Virulence Potential of *Staphylococcus aureus* Strains Isolated From Diabetic Foot Ulcers

A new paradigm

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OBJECTIVE — The purpose of this study was to assess the virulence potential of *Staphylococcus aureus* strains isolated from diabetic foot ulcers and to discriminate noninfected from infected ulcers.

RESEARCH DESIGN AND METHODS — Diabetic patients hospitalized in a diabetic foot department with a foot ulcer were prospectively enrolled if they had been free of antibiotic treatment over the previous 6 months. At admission, ulcers were classified as infected or noninfected on the basis of clinical examination, according to the International Working Group on the Diabetic Foot system. Only patients carrying *S. aureus* as the sole pathogen were included. In individuals with a grade 1 ulcer, a second bacterial specimen was obtained 1 month later. Using virulence genotyping markers, clonality tools, and an in vivo *Caenorhabditis elegans* model, we correlated the virulence of 132 *S. aureus* strains with grade, time of collection, and ulcer outcome.

RESULTS — Among virulence genes, the most relevant combination derived from the logistic regression was the association of *cap8*, *sea, sei, lukE*, and *hlgv* (area under the curve 0.958). These markers were useful to distinguish noninfected (grade 1) from infected (grades 2–4) ulcers and to predict wound status at the follow-up. With use of the nematode model, *S. aureus* strains isolated from grade 1 ulcers were found to be significantly less virulent than strains from ulcers at or above grade 2 ($P < 0.001$).

CONCLUSIONS — This study highlights the coexistence of two *S. aureus* populations on diabetic foot ulcers. A combination of five genes that may help distinguish colonized grade 1 from infected grade ≥2 wounds, predict ulcer outcome, and contribute to more appropriate use of antibiotics was discovered.

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Foot ulcers are common in diabetic patients, with prevalence as high as 25% (1). These ulcers frequently become infected, and spread of infections to soft tissue and to bony structures is a major causal factor for lower-limb amputation (2). Early diagnosis and adequate treatment are essential. Because microorganisms are always present on skin wounds, diagnosis of infection must be based not on microbiological findings but on clinical criteria, as emphasized by the Infectious Diseases Society of America and the International Working Group on the Diabetic Foot (IWGDF) and more recently by the French Society for Infectious Pathology (3–5). However, because of the confounding impact of neuropathy and ischemia on local and systemic inflammatory response, diagnosing foot infection at an early stage in diabetic individuals may be difficult.

Recently, we demonstrated the value of using a miniaturized oligonucleotide array covering different genes of *Staphylococcus aureus*, by far the most common and virulent pathogen in diabetic foot infection (3), and we showed that the virulence gene profile of *S. aureus* enables us to distinguish grade 1 from grades 2–4 ulcers because the former generally displayed a very low level of virulence genes (6). One of the main limitations in that work was the limited panel of genes used. Here we analyzed the most prevalent virulence-associated genes and the in vivo virulence potential of the different *S. aureus* strains isolated from diabetic foot ulcers. The aim was to detect genetic markers to distinguish noninfected and infected ulcer and to predict outcome of grade 1 ulcers.

RESEARCH DESIGN AND METHODS

— From 1 March 2004 through 31 July 2007, a prospective longitudinal study of patients with diabetic foot ulcers at Nîmes University Hospital was conducted as described previously (6). Seventy-four patients (63%), hospitalized before or during April 2006, were

enrolled previously (6). This study was approved by the local ethics committee and performed in accordance with the Declaration of Helsinki as revised in 2000.

Bacterial isolation

After wound debridement, samples for bacterial culture were obtained by swabbing the wound base, needle aspiration, or tissue biopsies and were sent immediately to the bacteriology department. Only patients with monomicrobial cultures positive for *S. aureus* were included in the study. Patients with grade 1 ulcers were closely followed over a period of 6 months to confirm the wound status (infected/noninfected ulcer). If the wound healed, a microbiological specimen was obtained 1 month later. If the wound did not heal, antibiotic therapy was initiated and surgical debridement or minor amputation was performed according to the wound status; a sample for bacteriological culture was obtained before antibiotic treatment was begun and the ulcer grade was updated.

