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# Vancomycin-intermediate *Staphylococcus aureus* selected during vancomycin therapy of experimental endocarditis are not detected by culture-based diagnostic procedures and persist after treatment arrest

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**Objectives:** Laboratory detection of vancomycin-intermediate *Staphylococcus aureus* (VISA) and their heterogeneous VISA (hVISA) precursors is difficult. Thus, it is possible that vancomycin failures against supposedly vancomycin-susceptible *S. aureus* are due to undiagnosed VISA or hVISA. We tested this hypothesis in experimental endocarditis.

**Methods:** Rats with aortic valve infection due to the vancomycin-susceptible (MIC 2 mg/L), methicillin-resistant *S. aureus* M1V2 were treated for 2 days with doses of vancomycin that mimicked the pharmacokinetics seen in humans following intravenous administration of 1 g of the drug every 12 h. Half of the treated animals were killed 8 h after treatment arrest and half 3 days thereafter. Population analyses were done directly on vegetation homogenates or after one subculture in drug-free medium to mimic standard diagnostic procedures.

**Results:** Vancomycin cured 14 of 26 animals (54%; $P < 0.05$ versus controls) after 2 days of treatment. When vegetation homogenates were plated directly on vancomycin-containing plates, 6 of 13 rats killed 8 h after treatment arrest had positive cultures, 1 of which harboured hVISA. Likewise, 6 of 13 rats killed 3 days thereafter had positive valve cultures, 5 of which harboured hVISA. However, one subculture of vegetations in drug-free broth was enough to revert all the hVISA phenotypes to the susceptible pattern of the parent. Thus, vancomycin selected for hVISA during therapy of experimental endocarditis due to vancomycin-susceptible *S. aureus*. These hVISA were associated with vancomycin failure. The hVISA phenotype persisted *in vivo*, even after vancomycin arrest, but was missed *in vitro* after a single passage of the vegetation homogenate on drug-free medium.

**Conclusions:** hVISA might escape detection in clinical samples if they are subcultured before susceptibility tests.

**Keywords:** vegetations, population analyses, inoculum effect

## Introduction

The first *Staphylococcus aureus* with reduced susceptibility to vancomycin was described by Hiramatsu *et al.*[1] in 1997. The organism, called Mu50, had an MIC of vancomycin of 8 mg/L. At that time, the CLSI defined staphylococci for which the MIC of vancomycin was ≤4 mg/L as susceptible, isolates for which the MIC was 8–16 mg/L as intermediate and those for which the MIC was ≥32 mg/L as resistant.[2] Therefore, the Mu50 isolate was defined as a vancomycin- (or glycopeptide-)intermediate *S. aureus* (VISA). Concomitantly, the same author reported a second *S. aureus*, called Mu3, responsible for vancomycin treatment failure in an adult patient with pneumonia.[3] Although the vancomycin MIC for this isolate was 4 mg/L and the isolate was formally considered as susceptible at the time, Mu3 contained VISA subpopulations (≤$10^{-6}$ cfu) that grew in the presence of 5–9 mg/L vancomycin and were not detected by standard drug-susceptibility testing. The term heteroresistant VISA (hVISA) was coined to define the Mu3 phenotype. Since then, a number of cases of VISA and hVISA have been described worldwide[4] and have been associated with vancomycin treatment failures both in animal experiments[5–7] and in humans.[7–9] Revised CLSI breakpoints now classify isolates with an MIC between 4 and 8 mg/L of vancomycin as VISA.[2]

VISA and hVISA are different from fully vancomycin-resistant *S. aureus* (VRSA; MIC ≥32 mg/L), which have been

reported in the USA since 2002.[10,11] In VRSA, vancomycin resistance is due to the acquisition of the *vanA* determinant, which also mediates high-level resistance to vancomycin in enterococci,[12] and is situated on a conjugative transposon.[13,14] In contrast, the resistance phenotype in VISA and hVISA is associated with a thickened cell wall[15] and various chromosomal mutations that affect metabolic and cell wall synthesis pathways.[16–21]

Because of their relatively low vancomycin MIC, hVISA are notoriously difficult to identify by routine laboratory procedures.[4] For example, for Mu3 the rate of resistant subpopulations at a vancomycin concentration of 8 mg/L is in the order of $10^{-6}$, which is one to two orders of magnitude below the detection level of standard susceptibility tests (which use inoculum sizes of $10^{4-5}$ cfu). Moreover, the hVISA phenotype is reputed to be unstable, due to the overgrowth of slow-growing resistant subpopulations by faster-growing susceptible ones in the absence of the drug.[22] This is somewhat reminiscent of the early times of methicillin-resistant *S. aureus* (MRSA), where heterogeneous expression of methicillin resistance passed undetected by routine laboratory testing, and the organisms were falsely reported as methicillin susceptible.[23]

Vancomycin treatment failures of *S. aureus* infections have been reported repeatedly, even before the description of VISA and hVISA.[24] These were often attributed to the slow bactericidal effect of the drug[24] and/or to the severity of the patients' underlying conditions.[25] However, knowing the difficulty of hVISA and VISA detection, the question now arises as to whether some of these vancomycin failures were not due to the lack of identification of such organisms. The present experiments addressed this hypothesis both by studying the natural history of hVISA and VISA emergence during vancomycin treatment of experimental endocarditis and by testing the ability of standard culture-based laboratory procedures to detect the emerging hVISA or VISA. We also examined the ability of such selected hVISA or VISA to persist after treatment discontinuation.

