J Antimicrob Chemother 2023; **78**: 669–677
https://doi.org/10.1093/jac/dkac434 Advance Access publication 6 January 2023

Journal of Antimicrobial Chemotherapy

Emerging resistance in *Staphylococcus epidermidis* during dalbavancin exposure: a case report and *in vitro* analysis of isolates from prosthetic joint infections

Jasmina Al Janabi[1]†, Staffan Tevell [1,2]†, Raphael Niklaus Sieber[3], Marc Stegger[1,3] and Bo Söderquist [1,4]*

[1]*School of Medical Sciences, Faculty of Medicine and Health, Örebro University, Örebro, Sweden;* [2]*Department of Infectious Diseases, Karlstad Hospital and Centre for Clinical Research and Education, Region Värmland, Karlstad, Sweden;* [3]*Department of Bacteria, Parasites and Fungi, Statens Serum Institut, Copenhagen, Denmark;* [4]*Department of Laboratory Medicine, Faculty of Medicine and Health, Örebro University, Örebro, Sweden*

*Corresponding author. E-mail: bo.soderquist@oru.se
†These authors share first authorship.

Received 12 September 2022; accepted 7 December 2022

Background: Dalbavancin, a semisynthetic lipoglycopeptide with exceptionally long half-life and Gram-positive spectrum, is an attractive option for infections requiring prolonged therapy, including prosthetic joint infections (PJIs).

Objectives: To investigate the prevalence of reduced susceptibility to dalbavancin in a strain collection of *Staphylococcus epidermidis* from PJIs, and to investigate genomic variation in isolates with reduced susceptibility selected during growth under dalbavancin exposure.

Methods: MIC determination was performed on *S. epidermidis* isolates from a strain collection ($n=64$) and from one patient with emerging resistance during treatment ($n=4$). These isolates were subsequently cultured on dalbavancin-containing agar and evaluated at 48 h; MIC determination was repeated if phenotypical heterogeneity was detected during growth. Population analysis profile (PAP-AUC) was performed in isolates where a $\geq$2-fold increase in MIC was detected, together with corresponding parental isolates ($n=21$). Finally, WGS was performed.

Results: All strains grew at 48 h on agar containing 0.125 mg/L dalbavancin. PAP-AUC demonstrated significant differences between parental and derived strains in four of the eight analysed groups. An amino acid change in the *walK* gene coinciding with emergence of phenotypic resistance was detected in the patient isolates, whereas no alterations were found in this region in the *in vitro* derived strains.

Conclusions: Exposure to dalbavancin may lead to reduced susceptibility to dalbavancin through either selection of pre-existing subpopulations, epigenetic changes or spontaneous mutations during antibiotic exposure. Source control combined with adequate antibiotic concentrations may be important to prevent emerging reduced susceptibility during dalbavancin treatment.

Introduction

Dalbavancin is a semisynthetic intravenous lipoglycopeptide antibiotic whose mode of action is similar to that of vancomycin; it interferes with cell wall synthesis through inhibition of late stages of the peptidoglycan synthesis by binding of the D-Ala-D-Ala terminus of peptidoglycan precursors.[1] It has several properties that may be beneficial in the treatment of complex implant-associated infections.[2] The antibacterial spectrum covers common pathogens causing these infections, including MDR *Staphylococcus epidermidis*.[1] The MIC_{90} values are lower in *S. epidermidis* for dalbavancin (0.06–0.12 mg/L) compared with vancomycin (2–4 mg/L),[3–5] with a minimum biofilm inhibitory concentration ($MBIC_{90}$) of 0.25–0.5 mg/L and a minimum biofilm bactericidal concentration ($MBBC_{90}$) of 4 mg/L,[4] both lower than the corresponding values for vancomycin ($MBIC_{90}$ 4 mg/L, $MBBC_{90}$ >128 mg/L, respectively).[5] This is consistent with *in vitro* data, whereby dalbavancin was significantly more effective at

reducing cultivable *S. epidermidis* from biofilms compared with vancomycin.[6] The pharmakokinetic properties with exceptionally long half-life of dalbavancin (149–250 h)[1] together with clinically relevant synovial and bone concentrations including intra-osteoblastic activity[7,8] and a favourable safety profile[9,10] render dalbavancin an attractive option for prolonged outpatient antibiotic treatment of implant-associated orthopaedic infections such as prosthetic joint infection (PJI) or fracture-related infection.[2]

However, emergence of reduced susceptibility during dalbavancin exposure has been described *in vitro* for *Staphylococcus aureus*,[11] as well as after prolonged treatment in the presence of medical implants.[12,13] Although the mechanisms remain to be fully understood, WGS demonstrated alterations in the WalKR two-component regulatory system,[11] consistent with previously published data on both glycopeptide-intermediate *S. aureus* (GISA) and heteroresistant strains (hGISA).[14–16] Because glycopeptide heteroresistance was detected in 77.9% of clinical *S. epidermidis* PJI isolates in Sweden,[17] concern is warranted regarding whether prolonged exposure to dalbavancin, with waning concentrations over time, can promote mutations associated with heteroresistance or select for pre-existing resistant subpopulations during the course of treatment.[16,18]

In this article, we present a case of an MDR *S. epidermidis* PJI with emerging dalbavancin resistance during treatment. Furthermore, we investigated the MIC distribution to dalbavancin in a strain collection of *S. epidermidis* isolated from PJIs. If subpopulations with reduced susceptibility were selected during growth under dalbavancin exposure, these were compared with parental strains using WGS to explore if genomic alterations could be found and associated with reduced susceptibility to dalbavancin.

