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Presence of Genes Encoding Panton-Valentine Leukocidin Is Not the Primary Determinant of Outcome in Patients with Hospital-Acquired Pneumonia Due to *Staphylococcus aureus*

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The impact of Panton-Valentine leukocidin (PVL) on the outcome in *Staphylococcus aureus* pneumonia is controversial. We genotyped *S. aureus* isolates from patients with hospital-acquired pneumonia (HAP) enrolled in two registrational multinational clinical trials for the genetic elements carrying *pvl* and 30 other virulence genes. A total of 287 isolates (173 methicillin-resistant *S. aureus* [MRSA] and 114 methicillin-susceptible *S. aureus* [MSSA] isolates) from patients from 127 centers in 34 countries for whom clinical outcomes of cure or failure were available underwent genotyping. Of these, *pvl* was detected by PCR and its product confirmed in 23 isolates (8.0%) (MRSA, 18/173 isolates [10.4%]; MSSA, 5/114 isolates [4.4%]). The presence of *pvl* was not associated with a higher risk for clinical failure (4/23 [17.4%] versus 48/264 [18.2%]; $P = 1.00$) or mortality. These findings persisted after adjustment for multiple potential confounding variables. No significant associations between clinical outcome and (i) presence of any of the 30 other virulence genes tested, (ii) presence of specific bacterial clone, (iii) levels of alpha-hemolysin, or (iv) delta-hemolysin production were identified. This study suggests that neither *pvl* presence nor *in vitro* level of alpha-hemolysin production is the primary determinant of outcome among patients with HAP caused by *S. aureus*.

The Panton-Valentine leukocidin (PVL) is a bacteriophage-associated, bicomponent cytotoxin produced by some strains of *Staphylococcus aureus*. PVL induces host cell necrosis and apoptosis by producing pores in the cell membranes of neutrophils and other infected cells. The presence of PVL and the genetic elements coding for its production (two contiguous, cotranscribed genes, *lukS* and *lukF*, here referred to as *pvl*) has been strongly associated with a severe necrotizing pneumonia (13, 15). Although controversy persists, there is evidence that PVL is associated with severe disease in community-acquired pneumonia (CAP) due to *S. aureus* both in clinical reports (13, 15) and in some (10, 22), but not all (3, 20, 30, 49), *in vivo* model systems. However, the studies on the association between PVL and clinical outcomes in hospital-acquired pneumonia (HAP), a distinct clinical entity from CAP, are limited.

Hospital-acquired pneumonia is the leading cause of morbidity and mortality from nosocomial infections (9), and *S. aureus* is the leading cause of HAP in U.S. hospitals (12, 28, 34). In the current study, we tested the hypothesis that *pvl* presence in *S. aureus* isolates causing HAP was associated with a worse clinical outcome than the outcome of HAP caused by *pvl*-negative *S. aureus* counterparts. To test this hypothesis, we made use of a large international cohort of *S. aureus* isolates from patients with HAP. These isolates were collected in two identically designed phase III clinical trials for *S. aureus* HAP.

MATERIALS AND METHODS

Patients and study settings. The ATTAIN (Assessment of Telavancin for Hospital-Acquired Pneumonia) clinical trials were two identical phase III, randomized, double-blinded, parallel-group, multinational trials (ClinicalTrials.gov identifiers NCT00107952 and NCT00124020) studying the efficacy and safety of intravenous telavancin versus vancomycin for the treatment of hospital-acquired pneumonia (HAP) with a focus on patients with infections due to methicillin-resistant *S. aureus* (MRSA) (35). Following randomization, patients were treated for 7 to 21 days with the study drug. From January 2005 to June 2007, a total of 1,503 patients were enrolled from 235 clinical centers in 38 countries. Patients were included in the current study if all of the following criteria were met: (i) inclusion in the modified all treated (MAT) population ($n = 1,089$), (ii) had monomicrobial infection with *S. aureus* at baseline, and (iii) had a clinical response of either "cure" or "failure" for the test-of-cure analysis. All patients or their legal guardian provided written informed consent. This study was approved by Duke University Medical Center Institutional Review Board.

Clinical outcomes and definitions. Clinical outcomes were established by site investigators. Outcomes were defined as either "cure" or "failure." Cure was defined as (i) signs and symptoms of pneumonia improved to the point that no further antibiotics for pneumonia are required and (ii) baseline radiographic findings improved or did not progress. Failure was defined as (i) persistence or progression of signs and symptoms of pneumonia that still require antibiotic therapy within two calendar days of therapy with a potentially effective antistaphylococcal medication and/or (ii) death on or after day three attributable to primary infection. The MAT subgroup comprises all subjects who received at least one dose of study medication and who had a baseline respiratory pathogen identified from respiratory samples or blood cultures if no respiratory sample was positive.

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PCR assays for genotyping. *S. aureus* genomic DNA was extracted as described previously (5), using an ultraclean microbial DNA kit (Mo Bio Laboratories, Inc., Carlsbad, CA) in accordance with the manufacturer's instructions. PCR assays were used to screen the *S. aureus* genome for 31 putative bacterial virulence determinants, including adhesin genes (*fnbA*, *fnbB*, *clfA*, *clfB*, *cna*, *spa*, *sdrC*, *sdrD*, *sdrE*, *bbp*, *ebpS*, and *map-eap*), toxin genes (*pvl*, *eta*, *etb*, *tst*, *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *hlg*), *agr* groups I to IV, staphylococcal cassette chromosome *mec* element (SCC*mec*) types I to IV, and other virulence genes (*efb*, *icaA*, *chp*, and the V8 protease gene). The primers and PCR conditions used to amplify the genes of interest were used as described previously (1, 5).