## Materials and methods

### Microorganisms and growth conditions

A vancomycin-susceptible MRSA (strain M1V2, vancomycin MIC of 2 mg/L) recovered from a patient with endocarditis was used in animal experiments. For certain experiments, strain M1V2 was passaged with vancomycin in the laboratory, as described previously,[26] to generate the isogenic VISA M1V8 (vancomycin MIC of 8 mg/L). Additional reference strains used for susceptibility testing and *agr* typing included *S. aureus* ATCC 29213 (vancomycin susceptible), hVISA Mu3, VISA Mu50 (both generously provided by K. Hiramatsu, Juntendo University, Tokyo, Japan), *S. aureus* RN6390 (*agr* group I), RN6607 (*agr* group II), RN8465 (*agr* group III), RN4850 (*agr* group IV) and RN6911 (null *agr*) (all generously provided by F. Vandenesch, Lyon, France).[27,28] Unless otherwise stated, all the organisms were grown at 37°C either on brain heart infusion (BHI) agar plates (Becton Dickinson, Sparks, MD, USA) or in BHI broth (Becton Dickinson) with shaking at 120 rpm. Stocks were kept at −70°C in BHI broth supplemented with 10% (v/v) glycerol.

### Bacterial typing

Clonal identity between the parent M1V2 and *S. aureus* colonies recovered from vegetations of rats with treatment failures was assessed by PFGE using a CHEF-DR II apparatus (Bio-Rad Laboratories, Hercules, MA, USA), as described previously.[29] The *agr* group of the parent and control strains was determined by PCR using previously described primers.[27,28,30]

### Antimicrobial susceptibility testing

MICs were determined by the broth macrodilution method in cation-adjusted Mueller–Hinton broth (Becton Dickinson) according to CLSI standards, with an inoculum of $10^5$–$10^6$ cfu/mL, and by Etest on BHI agar plates, by applying an inoculum with a turbidity equivalent to that of a 2 McFarland standard.[31] The MIC was defined as the lowest concentration of antibiotic that yielded no visible growth after 24 h of incubation at 37°C. Time–kill studies were performed by adding vancomycin to mid-logarithmic phase cultures at final concentrations mimicking peak (40 mg/L) and trough (5 mg/L) drug concentrations achieved in the serum of humans during therapy. Samples were removed 0, 6, 12 and 24 h after drug addition, serially diluted and spread on agar plates. Colony counts were determined after 48 h of incubation at 37°C.

### In vitro population analysis

Population analyses were carried out by spreading serial dilutions of an overnight culture (~$10^9$ cfu/mL) of the test strains on agar plates containing arithmetic progressions of vancomycin concentrations from 1 to 8 mg/L. Colonies were counted after 48 h of incubation at 37°C. Population analysis curves were drawn by plotting the numbers of colonies growing on the plates against the concentrations of vancomycin in the plates.

### Production of endocarditis and installation of an infusion pump device

All animal experiments were carried out according to the Swiss Federal Act on Animal Protection. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Consumer and Veterinary Affairs Department of the State of Vaud (permit number 879.8). Induction of sterile aortic vegetations and installation of the infusion pump to deliver vancomycin were performed as previously described.[32,33] Infective endocarditis was induced 24 h later by intravenous (iv) challenge of the animals with 0.5 mL of saline containing $10^5$ cfu of the vancomycin-susceptible parent strain M1V2. This inoculum corresponded to 10 times the minimum size of bacterial inoculum producing endocarditis in ≥90% of the rats.

### Vancomycin treatment of experimental endocarditis

Treatment was started 18 h after inoculation and lasted for 2 days. Treated animals received doses of vancomycin that mimicked the pharmacokinetics seen in humans following iv administration of 1 g of the drug every 12 h.[34] Control animals received saline. Rates and severity of valve infection were determined in animals killed either just before treatment onset, in order to determine the vegetation load at the start of therapy (control rats), or 8 h (early evaluation) or 72 h (late evaluation) after the end of therapy. As an additional control, a few untreated animals were followed over the entire experiment and killed in parallel to the early and late evaluation timepoints. The vegetations were dissected under sterile conditions, weighed, homogenized in 1 mL of saline, serially diluted and plated for colony counts. The limit of detection was 2 $\log_{10}$ cfu/g of vegetation.

