Evaluation of Daptomycin Non-Susceptible *Staphylococcus aureus* for Stability, Population Profiles, *mprF* Mutations, and Daptomycin Activity

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ABSTRACT

Introduction: Despite studies examining daptomycin non-susceptible (DNS)

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Staphylococcus aureus, examination of the stability and population profiles is limited. The objective was to evaluate the stability, population profiles, and daptomycin activity against DNS isolates.

Methods: The stability of 12 consecutive clinical DNS strains was evaluated by minimum inhibitory concentration (MICs) and population analysis profiles before and after 5 days of serial passage. Two pairs of DNS *S. aureus* having the same daptomycin MIC but different daptomycin population profiles were evaluated via an in vitro pharmacokinetic/pharmacodynamic (PK/PD) model of simulated endocardial vegetations for 96 h against daptomycin 6 and 10 mg/kg/day. The sequence of *mprF* was determined for these isolates before and after 96 h of daptomycin exposure in the in vitro PK/PD model.

Results: Daptomycin MIC values were 2–4 mg/L (via Microscan) for the 12 clinical isolates; 9 were confirmed DNS and 3 were within 1 tube dilution of Microscan (daptomycin MIC 1 mg/L). All were stable to serial passage. There was variation in the isolates susceptibility to daptomycin on population analysis (daptomycin population AUC 14.01–26.85). The killing patterns of

daptomycin 6 and 10 mg/kg/day differed between isolates with a left-shift and right-shift population profile to daptomycin. Two strains developed additional *mprF* mutations during daptomycin exposure in the in vitro PK/PD model resulting in P314L, L826F, S337L and a novel Q326Stop mutation.

Conclusions: The collection of DNS isolates was stable and displayed variation in susceptibility to daptomycin on population profile. Further research examining this clinical relevance is warranted.

Keywords: Daptomycin; Daptomycin non-susceptible; Infectious diseases; mprF; *Staphylococcus aureus*

INTRODUCTION

Daptomycin is a cyclic lipopeptide antibiotic with activity against Gram-positive organisms that received approval from the United States Food and Drug Administration in September, 2003 [1]. It is a concentration-dependent bactericidal antibiotic that acts by binding to and inserting into the bacterial cytoplasmic membrane resulting in rapid depolarization and deregulation of several cell functions such as DNA, RNA and protein synthesis [2–4]. Daptomycin susceptibility in *Staphylococcus aureus* is defined as a minimum inhibitory concentration (MIC) of ≤1 mg/L and any strain with an MIC >1 mg/L is considered daptomycin non-susceptible (DNS) [5].

The development of DNS in *S. aureus* laboratory studies, clinical trials, and post-marketing surveillance has been relatively low. Spontaneous mutagenesis in *S. aureus* for DNS appears at a rate of less than 10^{10} [6]. *Staphylococcus aureus* with DNS can be obtained via extended serial passage with increasing daptomycin concentrations and via chemical mutagenesis. An in vitro model evaluated standard vancomycin and daptomycin dosing regimens against 5 clinical strains of *S. aureus* that developed DNS in vivo [7]. The DNS could only be replicated in vitro in 1/5 of these strains and with vancomycin but not daptomycin exposure. Interestingly, the DNS in this *S. aureus* strain was unstable and reverted back to susceptible upon passage on antibiotic free media. Only 7 of 120 patients in the phase III trial for *S. aureus* bacteremia and infective endocarditis trial developed isolates with DNS [8]. Evaluation of 22,858 *S. aureus* isolated in North America from 2005 to 2010 revealed only 14 strains with a daptomycin MIC of ≥2 mg/L, and no trend indicating increasing MICs was noted [9].

Daptomycin non-susceptibility in *S. aureus* does not appear to be an all or nothing phenomenon, but instead a series of incremental changes that increase the MIC [10–15]. To date, four main genetic changes (*mprF*, *yycG*, *rpoB/rpoC*, *dltABCD*) have been associated with increased MIC and DNS in *S. aureus*. Mutations in or overexpression of the *mpr*F gene is commonly found in both laboratory derived and clinical DNS isolates [11–14]. Increased quantities of the MprF protein, a lysylphosphatidylglycerol synthase, is thought to contribute to DNS by affecting the composition of the cell membrane via translocation of positively charged phospholipids to the outer side of the cytoplasmic membrane and by lysinylation of membrane phosphatidylglycerol (PG) to generate lysyl-PG (LPG) thereby increasing positive surface charge [14–16]. Recently mutations in the phospholipid biosynthesis genes cardiolipin synthase (*cls2*) and CDP-diacylglycerol-glycerol-3-phosphate 3