PVL Western blotting. *S. aureus* isolates were cultured overnight from low-passage frozen stocks in CCY medium (3% [wt/vol] yeast extract, 2% Bacto-Casamino Acids, 2.3% sodium pyruvate, 0.63% Na_2HPO_4, and 0.041% KH_2PO_4, pH 6.7). Culture supernatants were prepared from bacteria at the early or late stationary phase of growth as described previously (17). LukF-PV and LukS-PV present in CCY culture supernatants were detected by Western blotting (immunoblotting) as described by Graves et al. (17), except polyvinylidene difluoride (PVDF) membranes were used with the iBlot dry blotting system (Invitrogen, Carlsbad, California). The presence or absence of PVL subunits in *S. aureus* isolates was confirmed by two separate experiments. Quantitation of immunoblots from both experiments was performed using an Alpha Innotech gel documentation system (FluorChemFC2; Alpha Innotech Corp., San Leandro, CA) and AlphaView software version 3.0.3.

MLST. Multilocus sequence typing (MLST) was performed as described by Enright et al. (11). Sequences were analyzed in Seqman Pro (DNA STAR Inc., Madison, WI) and compared with those in the public database (www.mlst.net) to generate the sequence types (STs). STs were grouped into clonal complexes (CCs) by using eBURST analysis tools at http://eburst.mlst.net.

Alpha-hemolysin activity assay. Alpha-hemolysin activity was measured by quantitative analysis of rabbit red blood cell (RBC) hemolysis as described earlier (19, 42) with the following slight modifications. Ten milliliters of tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD) in a 50-ml falcon tube was inoculated with a loopful of culture from a fresh plate of each strain and incubated at 37°C/220 rpm for overnight culture. An appropriate amount of overnight culture was inoculated into 10 ml of Mueller-Hinton broth 2 (Sigma, St. Louis, MO) in a 50-ml falcon tube to normalize the starting OD_{600} to 0.1 ($\sim 10^7$ CFU/ml) and incubated at 37°C/220 rpm for 20 h. After 20 h of incubation, the culture was spun down at 4°C/3,100 rpm for 10 min to remove the pellets. The supernatant was then filter sterilized, transferred to a sterile tube, and stored at −80°C until further use.

The ability of the culture supernatant to lyse rabbit erythrocytes (RBCs) was tested in a 96-well format. To do this, 100 μl of 1:5-diluted culture supernatant (in 1× phosphate-buffered saline [PBS]) of each strain was loaded into the first well and then serially diluted up to 1:80 in duplicate. After the dilution of each sample, 100 μl of 1% rabbit RBCs (Innovative Research, Novi, MI) in 1× PBS was added to each well and incubated at 37°C for 1 h. Following incubation, plates were centrifuged for 5 min, 100 μl of the supernatant was removed gently to a new microtiter plate, and absorbance was read at 550 nm. Hemolytic units (HU) per milliliter of alpha-hemolysin were defined as the inverse of the dilution causing 50% of hemolysis. Sterile distilled water served as the 100% hemolysis control (positive control), and 1× PBS was a negative control. All experiments were performed in triplicate and the results averaged. Mean alpha-hemolysin levels were defined as high if they were >10 hemolytic units (HU)/ml and low if they were ≤10 HU/ml. To ensure that no bias was introduced with this stratification, we repeated the analyses using cut points of 5 HU/ml and 7 HU/ml. No differences in the overall findings were introduced by varying the biological cut point definitions.

Delta-hemolysin activity assay. A delta-hemolysin activity assay was performed to exclude the possibility of *agr* dysfunction as a potential cause of clinical outcome. Delta-hemolysin activity on sheep blood agar plates was determined as previously described (36). Briefly, on each sheep blood agar plate, the beta-hemolysin-producing strain *S. aureus* RN4220 was streaked vertically and test strains were streaked horizontally. After overnight incubation at 37°C, the plates were observed for the enhanced zone of hemolysis created by the interaction of the beta-hemolysin of RN4420 and the delta-hemolysin of the test strain. *S. aureus* NRS149 (RN6607), also named 502A (standard *agr* group II prototype, obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* [NARSA]), and *S. aureus* NRS 155 (RN9120, *agr*-null derivative of RN6607) were used as positive and negative controls, respectively.

Statistical methods. The goal of this study was to investigate associations between clinical outcome of patients with *S. aureus* HAP and the presence of *pvl* in the infecting strain. To accomplish this goal, genetically defined patient subgroups (i.e., *pvl*-positive versus *pvl*-negative subjects) were assessed with the two-sample *t* test for continuously distributed variables, Fisher's exact test for binomial variables, and the Fisher-Freeman-Halton test for more general categorical variables (Table 1).

The association between clinical outcome and *pvl* status was assessed, adjusting for potential confounding variables (Table 2). Multiple analyses were conducted, and each stratified on a different covariate. Exact methods were used to test the null hypothesis that the odds ratio equaled unity in all strata (that is, that there was no association between clinical outcome and *pvl* status), stratifying on the third variable.

The association between clinical outcome at test-of-cure and the presence/absence of each of a number of putative virulence genes (Table 3) was assessed using Fisher's exact test to test the null hypothesis of no association.

Due to small sample sizes, the association between clinical outcome at test-of-cure and the amount of alpha-lysin produced (>10 HU/ml versus ≤10 HU/ml) (Table 4) was tested, with the MRSA and MSSA strains pooled. Within PVL-positive and PVL-negative pathogens separately, an exact stratified Cochran-Mantel-Haenzel test was conducted via Monte Carlo simulation, stratifying on methicillin susceptibility (MRSA or MSSA). All reported *P* values are two-sided. No adjustments were made for multiple comparisons.

