MAJOR ARTICLE

Associations between *Staphylococcus aureus* Genotype, Infection, and In-Hospital Mortality: A Nested Case-Control Study

Heiman F. L. Wertheim,[1] Willem B. van Leeuwen,[1] Susan Snijders,[1] Margreet C. Vos,[1] Andreas Voss,[2] Christina M. J. E. Vandenbroucke-Grauls,[3] Jan A. J. W. Kluytmans,[4] Henri A. Verbrugh,[1] and Alex van Belkum[1]

[1]Department of Medical Microbiology and Infectious Diseases, Erasmus Medical Center, University Medical Center Rotterdam, Rotterdam, [2]Department of Medical Microbiology and Infectious Diseases, University Medical Center Nijmegen, Nijmegen, [3]Department of Medical Microbiology and Infectious Diseases, VU Medical Center, Amsterdam, and [4]Department of Medical Microbiology and Infectious Diseases, Amphia Hospital, Breda, The Netherlands

We screened 14,008 adult nonsurgical patients for *Staphylococcus aureus* nasal carriage at hospital admission and assessed them for invasive *S. aureus* disease and in-hospital mortality. Multilocus sequence typing was performed on endogenous invasive strains and nasal strains of matched asymptomatic carriers to investigate whether virulent clones could be identified in nasal carriers. Clonal complex (CC) 45 was significantly underrepresented (odds ratio [OR], 0.16 [95% confidence interval {CI}, 0.04–0.59]) and CC30 was overrepresented (not statistically significant) among invasive strains (OR, 1.91 [95% CI, 0.91–4.0]). The distribution of CCs of invasive *S. aureus* strains in noncarriers did not differ from that in carriers. Those infected with *S. aureus* strains belonging to a CC had higher mortality than those infected with strains not belonging to a CC ($P < .05$), which indicates the coevolution of *S. aureus* virulence and spread in humans.

Staphylococcus aureus is a leading cause of nosocomial infections, varying from superficial wound infections to invasive disease, such as deep abscesses, osteomyelitis, and bacteremia [1]. These infections lead to prolonged hospital stays, increased antibiotic use, and increased medical costs [2]. *S. aureus* nasal carriers have a 3-fold increased risk of acquiring nosocomial *S. aureus* bacteremia, compared with noncarriers, but they have lower mortality when infection occurs [3]. This higher survival of carriers may be due to partial immunity. Alternatively, *S. aureus* strains from asymptomatic nasal carriers of *S. aureus* may belong to a less virulent genotype, compared with strains from nasal carriers with proven invasive disease.

We, therefore, wanted to investigate whether certain *S. aureus* clones found in *S. aureus* nasal carriers are more likely than others to cause invasive disease. We also investigated whether invasive *S. aureus* disease in noncarriers may be caused by a specific clone acquired during hospitalization. We used a microarray-based method for multilocus sequence typing (MLST) to analyze a collection of *S. aureus* strains described elsewhere that were isolated from a cohort of patients admitted to the hospital [3–5].

PATIENTS, MATERIALS, AND METHODS

Study design

We performed a nested case-control study in a cohort of 14,008 adult nonsurgical patients who were screened for *S. aureus* nasal carriage at hospital admission [3, 5]. The study was performed in 4 teaching hospitals in separate regions of The Netherlands from 1 February 1999 to 1 February 2001. All patients were monitored for the development of invasive *S. aureus* disease by checking microbiological data on a weekly basis, as described elsewhere [3, 5]. Invasive disease was defined

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Reprints or correspondence: Dr. Heiman F. L. Wertheim, Dr. Molewaterplein 40, Erasmus MC, Dept. of Medical Microbiology, Rotterdam 3015GD, The Netherlands (h.wertheim@erasmusmc.nl)

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Table 1. Characteristics of the study population.

