

Reduced vancomycin susceptibility and increased macrophage survival in *Staphylococcus aureus* strains sequentially isolated from a bacteraemic patient during a short course of antibiotic therapy

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Abstract

Purpose. The purpose of the present study was to determine the relatedness of *Staphylococcus aureus* strains successively isolated over a 7-day period from a single bacteraemic patient undergoing antibiotic treatment with vancomycin.

Methods. The *S. aureus* strains had been isolated and sequenced previously. Antibiotic susceptibility testing, population analysis profiling, and lysostaphin sensitivity and phagocytic killing assays were used to characterize these clonal isolates.

Results. The seven isolates (MEH1–MEH7) were determined to belong to a common multilocus sequence type (MLST) and *spa* type. Within the third and fifth day of vancomycin treatment, mutations were observed in the *vraS* and *rpsU* genes, respectively. Population analysis profiles revealed that the initial isolate (MEH1) was vancomycin-susceptible *S. aureus* (VSSA), while those isolated on day 7 were mostly heteroresistant vancomycin-intermediate *S. aureus* (hVISA). Supporting these findings, MEH7 was also observed to be slower in growth, to have an increase in cell wall width and to have reduced sensitivity to lysostaphin, all characteristics of VISA and hVISA strains. In addition, MEH7, although phagocytosed at numbers comparable to the initial isolate, MEH1, survived in higher numbers in RAW 264.7 macrophages. Macrophages infected with MEH7 also released more TNF- α and IFN- 1β .

Conclusion. We report an increasing resistance to vancomycin coupled with daptomycin that occurred within approximately 3 days of receiving vancomycin and steadily increased until the infection was cleared with an alternative antibiotic therapy. This study reiterates the need for rapid, efficient and accurate detection of hVISA and VISA infections, especially in high-bacterial load, metastatic infections like bacteraemia.

INTRODUCTION

Vancomycin, a glycopeptide antibiotic, was discovered in 1953 and approved in 1958 by the US Food and Drug

Administration to treat penicillin-resistant staphylococci [1]. Vancomycin was an alternative to methicillin due to the exponential increase in methicillin-resistant *Staphylococcus aureus* (MRSA) infections [2–5]. Vancomycin is commonly used to

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Keywords: *Staphylococcus aureus*; vancomycin resistance; hVISA; resilience to immunoclearance.

Abbreviations: BHI, brain heart infusion; CC8, clonal complex 8; CSLI, Clinical and Laboratory Standards Institute; ELISA, enzyme-linked immunosorbent assay; hVISA, vancomycin-heteroresistant *S. aureus*; IFN- β , interferon-beta; MAC, membrane attack complex; MIC, minimal inhibitory concentration; MLST, multilocus sequence typing; MRSA, methicillin-resistant *Staphylococcus aureus*; OD, optical density; PAP-AUC, population analysis profile-area under the curve; PBS, phosphate-buffered saline; SCV, small colony variants; TEM, transmission electron microscope; TNF- α , Tumor necrosis factor-alpha; TSB, tryptic soy broth; VISA, vancomycin-intermediate *S. aureus*; VRSA, Vancomycin-resistant *S. aureus*; VSSA, Vancomycin-susceptible *S. aureus*.

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Supplementary material is available with the online version of this article.

treat bacteraemia and associated metastatic infections such as endocarditis [6] and spinal osteomyelitis [7]. However, an increasing incidence of vancomycin treatment failures due to increasing resistance to vancomycin has been observed [8–13]. One of the earliest reports of a post-surgical infection by *S. aureus* Mu50 recalcitrant to vancomycin occurred in Japan in 1997 [9].

The Clinical and Laboratory Standards Institute (CLSI) currently defines vancomycin-susceptible *S. aureus* (VSSA) as those strains of *S. aureus* with a minimal inhibitory concentration (MIC) $\leq 2 \mu\text{g ml}^{-1}$, vancomycin-intermediate *S. aureus* (VISA) as those with an MIC of $4\text{--}8 \mu\text{g ml}^{-1}$ and vancomycin-resistant *S. aureus* (VRSA) as those with an MIC of $\geq 16 \mu\text{g ml}^{-1}$ [14]. Vancomycin-heteroresistant *S. aureus* (hVISA) was reported first in 1996; it consisted of subpopulations with varying vancomycin MIC values (MIC_{van}) [15] and displayed mixed-colony morphology [16]. Most of the persistent bacteraemic cases during vancomycin treatment are associated with VISA/hVISA [17–20]. Several MRSA strains have been reported to convert to VISA strains [21, 22] by genetic mutations and physiological changes. Unfortunately, there is no defined biomarker that can predict the emergence of hVISA/VISA from MRSA, which makes the diagnosis and treatment of such infections challenging.

Studies have observed the evolution of MRSA to VISA over weeks [23], months [24, 25] or years [26] of treatment with vancomycin. We report here the emergence of hVISA and the associated genetic and phenotypic changes that occurred within days with a MRSA strain obtained from a bacteraemic patient undergoing vancomycin therapy. Isolates were obtained every 2 days from the positive blood cultures over a period of 7 days until a combinatorial therapy of ceftaroline and daptomycin successfully cleared the infection (day 8).

METHODS

Patient

The patient was a 68-year-old Caucasian male with diabetes mellitus, who presented to the Emergency Department of the University of Arkansas for Medical Sciences (UAMS) with fever and back pain. Physical examination indicated lower spinal tenderness and a regurgitant systolic murmur in the mitral valve area. He was initially treated with 15 mg kg^{-1} vancomycin every 12 h. He underwent a transesophageal echocardiogram, which showed small mobile echodensities on the mitral and aortic valves. Magnetic resonance imaging of the spine demonstrated L4–5 diskitis, osteomyelitis and a splenic abscess. His blood cultures remained positive on day 5, resulting in an antibiotic regimen change to 6 mg kg^{-1} daptomycin daily on day 7. His blood cultures of day 7 tested positive and hence he was started on $600 \text{ mg } 8 \text{ h}^{-1}$ ceftaroline along with daptomycin (10 mg kg^{-1} daily). The patient became afebrile and cleared the bacteraemia on day 8. Five months prior, the patient had been treated with a 6-week course of vancomycin during a previous hospital stay for MRSA bacteraemia and pneumonia.