Results were obtained using SAS 9.2 (TS2M3) (SAS Institute Inc., Cary, NC) run on a Windows-based server. When exact results could not be obtained from SAS procedures, they were obtained with either StatXact 9 PROCS run within the SAS environment or StatXact-8 run within Cytel Studio 8 (both from the Cytel Software Corporation, Cambridge, MA).

RESULTS

Study population and baseline characteristics. Out of 1,503 patients enrolled in the ATTAIN studies, 287 patients from 127 centers in 34 countries met inclusion criteria for the current investigation. Bacterial isolates were available for all 287 patients. Of the 287 isolates, 173 (60.3%) were MRSA and 114 (39.7%) were MSSA. Baseline demographic characteristics of these patients are outlined in Table 1.

Clinical characteristics and outcome according to *pvl* presence. Of the 287 isolates, 23 (8.0%) were positive for *pvl* (MRSA, 18/173 isolates [10.4%]; MSSA, 5/114 isolates [4.4%]). PVL protein was confirmed in all 23 isolates by Western blotting of culture supernatants (data not shown). No significant differences were identified in clinical characteristics of patients infected with *pvl*-positive and *pvl*-negative *S. aureus* with the exception of higher *pvl* prevalence in MRSA populations from North America (Table 1).

Next, we compared the outcomes of patients according to presence of *pvl*. No significant differences were identified in the clinical outcomes of patients with *pvl*-positive and *pvl*-negative *S. aureus* HAP, either overall (cure rates were 19/23 [82.6%] for *pvl*-positive cases versus 216/264 [81.8%] for *pvl*-negative cases; $P = 1.00$) or within methicillin susceptibility subgroups (for MRSA,

TABLE 1 Baseline characteristics of the study population according to the *pvl* gene status of the infecting pathogen among patients with hospital-acquired pneumonia

Parameter	Value by PVL status[a]							
	MRSA				MSSA			
	Total ($n = 173$)	*pvl* negative ($n = 155$)	*pvl* positive ($n = 18$)	*P* value	Total ($n = 114$)	*pvl* negative ($n = 109$)	*pvl* positive ($n = 5$)	*P* value
Demographic characteristics								
Mean age (SD) (yr)[b]	66.3 (15.91)	66.7 (15.40)	63.1 (20.04)	0.364	56.4 (19.79)	56.4 (20.21)	56.4 (6.43)	0.996
Male sex[c]	93 (53.8)	80 (51.65)	13 (72.2)	0.134	66 (57.9)	63 (57.8)	3 (60.0)	1.000
Region of enrollment[c]								
Europe	48 (27.7)	45 (29.0)	3 (16.7)	0.005	50 (43.9)	49 (45.0)	1 (20.0)	0.519
North America	46 (26.6)	35 (22.6)	11 (61.1)		27 (23.7)	25 (22.9)	2 (40.0)	
Other[d]	79 (45.7)	75 (48.4)	4 (22.2)		37 (32.5)	35 (32.1)	2 (40.0)	
Prior antimicrobial therapy[c]	127 (73.4)	116 (74.8)	11 (61.1)	0.259	46 (40.4)	42 (38.5)	4 (80.0)	0.156
MRSA risk factors[c]								
Hospitalization within previous 6 months	109 (63.0)	100 (64.5)	9 (50.0)	0.302	41 (36.0)	40 (36.7)	1 (20.0)	0.653
Antibiotic treatment within prior 3 months	118 (68.2)	108 (69.7)	10 (55.6)	0.285	37 (32.5)	36 (33.0)	1 (20.0)	1.000
Chronic illness	144 (83.2)	130 (83.9)	14 (77.8)	0.509	67 (58.8)	63 (57.8)	4 (80.0)	0.647
Prior infection with MRSA	19 (11.0)	17 (11.0)	2 (11.1)	1.000	2 (1.8)	2 (1.8)	0	1.000
Admission from a nursing home or long term care facility	34 (19.7)	30 (19.4)	4 (22.2)	0.757	10 (8.8)	10 (9.2)	0	1.000
Surgical procedure during current hospital stay	46 (26.6)	42 (27.1)	4 (22.2)	0.784	45 (39.5)	44 (40.4)	1 (20.0)	0.647
Residing in an area known to have a high prevalence of community-acquired MRSA	37 (21.4)	30 (19.4)	7 (38.9)	0.070	14 (12.3)	14 (12.8)	0	1.000
Smoking status[c]								
Current smoker	33 (19.3)	29 (19.0)	4 (22.2)	0.412	33 (29.2)	30 (27.8)	3 (60.0)	0.377
Ex-smoker	66 (38.6)	57 (37.3)	9 (50.0)		19 (16.8)	19 (17.6)	0	
Nonsmoker	72 (42.1)	67 (43.8)	5 (27.8)		61 (54.0)	59 (54.6)	2 (40.0)	
Data missing	2	2	0		1	1	0	
On hemodialysis[c]	6 (3.5)	6 (3.9)	0	1.000	0	0	0	
Currently in acute renal failure[c]	16 (9.2)	15 (9.7)	1 (5.6)	1.000	6 (5.3)	6 (5.5)	0	1.000
Patient operative status[c]								
Nonoperative	132 (76.3)	118 (76.1)	14 (77.8)	0.372	70 (61.4)	66 (60.6)	4 (80.0)	1.000
Emergency postoperative	27 (15.6)	23 (14.8)	4 (22.2)		29 (25.4)	28 (25.7)	1 (20.0)	
Elective postoperative	14 (8.1)	14 (9.0)	0		15 (13.2)	15 (13.8)	0	
With history of severe organ system insufficiency/immunocompromised[c]	10 (5.8)	9 (5.8)	1 (5.6)	1.000	4 (3.5)	4 (3.7)	0	1.000
Diabetes and cardiac comorbidity[c]	118 (68.2)	104 (67.1)	14 (77.8)	0.432	58 (50.9)	55 (50.5)	3 (60.0)	1.000
Respiratory insufficiency/failure[c]	105 (60.7)	93 (60.0)	12 (66.7)	0.799	76 (66.7)	72 (66.1)	4 (80.0)	0.663
On a ventilator at the time of randomization[c]	76 (43.9)	71 (45.8)	5 (27.8)	0.210	49 (43.0)	49 (45.0)	0	0.069
Baseline bacteremia with *S. aureus*[c]	13 (7.5)	10 (6.5)	3 (16.7)	0.139	8 (7.0)	8 (7.3)	0	1.000
In ICU[e] at baseline[c]	85 (49.1)	77 (49.7)	8 (44.4)	0.805	61 (53.5)	60 (55.0)	1 (20.0)	0.182
Mean body mass index (SD) (kg/m^2)[b]	25.9 (6.72)	25.9 (6.92)	25.6 (4.73)	0.819	26.8 (6.26)	26.9 (6.23)	24.8 (7.29)	0.467
Mean total APACHE II score (SD)[b]	15.5 (5.99)	15.6 (5.95)	14.5 (6.44)	0.449	13.4 (5.85)	13.4 (5.82)	12.4 (7.09)	0.697