Characteristic	Case patients (n = 60)	Control subjects (n = 118)[a]	Noncarriers (n = 34)
Male sex, no. (%)	33 (55.0)	65 (55.1)	24 (70.6)
Mean age (±SD), years	53 (±17)	53 (±17)	64 (±17)[b]
Site of hospital admission, no. (%)			
Nijmegen	17 (28.3)	33 (28.0)	12 (35.3)
Amsterdam	8 (13.3)	16 (13.6)	5 (14.7)
Breda	5 (8.3)	10 (8.5)	ND[c]
Rotterdam	30 (50.0)	59 (50.0)	17 (50.0)
Origin of strain, no. (%)			
Blood	55 (91.7)	0 (0.0)	34 (100.0)
Other sterile site	5 (8.3)	0 (0.0)	0 (0.0)

NOTE. Case patients are carriers who acquired invasive *Staphylococcus aureus* disease caused by their own nasal strain during hospitalization, control subjects are carriers who did not acquire *S. aureus* invasive disease, and noncarriers are those who were not colonized by *S. aureus* in the nose at admission but did develop invasive *S. aureus* disease during hospitalization. ND, not done.

[a] Two control subjects were found to have *S. aureus* infection at a later date and were excluded from further analysis.

[b] Noncarriers were significantly older than case patients ($P = .005$).

[c] The hospital in Breda did not store invasive *S. aureus* strains from noncarriers.

as being present when *S. aureus* was cultured from blood or from pus present in a normally sterile site. Medical ethical approval was obtained from all participating centers.

For each case patient with invasive disease, 2 control subjects were included who were matched by the presence of *S. aureus* nasal carriage, the hospital of admission, the date of admission (±1 month), sex, age group (±5 years), and the absence of *S. aureus* infection during follow-up (until 6 weeks after discharge). If >2 matched control subjects were found, those closest in age to the case patient were selected. Nasal strains from carriers and invasive strains from noncarriers were genotyped by pulsed-field gel electrophoresis (PFGE), and the resulting data were interpreted in accordance with standard criteria [6]. MLST was performed on genetically similar, as determined by PFGE, invasive strains from carriers and on nasal strains from carriers who did not develop invasive disease (asymptomatic carriers). In addition, invasive strains cultured from blood or deep foci of infection from noncarriers were analyzed by MLST.

MLST. We used an oligonucleotide array for MLST of *S. aureus,* as described elsewhere [4]. Briefly, DNA was extracted using lysostaphine and the QIAamp DNA Minikit (Qiagen). DNA was used in a multiplex polymerase chain reaction (PCR) with specific primers targeting the 7 housekeeping genes, as defined by Enright et al. [7]. PCR products were fragmented and labeled with a new DNA amplicon labeling technique (bioMérieux), were purified with the QIAquick Nucleotide Removal Kit (Qiagen), and were hybridized with the oligoprobe arrays in the GeneChip Fluidics Station (Affymetrix) [4]. Each probe array was stained with R-phycoerythrin–labeled streptavidin (Dako), and the signal was measured with a GeneArray scanner (Agilent). Probe array cell intensities, base call, sequence determination, and reports were generated by functions available in GeneChip (Affymetrix). A candidate allele selection index was determined by the percentage of identity between the experimentally derived sequence and the distinct reference sequence tiled on the array.

For some housekeeping genes, the oligomediated MLST procedure can generate ambiguous results, because polymorphisms can be present in the 10 nucleotides proximal to the 5′ and 3′ ends of the amplicons [7]. Genes with these polymorphisms are not recognized by the oligoprobes. In such cases, the entire housekeeping gene was reamplified, and both strands of the amplicons were sequenced to identify possible polymorphisms [4].

Statistical analysis

Comparison of MLST results was performed with BURST software, as described elsewhere [8]. Data were analyzed with SPSS (version 10.0; SPSS). Frequencies were compared with the χ^2 test, and continuous variables were compared with Student's t test. $P < .05$ was considered to be statistically significant. Odds ratios with 95% confidence intervals (CIs) were calculated for the case-control study.

RESULTS

General

The demographic data of the 60 case patients and 118 control subjects are summarized in table 1. Two control subjects were excluded, because they were later found to have *S. aureus* infection, and they were not replaced by new control subjects. Most invasive strains (91.7%) were cultured from blood. Additionally, 34 invasive strains that were cultured from blood

from hospitalized nonnasal carriers of *S. aureus* and were described elsewhere [3] were selected for MLST. Five strains from the original cohort were lost and therefore were not analyzed. None of the cultured *S. aureus* strains was methicillin resistant.