Materials and methods

Case presentation

An 89-year-old man underwent right-sided total hip arthroplasty for osteoarthrosis. As per Swedish guidelines,[19] three doses of cloxacillin as antibiotic prophylaxis were administered, and he was discharged 2 days after surgery. He was readmitted to hospital 13 days post-surgery due to ongoing wound discharge; his C-reactive protein (CRP) level was 117 mg/L and haemoglobin count was 93 g/L. A sterile puncture through uninfected skin yielded 5 mL of brown/red liquid suggestive of haematoma, cultures of which grew an MDR *S. epidermidis* (isolate P1) susceptible to only linezolid, vancomycin and dalbavancin (Table 1). Debridement, antibiotics and implant retention (DAIR), including exchange of mobile parts and five deep-tissue biopsies for microbiological cultures, was performed on day 7 after readmission. *S. epidermidis* with identical antibiotic susceptibility patterns to the strain isolated from the haematoma grew in all five tissue cultures (isolate P2).

Intravenous vancomycin was started post-surgery, with measured concentrations between 15 and 22 mg/L. Wound discharge continued after DAIR, but because the patient suffered a transient ischaemic attack during surgery, removal of the prosthesis was not performed due to high surgical risk. Vacuum-assisted closure (VAC) dressings were applied, and dalbavancin was started on day 10 after DAIR (1000 mg loading dose followed by 500 mg weekly). Staples were removed 3 weeks after DAIR, but wound closure was incomplete and VAC dressings were continued. On day 28 after DAIR, a haematoma was evacuated, cultures of which grew *S. epidermidis* (isolate P3) with a dalbavancin MIC 1.0 mg/L, classifying it as resistant according to EUCAST breakpoints (accessed 12 November 2019). Finally, a superficial revision was performed on day 35 after DAIR (isolate P4). Dalbavancin was discontinued after five doses, and the patient was started on long-term suppressive doxycycline therapy. Four months later the wound was healed, and CRP was 3 mg/L. At follow-up 18 months after DAIR the patient was pain-free and in good general condition on suppressive doxycycline.

Bacterial isolates

In total, 68 *S. epidermidis* isolates were included: 4 from the patient and 64 from a strain collection comprising *S. epidermidis* isolated from PJIs (hip or knee) between 2007 and 2018 in Region Örebro, Sweden. No clinical data were available for the isolates from this strain collection. Species were confirmed by MALDI-TOF MS. For isolates displaying phenotypical heterogeneity during growth on dalbavancin-containing agar, MALDI-TOF MS was repeated after subculturing to exclude contamination. All isolates were stored at −80°C in preservation medium (trypticase soy broth; BD Diagnostic Systems, Sparks, MD, USA) supplemented with 0.3% yeast extract (BD Diagnostic Systems) and 29% horse serum (SVA, Uppsala, Sweden) at the Department of Laboratory Medicine, Clinical Microbiology, Örebro University Hospital, Sweden.

For antibiotic susceptibility testing, isolates were subcultured on Mueller–Hinton II agar 3.8% w/v plates (BD Diagnostic Systems) at 36°C. The MICs for dalbavancin and vancomycin were determined on 0.5 McFarland bacterial suspension in 0.85% (w/v) NaCl on Mueller–Hinton II agar plates with gradient strips (Liofilchem, Roseto degli Abruzzi, Italy; and Etest, bioMérieux, Marcy l'Etoile, France, respectively) incubated for 20 h at 35°C. The DAL0.125 method, a modification of the VAN4 method,[20] was developed to screen for *S. epidermidis* isolates with reduced susceptibility to dalbavancin. Briefly, 10 μL of a 0.5 McFarland bacterial suspension was pipetted on four agar plates containing different antibiotic concentrations (0.064 mg/L, 0.125 mg/L, 0.25 mg/L and 0.5 mg/L). Isolates were incubated at 35°C, and subsequently evaluated according to a protocol of 'no growth', 'growth' and 'confluent growth' at 24 and 48 h. When phenotypical heterogeneity was detected during growth on dalbavancin-containing agar ($n=16$), the species was confirmed to be *S. epidermidis* with MALDI-TOF MS, and isolates were subcultured and re-evaluated by MIC gradient strip test.

Population analysis profile (PAP)-AUC

Ten derived strains with a ≥2-fold increase in MIC were observed from 7 of the 16 isolates displaying phenotypical heterogeneity. These derived strains, together with original (parental) strains and the four patient isolates, were further analysed by the PAP-AUC method modified after Wootton *et al.*[21] In brief, 10 μL of a 0.5 McFarland bacterial suspension was plated onto eight BHI agar plates (Brain Heart Infusion Agar; BD Diagnostics, Sparks, MD, USA) containing serial dilutions of dalbavancin at 0.064 mg/L, 0.125 mg/L, 0.25 mg/L, 0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2.0 mg/L, as well as agar plates without dalbavancin. Plates were incubated at 36°C for 48 h, and colonies were counted both manually and with the Interscience Scan 4000 colony counter (Interscience, Saint-Nom-la-Bretèche, France). All isolates were tested in triplicate. Subsequently, the total number of colonies growing on each plate was transformed to common logarithm cfu/mL through multiplying the counted number by 100 and then taking the logarithm. The chosen maximum of counted colonies was 1000, equalling 10^6 cfu/mL. An average with SDs of $\log_{10}$cfu/mL was calculated for every concentration. These results were plotted on a graph representing each bacterial isolate, using the parental strain as reference for the derived strains, and AUC was calculated with the trapezoidal rule. Finally, isolates that grew on 2 mg/L agar ($n=8$) were subcultured on Mueller–Hinton II agar 14 times, followed by an additional MIC determination.