[a] Excluding the *P* value columns, values shown are numbers (percentages) of patients unless otherwise indicated.
[b] Assessed by two-sample *t* test.
[c] Assessed by Fisher's exact test (for 2-by-2 tables) or the Fisher-Freeman-Halton test (for tables larger than 2 by 2).
[d] "Other" includes Argentina, Australia, Brazil, Chile, China, India, Israel, Lebanon, Malaysia, Peru, Philippines, South Africa, South Korea, Taiwan, and Thailand.
[e] ICU, intensive care unit.

16/18 [88.9%] for *pvl*-positive cases versus 123/155 [79.4%] for *pvl*-negative cases [$P = 0.532$]; for MSSA, 3/5 [60.0%] for *pvl*-positive cases versus 93/109 [85.3%] for *pvl*-negative cases [$P = 0.176$]) (Fig. 1). There was also no significant difference in mortality rates among the *pvl*-positive and *pvl*-negative groups (data not shown). These findings persisted after adjustment for a number of potentially confounding clinical characteristics (Table 2). To look for possible correlation between the amounts of PVL production and clinical outcome, we next quantified the levels of LukS and LukF components of PVL in all 23 *pvl*-positive isolates. There was no difference in clinical outcome with the amount of PVL production (data not shown).

Clinical outcome of HAP according to presence of other virulence genes. Next, we considered potential associations between

TABLE 2 Outcome for patients with *Staphylococcus aureus* hospital-acquired pneumonia stratified by clinically relevant characteristics

Parameter	Value by PVL status					
	MRSA			MSSA		
	Cure rate (%)			Cure rate (%)		
	pvl negative ($n = 155$)	*pvl* positive ($n = 18$)	*P* value[a]	*pvl* negative ($n = 109$)	*pvl* positive ($n = 5$)	*P* value[a]
Demographic characteristics						
Age			0.524			0.153
<65 yr	50/54 (92.6)	7/8 (87.5)		55/63 (87.3)	3/5 (60.0)	
≥65 yr	73/101 (72.3)	9/10 (90.0)		38/46 (82.6)	0/0	
Sex			0.362			0.178
Male	59/80 (73.8)	12/13 (92.3)		54/63 (85.7)	1/3 (33.3)	
Female	64/75 (85.3)	4/5 (80.0)		39/46 (84.8)	2/2 (100.0)	
Region of enrollment			0.349			0.215
Europe	37/45 (82.2)	3/3 (100.0)		44/49 (89.8)	0/1 (0.0)	
North America	26/35 (74.3)	9/11 (81.8)		20/25 (80.0)	1/2 (50.0)	
Other[b]	60/75 (80.0)	4/4 (100.0)		29/35 (82.9)	2/2 (100.0)	
Prior antimicrobial therapy			0.531			0.167
Yes	92/116 (79.3)	10/11 (90.9)		37/42 (88.1)	2/4 (50.0)	
No	31/39 (79.5)	6/7 (85.7)		56/67 (83.6)	1/1 (100)	
MRSA risk factor			0.531			0.145
Yes	122/153 (79.7)	16/18 (88.9)		81/97 (83.5)	2/4 (50.0)	
No	1/2 (50.0)	0/0		12/12 (100)	1/1 (100.0)	
Hospitalization within previous 6 months			0.532			0.193
Yes	80/100 (80.0)	8/9 (88.9)		35/40 (87.5)	1/1 (100.0)	
No	43/55 (78.2)	8/9 (88.9)		58/69 (84.1)	2/4 (50.0)	
Antibiotic treatment within prior 3 months			0.531			0.175
Yes	84/108 (77.8)	9/10 (90.0)		31/36 (86.1)	0/1 (0.0)	
No	39/47 (83.0)	7/8 (87.5)		62/73 (84.9)	3/4 (75.0)	
Chronic illness			0.531			0.197
Yes	101/130 (77.7)	13/14 (92.9)		53/63 (84.1)	2/4 (50.0)	
No	22/25 (88.0)	3/4 (75.0)		40/46 (87.0)	1/1 (100.0)	
Prior infection with MRSA			0.533			0.165
Yes	15/17 (88.2)	1/2 (50.0)		1/2 (50.0)	0/0	
No	108/138 (78.3)	15/16 (93.8)		92/107 (86.0)	3/5 (60.0)	
Admission from a nursing home or long term care facility			0.533			0.206
Yes	23/30 (76.7)	3/4 (75.0)		10/10 (100.0)	0/0	
No	100/125 (80.0)	13/14 (92.9)		83/99 (83.8)	3/5 (60.0)	
Surgical procedure during current hospital stay			0.532			0.137
Yes	33/42 (78.6)	3/4 (75.0)		35/44 (79.5)	0/1 (0.0)	
No	90/113 (79.6)	13/14 (92.9)		58/65 (89.2)	3/4 (75.0)	
Residing in an area known to have a high prevalence of community-acquired MRSA			0.356			0.143
Yes	21/30 (70.0)	5/7 (71.4)		10/14 (71.4)	0/0	
No	102/125 (81.6)	11/11 (100.0)		83/95 (87.4)	3/5 (60.0)	
Smoking status			0.530			0.182
Nonsmoker	54/67 (80.6)	5/5 (100.0)		52/59 (88.1)	2/2 (100.0)	
Current or ex-smoker	69/86 (80.2)	11/13 (84.6)		41/49 (83.7)	1/3 (33.3)	
On hemodialysis			0.531			0.176
Yes	5/6 (83.3)	0/0		0/0	0/0	
No	118/149 (79.2)	16/18 (88.9)		93/109 (85.3)	3/5 (60.0)	