phosphatidyltransferase (*pgsA*) have been found in clinical DNS strains [17]. Another altered protein sometimes found in DNS strains is YycG, which is one of two components of a response regulator system involved in the metabolism of the cytoplasmic membrane and cell wall [11]. The proteins RpoB and RpoC, which comprise the β and β' subunit of RNA polymerase, have also been found with amino acid substitutions in DNS *S. aureus* strains [11]. Recently, a single nucleotide polymorphism in *rpoB* from a laboratory derived DNS *S. aureus* was associated with decreased negative surface charge, increased cell wall thickness, and both vancomycin and daptomycin heteroresistance [18]. Additionally, increased expression of the *dltABCD* operon increases D-alanylation of cell wall teichoic acids contributing to an increase in positive surface charge [13]. Recent work has also suggested membrane proteins may augment the bactericidal effects of daptomycin, and alteration or loss of these proteins may contribute to DNS [15]. It has also been proposed that changes in carotenoid biosynthesis in *S. aureus* can increase membrane rigidity and contribute to increases in daptomycin MIC values [19]. Overall, DNS *S. aureus* strains show altered membrane potential, changes in membrane fluidity, increased positive membrane surface charge, and decreased membrane depolarization [10–15]. It is hypothesized that the increase in cytoplasmic membrane surface charge repels the active daptomycin-Ca^{2+} complex and therefore impedes interaction of daptomycin with the membrane [10, 20]. There are likely other genetic changes that contribute to DNS in *S. aureus* as strains exhibiting elevated MICs often have only some of the changes mentioned above [21–24]. There is still much room for discovery of novel cell membrane and genetic changes in DNS strains of *S. aureus*.

We have observed that some of the *S. aureus* strains identified as DNS by the clinical microbiology laboratory at our institution using Microscan® (Dade Behring, Deerfield, IL, USA) were actually susceptible via broth microdilution following passage on antibiotic free agar or time being stored at −80 °C. This observation led us to question the stability of these isolates. Additionally, previous in vitro work we have done with DNS strains has demonstrated variable activity of daptomycin [25, 26]. In some cases, daptomycin regimens of 10 mg/kg per day maintain antibacterial activity and led us to hypothesize that some *S. aureus* strains with DNS may still be treatable with daptomycin. The objectives of this study were to evaluate the stability of DNS strains from the clinical microbiology laboratory and to evaluate the activity of daptomycin regimens against DNS *S. aureus* strains with differing daptomycin population profiles.

MATERIALS AND METHODS

Bacterial Strains

Twelve consecutive clinical *S. aureus* strains, each having a daptomycin MIC of ≥2 mg/L, were collected from the clinical microbiology laboratory beginning in May 2009 and were evaluated for stability of DNS. All isolates were transported from the clinical microbiology laboratory to our laboratory on the original blood agar isolation plate within hours of obtaining the clinical microbiology laboratory susceptibility results to prevent any passes.

Antimicrobials

Daptomycin analytical grade powder was obtained from Cubist Pharmaceuticals, Lexington, MA, USA.

Media

Mueller–Hinton broth II (MHBII, Difco, Detroit, MI, USA) supplemented to 50 mg/L calcium was used for daptomycin susceptibility testing according to Clinical and Laboratory Standards Institute (CLSI) guidelines and MHBII supplemented to 75 mg/L was used for in vitro model experiments to account for calcium binding to albumin. Colony counts were determined using Tryptic Soy Agar (TSA; Difco, Detroit, MI, USA) plates. Mueller–Hinton agar prepared from MHBII supplemented with 50 mg/L of calcium and 15 g/L of Bacto[TM] Agar (Beckton, Dickson & Company, Sparks, MD, USA) was used for population analysis profiles (PAP).

Serial Passage

All isolates confirmed as DNS by our laboratory were passed on TSA five consecutive times. Isolates with a daptomycin MIC remaining ≥2 mg/L (±1 tube dilution standard error) after 5 serial passages were defined as stable DNS *S. aureus* strains and isolates reverting back to a daptomycin MIC of <1 mg/L were defined as unstable DNS *S. aureus* strains.

Susceptibility Testing

The MICs of daptomycin obtained by Microscan and for the isolates obtained with each serial passage were confirmed by broth microdilution (BMD) using an inoculum of 10^6 CFU/milliliter (mL) in duplicate according to CLSI standard methods and by Etest according to the manufacturer's guidelines [5]. *S. aureus* ATCC 25923 was used as a control strain. After greater than 2 years of storage at −80 °C the daptomycin MIC of all isolates was retested to assess the effect of storage on the stability of the MIC.

Molecular Biology

All strains were characterized for SCCmec type, Panton-Valentine Leukocidin (PVL) status, and agr function and group by previously described methods [27–31]. *S. aureus* isolates were evaluated by pulse field gel electrophoresis (PFGE) using *Sma*I-digested DNA, as described previously [32]. Gels were run at 6 V/cm, 14 °C, at an included angle of 120°, on a 1.2% agarose gel with pulse times of 5–35 s for 21 h. Strain relatedness was determined by visual inspection of the gel using the criteria of Tenover et al. and DICE coefficient using BioNumerics Software (Version 4.6, Applied Maths, Saint-Martens-Latem, Belgium) [33].