Microbiological study

Genus, species, and antibiotic susceptibilities were determined using the Vitek 2 card (bioMérieux, Marcy-l'Etoile, France) and interpreted according to the recommendations of the French Society for Microbiology (7). Susceptibility to methicillin was screened by agar diffusion using cefoxitin disks (Bio-Rad, Marnes-La-Coquette, France) (7).

Virulence profile of *S. aureus* strains

To assess the virulence potential of strains, the presence of 31 among the most prevalent virulence-associated genes was evaluated by PCR as described previously (8,9): staphylococcal enterotoxins A, B, C, D, E, G, H, I, J, K, and Q (*se*), toxic shock syndrome toxin 1 (*tst*), exfoliative toxins A and B (*etA* and *etB*), Panton Valentine leukocidin (PVL) (*luk*S-PV_*luk*F-PV), LukDE leukocidin (*lukE*), β- and two γ-hemolysins (*hlb, hlg*, and *hlgv*), epidermal cell differentiation inhibitor (*edinC*), nine microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (*bbp, cna, ebpS, clfA, clfB, fib, fnbA, fnbB,* and *eno*), and capsular types 5 and 8 (*cap5* and *cap8*). The accessory gene regulator (*agr*) allele group was determined by multiplex PCR (10).

Clonality of *S. aureus* strains

To eliminate a bias in the distribution of virulence genes due to the presence of clonal bacteria in each grade, we used different epidemiological methods. This analysis also allowed us to compare strains isolated at admission and during follow-up to determine whether they were identical.

Macrorestriction analysis of *Sma*I-digested chromosomal DNA was performed by pulsed field gel electrophoresis (PFGE) with the CHEF DRII system (Bio-Rad) (11). The PFGE patterns were analyzed by GelCompar software (Applied Math, Kortrijk, Belgium) and compared by the algorithmic clustering method known as the unweighted-pair group method using arithmetic averages with the Dice coefficient of similarity.

Staphylococcal chromosomal cassette (SCC*mec*) type was determined by PCR typing according to a simplified strategy of Kondo's typing system without determining the differences in the junkyard region (see supplemental glossary, available in an online appendix at http://dx.doi.org/10.2337/dc08-1010) (12). The *spa* sequence (see supplemental glossary) typing was performed according to the Ridom Staph Type standard protocol (http://www.ridom.com) and by using the Ridom SpaServer, which automatically analyzes *spa* repeats, assigns *spa* types, and clusters related *spa* types in a *spa* group (http://spa.ridom.de/index.shtml).

Caenorhabditis elegans in vivo model

C. elegans (see supplemental glossary) has been used to develop an easy model system of host-pathogen interactions to identify basic evolutionarily conserved pathways associated with microbial pathogenesis. This test is based on the capacity of pathogens ingested by *C. elegans* nematodes to infect and ultimately kill the worms (13). The survival of nematodes fed on different *S. aureus* strains is an indirect marker of their virulence potential. Fer-15 worms were infected as previously described (14). In brief, nematode growth medium plates were inoculated with 10 μl of an overnight culture of *S. aureus* strains and incubated at 37°C for 8 h. Between 25 and 30 *C. elegans* worms were transferred from a lawn of *Escherichia coli* OP50 to a lawn of the bacterium to be tested, incubated at 25°C, and examined at 24-h intervals with a stereomicroscope (Leica MS5) for viability. Nematodes were classified as dead if they failed to respond to touch. All experiments were conducted in triplicate and repeated at least five times for each selected strain. *S. aureus* virulence was assessed using the nematode survival curve and calculating the LT50 and LT100 (the times required to kill 50 and 100% of the worms, respectively).