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TABLE 2 (Continued)

Parameter	Value by PVL status					
	MRSA			MSSA		
	Cure rate (%)			Cure rate (%)		
	pvl negative ($n = 155$)	*pvl* positive ($n = 18$)	*P* value[a]	*pvl* negative ($n = 109$)	*pvl* positive ($n = 5$)	*P* value[a]
Currently in acute renal failure			0.532			0.193
Yes	12/15 (80.0)	1/1 (100.0)		6/6 (100.0)	0/0	
No	111/140 (79.3)	15/17 (88.2)		87/103 (84.5)	3/5 (60.0)	
Patient was nonoperative			0.532			0.150
Yes	95/118 (80.5)	13/14 (92.9)		58/66 (87.9)	3/4 (75.0)	
No	28/37 (75.7)	3/4 (75.0)		35/43 (81.4)	0/1 (0.0)	
History of severe organ system insufficiency/ immunocompromised			0.530			0.187
Yes	5/9 (55.6)	1/1 (100.0)		4/4 (100.0)	0/0	
No	118/146 (80.8)	15/17 (88.2)		89/105 (84.8)	3/5 (60.0)	
Diabetes and cardiac comorbidity			0.361			0.203
Yes	76/104 (73.1)	13/14 (92.9)		43/55 (78.2)	1/3 (33.3)	
No	47/51 (92.2)	3/4 (75.0)		50/54 (92.6)	2/2 (100.0)	
Respiratory insufficiency/failure			0.532			0.196
Yes	71/93 (76.3)	10/12 (83.3)		60/72 (83.3)	2/4 (50.0)	
No	52/62 (83.9)	6/6 (100.0)		33/37 (89.2)	1/1 (100.0)	
On a ventilator at the time of randomization			0.530			0.137
Yes	54/71 (76.1)	4/5 (80.0)		40/49 (81.6)	0/0	
No	69/84 (82.1)	12/13 (92.3)		53/60 (88.3)	3/5 (60.0)	
Baseline bacteremia with *S. aureus*			0.532			0.181
Yes	7/10 (70.0)	3/3 (100.0)		7/8 (87.5)	0/0	
No	116/145 (80.0)	13/15 (86.7)		86/101 (85.1)	3/5 (60.0)	
In ICU[c] at baseline			0.532			0.124
Yes	59/77 (76.6)	7/8 (87.5)		48/60 (80.0)	1/1 (100.0)	
No	64/78 (82.1)	9/10 (90.0)		45/49 (91.8)	2/4 (50.0)	
APACHE II score			0.525			0.184
0-13 points	59/68 (86.8)	8/8 (100.0)		55/61 (90.2)	2/3 (66.7)	
14-19 points	41/51 (80.4)	4/6 (66.7)		26/30 (86.7)	1/1 (100.0)	
≥20 points	23/36 (63.9)	4/4 (100.0)		12/18 (66.7)	0/1 (0.0)	

[a] Two-sided *P* value from an exact test of the null hypothesis of no association between clinical outcome and *pvl* status, stratifying on the covariate.
[b] "Other" includes Argentina, Australia, Brazil, Chile, China, India, Israel, Lebanon, Malaysia, Peru, Philippines, South Africa, South Korea, Taiwan, and Thailand.
[c] ICU, intensive care unit.

other virulence genes and clinical outcome. The results of these comparisons are demonstrated in Table 3. After adjustment for multiple comparisons (data not shown) to control the false discovery rate for the family of all the tests, no significant associations between clinical outcome and presence or absence of any of the 30 other putative virulence genes were detected for patients with either MRSA or MSSA HAP (Table 3).

Alpha-hemolysin production and outcome of *S. aureus* HAP. Because alpha-hemolysin has also been identified as a virulence factor in *S. aureus* pneumonia (2, 3) and might be acting in concert with PVL to augment pulmonary inflammation (4), we quantified alpha-hemolysin activity in the culture supernatant of all 23 *pvl*-constitutive isolates, as well as 23 randomly selected *pvl*-negative *S. aureus* isolates matched according to methicillin susceptibility. There was no evidence that clinical cure rates were related to high or low levels of alpha-hemolysin production *in vitro* (Table 4).

Effect of *agr* dysfunction in clinical outcome. Because previous reports have suggested that dysfunction of the *agr* locus could result in attenuation in virulence (14, 39, 40, 46, 48), we evaluated *agr* function in all 286 *S. aureus* HAP isolates by using a delta-hemolysin activity/phenotyping assay. Of the 286 isolates, 191 (66.8%) exhibited a functional *agr* by the delta-hemolysin phenotyping assay (MRSA, 54.1% [93/172]; MSSA, 86.0% [98/114]). No significant association was identified between *agr* function and clinical outcome in either MRSA or MSSA HAP (Fig. 2).