Daptomycin Population Susceptibility Profiles

Fifty microliters of a $\sim 10^8$ CFU/mL suspension of each strain was plated onto MHA plates with calcium containing daptomycin (concentrations ranging from 0.5 to 6 mg/L) using an automatic spiral plating device (WASP; DW Scientific, West Yorkshire, UK). After 48 h of incubation at 37 °C, colony counts were determined using an automated colony counter (Synoptics Limited, Frederick, MD, USA). The lower limit of detection for colony count was 2 $\log_{10}$ CFU/mL. Curves were constructed by plotting colony counts ($\log_{10}$ CFU/mL) versus concentration. Strain SA-684, previously determined to be stable to passage, was used as a control strain [15].

In Vitro Model Experiment

Two pairs of DNS *S. aureus* strains with the same MIC values by Microscan and BMD but displaying different PAPs (left shift vs. right shift) were evaluated in an in vitro

pharmacokinetic/pharmacodynamic (PK/PD) model of simulated endocardial vegetations.

Simulated Endocardial Vegetations

Organism stocks were prepared by creating lawns on TSA plates and incubating at 37 °C overnight. Organisms were swabbed from the growth plates into five mL test tubes of MHBII, diluted 1:10 and resulting in a concentration of approximately 10^{10} CFU/mL. Simulated Endocardial Vegetations (SEVs) were prepared in 1.5 mL siliconized eppendorf tubes by mixing 0.05 mL of organism suspension (final inoculum 10^9 CFU/0.5 g), 0.5 mL of human cryoprecipitate from volunteer donors (American Red Cross, Detroit, MI, USA), 0.025 mL of platelets. Bovine thrombin (5,000 units/mL) 0.05 mL, was added to each tube after insertion of a sterile monofilament line into the mixture. The resultant SEVs were then removed from the eppendorf tubes with a sterile 21-gauge needle and introduced into the model. This methodology results in SEVs consisting of approximately 3–3.5 g/dL of albumin and 6.8–7.4 g/dL of total protein.

In Vitro PK/PD Model

An in vitro model, consisting of a 250 mL two compartment glass apparatus with ports where the SEVs were suspended, was utilized for all simulations. The apparatus was prefilled with media and antibiotics were administered as boluses over a 96 h time period into the central compartment via an injection port. Antibiotic regimens evaluated included daptomycin 6 mg/kg every 24 h (peak, 98.6 mg/L; average half-life, 8 h) and daptomycin 10 mg/kg every 24 h (peak 141.1 mg/L; average half-life 8 h) [34]. The model apparatus was placed in a 37 °C water

bath throughout the procedure and a magnetic stir bar was placed in the media for thorough mixing of the drug in the model. Fresh media was continuously supplied and removed from the compartment along with the drug via a peristaltic pump (Masterflex, Cole-Parmer Instrument Company, Chicago, IL, USA) set to simulate the half-lives of the antibiotics. All models were performed in duplicate to ensure reproducibility.

Pharmacodynamic Analysis

Two SEVs were removed from each model (total of 4) at 0, 4, 8, 24, 32, 48, 56, 72 and 96 h. The SEVs were homogenized and diluted in cold saline and then plated onto TSA plates. Plates were incubated at 37 °C for 24 h at which time colony count was performed. The total reduction in $\log_{10}$ CFU/g over 96 h was determined by plotting time kill curves. Bactericidal activity (99.9% kill) was defined as a ≥ 3 $\log_{10}$ CFU/g reduction in colony count from the initial inoculum, bacteriostatic activity was defined as a <3 $\log_{10}$ CFU/g reduction in colony count from the initial inoculum, and inactive was defined as no observed reductions in initial inocula. The time to achieve a 99.9% reduction was determined by linear regression or visual inspection (if $r^2 \geq 0.95$). Susceptibility was performed on the 96 h sample by broth microdilution.

Pharmacokinetic Analysis

Pharmacokinetic samples were obtained in duplicate through the injection port of each model at 0.5, 1, 2, 4, 8, 24, 32, 48, 56, 72 and 96 h for verification of target antibiotic concentrations. All samples were stored at −70 °C until ready for analysis. Concentrations of daptomycin were determined by

microbioassay utilizing *Micrococcus luteus* ATCC 9341. Briefly, blank ¼″ disks were placed on a pre-swabbed plate of appropriate antibiotic medium and spotted with 10 μL of the standards or samples. Each standard was tested in duplicate. Plates were incubated for 18–24 h at 37 °C at which time the zone sizes were measured. The half-lives, area under the curve (AUC), AUC/MIC and peak concentrations of the antibiotics were determined by the trapezoidal method utilizing PK Analyst software (Version 1.10, MicroMath Scientific Software, Salt Lake City, UT, USA).

Resistance

Development of resistance in the SEV model was evaluated at multiple time points throughout the simulation at 24, 48, 72, and 96 h. 100 μL samples from each time point were plated on MHA plates containing three times the drug's MIC to assess the development of resistance. Plates were then examined for growth after 24–48 h of incubation at 37 °C. MICs were determined for all mutants identified via this method (by microdilution and Etest as described above).

