/148 (33.8)	10/64 (15.6)	2.16 (1.17–3.99)
Gram-negative coverage at baseline			
No	1/4 (25.0)	0/3 (0.0)	. . .
Yes	84/269 (31.2)	14/105 (13.3)	2.34 (1.39–3.93)
Service			
Medical			
Yes	55/162 (34.0)	9/49 (18.4)	1.85 (.99–3.46)
No	30/111 (27.0)	5/59 (8.5)	3.19 (1.31–7.79)
Surgical	20/68 (29.4)	3/24 (12.5)	2.35 (.77–7.22)
Trauma	10/43 (23.3)	2/35 (5.7)	4.07 (.95–17.37)
Comorbidity			
Cardiac			
No	23/98 (23.5)	5/54 (9.3)	2.53 (1.02–6.29)
Yes	62/175 (35.4)	9/54 (16.7)	2.13 (1.13–3.99)
Diabetes			
No	41/152 (27.0)	10/79 (12.7)	2.13 (1.13–4.02)
Yes	44/121 (36.4)	4/29 (13.8)	2.64 (1.03–6.75)
Neoplastic			
No	73/244 (29.9)	10/96 (10.4)	2.87 (1.55–5.32)
Yes	12/29 (41.4)	4/12 (33.3)	1.24 (.50–3.08)
Renal/urinary			
No	37/149 (24.8)	10/78 (12.8)	1.94 (1.02–3.68)
Yes	48/124 (38.7)	4/30 (13.3)	2.90 (1.14–7.42)

Table 3   continued.

| | Patients Who Died/Patients Evaluated (%) | | |
Covariate	Low Cytotoxic Activity (n = 273)	High Cytotoxic Activity[a] (n = 108)	RR (95% CI)
Pulmonary			
No	22/86 (25.6)	5/34 (14.7)	1.74 (.72–4.22)
Yes	63/187 (33.7)	9/74 (12.2)	2.77 (1.45–5.28)
Hepatobiliary			
No	72/232 (31.0)	9/95 (9.5)	3.28 (1.71–6.28)
Yes	13/41 (31.7)	5/13 (38.5)	0.82 (.36–1.87)
Gastrointestinal			
No	24/116 (20.7)	5/47 (10.6)	1.94 (.79–4.79)
Yes	61/157 (38.9)	9/61 (14.8)	2.63 (1.40–4.97)
LTCF stay at baseline			
No	82/261 (31.4)	13/104 (12.5)	2.51 (1.47–4.31)
Yes	3/12 (25.0)	1/4 (25.0)	1.00 (.14–7.10)

Data associated with creatinine clearance and Clinical Pulmonary Infection Score are not shown.

Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation; CI, confidence interval; LTCF, long-term care facility; MIC, minimum inhibitory concentration; NA, not applicable; NT, not typable; RR, relative risk; SCC*mec*, staphylococcal cassette chromosome *mec*.

[a] Defined as a mean of ≥90% killing of PMN-HL60 cells within 2 hours.

[b] Semiquantitative scoring of δ-hemolysin on sheep blood agar: 0, nonhemoltyic; 1, weakly hemolytic; 2, strongly hemolytic.

The initial multivariate logistic regression analysis identified low cytotoxic activity and clonality as the only significant (and negative) bacterial predictors within the candidate predictor pool, which included all univariate predictor variables. Attenuated cytotoxic activity was more frequently observed in isolates from patients with comorbidities, such as cardiac and renal/urinary disease (Table 1). However, modeling data indicated that the importance of cytolysin for NP did not vary much by genotype, host comorbidity, or age. As noted above, there was a very clear correlation between low cytotoxicity and the presence of non-SCC*mec*IV type, which suggests that the relationship between cytotoxicity and mortality is based in part on a strain's association with the healthcare environment. However, when SCC*mec* and cytotoxic activity were entered into a multivariate logistic regression model, low cytotoxic activity appeared as a strong predictor of mortality, whereas SCC*mec* appeared as a weak predictor. These findings are consistent with several reports indicating that the presence of CA-MRSA and *pvl* per se do not confer a worse clinical course in all forms of infection caused by *S. aureus* [10–12, 40, 41]. Finally, analysis of treatment showed that the deleterious effect of low cytotoxic activity (reduced probability of survival) was seen in both patients treated with vancomycin and patients treated with linezolid (relative risk, 1.08 [95% confidence interval {CI}, .76–1.54]; Supplementary Table 2).

Overall mortality did not differ significantly among patients infected with CC8 strains and those infected with other strains. However, when mortality was adjusted for an overall high level of cytotoxic activity by using logistic regression, CC8 isolates were associated with increased mortality. Thus, differences in cytotoxic activity and genotype may both affect mortality, with one countering the other. The effect of genotype on mortality was also apparent for patients infected with CC8/239 isolates. A recent study suggested that the acquisition of a virulence factor, *sasX*, by ST239 (a major sequence type of CC239) resulted in enhanced virulence and an epidemiological outbreak in eastern China [29]. However, mortality distinctions between the *sasX* CC239–positive subgroup (n = 19) and the *sasX* CC239–negative subgroup (n = 19) were not observed in our population (OR, 1.71 [95% CI, .65–4.48]; P = .2728).

Previous studies have reported an association between reduced virulence, reduced vancomycin susceptibility, and outcomes [34]; the present work supports such an interaction between these 3 important variables. However, high vancomycin MIC and vancomycin heteroresistance were not risk factors for mortality or poor clinical outcome, even in patients treated with vancomycin.