MLST and clinical outcome. To consider the possibility that *pvl* presence could serve as a surrogate marker for a more virulent *S. aureus* clone, we used MLST to genotype all *pvl*-positive *S. aureus* isolates and a collection of randomly selected *pvl*-negative *S.*

TABLE 3 Association between putative virulence genes and clinical outcome among patients with hospital-acquired pneumonia due to methicillin-resistant or methicillin-sensitive *Staphylococcus aureus*

Gene	MRSA ($n = 173$)				MSSA ($n = 114$)			
	No. (%) of patients with genotype	Cure rate (no./total [%]) with:		*P* value[a]	No. (%) of patients with genotype	Cure rate (no./total [%]) with:		*P* value[a]
		Gene absent	Gene present			Gene absent	Gene present	
Adhesin genes								
fnbA	173/173 (100.0)	0/0	139/173 (80.3)		114/114 (100.0)	0/0	96/114 (84.2)	
clfA	142/173 (82.1)	24/31 (77.4)	115/142 (81.0)	0.625	114/114 (100.0)	0/0	96/114 (84.2)	
clfB	125/173 (72.3)	39/48 (81.3)	100/125 (80.0)	1.000	43/114 (37.7)	62/71 (87.3)	34/43 (79.1)	0.292
cna	75/173 (43.4)	79/98 (80.6)	60/75 (80.0)	1.000	52/114 (45.6)	48/62 (77.4)	48/52 (92.3)	0.039
spa	173/173 (100.0)	0/0	139/173 (80.3)		114/114 (100.0)	0/0	96/114 (84.2)	
sdrC	157/173 (90.8)	15/16 (93.8)	124/157 (79.0)	0.202	49/114 (43.0)	56/65 (86.2)	40/49 (81.6)	0.607
sdrD	154/173 (89.0)	16/19 (84.2)	123/154 (79.9)	1.000	74/114 (64.9)	35/40 (87.5)	61/74 (82.4)	0.595
sdrE	148/173 (85.5)	19/25 (76.0)	120/148 (81.1)	0.588	68/114 (59.6)	38/46 (82.6)	58/68 (85.3)	0.795
bbp	160/173 (92.5)	9/13 (69.2)	130/160 (81.3)	0.288	107/114 (93.9)	4/7 (57.1)	92/107 (86.0)	0.077
ebpS	173/173 (100.0)	0/0	139/173 (80.3)		114/114 (100.0)	0/0	96/114 (84.2)	
map-eap	58/173 (33.5)	92/115 (80.0)	47/58 (81.0)	1.000	14/114 (12.3)	85/100 (85.0)	11/14 (78.6)	0.462
fnbB	120/173 (69.4)	47/53 (88.7)	92/120 (76.7)	0.096	36/114 (31.6)	66/78 (84.6)	30/36 (83.3)	1.000
Toxin genes								
pvl	18/173 (10.4)	123/155 (79.4)	16/18 (88.9)	0.532	5/114 (4.4)	93/109 (85.3)	3/5 (60.0)	0.176
eta	115/173 (66.5)	51/58 (87.9)	88/115 (76.5)	0.104	63/114 (55.3)	44/51 (86.3)	52/63 (82.5)	0.617
etb	11/173 (6.4)	130/162 (80.2)	9/11 (81.8)	1.000	10/114 (8.8)	89/104 (85.6)	7/10 (70.0)	0.193
tst	80/173 (46.2)	74/93 (79.6)	65/80 (81.3)	0.849	47/114 (41.2)	60/67 (89.6)	36/47 (76.6)	0.072
sea	104/173 (60.1)	53/69 (76.8)	86/104 (82.7)	0.435	59/114 (51.8)	45/55 (81.8)	51/59 (86.4)	0.610
seb	4/173 (2.3)	135/169 (79.9)	4/4 (100.0)	1.000	7/114 (6.1)	90/107 (84.1)	6/7 (85.7)	1.000
sec	36/173 (20.8)	109/137 (79.6)	30/36 (83.3)	0.814	32/114 (28.1)	67/82 (81.7)	29/32 (90.6)	0.391
sed	63/173 (36.4)	88/110 (80.0)	51/63 (81.0)	1.000	24/114 (21.1)	76/90 (84.4)	20/24 (83.3)	1.000
see	56/173 (32.4)	92/117 (78.6)	47/56 (83.9)	0.540	19/114 (16.7)	80/95 (84.2)	16/19 (84.2)	1.000
seg	109/173 (63.0)	51/64 (79.7)	88/109 (80.7)	1.000	60/114 (52.6)	45/54 (83.3)	51/60 (85.0)	1.000
seh	8/173 (4.6)	131/165 (79.4)	8/8 (100.0)	0.358	16/114 (14.0)	83/98 (84.7)	13/16 (81.3)	0.716
sei	160/173 (92.5)	11/13 (84.6)	128/160 (80.0)	1.000	99/114 (86.8)	11/15 (73.3)	85/99 (85.9)	0.252
hlg	172/173 (99.4)	1/1 (100.0)	138/172 (80.2)	1.000	108/114 (94.7)	5/6 (83.3)	91/108 (84.3)	1.000
Others								
efb	173/173 (100.0)	0/0	139/173 (80.3)		114/114 (100.0)	0/0	96/114 (84.2)	
icaA	170/173 (98.3)	3/3 (100.0)	136/170 (80.0)	1.000	113/114 (99.1)	1/1 (100.0)	95/113 (84.1)	1.000
chp	128/173 (74.0)	37/45 (82.2)	102/128 (79.7)	0.829	91/114 (79.8)	19/23 (82.6)	77/91 (84.6)	0.758
V8	160/173 (92.5)	11/13 (84.6)	128/160 (80.0)	1.000	85/114 (74.6)	26/29 (89.7)	70/85 (82.4)	0.556
Agr group II vs. all others	71/173 (41.0)	85/102 (83.3)	54/71 (76.1)	0.249	34/114 (29.8)	67/80 (83.8)	29/34 (85.3)	1.000
SCC type II (4/non-4)	29/173 (16.8)	114/144 (79.2)	25/29 (86.2)	0.454	0/114 (0.0)	96/114 (84.2)	0/0	