Statistical Analysis

Changes in CFU/g at 24, 48, 72, and 96 h were compared by two-way analysis of variance with Tukey's post hoc test. A P value of ≤ 0.05 was considered significant. Paired continuous data was evaluated with a paired t test. All statistical analyses were performed using SPSS Statistical Software (Release 19.0, SPSS, Inc., Chicago, IL, USA).

mprF Sequencing

All 4 isolates placed in the SEV in vitro model and the isolates recovered at 96 h were evaluated for mutations in the *mprF* gene. The *mprF* genes were amplified by PCR using previously described primers [12]. The products were sequenced in both directions by an automated dideoxy chain termination method by the Applied Genomics Technology Center, Wayne State University. Nucleotide sequence analysis was performed with DS Gene 1.5 (Accelrys, Inc. San Diego, CA, USA).

Membrane Potential Measurements

Membrane potential was compared between the stable DNS *S. aureus* strains and the daptomycin susceptible parent (when available and confirmed isogenic by PFGE). Strains were grown to early exponential phase in 20 mL of SMHB, pelleted, washed twice with HEPES buffer (pH 7.2, containing 50 mg/L Ca^{2+}) and then re-suspended in HEPES ($OD_{600} = 0.2$). Aliquots were transferred to a cuvette containing a stir bar, then KCl (100 mM) was added and the cuvette was placed in the heated chamber of a FluoroMax-3 spectrofluorometer ($\lambda_{ex} = 622$ nm and λ_{em} 670 nm at 37 °C) (Horiba Jobin–Yvon Inc., Edison, NJ, USA). Cells were incubated with the membrane potential-sensitive dye $DiSC_3$ (0.1 mg/mL) for 10 min. Conditions included no antibiotic, nisin (25 mg/L) and daptomycin (8 mg/L). The membrane-depolarizing activity of daptomycin over 60 min was calculated as follows: % depolarization = $[(F_d - F_c)/(F_n - F_c)] \times 100$, where F_d, F_c and F_n are fluorescence measurements with daptomycin, no antibiotic and nisin, respectively. Results are expressed as the mean of two independent experiments.

This article does not contain any studies with human or animal subjects performed by any of the authors.

Table 1 Minimum inhibitory concentration values, daptomycin population analysis area under curve (AUC) values and molecular characteristics

| Isolate | MIC value (mg/L) | | | DAP AUC | SCCmec | USA | PVL | AGR | |
| | Initial | | Storage | | | | | | |
	Microscan	BMD	BMD					Type	Function
R6297	2	1	1	14.01	2	−	−	2	−
R6515	2	1	1	16.87	2	−	−	2	−
R6738	2	1	1	18.08	4	300	+	1	−
R6212	2	2	2	18.45	2	−	−	2	+
R6737	2	2	2	20.03	2	−	−	2	−
R6516[a]	2	2	2	22.09	4	−	−	1	−
R6003	4	2	4	22.14	2	−	−	2	−
R6253	4	2	2	23.66	4	300	+	1	+
R6747	4	2	2	22.28	2	−	−	2	−
R6219	4	4	2	20.68	4	300	−	1	+
R6255	4	4	4	26.85	2	−	+	2	−
R6827	4	4	0.5	21.24	4	300	+	1	+

BMD broth microdilution, *DAP* daptomycin, *SCCmec* Staphylococcal cassette chromosome mec, *USA* Staphylococcal USA clone type, *PVL* Panton-valentine leukocidin (+present, −absent), *AGR* accessory gene regulator (function: +functional, −dysfunctional)

[a] USA clone type could not be interpreted from PCR

RESULTS

MICs as determined by both Microscan and BMD for the twelve DNS *S. aureus* isolates are displayed in Table 1. As can be seen, all isolates had the Microscan MICs confirmed by BMD (within 1 tube dilution standard error). The spread for MIC values was as follows: three isolates with 2 mg/L (Microscan) and 1 mg/L (BMD), three isolates with 2 mg/L (Microscan/ BMD), three isolates with 4 mg/L (Microscan) and 2 mg/L (BMD), and three isolates with 4 mg/L (Microscan/BMD). All isolates were stable over five serial passages on drug free TSA. Etest MICs confirmed the daptomycin MIC value within 1 tube dilution. Examination of the twelve isolates by daptomycin population analysis revealed both left-shift and right-shift profiles within the 4 MIC value groups (Fig. 1a–d). Additionally, the daptomycin AUC values (Table 1) increase as the MIC values increase (Table 1). Retesting of the isolate's daptomycin MIC values by BMD after greater than 2 years of storage at −80 °C revealed that the MIC values were stable (±1 tube dilution standard error) for 11/12 isolates (Table 1). One isolate, R6827, displayed a daptomycin MIC decrease from 4 to 0.5 mg/L on retesting after storage.