Bacterial isolates and culture conditions

Blood was obtained from the bacteraemic patient every 2 days. The colonies isolated from the subsequent cultures grown from the positive blood cultures were named MEH1–7. These isolates were determined to be MRSA by the Alere PBP2a SA Culture Colony Test (Alere Inc., Waltham, MA, USA). The colonies of day 1 and day 3 were uniform in size when grown in the microbiology laboratory of UAMS and one colony was procured from each of these days, constituting MEH1 and MEH2. The colonies of days 5 and 7 were not uniform in size, hence more than one colony were chosen from day 5 (MEH3–5) and day 7 (MEH6–7). *S. aureus* Mu50 [15] and Mu3 [9] strains were used as positive controls for VISA and hVISA, respectively.

All isolates were grown in tryptic soy broth (TSB; Becton, Dickinson and Co., Sparks, MD, USA) with orbital shaking (200 r.p.m.) at 37°C or on TSB+1.5 % agar (TSA) plates.

Growth assessment

Briefly, overnight cultures (15–18 h) of *S. aureus* were used to inoculate sterile medium to an optical density (OD) of 0.05 at 550 nm. These normalized cultures were incubated with orbital shaking (200 r.p.m.) at 37°C and growth was spectrophotometrically determined every 30 min for 6 h.

Antibiotic susceptibility profile

Susceptibility to antibiotics was determined by the clinical microbiology laboratory at UAMS. The MICs of ceftaroline, clindamycin, daptomycin, erythromycin, oxacillin, tetracycline, trimethoprim/sulfamethoxazole and vancomycin were tested by E-test (bioMérieux, St Louis, MO, USA) using the CLSI guidelines [14].

Population analysis profile

hVISA was determined by calculating the population analysis profile/area under the curve (PAP-AUC) ratios [27–29]. Briefly, overnight cultures normalized to an OD_{550} of 0.3 were serially diluted and plated on brain heart infusion (Becton, Dickinson and Co., MD, USA) agar plates containing vancomycin (0, 0.5, 1.0, 2.0, 4.0 and $8.0 \mu\text{g ml}^{-1}$) (Sigma-Aldrich Chemical Co., St Louis, MO, USA). The VISA strain Mu50 (MIC_{van} of $8 \mu\text{g ml}^{-1}$) [15] and the hVISA strain Mu3 (MIC_{van} of $2 \mu\text{g ml}^{-1}$) [9] were included as controls. The plates were incubated for 48 h at 37°C . Colonies were counted and the colony-forming unit (c.f.u.) ml^{-1} values were plotted against the vancomycin concentrations to obtain the PAP. The PAP-AUC was calculated by determining the ratio of the AUC for each isolate to the AUC for Mu3. Strains with AUC ratios ≥ 0.9 were considered to be hVISA and those with ≥ 1.3 were VISA [29–31].

Genomic profiling

The genomes of MEH1 through 7 (three biological replications per isolate) were sequenced previously by Basco et al. [30]. The mutations were detected by genome comparisons of all strains to MEH1 using VarScan2 [31].

Multilocus sequence typing (MLST) and *spa* typing

The MLST based on sequences of the seven housekeeping genes was conducted at <https://cge.cbs.dtu.dk/services/MLST/> [32]. The *spa* types were determined using the Ridom SpaServer (<http://www.spaserver.ridom.de>) [33].

RNA transcriptomics

The bacterial isolates were grown in TSB to mid-logarithmic phase. Cells were collected by centrifugation and treated with RNAProtect bacteria reagent (Qiagen, Inc., Germantown, MD, USA). Extraction of RNA from cells, cDNA synthesis and gene expression analysis (details are available in the Supplementary Material, available in the online version of this article) were performed by EA, Q2 Solutions Company, NC, USA.

Cell Wall thickness by transmission electron microscopy

Overnight TSB cultures were used to inoculate fresh TSB to an initial optical density of 0.05 at 550 nm. Cultures were grown for 3.5 h at 37 °C with orbital shaking (200 r.p.m.). Cultures were standardized with respect to optical density, centrifuged (5000 g for 5 min) and washed in phosphate-buffered saline (PBS; 0.1 M, pH 7.2). The bacteria were fixed by incubation in 2.5 % glutaraldehyde [Electron Microscopy Sciences (EMS), Hatfield, PA, USA] for an hour and then washed with 0.1 M PBS buffer (pH 7.2) before incubation with osmium tetroxide (EMS, Hatfield, PA, USA). Samples were washed and dehydrated using increasing concentrations of ethanol. Samples were embedded in Araldite/Embed 812 resin (EMS, Hatfield, PA, USA), sectioned and stained with uranyl acetate and lead citrate. The FEI Tecnai F20 transmission electron microscope (FEI Company, Hillsboro, OR, USA) was employed to image the samples at UAMS.

The cell wall thickness of *S. aureus* isolates was measured by employing FEI Tecnai Imaging and Analysis (TIA) software

(FEI Company, Hillsboro, OR, USA). Sixty cells per isolate were measured to obtain the mean thickness of the cell wall.

Lysostaphin-induced autolysis assay

A lysostaphin-induced lysis assay was conducted as described by Berscheid *et al.* [34]. Briefly, normalized cultures were collected by centrifugation when the OD₅₅₀ reached 1.0. The cells were washed and suspended in PBS pH 7.4 (Thermo Fisher Scientific, Grand Island, USA) containing 200 ng ml⁻¹ of lysostaphin (AMBI Products, LLC, Lawrence, NY) to an OD₅₆₀ of 0.8. The lysis of the cell suspension was measured spectrophotometrically as a decrease in OD over time (5 min intervals).