Statistical analysis

For each qualitative variable (virulence genotyping), comparison between ulcer grades was assessed using Fisher's exact test. The ability to diagnose infection of a wound was expressed by sensitivity, specificity, and positive and negative predictive values; area under the receiver operating characteristic (AUC_{ROC}) curve was calculated by the nonparametric Hanley method. To assess the utility of combining several virulence markers, we used a logistic regression with a backward procedure to select the most relevant markers; only markers for which AUC_{ROC} was >0.80 were initially entered as explanatory variables in the regression analysis. An ROC curve was then generated for the combination derived from the regression model, and its area was compared with that of every single virulence marker by a nonparametric method adapted to paired data (15). To compare overall survival curves in the nematode killing assay, a Cox regression was used. Statistical analysis was performed using S-Plus 2000 software (Insightful, Seattle, WA), and results were considered significant for $P < 0.05$.

RESULTS

Clinical and bacteriological data

From 513 selected patients, 118 were included because they had been free of any antibiotic treatment for at least 6 months, and *S. aureus* was the single organism isolated from the bacterial culture of their wound (Fig. 1). In 69, the current wound was the first episode of ulceration, whereas in 49 it was a recurrence. The characteristics of the study population are shown in Fig. 1 and Table 1. Of the wounds, 24 (20%) were classified as grade 1 and were followed for 6 months. Of the 118 *S. aureus* strains, 48 (41%) were methicillin-resistant (MRSA). During the follow-up period, 9 grade 1 ulcers healed (38%), whereas 15 worsened. In two healing ulcers, samples remained positive for *S. aureus* compared with positive results for 12 of the 15 nonhealed ulcers. In total, 132 *S. aureus* strains were