Table 1. Antimicrobial susceptibility testing of four *S. epidermidis* strains isolated over 42 days from an 89-year-old man with PJI performed by disc diffusion tests, except where noted. Numbers within parentheses are zone diameter for disc diffusion tests or MIC values for gradient tests or broth dilutions

Isolate	P1	P2	P3	P4
Day post-arthroplasty	13	20	48	55
Sample type	Sterile puncture	Tissue biopsy (DAIR)	Evacuated haematoma	Superficial revision
SNP (P1 is reference)	0	1	18	19
Cefoxitin	R (6)	R (6)	R (6)	R (6)
Erythromycin	R (6)	R (6)	R (6)	R (6)
Clindamycin	R (6)	R (6)	R (6)	R (6)
Gentamicin	R (6)	R (6)	R (6)	R (6)
Fusidic acid	R (8)	R (11)	R (6)	R (10)
Trimethoprim/ sulfamethoxazole	R (6)	R (10)	R (6)	R (6)
Norfloxacin	R (11)	R (14)	R (6)	R (6)
Rifampin	R (6)	R (13)	R (6)	R (10)
Linezolid	S (29)	S (32)	S (32)	S (36)
Tetracycline				S (25)
Daptomycin (gradient test)	S (1)	S (1)	S (1)	R (2)
Vancomycin (gradient test)	S (2)	S (2)	R (4)	R (4)
Vancomycin (broth)		R (8)	R (8)	R (8)
Teicoplanin (broth)		R (8)	S (4)	R (8)
Dalbavancin (gradient test)	S (0.064)	S (0.064)	R (1.0)	R (0.5)
Dalbavancin (broth)			R (1.0)	

R, resistant; S, susceptible; SNP, single nucleotide polymorphism.

Genome sequencing and genomic analysis

The 21 isolates where PAP-AUC was performed were further analysed by WGS. All parental isolates of a lineage were assembled using SPAdes,[22] and single nucleotide polymorphisms (SNPs) were called by mapping reads of subsequent isolates against this assembly using the Burrows–Wheeler Aligner (BWA-MEM) as implemented in version 1.0.0 of NASP.[23] The GATK Unified Genotyper[24] was used with filters set to remove SNPs with <10-fold sequencing depth and 90% unambiguous variant calls after duplicated regions of the assemblies were excluded using NUCmer.[25]

To identify differences in gene presence/absence, all genomes were assembled using SPAdes,[22] filtered for contigs with minimum coverage of 10× and a minimum length of 200 bp, and annotated using Prokka.[26] The pan-genome among all isolates was then identified using Roary,[27] and raw sequencing reads were mapped back to the reference sequence of each gene using KMA[28] to control for spurious assembly discrepancies.

Statistics

The AUC for individual isolates in each test round was compared within the group of parent and derived isolates for each strain using either an independent samples *t*-test, one-way analysis of variance (ANOVA) or one-way non-parametric ANOVA (Kruskal–Wallis test) depending both on the number of isolates in the group and on whether the assumption of homogeneity of variance was met. Version 17 of the SPSS software package (SPSS Inc., Chicago, IL, USA) was used, and the statistical significance level was set at $P < 0.05$.

Ethics

The research was conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from the patient.

Results

Antibiotic susceptibility testing

MIC values in the strain collection of *S. epidermidis* isolated from PJIs ranged from 0.006 to 0.125 mg/L for dalbavancin and from

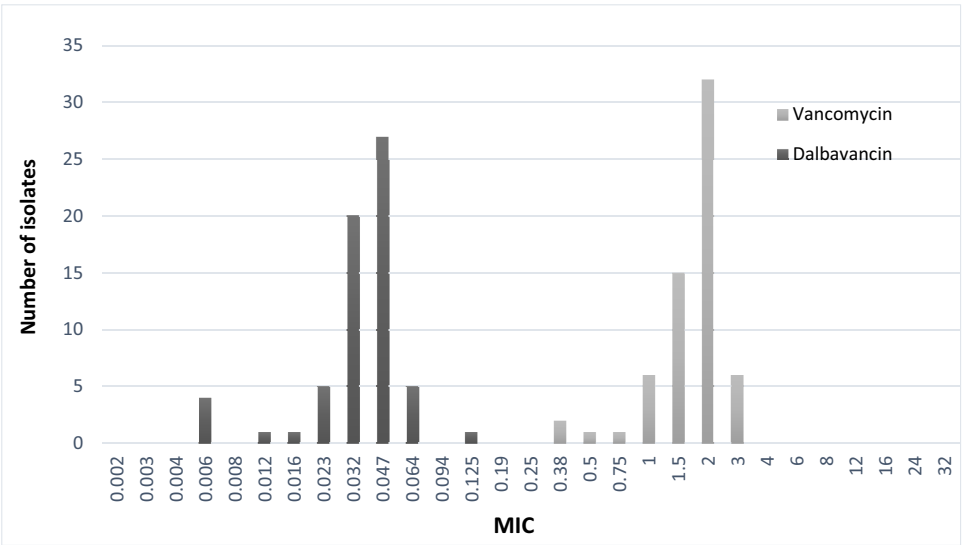

Figure 1. Distribution of MIC values determined by gradient tests for both dalbavancin and vancomycin on 64 isolates from a strain collection of *S. epidermidis* from knee and hip PJIs. EUCAST breakpoints (accessed 12 November 2019) (S): dalbavancin ≤0.125 mg/L, vancomycin ≤2 mg/L.

Table 2. Results from DAL0.125 screening for reduced susceptibility after 24 and 48 h of incubation for isolates of *S. epidermidis* from hip and knee PJIs ($n=64$) tested on agar plates containing four different concentrations of dalbavancin. The result for one isolate at 24 h was not available

	No growth		Growth		Confluent growth	
	24 h	48 h	24 h	48 h	24 h	48 h
0.064 mg/L	0	0	3	1	60	63
0.125 mg/L	0	0	6	6	57	58
0.25 mg/L	4	4	17	9	42	51
0.5 mg/L	13	10	48	50	2	4

0.38 to 3 mg/L for vancomycin (Figure 1). Results for DAL0.125 screening for reduced susceptibility were evaluable for 63 isolates after 24 h and for 64 isolates after 48 h (Table 2). Growth at 24 h at a dalbavancin concentration of 0.125 mg/L was detected for isolates with an initial MIC as low as 0.006 mg/L. At 48 h, the number of isolates with growth or confluent growth had increased. Initial MIC values for isolates displaying confluent growth at 48 h on agar containing dalbavancin 0.5 mg/L were 0.032 mg/L, 0.047 mg/L and 0.125 mg/L, respectively. A repeated MIC determination on isolates displaying confluent growth on dalbavancin 0.5 mg/L agar showed no significant differences from initial MIC values.