Sequence types, invasive disease, and mortality

Overall, 32 different sequence types (STs) were identified (figure 1). Nine STs accounted for 80% of all tested strains, and 3 STs (30, 15, and 45) were the most prevalent (20%, 15%, and 12%, respectively). There were no significant differences in the distribution of STs per hospital (data not shown). STs were grouped by BURST analysis into clonal complexes (CCs), as is shown in table 2. Although the results of the BURST analysis regarded STs 5, 9, 12, 15, and 22 as singletons, we classified them as CCs, as was done in a previous study [8]. Only CC45 was significantly more prevalent in noninvasive strains (odds ratio [OR], 0.2 [95% CI, 0.04–0.6]). Most invasive strains belonged to CC30, and many were singletons. Invasive strains from noncarriers did not differ markedly from invasive strains from carriers in the distribution of CCs (table 2).

The overall mortality in noncarriers was higher than that in carriers (table 3) ($P < .001$), as described elsewhere [3]. By use of MLST analysis, we could not identify a specific clone in noncarriers that could explain the higher in-hospital mortality in this group. However, there was significantly higher mortality in carriers and noncarriers infected with *S. aureus* strains belonging to a CC (20/69 [29.0%]), compared with that in those infected with strains classified as singletons or new STs (1/25 [4.0%]; $P < .05$).

DISCUSSION

In a previous study, patients were screened for *S. aureus* nasal carriage at admission in 4 teaching hospitals and were followed for the development of nosocomial invasive *S. aureus* disease. In the present study, invasive *S. aureus* strains from carriers were compared, by MLST, with nasal strains isolated from matched control subjects who did not develop invasive *S. aureus* disease. The STs of invasive *S. aureus* strains from noncarriers were defined as well. The distribution of the CCs was comparable with that found in other studies [8, 9].

Overall, no major CC could be identified that was responsible for invasive *S. aureus* disease. However, 35.0% of invasive