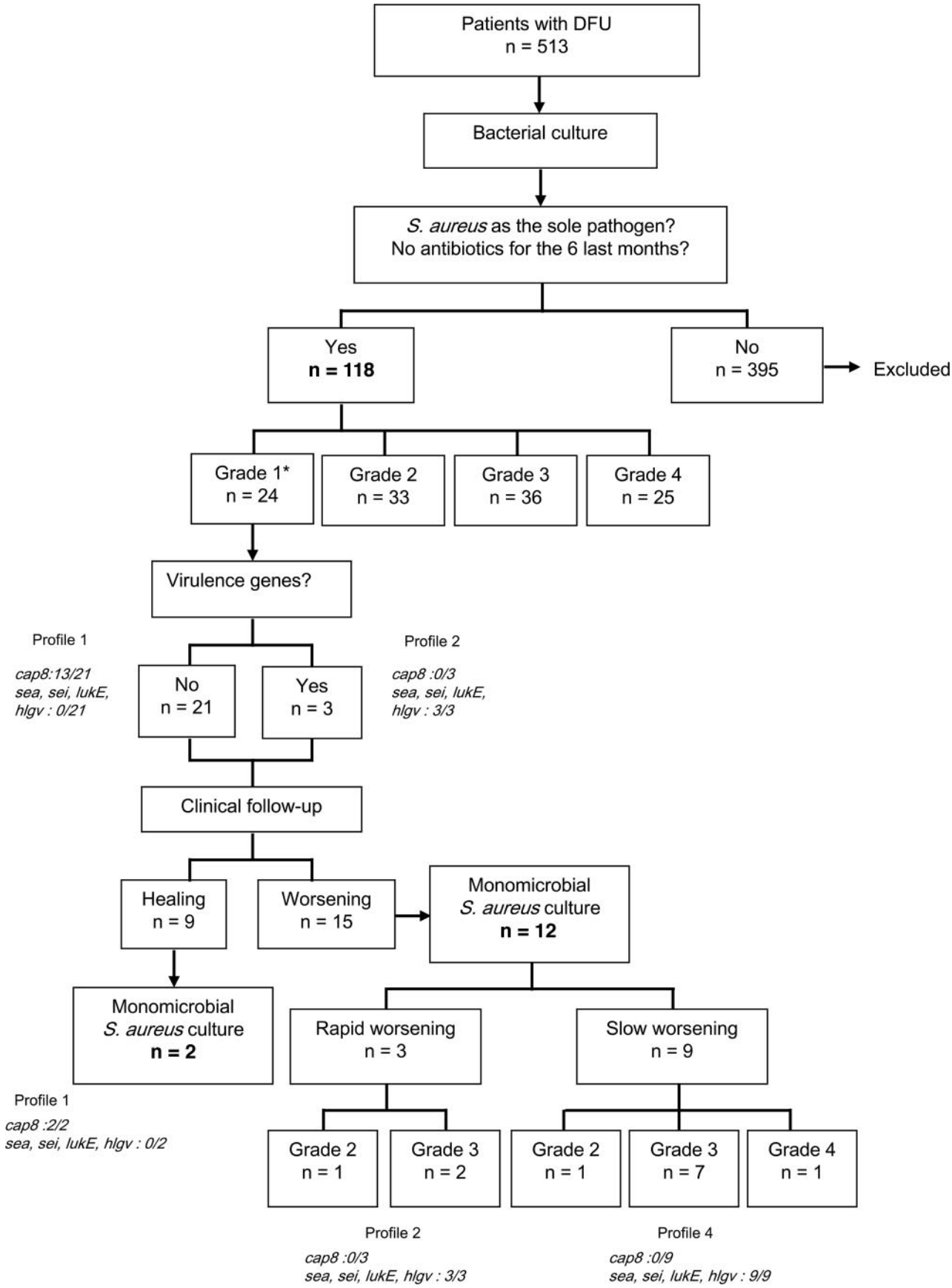

Figure 1—*Flow of patients with diabetic foot ulcers (DFU) through the study and the genotyping determination obtained during grade 1 inclusion and follow-up. In grade 1 ulcers, two genotyping profiles were obtained: profile 1, without the four infection markers and with the noninfected ulcer*

Table 1—*Demographic and clinical characteristics of study patients*

Characteristics	Value
Age (years)	68.0 (43–95)
Men/women	72 (61)/46 (39)
Type 1/type 2 diabetes	10/108
Diabetes duration (years)	18.4 (2–35)
A1C on admission (%)	7.4 (4.9–9.3)
Complications and comorbidities	
Cardiovascular disease	71 (60)
Peripheral vascular disease	46 (39)
Neuropathy	95 (81)
Nephropathy	73 (62)
Retinopathy	65 (55)
Previous hospitalization <1 year	11 (15)
First presentation/recurrence	69/49
Wound depth	
Superficial	57 (48)
Deep	61 (52)
IWGDF grade	
1	24 (20)
2	33 (28)
3	36 (31)
4	25 (21)
Microbial samples	
Swab	65 (55.1)
Aspiration	29 (24.6)
Tissue biopsy	24 (20.3)
Wound outcome (6-month follow-up)	
Healing	52 (44)
No healing	25 (21)
Revascularization procedure	29 (25)
Amputation	6 (5)
Death	2 (2)
No follow-up	0 (0)

Data are median (interquartile range: 25th–75th percentile) or *n* (%).

isolated (118 on the initial cultures and 14 during the follow-up period) (Fig. 1).

All recurrent ulcers were positive for MRSA, whereas methicillin-susceptible *S. aureus* (MSSA) was isolated in all but one of the ulcers appearing as the first episode. A higher prevalence of MRSA with increasing severity of infection was also noted, with a statically significant difference for grade 1 compared with grades 2–4 ($P = 0.021$).

Virulence profile

Virulence genotyping of the 132 strains evaluated by PCR is shown in Table 2. Individual gene analysis showed that the prevalence rates of 10 genes (*sea, seb, sec, sei, sej*, *hlb, hlg, hlgv*, *cap5,* and *lukE*) were significantly more often associated with strains isolated from grades 2–4 ulcers, whereas the *cap8* gene was most frequently found in strains from grade 1 ulcers ($P < 0.05$). From the logistic model analysis, a five-gene combination of *sea, sei, lukE, hlgv*, and *cap8* was the most predictive for differentiating grade 1 from grades 2–4 ulcers: mean ± SD AUC_{ROC} was 0.940 ± 0.028 (95% CI 0.885–0.995), which is significantly greater than that from combining all of the 30 virulence genes (0.810 ± 0.078) ($P < 0.05$); sensitivity was 0.977 ± 0.025, specificity was 0.871 ± 0.063, and positive and negative predictive values were 0.884 and 0.975, respectively. Using the logistic regression equation, three grade 1 ulcers were misclassified owing to absence of *cap8* but presence of *sea, sei, lukE,* and *hlgv* genes; interestingly, these ulcers rapidly worsened. Conversely, 17 grades 2–4 ulcers were misclassified owing to absence of *sea, sei, lukE,* and *hlgv* genes.