[a] Two-sided *P* value from Fisher's exact test of the null hypothesis of no association between clinical outcome and the presence/absence of the putative virulence genes. After adjustment for multiple comparisons to control the false discovery rate for the family of all the tests, there was no significant difference between clinical outcome and PVL status.

aureus isolates matched 1:1 (23 *pvl*-positive and 23 *pvl*-negative *S. aureus* isolates) (Table 5). Most *pvl*-positive *S. aureus* isolates belonged to CC8 (12/23 [52.2%]), whereas the most common CC among *pvl*-negative isolates was CC5 (11/23 [47.8%]). No clones were significantly associated with worse clinical outcome.

DISCUSSION

The impact of *pvl* on the severity of *S. aureus* pneumonia is unknown. Using *S. aureus* isolates from a large collection of contemporary, geographically diverse, clinically well-characterized HAP patients, the current investigation found no evidence that *pvl* presence is associated with a more severe clinical course. This finding has several key implications.

The results of the present study demonstrate that the simple presence of *pvl* is not the primary outcome determinant in *S. aureus* HAP. Our finding is consistent with that of a recent study from Peyrani et al. (33). While an important hypothesis-generating observation, the Peyrani study was limited by its relatively small sample size, retrospective study design, limitation to MRSA-infected patients, geographically limited enrollment (4 U.S. centers), failure to confirm PVL production, and failure to consider the potential impact of other bacterial virulence characteristics. The current study overcomes all of these limitations to provide compelling evidence that factors other than the simple presence of PVL are the primary determinant of outcome in patients with HAP due to *S. aureus*. Our study findings persisted after adjusting for multiple patient and bacterial characteristics and are consistent with several other lines of evidence. First, the results of this study are consistent with three previous reports by our group that show a similar lack of association between *pvl*

TABLE 4 Clinical outcome versus alpha-lysin production among patients with *Staphylococcus aureus* hospital-acquired pneumonia[b]

S. aureus type and amt of alpha-lysin[a]	PVL$^+$			PVL$^-$		
	No. of isolates			No. of isolates		
	Cure	Failure	Total	Cure	Failure	Total
MRSA						
High	4 (80.0)	1 (20.0)	5	1 (100.0)	0	1
Low	12 (92.3)	1 (7.7)	13	13 (76.5)	4 (23.5)	17
Total	16 (88.9)	2 (11.1)	18	14 (77.8)	4 (22.2)	18
MSSA						
High	2 (100.0)	0	2	1 (50.0)	1 (50.0)	2
Low	1 (33.3)	2 (66.7)	3	1 (33.3)	2 (66.7)	3
Total	3 (60.0)	2 (40.0)	5	2 (40.0)	3 (60.0)	5

[a] High, >10 HU/ml; low, ≤10 HU/ml.
[b] The *P* value for MRSA and MSSA pooled was 1.00, as calculated by using an exact Monte Carlo Cochran-Mantel-Haenszel test, with stratification on methicillin susceptibility (MRSA or MSSA).

presence and higher likelihood of worse patient outcome in complicated skin and skin structure infections (1, 5, 44). In all of those studies, *pvl* presence was not associated with more severe infection, and in two of these studies (1, 5), *pvl* presence was actually associated with a significantly higher cure rate. Second, levels of PVL production do not appear to correlate with severity of infection (18). By enzyme-linked immunosorbent assay (ELISA), *S. aureus* strains from severe infections like necrotizing pneumonia were not the hyperproducers of PVL toxin compared to those associated with comparatively minor infections. Third, severe necrotizing pneumonia has been associated with *pvl*-negative community-acquired MRSA (CA-MRSA) strains (43). Collectively, these findings support the notion that factors other than the simple presence of *pvl* are responsible for influencing severity in a variety of *S. aureus* infections (32), including HAP.

Alpha-hemolysin is an important virulence factor in pneumonia (2, 3). Because it may act in concert with PVL to induce pulmonary inflammation (4), we considered its additive impact on clinical severity of *S. aureus* HAP. No significant differences in cure rates were identified among the high alpha-lysin producers within *pvl*-positive or *pvl*-negative isolates. These findings suggest that our understanding of the pathogenesis of *S. aureus* pneumonia remains incomplete and that the determinants of infection severity are far more complex than the presence or absence of a few virulence characteristics.

Approximately one-third of the HAP isolates in the present study were delta-hemolysin deficient, suggesting that *agr* dysfunction did not reduce the capacity of *S. aureus* to cause invasive infection. The expression of virulence factors, including toxins and adhesins, in *S. aureus* is controlled by a global polycistronic regulatory locus, *agr*. This regulatory network encodes the quorum-sensing system that coordinates the expression of secreted and cell-associated virulence factors in a cell-density-dependent manner (29). Because delta-hemolysin is encoded by *hld* within the *agr* locus and is derived from the translation of RNAIII, the effector of *agr* regulon, its production is a marker of

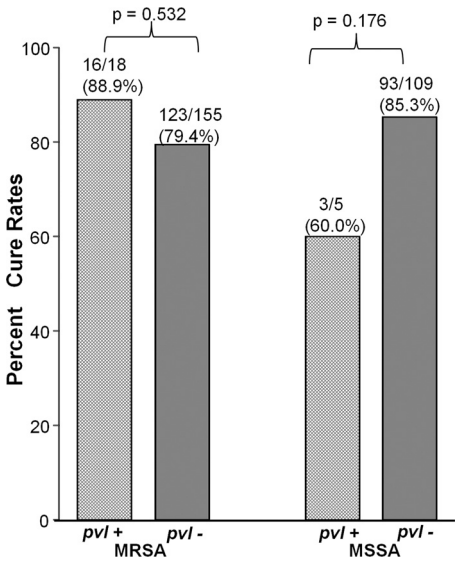

FIG 1 Cure rates among patients due to methicillin-resistant (MRSA) or methicillin-sensitive (MSSA) *Staphylococcus aureus* hospital-acquired pneumonia according to presence or absence of *pvl*.