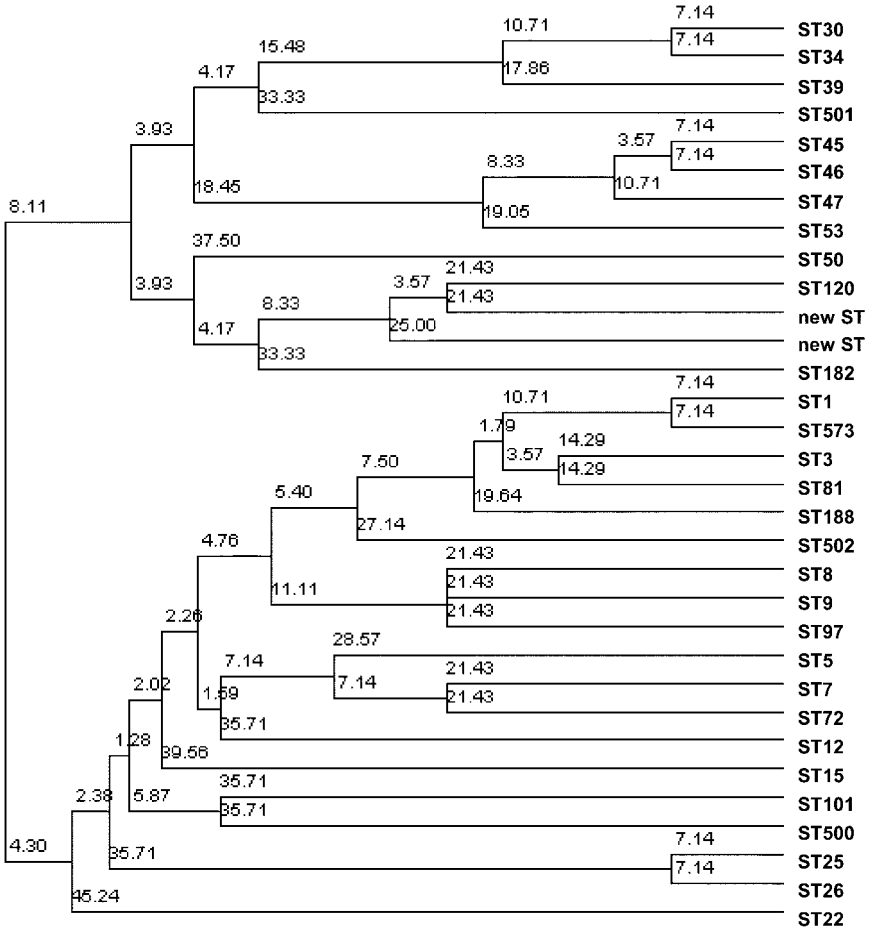

Figure 1. Dendrogram showing the relatedness of *Staphylococcus aureus* sequence types

Table 2. Distribution of identified clonal complexes (CCs).

CC	Case patients	Control subjects	OR (95% CI)	Noncarriers
1	1 (1.7)	4 (3.4)	0.5 (0.02–4.8)	4 (11.7)
5	6 (10.0)	5 (4.2)	2.5 (0.6–10.0)	1 (2.9)
9	1 (1.7)	1 (0.8)	2.0 (0.01–74.0)	1 (2.9)
12	1 (1.7)	0 (0.0)	…	0 (0.0)
15	8 (13.3)	17 (14.4)	0.9 (0.3–2.4)	7 (20.6)
22	0	2 (1.7)	0.0 (0.0–8.1)	0 (0.0)
25	1 (1.7)	6 (5.1)	0.3 (0.01–2.8)	3 (8.8)
30	21 (35.0)	26 (22.0)	1.9 (0.9–4.0)	6 (17.6)
45	3 (5.0)	29 (24.6)	0.2 (0.04–0.6)[a]	5 (14.7)
Singletons	17 (28.3)	28 (23.7)	1.3 (0.6–2.7)	6 (17.6)
New ST	1 (1.7)	0 (0.0)	…	1 (2.9)
Total	60	118	…	34

NOTE. Data are no. (%) of strains, unless otherwise indicated. CC1 includes sequence types (STs) 1, 3, 81, 188, and 573; CC25 includes STs 25 and 26; CC30 includes STs 30, 34, and 39; CC45 includes STs 45, 46, 47, and 53; singletons include STs 7, 8, 50, 97, 120, 182, 500, 501, and 502; and new STs include the following allelic profiles: 18-33-6-2-7-13-2 and 3-14-6-2-14-13-x. CI, confidence interval; OR, odds ratio.

[a] $P = .001$.

strains belonged to ST30 (not statistically significant). Interestingly, we did identify a CC (45) that was significantly more prevalent among nasal strains. This finding is supported by data in a study by Melles et al. [9]. They found that CC45 was present in 21% of asymptomatic carriers and in only 13% of invasive strains (not statistically significant) [9]. We could not identify a CC that was significantly more prevalent in invasive strains from noncarriers than in other strains.

We reported elsewhere that there was considerably higher mortality in noncarriers with invasive *S. aureus* strains, compared with that in carriers with invasive disease in the same cohort [3]. No single CC could be identified that could explain the higher mortality in this patient category. But this analysis is not definite, because some CCs had small numbers. However, in the present study, mortality was significantly higher in carriers and noncarriers infected with *S. aureus* strains belonging to a CC, compared with mortality in those infected with strains not belonging to a CC (29.0% vs. 4.0%). A multivariate analysis did not alter this finding (data not shown).

There is some controversy regarding whether ST8 belongs to a CC (CC8). A recent overview of the clonality of *S. aureus* classifies this ST as a singleton, like we did [8]. Other studies classify ST8 as a CC [10]. In the present study, when we regarded ST8 as a CC, we still found much higher mortality in carriers and noncarriers infected with *S. aureus* strains belonging to a CC (20/77 [26.0%]), compared with mortality in those infected with strains classified as singletons or new STs (1/17 [5.9%]; $P = .07$).