Phagocytic survival assay

Overnight TSB cultures were used to inoculate fresh TSB to an initial optical density of 0.05 at 550 nm. Cultures were grown for 3.5 h at 37 °C with orbital shaking (200 r.p.m.). Cultures were standardized with respect to optical density, centrifuged as before, washed in PBS and suspended in PBS to yield a viable cell count of approximately 3×10⁷ c.f.u. ml⁻¹. Suspended cells were used in the phagocytic survival assay. RAW 264.7 macrophages (American Type Culture Collection, Manassas, VA, USA) were exposed to the bacteria at a multiplicity of infection (m.o.i.) of 10.

After 15 min of incubation with the macrophages, the cells were washed twice with PBS, lysed with PBS containing 1 % Triton X-100 and plated on TSA. Phagocytic uptake was calculated by enumeration of the c.f.u. Intracellular survival was measured by the percentage of bacterial cells surviving at 30, 60 and 90 min, as recorded by plating lysates of the macrophages at the respective time points.

ELISA

The DuoSet ELISA Development kit (R and D Systems, Minneapolis, MN, USA) was used for the detection of

Table 1. Antibiotic susceptibility profiles for *S. aureus* clinical strains isolated on days 1 through 7

Day (isolate number)	Minimal inhibitory concentration (µg ml ⁻¹)*						
	Cef	Cli	Dap	Ery	Oxa	Tet	Tri/Suf
1 (MEH1)	1	≤0.25	1	≥8	≥4	≤1	≤10
3 (MEH2)	1	≤0.25	0.5	≥8	≥4	≤1	≤10
5 (MEH3)	1	≤0.25	2	≥8	≥4	≤1	≤10
5 (MEH4)	1	≤0.25	2	≥8	≥4	≤1	≤10
5 (MEH5)	1	≤0.25	2	≥8	≥4	≤1	≤10
7 (MEH6)	1	≤0.25	4	≥8	≥4	≤1	≤10
7 (MEH7)	1	≤0.25	4	≥8	≥4	≤1	≤10

*Minimal inhibitory concentration (MIC) determinations for Cef, Dap and Van were determined by E-test (bioMérieux). All other MIC values were determined by ViTek.

Cef, ceftaroline; Cli, clindamycin; Dap, daptomycin; Ery, erythromycin; Lin, linezolid; Oxa, oxacillin; Tet, tetracycline; Tri/Suf, trimethoprim/sulfamethoxazole; Van, vancomycin.

TNF- α and IL-1 β levels after 24 h infection of RAW 264.7 macrophages with MEH1–7. Briefly, wells of Nunc Maxisorp 96-well plates were coated with anti-mouse TNF- α and IL-1 β (R and D BioSystems, Minneapolis, MN, USA) overnight at room temperature (RT) according to the manufacturer's instructions. The plates were blocked with 1 % bovine serum albumin fraction V (Sigma-Aldrich Chemical Co., St Louis, MO, USA) in PBS for 2 h. The supernatants of the infected and uninfected controls were added to the wells and incubated for 2 h. The wells were washed four times with PBS containing 0.05 % Tween 20. The biotinylated monoclonal antibodies for each of the cytokines were added and incubated for 2 h. Horseradish peroxidase-conjugated streptavidin (R and D Biosystems, Minneapolis, MN, USA) was added per the manufacturer's recommendations and incubated for 30 min. Standard curves were generated using purified recombinant TNF α or IL-1 β using SoftMax Pro (Molecular Devices Co., San Jose, CA, USA), and a four-parameter curve fit was generated for each cytokine.

Statistical analysis

The data were plotted and statistical significance was assessed by *t*-tests (with a Bonferroni correction where appropriate) employing SigmaPlot version 13 (*P*-value <0.05).

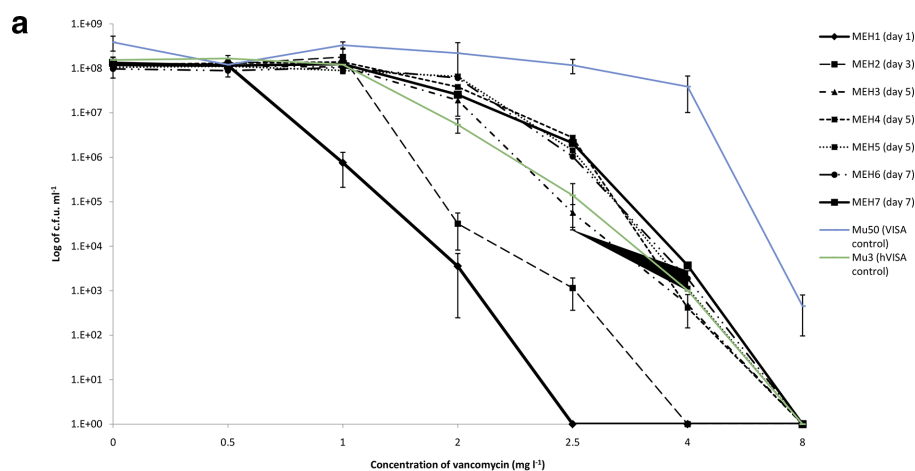
RESULTS

MEH1–7 share a common MLST and *spa* type

The successive isolates were analysed for relatedness by MLST. The genetic sequences of the seven housekeeping genes in MEH1–7 revealed that they belonged to a common sequence, type 8 (ST8) (3-3-1-1-4-4-3) and thus had no lineage diversification in 7 days. This was further supported by their common *spa* type, t008 (11-19-12-21-17-34-24-34-22-25). This suggested that the seven isolates probably emerged from a single clone in succession.