Clonality study

By using PFGE, a wide genomic diversity was shown among the 132 *S. aureus* isolates (data not shown), allowing us to exclude a bias in the statistical analysis. PFGE also revealed the spread of three clonal MRSA groups (44 strains; 33% of the isolates). A major group, clustering 29 strains (66% of MRSA strains) matched the Lyon clone (*agr* allele 1, *spa* type t008, and SCC*mec* type IV). The first minor clone (eight strains, 16% of MRSA) was the "classic pediatric" clone (*agr* allele 2, *spa* type related to t311, and SCC*mec* type IV). The second minor clone (three strains, 6% of MRSA) was the "new pediatric" clone (*agr2* allele, *spa* type t777, and SCC*mec* type VI).

Comparison between strains isolated from patients' ulcers at admission and follow-up demonstrated that those isolated from healing and slowly worsening ulcers had no clonal link. On the other hand, the strains from the three rapidly worsening ulcers were similar in each ulcer (data not shown).

S. aureus–mediated killing of *C. elegans*

When feeding on *E. coli* OP50, *C. elegans* has a 2-week life span (LT100 of worms varying between 11 and 14 days and LT50 between 3 and 5 days). When feeding on a pathogen, worms die far more rapidly, with a life span between 3 and 7 days (LT50 between 1 and 2 days).

Three strains (two MSSA and one MRSA) chosen at random among those isolated at admission in each of the four ulcer grades were tested for their capacity to kill *C. elegans*. In addition, strains initially isolated from grade 1 ulcers were compared with those isolated at the follow-up: strains from three ulcers with different outcomes were chosen: one that healed, one that worsened rapidly, and

*marker; and profile 2, with presence of infection markers. During the follow-up, if the wound healed, the genotyping profiles (profile 1) are the same as the inclusion profiles. Also if a wound presented a slowly worsening evolution, the genotyping profiles (profile 4) are different from the inclusion profile (profile 1), with the four infection markers in all of the follow-up cases. On the other hand, if the wound presented a rapidly worsening evolution, the genotyping profiles (profile 2) are exactly the same in inclusion and follow-up with the four infection markers. *Grades according to the IWGDF classification system (3,4). Number of monomicrobial cultures for* S. aureus *(132 = 118 + 12 + 2) is in bold type.*

Table 2—*Prevalence of virulence determinants among the 132* S. aureus *strains isolated from diabetic foot ulcers during inclusion and follow-up*