In 16 isolates, phenotypic differences in colony morphology were observed after subculturing on agar containing dalbavancin. MALDI-TOF MS confirmed that these isolates were *S. epidermidis*. On repeated MIC determination, seven of these parental isolates generated 10 derived strains with ≥2-fold changes in MIC values (Table 3), implying an altered status from dalbavancin susceptible to resistant according to EUCAST breakpoints (≤0.125 mg/L, accessed 12 November 2019). These, together with the four patient isolates (one parental and three derived), comprised a collection of 21 isolates (8 parental strains, 13 derived isolates) on which PAP-AUC was performed.

In addition, MIC determination for daptomycin was performed by gradient test on both the four isolates obtained from the patient (Table 1), and the 17 investigated isolates from the collection (Table S1, available as Supplementary data at *JAC* Online).

PAP-AUC

All 21 isolates displayed growth of colonies on all agar plates containing dalbavancin up to a concentration of 1.5 mg/L. At a concentration of 2 mg/L, nine isolates were inhibited so that no growth was seen. The remaining 10 were also tested on agar plates with a dalbavancin concentration of 4 mg/L, which inhibited an additional eight isolates whereas growth was still detected in two. In general, parental isolates displayed fewer colonies on the dalbavancin-containing agar plates compared with the derived strains. Four isolates (L1, L4, L6 and P1; Figure 2b, c, g and h) showed significant differences, with lower AUCs for parental isolates compared with derived strains.

For the eight isolates that grew on plates containing 2 mg/L of dalbavancin during PAP-AUC, renewed MIC determination was performed, both after PAP-AUC and after an additional 14 subcultures on agar plates without dalbavancin (Table 4). This revealed decreased MIC values for six of the eight isolates after subculturing.

Table 3. Results from MIC determination of dalbavancin by gradient test on 16 isolates of *S. epidermidis* from PJIs from knee and hip arthroplasties displaying phenotypic differences in colony morphology after subculturing on agar containing dalbavancin

Parental strain MIC (mg/L)	Derived isolate A MIC (mg/L)	Derived isolate B MIC (mg/L)	Derived isolate C MIC (mg/L)	Derived isolate D MIC (mg/L)
0.012	0.016	0.023	—	—
0.023	0.032	0.032	0.032	—
0.032	0.064	0.023	0.38[a]	—
0.032	0.032	0.5[a]	—	—
0.032	0.75[a]	0.25[a]	0.094	—
0.032	0.047	0.023	—	—
0.047	0.38[a]	0.032	—	—
0.047	0.19[a]	0.38[a]	—	—
0.047	0.032	0.38[a]	0.094	—
0.047	1.5[a]	0.38[a]	0.032	—
0.047	0.064	0.023	—	—
0.047	0.064	0.094	0.047	—
0.047	0.047	0.064	0.032	0.032
0.047	0.032	0.094	0.094	—
0.047	0.032	0.047	0.047	—
0.064	0.047	0.25[a]	0.094	—

[a]Denotes alterations with a ≥2-fold increase in MIC.

WGS and genomic analysis

Genomic comparison of the patient isolates (P1–4) revealed several SNPs in the core genome (Table S2). It is worth noting that one SNP in the *walK* gene causing an amino acid change from tyrosine to alanine was present at position 272; mutations in this gene have previously been linked to reduced dalbavancin susceptibility.[11,29] Analysis of the accessory genome furthermore revealed the acquisition of the erythromycin resistance gene *ermC* in conjunction with the replication protein *repL* in the three subsequent patient isolates (P2–4), as well as a 30 404 bp region in the final two patient isolates (P3–4). This element carries several genes (Table S3) including *blaZ* and *blaR1*, which are known to confer resistance to β-lactams, and genes involved in biofilm formation. These latter genes include *epsH*, which has been speculated to be involved in production of the exopolysaccharide component of the extracellular matrix during biofilm formation.[30,31] The presence of the tyrosine recombinase *xerC* further suggests that this may be a phage-like mobile genetic element. However, searching the public databases did not result in any obvious hits besides other *S. epidermidis* genomes.

Despite these genomic changes in the patient isolates, we saw no changes in isolates from the strain collection where PAP-AUC was performed. Pairwise comparison of derived isolates with their parental *S. epidermidis* from the strain collection only showed absence of *ermC* in isolate L3_d, and no SNPs.