Molecular characterization of the twelve strains is displayed in Table 1. The activity of daptomycin against 2 selected pairs (4 isolates total) in the in vitro PK/PD model of SEVs with the same MIC values but differing daptomycin PAPs is shown in Fig. 2a–d. A daptomycin dose response relationship was observed for all four strains. The daptomycin 6 mg/kg regimen
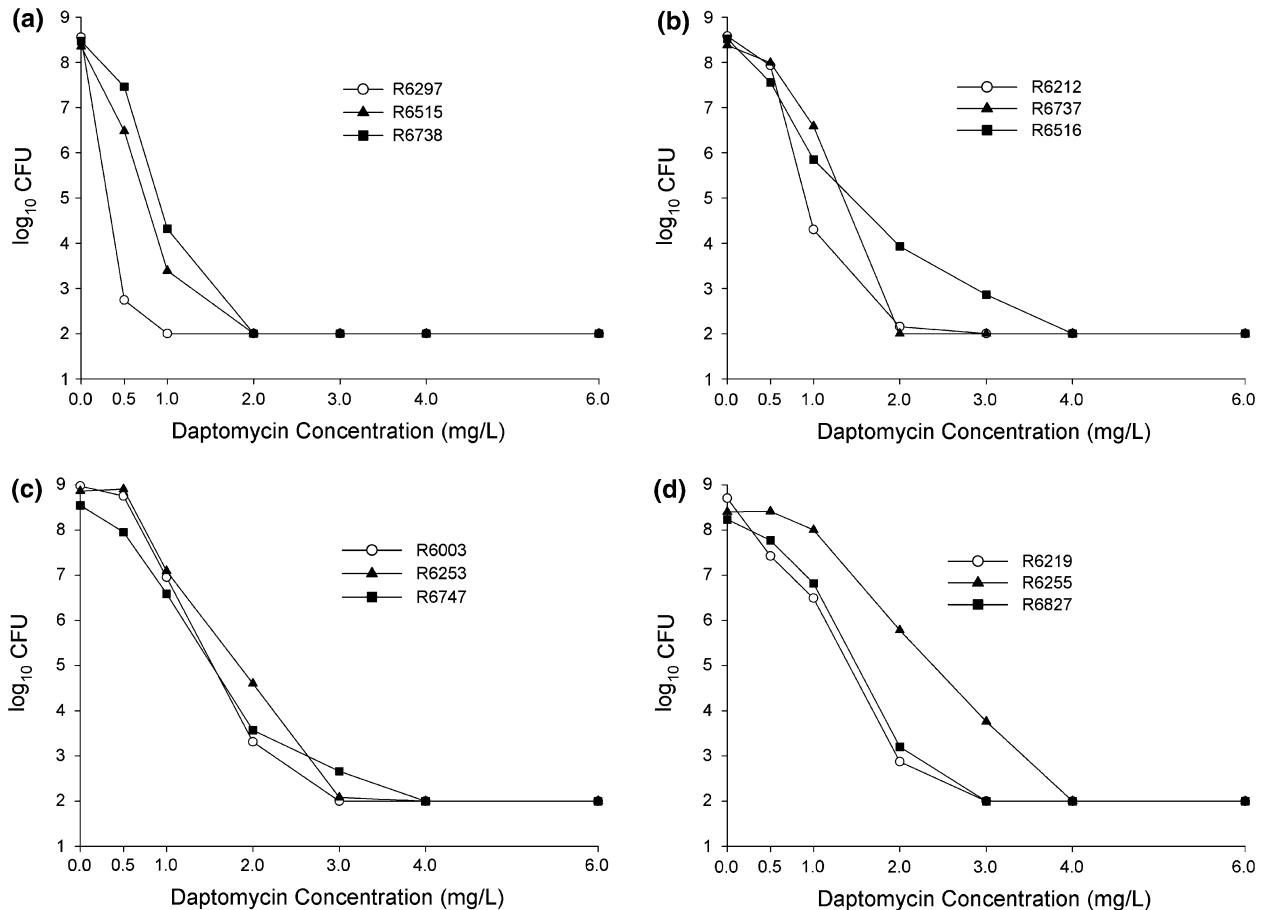
Fig. 1 Population analysis profiles for **a** Isolates with MIC values of 2 mg/L (Microscan) and 1 mg/L (Broth Microdilution, BMD). **b** Isolates with MIC values of 2 mg/L (Microscan/BMD). **c** Isolates with MIC values of 4 mg/L (Microscan) and 2 mg/L (BMD). **d** Isolates with MIC values of 4 mg/L (Microscan/BMD)

initially had sustained bactericidal activity in the first 24 h against isolates with a left-shift population profile (R6003 and R6219) (Fig. 2a). In contrast, isolates with the same MIC value and a right-shift profile (R6253 and R6255) displayed bactericidal activity at 8 h but regrowth at 24 h. The two left-shift isolates (R6003 and R6219) began to gradually regrow after 24 h eventually losing their bactericidal activity. In contrast, the two right-shift isolates displayed substantial killing and a more rapid regrowth with the 24 h dose before leveling off. The regimen of daptomycin 6 mg/kg maintained bactericidal activity against R6255

at 96 h. No mutants were recovered. Observed pharmacokinetic parameters were 94.23–109 mg/L and 6.78–7.42 h.