Virulence genotyping	Grade 1	Grade 2	Grade 3	Grade 4	*P* value: Grade 1 vs. Grade 2	*P* value: Grade 1 vs. G*
n	26	35	45	26		
sea	3 (12)	16 (46)	29 (64)	14 (54)	0.015	<0.001
seb	0 (0)	2 (6)	7 (16)	7 (27)		0.041
sec	0 (0)	5 (14)	4 (9)	9 (35)		0.023
sed	7 (27)	16 (46)	18 (40)	12 (46)		
see	0 (0)	0 (0)	0 (0)	0 (0)		
seg	6 (23)	10 (29)	18 (40)	16 (62)		
seh	1 (4)	3 (9)	3 (7)	1 (4)		
sei	5 (19)	12 (34)	20 (44)	18 (69)		0.014
sej	4 (15)	14 (40)	12 (27)	8 (31)	0.049	
sek	0 (0)	3 (9)	1 (2)	3 (12)		
seq	0 (0)	3 (9)	1 (2)	2 (8)		
tst	2 (8)	4 (11)	7 (16)	12 (46)		
etA	0 (0)	1 (3)	2 (4)	1 (4)		
etB	0 (0)	0 (0)	0 (0)	0 (0)		
luk-PV	0 (0)	0 (0)	2 (4)	3 (12)		
lukE	7 (27)	23 (66)	23 (51)	17 (65)	0.004	0.004
hlb	7 (27)	17 (49)	29 (64)	17 (65)		0.004
hlg	3 (12)	23 (66)	36 (80)	21 (81)	<0.001	<0.001
hlgv	2 (8)	21 (60)	35 (78)	20 (77)	<0.001	<0.001
edinC	0 (0)	0 (0)	0 (0)	0 (0)		
bbp	1 (4)	5 (14)	7 (16)	6 (23)		
cna	3 (12)	4 (11)	5 (11)	8 (31)		
ebpS	3 (12)	7 (20)	13 (29)	12 (46)		
clfA	22 (85)	30 (86)	36 (80)	23 (89)		
clfB	20 (77)	31 (89)	32 (71)	24 (92)		
fib	16 (62)	20 (57)	27 (60)	20 (77)		
fnbA	5 (19)	14 (40)	13 (29)	13 (50)		
fnbB	13 (50)	21 (60)	19 (42)	17 (65)		
eno	26 (100)	35 (100)	45 (100)	26 (100)		
cap5	9 (35)	23 (66)	28 (62)	16 (62)	0.021	0.014
cap8	15 (58)	5 (14)	6 (13)	3 (12)	0.019	0.002
agr1	10 (39)	24 (69)	21 (47)	13 (50)		
agr2	10 (39)	5 (14)	11 (24)	6 (24)		
agr3	6 (23)	4 (11)	7 (16)	7 (27)		
agr4	0 (0)	2 (6)	6 (13)	0 (0)		
MRSA	5 (19)	12 (34)	20 (44)	14 (54)		0.021

Data are *n* (%) unless otherwise indicated. *G corresponds to grades 2–4 according to the IWGDF classification system (3,4).

one that degraded slowly (Fig. 2). According to their killing ability, two populations of bacteria can be observed: the first with LT50 <2 days, suggesting a high virulence potential, and the second with LT50 >3 days, suggesting lower virulence ($P < 0.001$). All of the strains with LT50 <2 days were isolated from ulcers grades 2–4 except for one (NSA22465); conversely, all of the strains but one with LT50 >3 days were isolated from grade 1 ulcers. Interestingly, no significant difference in the killing potentials of MRSA and MSSA was observed within ulcers of the same grade: both strains from grade 1 had a similarly long LT50 (3.5 ± 0.3 days), and MRSA and MSSA from ulcers grades 2–4 had a similarly short LT50 (1.7 ± 0.2 days). Finally, both at entry and at follow-up, the LT50 for strains isolated from healing ulcers was relatively long (3.7 vs. 3.6 days, NS), whereas it was short for the strains from rapidly worsening ulcers (1.67 vs. 1.71 days, NS). For the strain from a slowly worsening ulcer, the LT50 was significantly shorter at follow-up than at admission (1.8 vs. 3.3 days, $P < 0.001$).

CONCLUSIONS — This study demonstrated for the first time the existence of two populations of *S. aureus* strains in diabetic foot ulcers: strains isolated from noninfected ulcers with a low virulence potential (as shown by the in vivo nematode model results) as opposed to strains isolated from infected ulcers with a high virulence potential. Moreover, the presence or absence of five virulence genes separated the two populations and allowed us to distinguish noninfected from infected wounds.