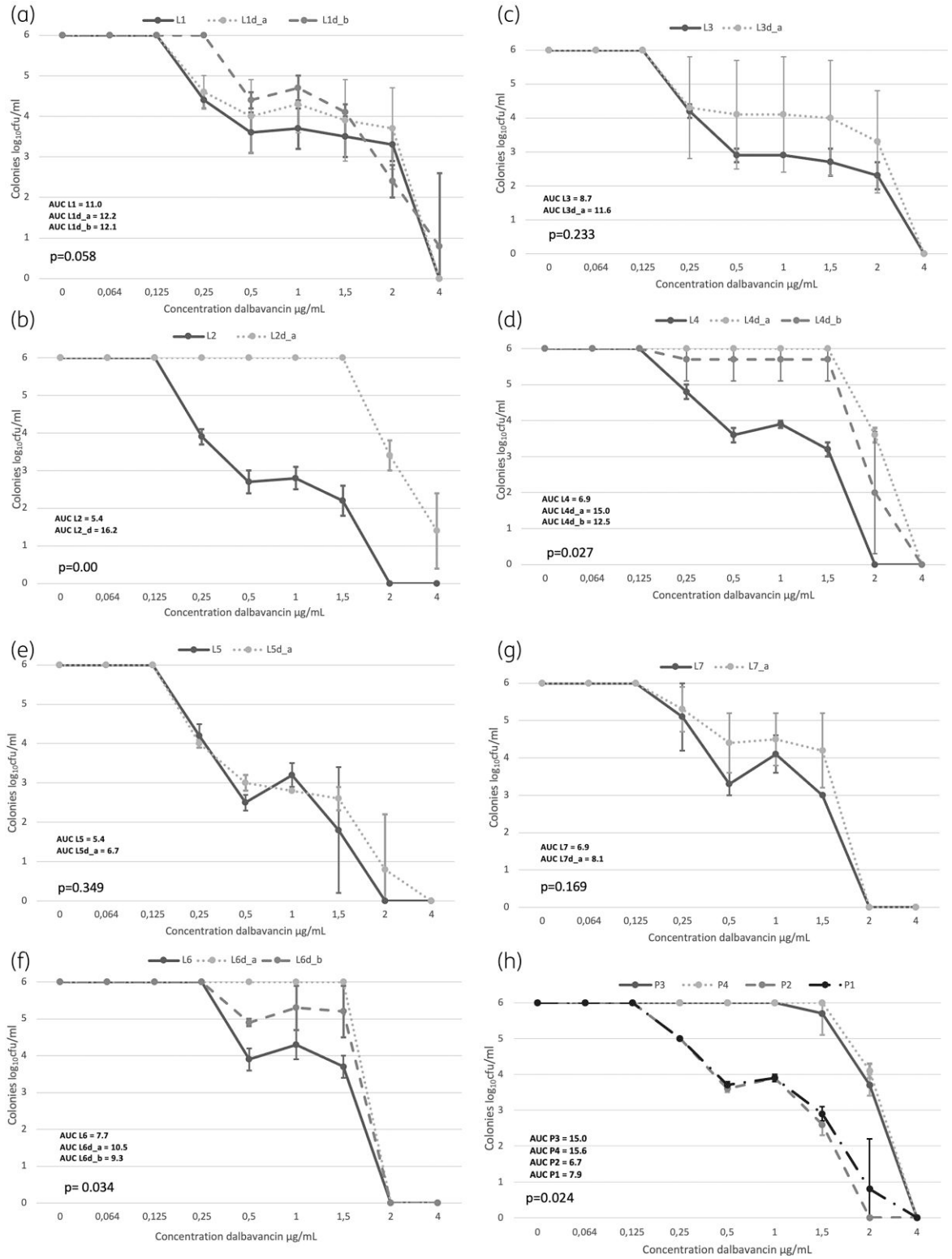

Figure 2. PAP performed on *Staphylococcus epidermidis* isolated from prosthetic joint infections. (a–g) Isolates from the strain collection that developed an increased MIC value during subculturing on dalbavancin-containing agar. (h) Clinical isolates from the patient isolated at different dates during the clinical course (see Table 1). Each graph represents the change of growth of total colonies (log_{10}cfu/mL) at different antibiotic concentrations (mg/L) of dalbavancin. In every part of the figure, the first isolate listed in the legend is the parent strain whereas the others represent the derived isolates. Error bars indicate SD. The *P* value for the difference between the groups is presented at the bottom left.

Table 4. MIC determination of eight *S. epidermidis* isolates that displayed growth on agar plates containing 2 mg/L of dalbavancin. Analysis performed after the PAP-AUC and repeated after being subcultured 14 times on agar plates without any antibiotic

	Initial MIC (mg/L)	MIC after PAP-AUC (mg/L)	MIC after 14 subcultures (mg/L)
L1	0.047	1	1
L2d_a	0.38	1.5	1
L3	0.032	0.5	0.5
L4d_a	0.19	0.75	0.125
L5d_a	0.38	0.25	0.094
P1	0.064	0.094	0.064
P3	1	1	0.5
P4	0.5	1	0.38

Discussion

We here present a case with emerging dalbavancin resistance in *S. epidermidis* during PJI treatment, resulting in treatment failure and consequently long-term suppressive antibiotic therapy. Further analysis of *S. epidermidis* isolates from a PJI strain collection demonstrated that despite being fully susceptible according to EUCAST breakpoints, all 64 tested strains demonstrated growth at 48 h on agar containing dalbavancin up to 0.125 mg/ L, and 54 still grew at a concentration of 0.5 mg/L. Because the long half-life of dalbavancin has been presented as a risk factor for development of reduced antibiotic susceptibility by prolonging the mutant selection window,[11,32] these data are of concern.

Pharmacokinetic/pharmacodynamic data suggest that two 1500 mg doses 1 week apart result in adequate bone and synovial fluid concentrations above the staphylococcal MIC of 0.125 mg/L for 6–8 weeks, and that the simulated AUC would be similar to that of a 1000 mg loading dose followed by four 500 mg weekly doses.[8] However, because the $MBIC_{90}$ and $MBBC_{90}$ reported for dalbavancin are considerably higher than the MIC_{90},[2] there might be a risk of introducing an evolutionarily selective pressure on staphylococci in orthopaedic biofilm-associated infections such as PJIs. A similar scenario could occur in abscesses during the last few days before administration of the next dose in a weekly dosing strategy, because animal data have shown that staphylococcal regrowth in tissue fluid commenced 4–5 days after one dose.[33] Because the inoculum size matters both in the selection of subpopulations with reduced susceptibility and in the stepwise accumulation of mutations proposed in staphylococcal glycopeptide heteroresistance,[14,32] adequate source control is important in preventing the emergence of reduced susceptibility during long-term dalbavancin treatment. In the present case, it is possible that the rivaroxaban-associated haematoma provided beneficial bacterial growth conditions leading to the presence of a large inoculum during treatment with vancomycin and dalbavancin, thus facilitating the evolution of dalbavancin resistance. Because the underlying genetic alterations in reduced susceptibility to dalbavancin are similar to those reported in other staphylococci with reduced glycopeptide susceptibility,[11] the risk posed by antibiotic pressure may be comparable for glycopeptides and lipoglycopeptides. Still, the MIC values for dalbavancin are significantly lower than those for vancomycin, and 61% (39/64) of isolates in this study showed a vancomycin MIC of ≥2 mg/L whereas only 2% (1/64) showed a dalbavancin MIC of ≥0.125. Therapeutic drug monitoring is not currently used in clinical routine for dalbavancin in the way it is for vancomycin. However, in an open label randomized clinical trial,[34] a dosing regimen of 1000 mg dalbavancin followed by 500 mg on day 8 led to a mean concentration of 21.2 mg/L, but an interindividual variation with a range of 7.6–40.1 mg/L at day 12 after the second dose. Thus, determining serum concentrations during dalbavancin treatment could lead to improved and individualized dosages and dose intervals in order to reduce the mutant selection window.