The isolates recovered at 96 h from the simulations of daptomycin 6 mg/kg did not have any change in MIC value from the initial isolates. However, examination of the population profiles revealed a rightward shift and increase in AUC. The AUC increased from 0 to 96 h for both R6003 (22.4 vs. 27.3) and R6219 (20.68 vs. 26.15). For isolates with an initial profile with a right shift, the AUC increase from 0 to 96 h for R6253 (23.66 vs. 27.31) and for R6253 (26.85 vs. 27.43) was less pronounced.
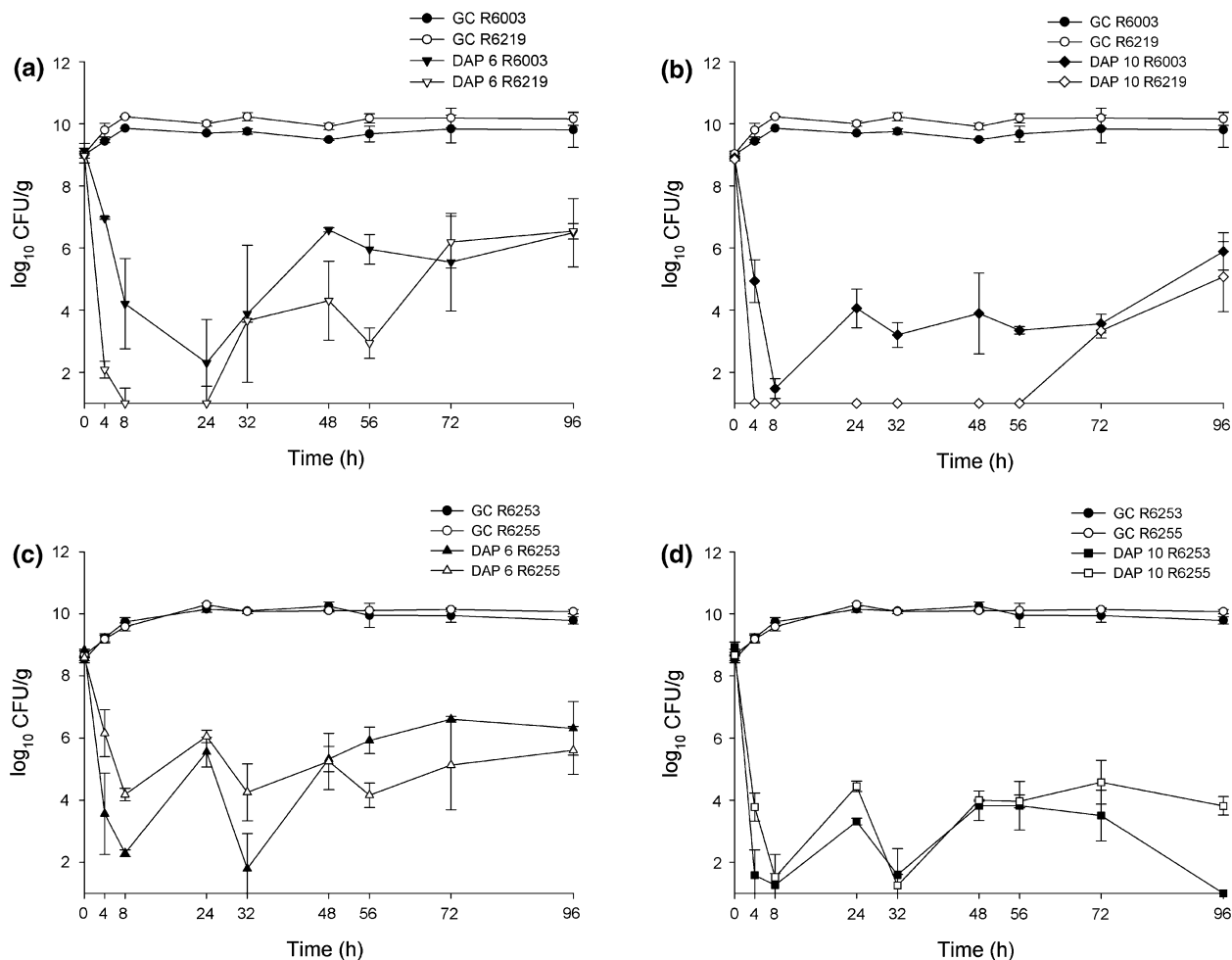
Fig. 2 a Activity of daptomycin 6 mg/kg against daptomycin left-shift strains R6003 & R6219. b Activity of daptomycin 10 mg/kg against daptomycin left-shift strains R6003 and R6219. c Activity of daptomycin 6 mg/kg against daptomycin right-shift strains R6253 & R6255. d Activity of daptomycin 10 mg/kg against daptomycin right-shift strains R6253 and R6255. *DAP 6* Daptomycin 6 mg/kg/day, *DAP 10* daptomycin 10 mg/kg/day, *GC* growth control

All initial isolates evaluated in the in vitro PK/PD SEV model (R6003, R6219, R6253, and R6255), and derivatives recovered after 96 h of exposure to a simulated regimen of daptomycin 6 mg/kg/day, underwent sequence analyses of mprF. Both R6003 (P314L and E692Q) and R6253 (P314L) initially possessed mutations with no additional changes developing during further daptomycin exposure. Strain R6219 had a L826F mutation initially and acquired a Q326Stop mutation during exposure to the simulated regimen of 6 mg/kg/day. Lastly, R6255 initially possessed an E692Q mutation and acquired the S337L mutation during daptomycin exposure.