The fact that determining five virulence genes may help to differentiate noninfected from infected wound is an attractive result. Among infection-associated genes, four corresponded to MRSA markers (*sea, sei, lukE,* and *hlgv*) and *hlgv*, *sea,* and *lukE-lukD* genes are shared in the Lyon clone. Moreover, hospital-acquired MRSA strains shared the enterotoxin gene cluster locus, notably *sei* (16). *sea* is the most studied and interesting gene; its product has a strong proinflammatory effect (17). In our study, this gene was not exclusively related to the virulence of MRSA infection, as it was also detected in MSSA strains. Recently, an innate immune evasion cluster located on β-hemolysin–converting bacteriophages and carrying *sea* was discovered. It is easily transferred among strains (18). This potential of transfer via bacteriophage could explain our results and, notably, the presence of the *sea* gene in MSSA strains. The higher prevalence of *sea, sei, lukE,* and *hlgv* genes among the strains isolated from grades 2–4 compared with grade 1 ulcers and the absence of differences in MRSA clones between grade 1 and grades 2–4 ulcers suggest that these markers are really interesting, as they actually could be virulence markers. Finally, a number of studies demonstrated that the noninfected ulcer marker, capsular polysaccharide T8 (Cap8) was strongly associated with MSSA strains, as suggested by our study (19). However, its role in virulence has not been clearly defined. Production of Cap8 appears to be regulated by various environmental cues, and its overproduction might be implicated in *S. aureus* virulence (20,21).

Another interesting point is the low level of clonal strains isolated in this study (33%). Among MRSA strains, results indicated that a major clone matching the Lyon clone (66%) was widely distributed. Our study is in accordance with a recent report showing that this clone is currently the most prevalent MRSA clone in France