MIC creep and the transition of VSSA to hVISA

We observed an increasing MIC_{van} and MIC_{dap} (Table 1) of the isolates procured from the patient over a period of 7 days. The MIC_{van} was 1 $\mu\text{g ml}^{-1}$ for MEH1 and MEH2 isolated from days 1 and 3, respectively, but the MIC of the subsequently procured strains showed an increase. MEH3–5 (day 5) had varying MIC values between the vancomycin-susceptible range of 2 $\mu\text{g ml}^{-1}$ and the vancomycin-intermediate range of 4 $\mu\text{g ml}^{-1}$, which, based on the definition by Hiramatsu *et al.* [35], made them hVISA. Both MEH6 and MEH7 (day 7) showed a uniform MIC_{van} of 4 $\mu\text{g ml}^{-1}$. Thus, we not only observed increasing resistance to vancomycin, but also a brief intermediate period during which we observed heteroresistance to vancomycin in these bacteraemic isolates. A similar trend of increasing MIC with time was observed



b

Isolate no.	MEH1	MEH2	MEH3	MEH4	MEH5	MEH6	MEH7
Day of procurement	1	3	5	5	5	7	7
PAP-AUC	0.4	1.1	0.8	1.1	0.9	0.9	0.9

Fig. 1. Population analysis profiles of MEH1–7 show a transition of strains from VSSA to hVISA. (a) Population analysis profiles (PAP) of *S. aureus* isolates MEH1–7. (b) PAP-area under the curve values (PAP-AUC) reveal a trend of increasing resistance to vancomycin and a heteroresistant population among isolates procured from the same day. This also indicates isolates MEH2–7 to be mostly hVISA and MEH1 to be a VSSA strain. The values represent the mean \pm SD of a minimal of three independent biological determinations.

with daptomycin (except that MEH2 had an MIC_{dap} of 0.5 µg ml⁻¹). Based on the MIC definitions by the CLSI [36], resistance to daptomycin was observed from day 5 onwards. Thus, we report a shortest time of 5 days in the MIC creep of vancomycin and daptomycin.

The results from the PAP-AUC determinations indicated increasing resistance to vancomycin with strains isolated later in the progression of treatment (Fig. 1a). MEH1 and MEH2 were fully susceptible at 2.5 and 4 µg ml⁻¹ of vancomycin, while MEH3–7 (day 5 onwards), like Mu3 (hVISA control), were susceptible at 8 µg ml⁻¹. The PAP-AUC values indicated a similar trend, with the value for VSSA (MEH1) being 0.4, while the values for the hVISA isolates (MEH2–7) ranged from 0.8 to 1.1 (Fig. 1b).

In addition, the microbiology laboratory at UAMS observed that day 5 and day 7 isolates grew in varying colony sizes (called small colony variants, SCVs) on blood plates without antibiotics. We observed SCVs with all isolates (Fig. 2) on BHI agar with vancomycin.

Single-nucleotide variants (SNV) emerged in *vraS* on day 3 and *rpsU* on day 5 of vancomycin treatment

While comparing the genomic sequences of the seven isolates with each other, we detected that one non-synonymous and one frameshift mutation caused by SNVs had occurred during the course of antibiotic treatment. A unique missense single-nucleotide mutation (G45V) was detected in the two-component histidine kinase sensor gene, *vraS*, in strains MEH2–7 (day 3 onwards). The location of the mutation in *vraS* has previously been reported among VISA strains [37–41]. However, the type of mutation and the relatively short period (by day 3 of vancomycin exposure)

for appearance with respect to previously published studies regarding the *vraS* mutation suggest that the genetic event leading to hVISA occurs at a much faster rate than previously thought. We also detected a nucleotide insertion in the 30S ribosomal protein S21 gene (*rpsU*) that led to a frameshift mutation from the fourth amino acid onwards in RpsU of strains MEH3–7 (day 5 onwards). This type of mutation and location and the short time for acquisition of the mutation (day 5) make this unlike previously reported cases of vancomycin resistance.

Characteristic features of VISA observed in hVISA strain MEH7

We further confirmed transformation to a hVISA strain by assessing MEH7 in comparison to MEH1 using assays that reveal the salient features of a VISA strain, such as slower growth rate [42, 43], thicker cell walls [18, 42, 44–49] and increased resistance to lysostaphin-mediated cell wall lysis [42, 50, 51]. Growth monitoring during a time-course study revealed that MEH7 grew at a slower rate than the first isolate, MEH1 (Fig. 3a). In addition, a comparative analysis of the cell walls of MEH1 and MEH7 by transmission electron microscopy (TEM) indicated that the cell wall of MEH7 (72.16±6.85 nm) was considerably thicker than that of MEH1 (41.82±3.29 nm) (Fig. 3b). Furthermore, MEH7 was also more resistant to lysostaphin-induced lysis than MEH1 (Fig. 3c).

Transcriptional differences between MEH1 and MEH7

We observed the transcription of 57 genes to be significantly downregulated and that of 37 genes to be significantly upregulated in MEH7 compared to MEH1 (Table 2).

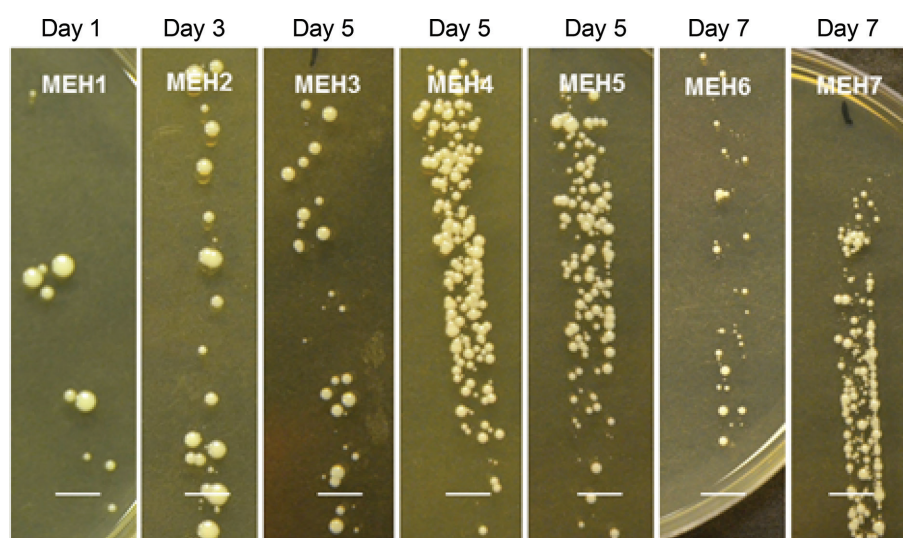


Fig. 2. Small colony variants (SCVs) observed. On plating all seven isolates (MEH1–7) on BHI agar with vancomycin, SCVs were consistently observed across all seven clinical isolates. Scale, bar=0.5 cm.