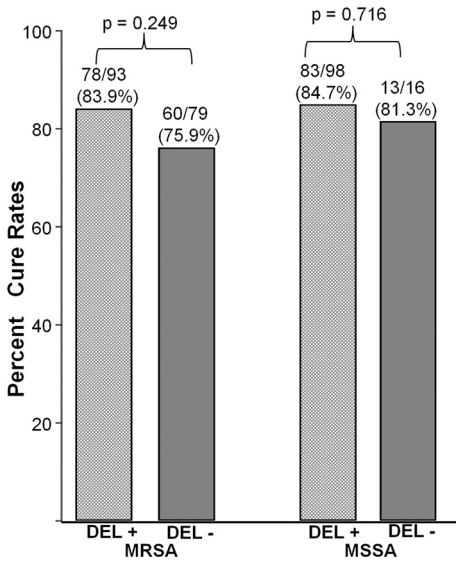

FIG 2 Cure rates among patients due to methicillin-resistant (MRSA) or methicillin-sensitive (MSSA) *Staphylococcus aureus* hospital-acquired pneumonia according to delta-lysin (DEL) production.

TABLE 5 Clonal complex distribution among *Staphylococcus aureus* hospital-acquired pneumonia *pvl*-positive and *pvl*-negative isolates

Clonal complex	No. of isolates		
	Total	Cure	Failure
pvl positive			
CC5	1	1	0
CC8	12	9	3
CC15	1	1	0
CC30	3	3	0
CC59	2	2	0
CC88	1	1	0
CC121	2	1	1
Singleton	1	1	0
Total	23	19	4
pvl negative			
CC5	11	10	1
CC8	6	3	3
CC15	1	1	0
CC22	1	1	0
CC30	1	1	0
CC97	2	0	2
CC121	1	0	1
Total	23	16	7

agr function (36). Loss of *agr* function has been linked to attenuated virulence (48) and has global effects on bacterial phenotypes (14, 40, 46) and increased mortality among *S. aureus* bacteremia patients (39). However, we found no evidence in the current investigation that *agr* dysfunction is associated with the outcome of HAP.

The prevalence of *pvl* in *S. aureus* varies widely among different infection types (7, 8, 21, 23–27, 33, 37, 38, 41, 45). Less than 10% of the *S. aureus* HAP isolates in this study contained *pvl*. This relatively low prevalence stands in sharp contrast to trends seen in soft tissue infections (1, 45) as well as in a pneumonia study in children (6).

Our study had several limitations. First, our study focused by design on patients with *S. aureus* HAP, while most prior reports that link PVL with pneumonia involved community-acquired *S. aureus* necrotizing pneumonia (15, 16). Thus, our findings would not apply to community-acquired *S. aureus* necrotizing pneumonia. This is an important distinction because of the prior health status of the infected individuals, i.e., those with community-acquired *S. aureus* necrotizing pneumonia were typically otherwise healthy, whereas patients with *S. aureus* HAP had risk factors for infection (and were thus highly susceptible to infection). Previously existing susceptibility of the HAP patients to infection could in part explain the finding that there was no significant association between clinical outcome and presence or absence of putative virulence genes (Table 3). Such molecules might simply be unnecessary to cause infection in these immunocompromised patients. Second, the proportion of isolates harboring and expressing PVL was low (<10%) and thus limited the statistical power of finding associations. Although we must remain cautious in the conclusions drawn from the present study, the fact that other investigators (33) have recently reported the same result strengthens its generalizability. Third, we evaluated alpha-hemolysin levels *in vitro*, and it remains unclear whether *in vitro* activity is a reflection of relative toxin production *in vivo*.

Study strengths include the fact that we have utilized one of the largest cohorts of patients with HAP, including large subgroups with *S. aureus* and MRSA. Therefore, although proportionally there were few *pvl*-positive isolates, this is likely to be one of the largest cohorts of patients with staphylococcal HAP to allow any comparison between *pvl*-positive and *pvl*-negative groups. Besides the larger size of the cohort, the other strengths of this investigation included its contemporary nature, multinational design, and detailed clinical and laboratory data. Finally, confirmation of *pvl* genotyping data by PVL Western blotting further strengthens the study findings.

In summary, this study provides evidence that PVL presence is not the primary determinant of outcome among patients with *S. aureus* HAP. This finding suggests that clinical outcome may be more significantly influenced by the presence of several bacterial virulence factors acting in concert than by the mere presence of *pvl*. Alternately, currently unrecognized or recently discovered virulence factors (e.g., phenol soluble modulins [31] or the novel bicomponent leukotoxin LukGH [47]) as well as the host genetic factors may also contribute to clinical outcome. The discovery of an exact virulence determinant needs further elucidation using appropriate *in vivo* models and using a collection of widely distributed well characterized strains. We anticipate future studies will combine clinical data with not only the presence/absence of any genotype but also technologies such as RNASeq to determine the relationships between pathogen transcriptome and clinical outcomes.

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