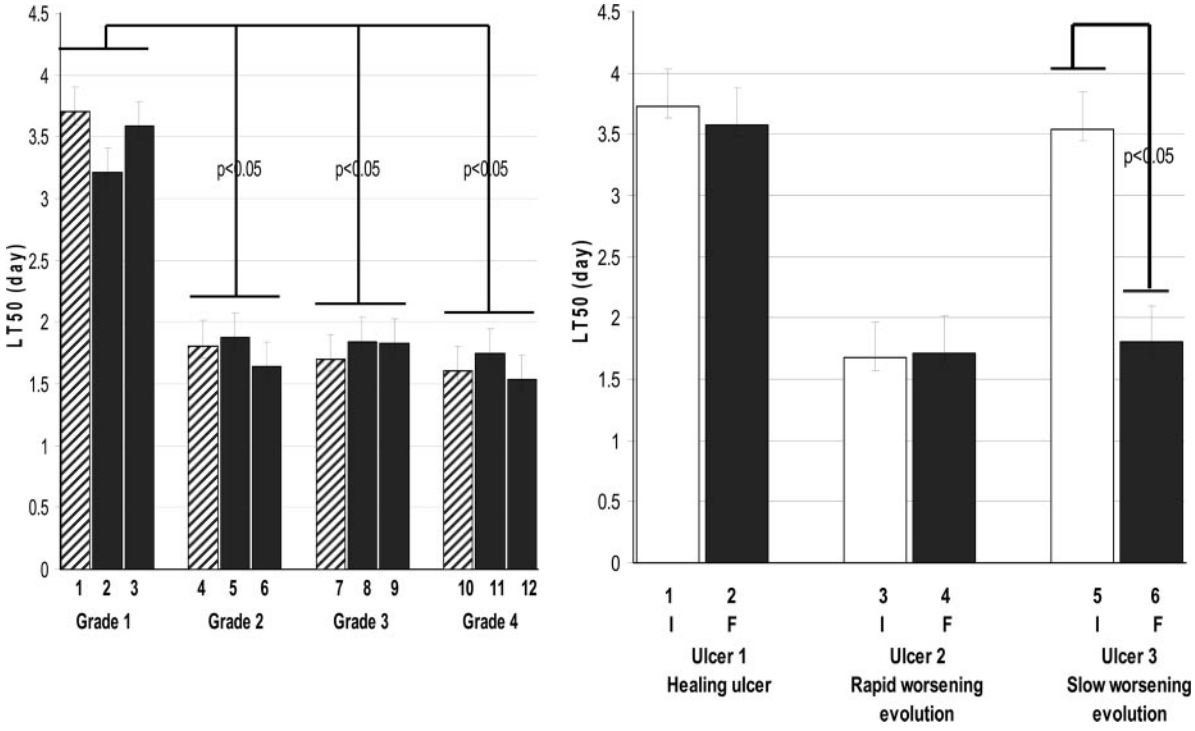

Figure 2— C. elegans *killing assay*. A: *Comparison of LT50 according to IWGDF grade and susceptibility of* S. aureus *to methicillin*. B: *Comparison of LT50 of strains isolated from grade 1 ulcers at inclusion and on the same wound after follow-up: ulcer 1, healing ulcer (grade 1 followed by grade 1); ulcer 2, rapid worsening ulcer (grade 1 followed by grade 2); and ulcer 3, slow worsening ulcer (grade 1 followed by grade 3). Virulent bacteria (isolated from ulcers grades 2–4) kill worms more quickly than avirulent strains (isolated from grade 1 ulcers). No statistically significant differences in LT50 were noted between MSSA and MRSA isolated from ulcers of the same grade. LT50 corresponds to the time for half of the worms to die.* A: ▨, *MRSA;* ■, *MSSA. 1, NSA6759* (n = *6 virulence genes); 2, NSA43233* (n = *3); 3, NSA56348* (n = *2); 4, NSA443564* (n = *15); 5, NSA16210* (n = *15); 6, NSA454193* (n = *13); 7, NSA369602* (n = *14); 8, NSA26758* (n = *15); 9, NSA19308* (n = *14); 10, NSA4281* (n = *18); 11, NSA29197* (n = *15); and 12, NSA41007* (n = *10).* B: □, *at admission;* ■, *after follow-up. 1, NSA11260* (n = *4 virulence genes); 2, NSA739* (n = *6); 3, NSA22465* (n = *9); 4, NSA27333* (n = *9); 5, NSA18026* (n = *5); and 6, NSA388104* (n = *12). The results are representative of at least five independent trials for each group of strains. I, strains isolated at inclusion; F, strains isolated at follow-up.*

(16). Interestingly, although dissemination of PVL-producing clones has been extensively reported and discussed since the beginning of the new millennium, these strains were not detected in our study.

The use of the *C. elegans* model demonstrated that *S. aureus* virulence was not dependent on methicillin resistance as suggested previously (22,23). This result is interesting because nearly half of the *S. aureus* strains isolated were methicillin resistant. We can speculate that within the same bacterial species there are pathogens with different virulence potential against the host. These bacterial populations with variable virulence represent a new challenge in terms of pathogenicity, treatment, and prevention of transmission.

Our study suggests that testing for the presence of five genes may not only help clinicians to distinguish grade 1 from grades 2–4 ulcers but will also predict wound outcome. At follow-up, *S. aureus* was isolated from 13 grade 1 ulcers. *cap8* was detected in *S. aureus* from the two healing ulcers, but the strains had pulsotypes and genotypes different from those of the baseline sample. Three of the 15 worsening ulcers (corresponding to the three false-positive results) rapidly degraded; both pulsotype and virulence profiles were found to be unchanged, suggesting that the isolates were the same and the wound was actually infected at baseline and not simply colonized. Finally, *S. aureus* from slowly worsening recalcitrant ulcers harbored virulence markers that were absent at baseline: pulsotypes and genotypes were different in every case, suggesting that new, more virulent *S. aureus* strains had colonized the ulcer (Fig. 1).

In summary, the increasing prevalence of resistant staphylococci and the small number of new antimicrobial drugs must stimulate the discovery of new solutions for diabetic foot infections in the near future. Testing for the presence of five genes will be a useful tool in management of diabetic foot ulcers. One-step multiplex PCR assays are relatively easy and rapid to perform (2 h after obtaining a specimen) at low cost (~5 USD). This testing will allow early discrimination between noninfected grade 1 and infected grades 2–4 diabetic foot ulcers, such that antibiotic treatment is prescribed for those most likely to benefit.

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