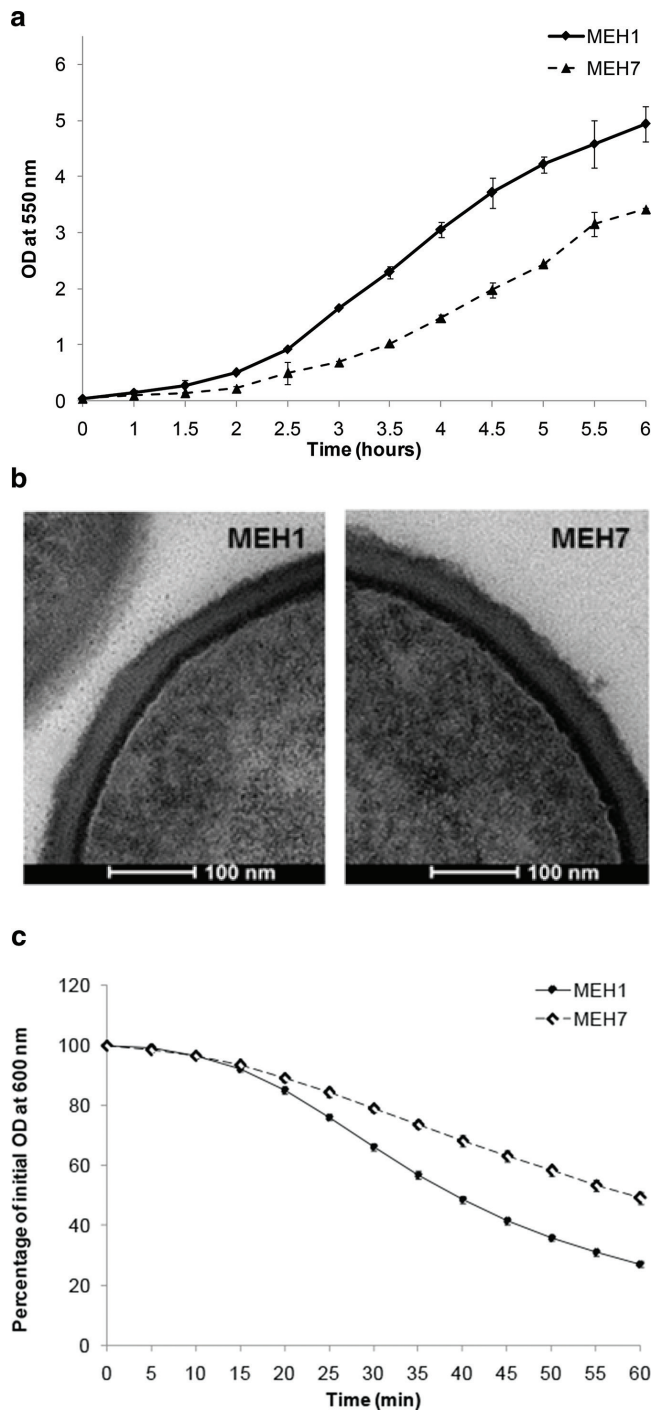


Fig. 3. Development of typical features of hVISA in isolate MEH7. Comparison of (a) growth curves of isolates MEH1 and MEH7 in TSB at 37 °C, (b) cell wall thickness by transmission electron microscopy and (c) lysostaphin-induced assay, revealing MEH7 (72.16 nm±6.85) to have the characteristic features of VISA, as they are slow growers with thicker cell walls that are more resistant to lysostaphin than MEH1 (41.82 nm±3.29). The values are representative of at least three biological determinations. The cell wall thickness recorded is the mean±SD of 60 cells.

Table 2. Transcriptional differences observed between MEH1 and MEH7. Relative quantitative expression of genes of MEH7 as compared to MEH1. The transcriptional differences represent a threefold change and the values were statistically significant with a *P*-value <0.05

Gene ID	Description	Fold Change
<i>Cell wall synthesis and metabolism</i>		
SAV1439	Alanine dehydrogenase	−42.94
SAV1438	Serine/threonine dehydratase	−42.21
SAV1437	Amino acid permease	−39.38
SAV2095	Transglycosylase sceD	9.33
SAV2356	Teicoplanin resistance-associated protein, tcaA	3
SAV2285	Acyl-CoA dehydrogenase required for lipid transport and metabolism, caiA	−4.72
SAV0148	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase, adhE	−6.76
SAV0605	Zinc-dependent alcohol dehydrogenase, adh1	−14.58
SAV2524	D-lactate dehydrogenase	−3.79
SAV2602	L-lactate dehydrogenase	−4.38
SAV1300	Glycerol uptake facilitator, glpF	5.29
SAV1302	Aerobic glycerol-3-phosphate dehydrogenase, glpA	3.99
SAV0095	1-phosphatidylinositol phosphodiesterase, plc	−6.33
SAV2524	Lactate dehydrogenase	−3.79
SAV0465	LysM	−3.12
SAV0226	Formate acetyltransferase	−13.42
SAV0227	Pyruvate formate-lyase-activating enzyme	−13.99
SAV0474	PTS ascorbate transporter subunit IIA	−3.31
SAV0632	Membrane protein involved in nutrient transport	−3.48
SAV0959	Glycerophosphoryl diester phosphodiesterase	−3.68
SAV1529	Peptidase M24, pepP	−3.07
SAV1683	Threonine—tRNA ligase	−3.31
SAV2206	α-acetolactate decarboxylase	−3.63
SAV2207	Acetolactate synthase	−3.83
SAV2403	Formate/nitrite transporter	−3.42
SAV2416	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	−8.04
SAV2521	N-acetyltransferase	−3.42
SAV0276	Glycyl-glycine endopeptidase lytM	4.29
SAV2299	CHAP domain-containing protein (secretory antigen precursor SsaA-like protein)	4.46
SAV2304	CHAP domain-containing protein (secretory antigen SsaA-like protein)	7.66