True *vanA*-mediated glycopeptide resistance is rare among *S. epidermidis*, whereas reduced susceptibility due to alterations in cell wall metabolism is more common.[35] Heteroresistance comprises a mixed range of susceptibility in what appear to be isogenic bacterial cultures.[16] However, MIC determination is insufficient for the detection of heteroresistance in *S. epidermidis*.[18] Further analysis with PAP-AUC (Figure 2a–h) demonstrated significant alterations between the fully susceptible patient strains (P1, P2) and those isolated during treatment failure (P3, P4). This pattern was also seen in three of the *in vitro* generated groups (L2, L4 and L6), whereas no significant alterations could be detected in the remaining groups.

The development of heteroresistance is not linked to only one mechanism, but rather is a stepwise process. Among the most commonly described alterations are non-synonymous mutations in the genes encoding the WalKR two-component system that contribute to alterations in cell wall metabolism,[11,15,36] although several other genes may also be involved.[37] Analyses of the genomic data for the patient isolates revealed a novel mutation in *walK* that, in combination with the detection of other genetic variations across the genomes, was linked to increased resistance. We also observed other genetic variability between these closely related isolates, similar to a recent study in heterogeneity in *S. epidermidis* PJIs.[38] These differences include a likely acquisition of other resistance markers that could indicate the selection pressure under which these isolates thrived.

However, no genetic differences were observed among the *in vitro* collection, indicating that the regulatory, epigenetic or extracellular mechanisms of persister cells[39] could be involved in the observed reduced susceptibility. Consequently, subculturing strains 14 times post-exposure led to a decrease in MIC values for six of eight isolates, even though most isolates still had higher MIC than before PAP-AUC. This could be explained by adaptive resistance, a phenomenon initiated by external stress (e.g. environmental factors such as antibiotic exposure) but reversed when the trigger factor was removed, thus making the bacteria return to a more susceptible phenotype.[40] The mechanisms behind this adaptive resistance are believed to include intricate molecular processes, but also epigenetic changes because genetic changes would neither appear, nor reverse, at such a quick pace. Epigenetic mechanisms of the bacterial genome differ from the systems that have been studied in eukaryotes, because the bacterial DNA is not packed in histones. The mechanisms set in place for epigenetic changes in bacteria are associated with modifications of their DNA and RNA, such as methylation of the DNA. This is a defence mechanism that enables bacterial endonucleases to

recognize foreign unmethylated DNA and to degrade it.[41] Because epigenetic processes are dynamic and dependent on environmental factors, this could be a plausible explanation for the reduced susceptibility to dalbavancin among isolates where no genetic alterations were detected.

Because previous studies[42,43] of *S. aureus* have demonstrated cross-resistance between glycopeptides and daptomycin, and the molecular basis for the genomic evolution has been reported,[44] we included MIC determination for daptomycin of the *S. epidermidis* isolates investigated in the present study. When comparing parental isolates and derived isolates no significant alteration of the MIC values, i.e. ≤1-fold difference in MIC, were found, with one exception, L4.

There are several limitations of the present study. We used a gradient test method rather than broth dilution method for the MIC determination. Gradient tests have been reported to overestimate MIC values for glycopeptides,[45] though a recent study found no large discrepancies.[46] However, the isolates obtained from the patient reported in the present study displayed 1–2-fold lower MIC values for vancomycin when determined by gradient test compared with the broth microdilution method. Still, all analyses in this study were performed by the same method. Another limitation was that only a subset of the strain collection was analysed by PAP-AUC and WGS. Because these methods are laborious, the strategy was to select strains with a ≥2-fold increase in MIC after subculturing on dalbavancin-containing media for further analysis. Furthermore, Illumina sequencing does not allow for detecting epigenetic changes in derived strains; this would have required sequencing and extensive analyses of data from other next-generation sequencing platforms. Still, the demonstration of emergence of resistance to dalbavancin *in vivo* in a patient as well as the development of *S. epidermidis* subpopulations with reduced susceptibility *in vitro* is regarded as a strength.

In conclusion, dalbavancin exposure both *in vitro* and *in vivo* can lead to reduced antibiotic susceptibility in *S. epidermidis*. The mechanisms behind this might include selection of pre-existing heteroresistant subpopulations or persister cells, epigenetic changes or spontaneous mutations in regions involved in cell wall synthesis during antibiotic pressure. Considering the long half-life of dalbavancin in combination with specific challenges in the treatment of PJIs, adequate source control in combination with assurance of adequate serum concentrations may be required to reduce the risk of emergence of reduced dalbavancin susceptibility during treatment.

Data availability

The datasets generated and analysed during the current study are available from the European Nucleotide Archive (ENA) under BioProject Accession number PRJEB55821.

Funding

This work was supported by the research committee of Region Värmland (grant numbers LIVFOU-968115, LIVFOU-939253, LIVFOU-939836); ALF funding for Region Örebro County; and Nyckelfonden at Örebro University Hospital (grant number OLL-935525).

Transparency declarations

B.S. is a member of an advisory board at ADVANZ PHARMA. B.S. also gave a lecture about prosthetic joint infections at Correvio Pharma Corp. in 2019, receiving financial compensation. The other authors declare no conflicts of interest.

Author contributions

B.S., S.T. and M.S. contributed to the design of the work. S.T. collected the medical data. J.A.J. and B.S. performed the microbiological analysis. M.S. and R.N.S. performed the genome sequencing and the bioinformatic analyses. S.T. and J.A.J. wrote the manuscript draft, and M.S., R.N.S. and B.S. made critical revisions. All authors have read and approved the final manuscript.