This finding indicates that staphylococcal clones that have successfully spread in humans—that is, have evolved into prevalent CCs or lineages—are those that may have more virulence factors associated with lethality of *S. aureus* disease. Additional screening of the staphylococcal genome for virulence factors could aid in identifying the putative factor(s) responsible for the higher mortality caused by strains belonging to CCs. Although we did not correct for underlying disease in the present study (data not available), recent data indicate that underlying disease is not as important in predicting mortality as was previously thought [3]. Control subjects who fit the criteria for the present study were picked at random, to exclude a biased selection.

Feil et al. also identified CC30 as a major CC in invasive nosocomial disease [8]. They attributed this observation to the widespread presence of EMRSA-16 within this CC [7, 8]. However, in the present study, no methicillin-resistant *S. aureus* (MRSA) strains were identified—and the prevalence of MRSA strains is very low in The Netherlands [11]—so methicillin resistance does not explain this finding. Clearly, CC30 is a successful *S. aureus* lineage, irrespective of its methicillin resistance. Due to the abundance of strains belonging to CC30, as was found in our study and by Melles et al. [9], the chances that this lineage will acquire a *Staphylococcus* cassette chromosome *mec* (SCC*mec*) are likely high. Once a SCC*mec* is acquired, these CC30 MRSA strains can replace CC30 methicillin-susceptible strains easily in settings with high antibiotic use, including hospitals.

Peacock et al. [12] compared 155 *S. aureus* isolates from patients with invasive disease with carriage isolates from healthy individuals in the same cohort that Feil et al. studied [8]. Peacock et al. proposed that allelic variants of a polymorphic locus can make different contributions to the disease process, although it remains unclear how this is done. They also found

Table 3. Mortality data of carriers and noncarriers with invasive *Staphylococcus aureus* disease, by clonal complex (CC).

CC	In-hospital mortality		
	Carriers	Noncarriers	Total
1	1/1 (100.0)	2/4 (50.0)	3/5 (60.0)
5	1/6 (16.7)	0/1 (0.0)	1/7 (14.3)
9	0/1 (0.0)	1/1 (100.0)	1/2 (50.0)
12	0/1 (0.0)	0/0 (0.0)	0/1 (0.0)
15	1/8 (12.5)	3/7 (42.9)	4/15 (26.7)
25	1/1 (100.0)	1/3 (33.3)	2/4 (50.0)
30	2/21 (9.5)	4/6 (66.7)	6/27 (22.2)
45	0/3 (0.0)	3/5 (60.0)	3/8 (37.5)
Singletons	0/17 (0.0)	1/6 (16.7)	1/23 (4.3)
New ST	0/1 (0.0)	0/1 (0.0)	0/2 (0.0)
Total	6/60 (10.0)	15/34 (44.1)	21/94 (22.3)

NOTE. Data are no./total (%) of strains. There was significantly higher mortality in noncarriers, compared with that in carriers ($P < .001$, χ^2 test). No specific CC could be identified that was associated with higher mortality in noncarriers. CC1 includes sequence types (STs) 1, 3, 81, 188, and 573; CC25 includes STs 25 and 26; CC30 includes STs 30, 34, and 39; CC45 includes STs 45, 46, 47, and 53; singletons include STs 7, 8, 50, 97, 120, 182, 500, 501, and 502; and new STs include the following allelic profiles: 18-33-6-2-7-13-2 and 3-14-6-2-14-13-x.

evidence for considerable horizontal transfer of genes against a clonal background. It is now well established that, within and between *S. aureus* clones, there is a significant level of exchange of mobile DNA that codes for virulence and resistance [13–15]. Melles et al. showed that all clones can cause life-threatening infections, but certain clones are more virulent than others [9]. It would be interesting to investigate whether there is a difference in competence for the uptake of (mobile) DNA between clones.

By MLST, we could not identify any *S. aureus* CC that was more likely than other CCs to cause invasive disease. We did find that CC45 was more prevalent in asymptomatic carriers. CC30 was also prevalent, and this prevalence was independent of methicillin resistance. There were no prevalent clones of invasive *S. aureus* strains in noncarriers and no specific clone that could explain the higher in-hospital mortality. However, overall mortality, irrespective of carriage status, was significantly higher for carriers and noncarriers infected with strains belonging to a CC, which indicates the coevolution of *S. aureus* virulence and spread in humans.

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