Continued

Table 2. Continued

Gene ID	Description	Fold Change
SAV2566	CHAP domain-containing protein (secretory antigen precursor SsaA-like protein)	4.26
SAV1606	Acetyl-CoA carboxylase biotin carboxylase subunit	5.22
SAV1607	Acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	5.11
SAV1575	30S ribosomal protein S21	11.94
SAV1885	Two-component sensor histidine kinase, vraS	3.22
SAV1608	Allophanate hydrolase	5.55
SAV1609	Allophanate hydrolase	5.29
SAV1778	Competence protein, comK	3.88
SAV1874	Monofunctional glycosyltransferase, mgtB	3.48
<i>Nucleoside/nucleotide metabolism</i>		
SAV0136	Purine-nucleoside phosphorylase	−4.1
SAV0307	5'-nucleotidase, lipoprotein e(P4) family	−5.61
SAV2136	Pyrimidine-nucleoside phosphorylase	−3.59
SAV2616	Anaerobic ribonucleoside-triphosphate reductase-activating protein	−19.68
SAV2617	Anaerobic ribonucleoside-triphosphate reductase	−16.23
SAV2137	2-deoxyribose-5-phosphate aldolase	−3.54
SAV2589	Dihydroorotate dehydrogenase (quinone)	−3.46
<i>Osmoprotection/oxidative stress protection</i>		
SAV2612	Oxygen-dependent choline dehydrogenase, betA	3.81
SAV2613	Betaine-aldehyde dehydrogenase, gbsA	3
SAV2584	Baeyer–Villiger Flavin containing monooxygenase	3.22
SAV0133	Superoxide dismutase [Mn/Fe] 2, sodM	−3.88
SAV0211	FMN-dependent NADH-azoreductase, acpD	−3.33
SAV0240	Nitric oxide dioxygenase	−5.35
SAV2523	NAD(P)H-dependent oxidoreductase	−5.17
<i>Transporters</i>		
SAV1436	Quinolone resistance protein, norB	−31.51
SAV1886	Transporter, yvqF	3.26
SAV2448	Glycine/betaine ABC transporter ATP-binding protein	−4.89
SAV2447	Choline ABC transporter permease	−4.89
SAV2279	Molybdate ABC transporter substrate-binding protein	−4.73
SAV2446	ProX, glycine betaine/carnitine/choline ABC transporter substrate-binding protein	−4.49

Continued

Table 2. Continued

Gene ID	Description	Fold Change
SAV2445	Amino acid ABC transporter permease	−4.45
SAV0633	Phosphonate ABC transporter ATP-binding protein	−3.84
SAV0631	Metal ABC transporter substrate-binding protein	−3.71
SAV2278	Molybdenum ABC transporter permease subunit	−3.19
SAV2277	Molybdenum ABC transporter ATP-binding protein	−3.02
SAV1300	Glycerol transporter	5.29
<i>Regulators</i>		
SAV2705	Transcriptional regulator	−5.66
<i>Microbial surface component recognizing adhesive matrix molecules (MSCRAMMs)</i>		
SAV0111	Protein A, spa	−5.77
SAV0134	Hypothetical protein, 98 % identical to cell wall-anchored protein SasD	−3.72
SAV2630	Clumping factor B, clfB	3.13
<i>Iron sulfur proteins</i>		
SAV0259	Iron-sulfur cluster repair protein, ScdA	−8.43
SAV1604	Iron transporter	4.9
<i>Secretion systems</i>		
SAV0284	EssA, type 6 secretion	3.06
SAV0282	EsxA, type 6 secretion	3.96
SAV0283	EsaA, type 6 secretion	3.54
SAV2587	Type VII secretion effector	4.29
SAV2585	Type II secretion system	3.96
SAV0579	VraX	6.58
<i>Hypothetical proteins</i>		
SAV1037, SAV1489, SAV1490, SAV1605, SAV1846, SAV1887, SAV2322, SAV2556, SAV2586, SAV0279		Upregulated
SAV0238, SAV0239, SAV2404, SAV2480, SAV2590, SAV2706, SAV2707, SAV2373		Downregulated

This modulation was mostly observed with genes associated with amino acid metabolism, cell wall biosynthesis, nucleotide metabolism, transport and stress response. The highest expressional change of −42.94-, −42.21-, −39.38- and −31.51-fold was observed with genes of amino acid metabolism and cell fitness, namely, alanine dehydrogenase, serine/threonine dehydratase, amino acid permease and quinolone resistance protein (NorB), respectively. Ten hypothetical proteins were upregulated and eight were downregulated. The transcription of the mutated *vraS* and *rpsU* genes was upregulated in MEH7.

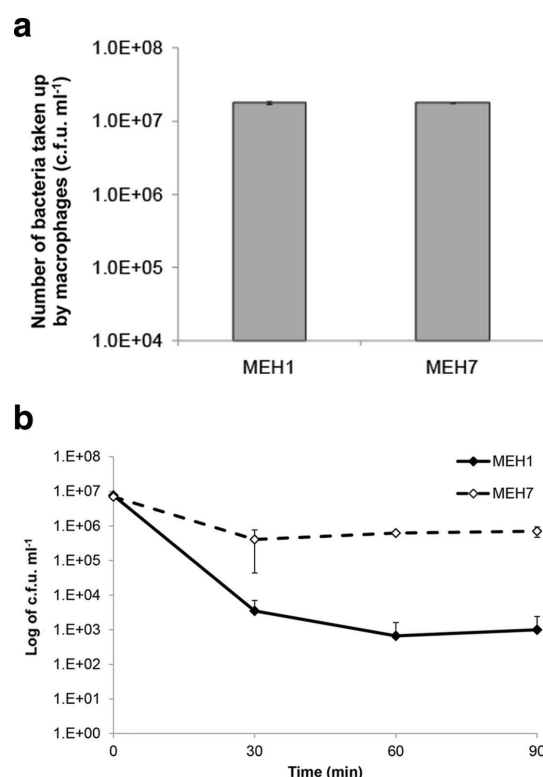


Fig. 4. Isolate MEH7 is resistant to intracellular phagocytic killing. (a) Phagocytic uptake assay showing that equal numbers of bacteria were taken up in 15 min when isolates MEH1 and MEH7 were exposed to RAW 264.7 macrophages. (b) Intracellular survival assay of isolates MEH1 and MEH7 show that isolate MEH7 survives in greater numbers than MEH1. The values represent the mean \pm SD of three independent biological determinations. Employing a Student *t*-test (with Bonferroni correction), statistical significance was observed at the 30, 60 and 90 min time points of intracellular survival assessed ($P<0.01$).