Modeling of Clinical Cure

Mortality modeling suggests that invasive virulence is not key to outcome in nosocomial NP, unless there are alternate pathways that selectively activate cytolysin genes in vivo. However, it is possible that cytotoxic activity correlates directly with clinical outcome from pneumonia and that the onset of its action is too rapid to be measured by mortality at the end of study. We therefore studied clinical outcomes at end of treatment and the end of the study (Supplementary Table 3). In contrast to mortality, cytotoxic activity was not associated with clinical cure at any time point. Consistent with this observation, cytotoxicity

was not associated with severity of illness score (APACHE II or Clinical Pulmonary Infection Score), bacteremia, or vasopressor use (Table 1). However, the association of a cytotoxin, PVL, with a particular CA-MRSA strain predominant in the United States (USA300) correlated with poor outcomes at the end of treatment (OR, 0.34 [95% CI, .17–.69]; $P = 0.0025$) and at the end of the study (OR, 0.37 [95% CI, .15–.92]; $P = .0318$). Additionally, CC8/239 and SCCmecIV were significantly associated with poor outcome at the end of the study. Thus, both virulence profiles and the genetic background provide discriminatory power for outcomes of MRSA NP. Finally, as in the parent study, treatment with linezolid was associated with improved outcome at the end of treatment (OR, 1.85 [95% CI, 1.10–3.12]; $P = .02$; Supplementary Table 3).

DISCUSSION

The results show that crude mortality rates for adequately treated patients with MRSA NP due to strains of low cytotoxic activity was 4-fold higher than that for patients infected with strains of high virulence, even after multivariate logistic regression analysis with careful adjustment for bacterial and host factors. The high prevalence of strains with low cytotoxic activity and their association with HA-MRSA and mortality suggests that these strains are, paradoxically, more virulent than other strains, at least under some circumstances. Elevated mortality may be due to the presence of unmeasured cytolysins or other factors. An alternative but nonmutually exclusive hypothesis is that cytolysins act as positive virulence factors but also enhance or trigger activation of the immune system [42–44].

Although it is not possible to state definitively that enhanced virulence does not play a role in mortality associated with strains having repressed cytotoxic activity, 3 observations suggest that such strains are actually less virulent. First, in contrast to mortality, low cytotoxic activity did not modify the effect of MRSA NP on clinical outcome. The treatment effect for an end point such as clinical failure should be larger than that seen with a mortality end point. Second, MRSA strains of hospital origin were significantly less cytotoxic than strains of community origin, and such strains are generally less virulent than those of community clades in most animal models of infection [45–47]. Finally, substantial differences in clinical characteristics (eg, age and comorbidities) were observed between the group with low cytolysin levels and the group with high cytolysin levels. These data suggest that infections due to the former occur in sicker patients. Collectively, these observations suggest that the discrepancies in clinical outcome and survival of low virulence strains stem from confounding factors related to differences in populations of patients infected with highly cytotoxic and weakly cytotoxic MRSA. In this scenario, cytotoxic activity is a proxy for a subtle factor that makes hospitalized patients more susceptible to infection with bacteria of low virulence. We call that factor "host quality."

In contrast to results for cytotoxic activity, both mortality and poor clinical cure were concentrated within CC8/239, a weakly cytotoxic lineage. One potential explanation for the observed link between outcome and phylogeny is the importance of compatibility between genomes and virulence genes for the emergence of a virulent clone. For example, in the case of CC 8/239, compensatory mutations that help maintain virulence not measured by cytotoxic activity may have appeared in this lineage, which initially may not have been particularly adaptive for its repertoire of virulence genes. Indeed, CC 8/239 is a hybrid clone [48] resulting from the integration of a genomic fragment from CC30, which is associated with invasive infection and poor outcome [49], into a CC8 parent strain. Thus, integration of CC30 genes may have been an evolutionary route to a hypervirulence that kills patients at high frequency.

The present work has several limitations. First, correlations between bacterial factors and outcome may reflect factors we did not account for, such as duration of stay or mechanical ventilation before diagnosis of NP. Second, our data are limited to NP; whether cytotoxic patterns predict outcome in different syndromes will need to be tested. Third, the diagnosis of pneumonia was based on clinical criteria despite the fact that lower respiratory tract specimens were collected for culture from all patients before antibiotic therapy. Using clinical criteria for the diagnosis of NP may lead to overdiagnosis, potentially increasing the rate of overall disease detected and the proportion of low virulence strains that may be linked to mortality. Finally, the diagnosis was based on analysis of respiratory samples, including sputum and tracheal aspirates; the use of samples other than bronchoalveolar lavage to characterize the etiology of pneumonia may also have resulted in inclusion of patients colonized but not infected with MRSA.

In conclusion, we identified low cytotoxic activity as a predictor of mortality in MRSA NP, but deeper investigation revealed it to be a marker of the host. Although it may not be possible to identify what information is embedded in cytotoxic activity and exactly why it is associated with mortality, the results provide a framework with which to further explore the relationship between strain variation in MRSA and clinical outcomes. The issue is that patients who are infected with low virulence MRSA are inherently different from those infected with virulent MRSA and, in turn, that both the natural history of disease and treatment effects between some drugs may be different in these patients. Thus, if additional studies substantiate these results, future outcome studies should be designed so that this factor is assessed and evenly balanced between study groups.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary

data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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