Supplementary data

Tables S1 to S3 are available as Supplementary data at *JAC* Online.

References

1 Billeter M, Zervos MJ, Chen AY *et al*. Dalbavancin: a novel once-weekly lipoglycopeptide antibiotic. *Clin Infect Dis* 2008; **46**: 577–83. https://doi.org/10.1086/526772

2 Buzón-Martín L, Zollner-Schwetz I, Tobudic S *et al*. Dalbavancin for the treatment of prosthetic joint infections: a narrative review. *Antibiotics (Basel)* 2021; **10**: 656. https://doi.org/10.3390/antibiotics10060656

3 Sader HS, Carvalhaes CG, Streit JM *et al*. Antimicrobial activity of dalbavancin against clinical isolates of coagulase-negative staphylococci from the USA and Europe stratified by species. *J Glob Antimicrob Resist* 2021; **24**: 48–52. https://doi.org/10.1016/j.jgar.2020.11.020

4 Fernandez J, Greenwood-Quaintance KE, Patel R. In vitro activity of dalbavancin against biofilms of staphylococci isolated from prosthetic joint infections. *Diagn Microbiol Infect Dis* 2016; **85**: 449–51. https://doi.org/10.1016/j.diagmicrobio.2016.05.009

5 Schmidt-Malan SM, Greenwood Quaintance KE, Karau MJ *et al*. In vitro activity of tedizolid against staphylococci isolated from prosthetic joint infections. *Diagn Microbiol Infect Dis* 2016; **85**: 77–9. https://doi.org/10.1016/j.diagmicrobio.2016.01.008

6 Di Pilato V, Ceccherini F, Sennati S *et al*. In vitro time-kill kinetics of dalbavancin against *Staphylococcus* spp. biofilms over prolonged exposure times. *Diagn Microbiol Infect Dis* 2020; **96**: 114901. https://doi.org/10.1016/j.diagmicrobio.2019.114901

7 Chauvelot P, Dupieux-Chabert C, Abad L *et al*. Evaluation of intraosteoblastic activity of dalbavancin against *Staphylococcus aureus* in an ex vivo model of bone cell infection. *J Antimicrob Chemother* 2021; **76**: 2863–6. https://doi.org/10.1093/jac/dkab299

8 Dunne MW, Puttagunta S, Sprenger CR *et al*. Extended-duration dosing and distribution of dalbavancin into bone and articular tissue. *Antimicrob Agents Chemother* 2015; **59**: 1849–55. https://doi.org/10.1128/AAC.04550-14

9 Monteagudo-Martinez N, Solis-Garcia Del Pozo J, Ikuta I *et al*. Systematic review and meta-analysis on the safety of dalbavancin. *Expert Opin Drug Saf* 2021; **20**: 1095–107.

10 Morata L, Cobo J, Fernández-Sampedro M *et al*. Safety and efficacy of prolonged use of dalbavancin in bone and joint infections. *Antimicrob Agents Chemother* 2019; **63**: e02280-18. https://doi.org/10.1128/AAC.02280-18

11 Werth BJ, Ashford NK, Penewit K *et al*. Dalbavancin exposure in vitro selects for dalbavancin-non-susceptible and vancomycin-intermediate

strains of methicillin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* 2021; **27**: 910.e1–8. https://doi.org/10.1016/j.cmi.2020.08.025

12 Werth BJ, Jain R, Hahn A *et al*. Emergence of dalbavancin non-susceptible, vancomycin-intermediate *Staphylococcus aureus* (VISA) after treatment of MRSA central line-associated bloodstream infection with a dalbavancin- and vancomycin-containing regimen. *Clin Microbiol Infect* 2018; **24**: e1–5. https://doi.org/10.1016/j.cmi.2017.07.028

13 Kussmann M, Karer M, Obermueller M *et al*. Emergence of a dalbavancin induced glycopeptide/lipoglycopeptide non-susceptible *Staphylococcus aureus* during treatment of a cardiac device-related endocarditis. *Emerg Microbes Infect* 2018; **7**: 202. https://doi.org/10.1038/s41426-018-0205-z

14 Howden BP, Peleg AY, Stinear TP. The evolution of vancomycin intermediate *Staphylococcus aureus* (VISA) and heterogenous-VISA. *Infect Genet Evol* 2014; **21**: 575–82. https://doi.org/10.1016/j.meegid.2013.03.047

15 Howden BP, McEvoy CR, Allen DL *et al*. Evolution of multidrug resistance during *Staphylococcus aureus* infection involves mutation of the essential two component regulator WalKR. *PLoS Pathog* 2011; **7**: e1002359. https://doi.org/10.1371/journal.ppat.1002359

16 El-Halfawy OM, Valvano MA. Antimicrobial heteroresistance: an emerging field in need of clarity. *Clin Microbiol Rev* 2015; **28**: 191–207. https://doi.org/10.1128/CMR.00058-14

17 Tevell S, Claesson C, Hellmark B *et al*. Heterogeneous glycopeptide intermediate *Staphylococcus epidermidis* isolated from prosthetic joint infections. *Eur J Clin Microbiol Infect Dis* 2014; **33**: 911–17. https://doi.org/10.1007/s10096-013-2025-3

18 Andersson DI, Nicoloff H, Hjort K. Mechanisms and clinical relevance of bacterial heteroresistance. *Nat Rev Microbiol* 2019; **17**: 479–96. https://doi.org/10.1038/s41579-019-0218-1

19 Tevell S, Christensson B, Nilsdotter-Augustinsson A *et al*. Handläggning av infektioner vid ortopediska implantat en utmaning för vården [Treatment of orthopaedic implant-associated infections]. *Lakartidningen* 2019; **116**: FR6C.