The activity of daptomycin 10 mg/kg against the four tested isolates revealed a similar pattern as the daptomycin 6 mg/kg regimen. Daptomycin 10 mg/kg was bactericidal at 4 and 8 h against the two left-shift profile isolates (R6003 and R6219) with slow regrowth occurring for both strains by 96 h (Fig. 2b). In
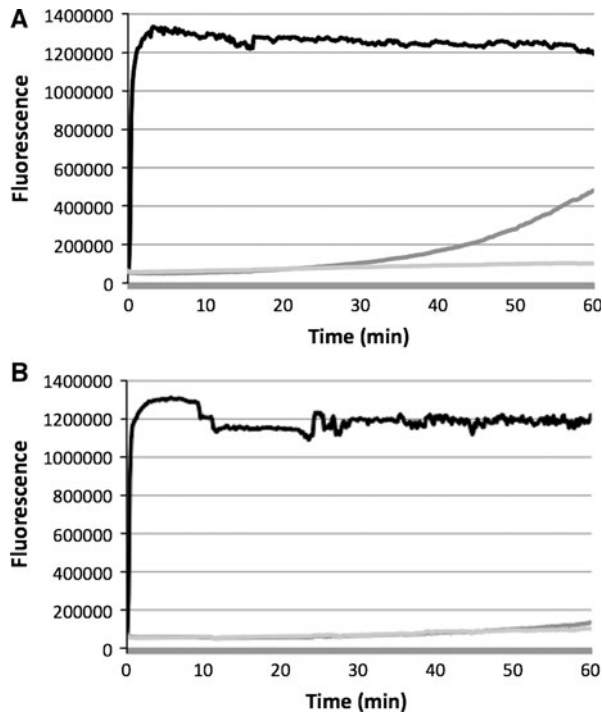
Fig. 3 Cytoplasmic membrane depolarization of the isogenic pair. **a** R6194 with daptomycin minimum inhibitory concentration of 0.25 mg/L and **b** R6212 with daptomycin minimum inhibitory concentration value of 2 mg/L. *Black lines* show results with nisin, *dark grey lines* show results with daptomycin, and *light grey lines* show results for control

contrast, against the right-shift isolates (R6253 and R6255) daptomycin 10 mg/kg resulted in multiple cycles of colony count decrease followed by regrowth. Bactericidal activity was maintained at 96 h for the two right-shift isolates. No mutants were recovered and isolates displayed no difference in MIC values at 96 h. Observed pharmacokinetic parameters ranged 139.8–144.3 mg/L and 6.9–8.3 h.

One daptomycin susceptible isogenic pair from the same patient (R6194, daptomycin MIC value 0.25 mg/L, and R6212 daptomycin MIC value 2 mg/L, clonality confirmed by PFGE) was available for depolarization testing. As can be seen in Fig. 3, the ability of daptomycin to depolarize the cytoplasmic membrane

decreased from $35.57 \pm 2.12\%$ for R6194 to $2.62 \pm 5.29\%$ for R6212, $P = 0.045$.

DISCUSSION

While the occurrence of DNS in *S. aureus* is relatively rare, there is still much room for discovery on mechanisms of resistance and optimal treatment. While multiple studies have examined both genetic and phenotypic changes found in both laboratory derived and clinical DNS *S. aureus*, limited work has examined the population profiles or stability of these strains. Additionally, to our knowledge no previous work has attempted to evaluate the relationship between daptomycin activity and the daptomycin PAPs of DNS *S. aureus* strains.

In the current study, we found all 12 of the clinical DNS *S. aureus* strains to be stable in nature as they did not revert to susceptible after serial passage on drug free agar. Previous work examining laboratory derived and clinical DNS *S. aureus* strains has revealed the occurrence of an unstable DNS *S. aureus* phenotype. A DNS *S. aureus* strain recovered previously from an in vitro PK/PD model reverted back to its susceptible state after serial passage on drug free agar [35]. Additionally, examination of the resistant subpopulations from a clinical isogenic daptomycin susceptible/DNS pair, SA-675 and SA-684, revealed that the resistant subpopulations were unstable [15]. Additionally, we found the majority (11/12) of strains to be stable in daptomycin MIC values after over 2 years of storage at −80 °C. A recent paper examining daptomycin susceptible *S. aureus* strains found an overall decrease in MIC values after storage when tested by Etest [36]. This is in contrast to our study in which all but one strain was stable on repeat testing over two years later. These differences may be due to the

testing method (Etest vs. BMD) or the MIC stability of daptomycin susceptible versus daptomycin non-susceptible *S. aureus*. While it appears from our work that the majority of all daptomycin non-susceptible clinical strains are indeed stable, further research in this area is needed to confirm these findings, as most studies to date have not examined the stability of DNS *S. aureus* clinical isolates.