Resilience to immunoclearance observed in MEH7

We compared the outcome of phagocytosis of MEH7 with the first isolate, MEH1. When RAW macrophages were infected with these isolates, both were phagocytized at comparable numbers (Fig. 4a). However, the extended survival rate of MEH7 suggests that this strain is considerably more recalcitrant to phagocytic killing as compared to MEH1 (Fig. 4b). In addition, the proinflammatory cytokines, TNF α and IL-1 β , were both elevated in MEH7-phagocytized macrophages to a greater extent than in macrophages containing MEH1 (Fig. 5).

DISCUSSION

We report here the successive isolation and characterization of MRSA strains from a single bacteraemic patient that appear to be clonal, exhibiting increased levels of vancomycin resistance over the course of 7 days. Approximately 5 months prior to the patient's emergency department visit and subsequent hospital admission, the patient had

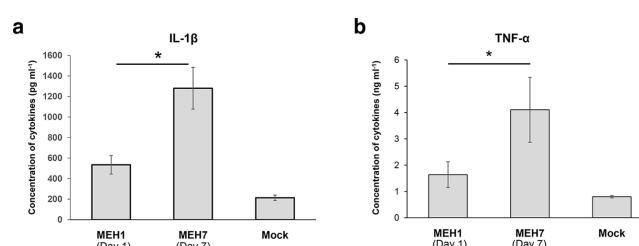


Fig. 5. Isolate MEH7 induces a pro-inflammatory response. Concentration of proinflammatory cytokines TNF- α (a) and IL-1 β (a) as quantified by ELISA. The values are the mean \pm SD of three independent biological determinations. The bar with asterisks represents statistical significance ($P<0.05$) by pairwise Student *t*-test.

been treated successfully with a 6-week course of vancomycin for a MRSA bacteraemia and pneumonia infection. While previous studies [17, 19, 21, 52] indicated that prior infections with MRSA and subsequent administration of vancomycin contribute to the development of vancomycin resistance, the data presented in this study suggest an independent occurrence of reduced susceptibility to vancomycin within 7 days of initiating vancomycin treatment. Positive blood culture isolates from days 1 and 3 were susceptible to vancomycin at 1 μ g ml⁻¹, but the day 3 isolate (MEH2) produced a PAP profile characteristic of hVISA. This isolate may represent the beginning of the occurrence of an hVISA strain that still exhibits, at the time of determination, a MIC in the susceptible range, a phenomenon known as vancomycin MIC 'creep', where treatment failures with MRSA isolates [26, 53] with vancomycin MICs of 1–2 μ g ml⁻¹ have been observed, resulting in low calculated clinical success rates (9.5%) [54–56].

In this study, a total of seven isolates were procured in succession from positive blood cultures and were subsequently shown to not only be members of the same common ancestral clonal cluster, CC8, including identical MLST (ST8) and *spa* (t008) types, but also to contain only two differences within their genomic sequences. These differences occurred sequentially in isolates obtained from cultures on the third (*vraS*) and fifth (*vraS rpsU*) day with respect to vancomycin treatment. The day 3 isolate and two of the three day 5 isolates exhibited PAP profiles of 0.9, which by definition characterizes these isolates as hVISA. By day 7, the isolates exhibited characteristics that are typically used to describe VISA strains. These isolates had vancomycin MICs of 4 μ g ml⁻¹ and exhibited PAP profiles of 0.9. In addition, these isolates contained thicker cell walls, a feature of low-level vancomycin-resistant *S. aureus* such as hVISA and VISA [18, 42, 44–49], grew slower [42, 43] and had increased resistance to lysostaphin [42, 50, 51] as compared to the strain isolated on day 1. Another important phenotype we observed was the appearance of SCVs, a common feature of hVISA and VISA [10, 16, 57–59]. However, it must be pointed out that all isolates when grown on BHI containing vancomycin exhibited SCVs. This was also

true for the day 1 isolate, which had a vancomycin MIC of $1 \mu\text{g ml}^{-1}$ but displayed a PAP profile of 0.4, suggesting that the source of the infecting MRSA isolated might have been from the patient's previous MRSA infection that was treated with vancomycin for 6 weeks 5 months prior to the current incident.

While isolation of *vraS* and *rpsU* mutant strains in a relatively short period of vancomycin treatment is remarkable, their occurrence in hVISA and VISA strains is not novel (reviewed in [41]). In fact, numerous mutated genetic determinants have been implicated in the hVISA and VISA phenotypes. The *vraS* gene codes for the two-component histidine kinase sensor [52], which is part of the *vraUTSR* operon reported to be an important upregulator of cell wall synthesis [60, 61] and has been shown to be a common mutation in clinical isolates of hVISA and VISA [41]. The SNV of *vraS* resulting in an amino acid change of glycine to valine at the 45th amino acid of the *VraS* protein is unique to our study, but this location of the SNV has previously been reported with reduced susceptibility to vancomycin and daptomycin [37–41, 62].

The *rpsU* mutation that occurred on the fifth day has also been reported [41]. This gene codes for the small subunit ribosomal protein, S21, and represents one of 32 genetic determinants known to occur as a single mutation resulting in a VISA phenotype [41]. The frameshift mutation at the fourth amino acid of *rpsU* is unique to our study. Previous studies have reported SNVs in the promoter region [63, 64] and in the 31st [41], 51st, or 53rd amino acid of *RpsU* [65].