20 Hiramatsu K, Aritaka N, Hanaki H *et al*. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet* 1997; **350**: 1670–3. https://doi.org/10.1016/S0140-6736(97)07324-8

21 Wootton M, Howe RA, Hillman R *et al*. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in *Staphylococcus aureus* in a UK hospital. *J Antimicrob Chemother* 2001; **47**: 399–403. https://doi.org/10.1093/jac/47.4.399

22 Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455–77. https://doi.org/10.1089/cmb.2012.0021

23 Sahl JW, Lemmer D, Travis J *et al*. NASP: an accurate, rapid method for the identification of SNPs in WGS datasets that supports flexible input and output formats. *Microb Genom* 2016; **2**: e000074.

24 McKenna A, Hanna M, Banks E *et al*. The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res* 2010; **20**: 1297–303. https://doi.org/10.1101/gr.107524.110

25 Delcher AL, Phillippy A, Carlton J *et al*. Fast algorithms for large-scale genome alignment and comparison. *Nucleic Acids Res* 2002; **30**: 2478–83. https://doi.org/10.1093/nar/30.11.2478

26 Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014; **30**: 2068–9. https://doi.org/https://doi.org/10.1093/bioinformatics/btu153

27 Page AJ, Cummins CA, Hunt M *et al*. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 2015; **31**: 3691–3. https://doi.org/10.1093/bioinformatics/btv421

28 Clausen P, Aarestrup FM, Lund O. Rapid and precise alignment of raw reads against redundant databases with KMA. *BMC Bioinformatics* 2018; **19:** 307. https://doi.org/10.1186/s12859-018-2336-6

29 Baseri N, Najar-Peerayeh S, Bakhshi B. Investigating the effect of an identified mutation within a critical site of PAS domain of WalK protein in a vancomycin-intermediate resistant *Staphylococcus aureus* by computational approaches. *BMC Microbiol* 2021; **21**: 240. https://doi.org/10.1186/s12866-021-02298-9

30 UniProt Beta. P71057 EPSH_BACSU. https://www.uniprot.org/uniprot/P71057

31 UniProt Consortium. UniProt: the universal protein knowledgebase in 2021. *Nucleic Acids Res* 2021; **49**: D480–9. https://doi.org/10.1093/nar/gkaa1100

32 Drlica K, Zhao X. Mutant selection window hypothesis updated. *Clin Infect Dis* 2007; **44**: 681–8. https://doi.org/10.1086/511642

33 Dorr MB, Jabes D, Cavaleri M *et al*. Human pharmacokinetics and rationale for once-weekly dosing of dalbavancin, a semi-synthetic glycopeptide. *J Antimicrob Chemother* 2005; **55** Suppl 2: ii25–30. https://doi.org/10.1093/jac/dki008

34 Seltzer E, Dorr MB, Goldstein BP *et al*. Once-weekly dalbavancin versus standard-of-care antimicrobial regimens for treatment of skin and soft-tissue infections. *Clin Infect Dis* 2003; **37**: 1298–303. https://doi.org/10.1086/379015

35 Howden BP, Davies JK, Johnson PD *et al*. Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clin Microbiol Rev* 2010; **23**: 99–139. https://doi.org/10.1128/CMR.00042-09

36 Delaune A, Dubrac S, Blanchet C *et al*. The WalKR system controls major staphylococcal virulence genes and is involved in triggering the host inflammatory response. *Infect Immun* 2012; **80**: 3438–53. https://doi.org/10.1128/IAI.00195-12

37 Alam MT, Petit RA 3rd, Crispell EK *et al*. Dissecting vancomycin-intermediate resistance in *Staphylococcus aureus* using genome-wide association. *Genome Biol Evol* 2014; **6**: 1174–85.

38 Widerstrom M, Stegger M, Johansson A *et al*. Heterogeneity of *Staphylococcus epidermidis* in prosthetic joint infections: time to re-evaluate microbiological criteria? *Eur J Clin Microbiol Infect Dis* 2022; **41**: 87–97. https://doi.org/10.1007/s10096-021-04352-w

39 Peyrusson F, Varet H, Nguyen TK *et al*. Intracellular *Staphylococcus aureus* persisters upon antibiotic exposure. *Nat Commun* 2020; **11**: 2200. https://doi.org/10.1038/s41467-020-15966-7

40 Fernández L, Breidenstein EB, Hancock RE. Creeping baselines and adaptive resistance to antibiotics. *Drug Resist Updat* 2011; **14**: 1–21. https://doi.org/10.1016/j.drup.2011.01.001

41 Ghosh D, Veeraraghavan B, Elangovan R *et al*. Antibiotic resistance and epigenetics: more to it than meets the eye. *Antimicrob Agents Chemother* 2020; **64**: e02225-19. https://doi.org/10.1128/AAC.02225-19

42 Tenover FC, Sinner SW, Segal RE *et al*. Characterisation of a *Staphylococcus aureus* strain with progressive loss of susceptibility to vancomycin and daptomycin during therapy. *Int J Antimicrob Agents* 2009; **33**: 564–8. https://doi.org/10.1016/j.ijantimicag.2008.12.010

43 Cui L, Tominaga E, Neoh HM *et al*. Correlation between reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2006; **50**: 1079–82. https://doi.org/10.1128/AAC.50.3.1079-1082.2006

44 Chen CJ, Huang YC, Chiu CH. Multiple pathways of cross-resistance to glycopeptides and daptomycin in persistent MRSA bacteraemia. *J Antimicrob Chemother* 2015; **70**: 2965–72. https://doi.org/10.1093/jac/dkv225

45 Rybak MJ, Vidaillac C, Sader HS *et al.* Evaluation of vancomycin susceptibility testing for methicillin-resistant *Staphylococcus aureus*: comparison of Etest and three automated testing methods. *J Clin Microbiol* 2013; **51**: 2077–81. https://doi.org/10.1128/JCM.00448-13

46 Leroy AG, Lavigne-Quilichini V, Le Turnier P *et al.* Accuracy of gradient diffusion method for susceptibility testing of dalbavancin and comparators. *Expert Rev Anti Infect Ther* 2022; **20**: 457–61. https://doi.org/10.1080/14787210.2021.1976143