In this study, we found variation in the susceptibility to daptomycin when the isolates were examined by population analysis with some isolates displaying prominent left or right shifts. Previous work has found the occurrence of daptomycin heteroresistance in both daptomycin susceptible and DNS *S. aureus* strains. Examination of the previously mentioned clinical isogenic pair, SA-675 and SA-684, by daptomycin population analysis revealed a heterogeneous profile [15]. Examination of a series of *S. aureus* isolates, ranging from daptomycin susceptible to DNS, recovered from a patient receiving high-dose daptomycin therapy by daptomycin population analysis revealed the presence of daptomycin heteroresistance on visual inspection both before and after the development of DNS [37]. In our study we also found a shift in the profile from the isolates recovered from the in vitro model after 96 h of exposure to daptomycin. This is consistent with the shift seen in clinical pairs analyzed after in vivo exposure to daptomycin [15, 37].

Examination of the impact of a DNS *S. aureus* daptomycin population profile on the activity of daptomycin in the in vitro PK/PD model of SEVs revealed unique killing patterns. The two isolates with left-shift profiles displayed one initial decrease in colony counts followed by a gradual regrowth, while the two right-shift profile isolates displayed multiple cycles of killing and regrowth. The extent of the antimicrobial activity may also be explained by the daptomycin PAPs. Compared to R6003, R6219 exhibited a greater decrease in colony counts when exposed to both daptomycin 6 and 10 mg/kg in the in vitro PK/PD SEV model despite having the same/higher daptomycin MIC value. These increases in susceptibility to daptomycin may be explained by the smaller AUC of the daptomycin PAP of R6219 (AUC 20.68) compared to R6003 (AUC 22.14). No correlation was observed, however, between the daptomycin PAP/AUC and the colony counts at 72–96 h in the in vitro PK/PD model.

Examination of our strains for mutations in the *mprF* gene revealed common mutations previously described including the E692Q, P314L, L826F and S337L. The finding of a premature stop codon, Q326Stop, in the R6219 96 h isolate after daptomycin 6 mg/kg exposure is interesting as its position would be expected to interrupt the functional C-terminal domain of the mprF protein. Previous work has shown the mprF protein is comprised of two functional domains, the C-terminal and N-terminal. While the C-terminal could independently complete lysinylation of membrane phospholipids, the N-terminal was incapable of completing functions without the assistance of the C-terminal domain. The Q326Stop mutation would logically render the mprF protein non-functional.

While our study is novel in examining a large collection of DNS *S. aureus* strains for stability and PAP, it does have limitations. Firstly, due to the relative rarity of DNS *S. aureus*, our collection of examined isolates is small at 12 and we were only able to obtain a single daptomycin susceptible—DNS isogenic pair for comparison evaluation. We also used standard inocula (log 10^6 CFU/mL) for broth microdilution and Etest susceptibility testing per CLSI and manufacturer's instructions,

respectively. The results may have been different if we employed a high inoculum for susceptibility testing (10^9 CFU/mL) as was done for the PAP and in vitro PK/PD model of SEVs. Our study is also limited as it focused on the most common gene mutation in DNS *S. aureus*, *mprf*, and did not examine the isolates for mutations or changes in expression of other genes known to be involved in DNS *S. aureus*. Lastly, our isolates are from a single geographic area (Detroit, MI, USA) with an established history of cutting edge resistance in *S. aureus* and may not be representative of resistance patterns in other areas of the country.

CONCLUSION

All 12 DNS *S. aureus* isolates were stable and displayed different degrees of susceptibility when examined by PAP. To our knowledge, this is the first study to examine such a large collection of clinical DNS *S. aureus* strains and confirm their stability. This is also the first study to examine the impact of the daptomycin PAP on the activity of both standard and high dose simulated daptomycin. Additionally, an organism with a unique mutation in *mprF*, Q326Stop, which would likely render the mprF protein non-functional, was discovered. The findings are clinically relevant because for some organisms the daptomycin AUC predicted antimicrobial activity or killing pattern better than the MIC value by BMD. This highlights the need to consider the whole population of bacteria when discussing susceptibility or the development of resistance. Despite previous reports that some aspects of DNS may be inducible and unstable, eleven of our twelve isolates displayed stable resistance even after 2 years of freezer storage confirming that DNS can frequently be a stable and not transient

phenomenon in *S. aureus*. Daptomycin should continue to be utilized appropriately to minimize resistance and preserve its efficacy.

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Compliance with ethics guidelines. This article does not contain any studies with human or animal subjects performed by any of the authors.

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