Accompanying the characteristic features of hVISA and VISA strains are transcriptional changes in genes responsible for cell wall thickening, energy biosynthesis, gene regulators and oxidative stress [66]. The only transcriptional changes observed in this study and common to other studies comparing transcriptional variations between pairs of VSSA and hVISA/VISA [67] were those of *sgtB*, *vraS*, *tcaA* and *yyqF* (*vraT*) and the hypothetical proteins SAV1887 and SAV2556. Among the genes that play a role in cell wall biosynthesis and response to cell wall-active antibiotics, we observed upregulation in the *sceD*, *lytM*, *sgtB*, *yyqF*, *tcaA* (teicoplanin resistance) and *vraS*, any or all of which could have contributed to the thicker cell wall of the hVISA isolate, MEH7. The increase in transcription of *sceD* and *lytM* in MEH7 has been shown in other VISA isolates to be associated with altered cell wall architecture and cell wall turnover [53]. The upregulation of the *vraS* gene in MEH7 could be due to the exposure to vancomycin and/or compensation for the G45V missense mutation. This upregulation of a mutated *vraS* has been previously reported [34]. The *VraRS* two-component system has been known to respond to cell wall-targeting antibiotics [54, 66]. The G45R SNV in *VraS* contributes to reduced susceptibility to glycopeptide antibiotics [55]. Congruent with this study, we observed (a) reduced susceptibility to vancomycin and (b) a SNV at the same location (45th amino acid) associated with no alteration in the transcription of *vraR*, *prsA*, *htrA1*, *pbp4* or *murZ* that are usually regulated by the *VraRS* system [55]. The autolysin-like protein *LysM* was the only autolysin

downregulated in MEH7, as observed when *S. aureus* was exposed to vancomycin [56, 66].

Surprisingly, the MEH7 isolate did not contain mutations within the genes of the accessory gene regulatory (*agr*) system. Likewise, the *agr* transcriptional levels were unaltered as compared to MEH1. The *agr* regulatory system is a two-component signal transduction system that is primarily responsible for controlling the expression of virulence genes in *S. aureus* [68, 69], and previous studies characterizing hVISA/VISA strains have demonstrated a reduction in *agr* and subsequently a reduction in a number of virulence genes, resulting in reduced pathogenesis in animal models [18, 70–73].

Vancomycin and daptomycin have different targets in the bacterial cell [35, 74–76]; however, exposure to these antibiotics commonly leads to a characteristic thickening of the bacterial cell wall [77, 78]. Interestingly, resistance to vancomycin due to exposure to vancomycin has been demonstrated to induce a decrease in susceptibility to daptomycin [62] due to the decrease in cell wall penetration by daptomycin [78]. We observed a similar trend of combined resistance in our study and suggest that the rapid acquisition of vancomycin resistance could have influenced the rapid resistance to daptomycin. This suggests that daptomycin might have limited success as an alternative to vancomycin treatments and further reduces the treatment options available for drug-resistant infections.

Consistent with other studies, protein A (*spa*) was downregulated [53, 56, 60, 61, 67, 79, 80] in the hVISA MEH7 strain. Protein A binds to the Fc region of immunoglobulins and prevents Fc-dependent phagocytosis [81, 82]. In addition, the *vraX* gene was upregulated in MEH7, as observed when *S. aureus* are exposed to cell wall-targeting antibiotics such as vancomycin [56, 83]. The increase in the *vraX* gene that inhibits the classical complement activation pathway [84] could inhibit the direct lysis of the bacteria by the membrane attack complex (MAC) and/or not contribute to the production of opsonins. Betaine-aldehyde dehydrogenase and oxygen-dependent choline dehydrogenase, which aid in acquisition of betaine to survive in macrophages [57], were also upregulated in MEH7.

The levels of the pro-inflammatory cytokines, $\text{TNF-}\alpha$ and $\text{IL-1}\beta$, were increased in MEH7 as compared to MEH1. Such an increase in the levels of these cytokines has been reported from surviving intracellular *S. aureus* [58] and in other sepsis cases [59]. We postulate that these factors worked in concert to aid in resisting phagocytic clearance of MEH7. Since MEH7 can survive intracellularly when phagocytized, we believe that it is advantageous to attract phagocytes with enhanced levels of proinflammatory cytokines and lower protein A levels. It is postulated that MEH7 can utilize the phagocyte as a vehicle to disseminate the infection.

In summary, we report that the successive transformation of a VSSA strain towards a VISA phenotype begins in as few as 3 days of vancomycin treatment with the appearance of hVISA strains. While much remains in determining the prevalence and clinical impact of hVISA and VISA

infections, the 30-day mortality rate associated with a hVISA infection appears to be similar to that encountered with a VSSA infection, even though treatment failures occurred more frequently with hVISA infections [70]. Nevertheless, we believe that the current study emphasizes the importance of monitoring MRSA infections treated with vancomycin by appropriate means other than strictly relying on MIC susceptibility testing. For example, incorporating colonial morphology observations for the occurrence of SCVs, using screening tests that incorporate a higher inoculum and longer incubation periods to detect the appearance of hVISA. This is particularly true for patients with increased risks for vancomycin failures, namely, older age, previous MRSA infection and prior vancomycin exposure, as well as host infection sites typically containing a higher bacterial burden [85].

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Author contributions

Study concept and design: M.D.S.B., S.S.A., J.R.R., M.E.H. Data acquisition: M.D.S.B., J.R.R., P.B.M., M.P.A., S.S.A., A.K. Data analysis and interpretation: M.D.S.B., J.R.R., P.B.M., M.E.H. Drafting manuscript: M.D.S.B., M.E.H. Critical review and editing of manuscript: M.D.S.B., M.E.H., J.R.R., P.B.M., S.S.A., M.P.A., A.K., M.S.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The study was originally approved by the UAMS IRB and deemed 'not human subject research determination'. The NCTR IRB agreed with this assessment.

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