### *Pharmacokinetic studies*

The concentration of vancomycin in the serum of rats was determined by an agar diffusion assay with antibiotic medium 1 (Difco) and *Bacillus subtilis* ATCC 6633 as the indicator organism. Standard curves were determined using pooled rat serum. The limit of detection of the assay was 0.7 mg/L. The linearities of the standard curves were assessed with a regression coefficient of $\geq$0.994. Intraplate and interplate variations were $\leq$10%.

### *Ex vivo population analysis*

The possible emergence of hVISA or VISA in animals was assessed either by direct plating of vegetation homogenates on agar containing serial dilutions of vancomycin or by plating the homogenates after overnight growth in antibiotic-free broth. For direct plating, 0.01 mL aliquots of the homogenates, plus serial dilutions of them, were used. For plating after overnight regrowth, 0.01 mL aliquots of the same homogenates were inoculated into 10 mL of drug-free broth and incubated overnight before processing as above. Colonies that grew on plates containing 8 mg/L vancomycin were picked at random and retested for vancomycin MIC.

### *In vitro and in vivo persistence of the vancomycin intermediate-resistant phenotype*

The *in vitro* and *in vivo* persistence of subpopulations with decreased susceptibility to vancomycin was tested using a laboratory-generated VISA derivative of parent M1V2, strain M1V8. For *in vitro* persistence examination, stationary phase cultures of the susceptible M1V2 strain and of its vancomycin-passaged VISA derivative M1V8 (vancomycin MIC of 8 mg/L) were left to stand at 37°C for 15 days. At several timepoints during this period, aliquots were removed and plated on agar containing increasing concentrations of vancomycin. To test the persistence of the VISA phenotype *in vivo*, animals with catheter-induced aortic vegetations were infected with either the vancomycin-susceptible M1V2 or its VISA derivative M1V8 and followed without therapy for 7 days. Animals were killed at several timepoints; their vegetations were processed as above and directly plated on vancomycin-containing agar.

### *Statistical analysis*

The rates of valve infections of the various groups were compared by the Fisher's exact test.

## Results

### *agr type and vancomycin susceptibility profile*

Test strain M1V2 was *agr* group II, which is a frequent *agr* type associated with vancomycin-intermediate resistance and vancomycin treatment failure.[35,36] Its basal MIC of vancomycin was 2 mg/L, as measured both by broth dilution and Etest. Accordingly, population analysis profiles indicated that the entire population of M1V2 was inhibited by 4 mg/L vancomycin and contained no resistant subpopulations (Figure 1). In contrast, its vancomycin-cycled derivative M1V8 had a vancomycin MIC of 8 mg/L and behaved like a true VISA in population analyses (Figure 1). In time–kill studies, parent M1V2 lost 2–2.5 $\log_{10}$ cfu/mL after 24 h of exposure to vancomycin concentrations mimicking peak and trough concentrations of the drug in the blood, i.e. 40 and 5 mg/L, respectively, whereas VISA M1V8 resisted killing and lost $\leq$1 $\log_{10}$ cfu/mL at 24 h in the same conditions (data not presented).

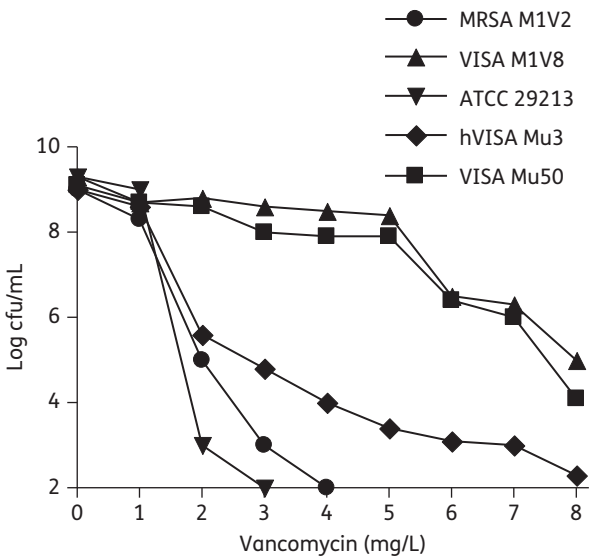


**Figure 1.** Population analyses of vancomycin-susceptible MRSA M1V2 parent strain and its VISA M1V8 derivative. *S. aureus* ATCC 29213, hVISA Mu3 and VISA Mu50 were used as reference strains. The curves are representative of at least two experiments with each strain. Population analysis was done with strains after overnight culture at 37°C. Cultures were serially diluted and plated on various concentrations of vancomycin-containing agar medium.

### *Pharmacokinetic studies*

The peak (30 min after drug injection) and trough (12 h after drug injection) concentrations (mean $\pm$ SD for three to six individual animals) of vancomycin were 42.2 $\pm$ 3.7 and 8.8 $\pm$ 2.3 mg/L, respectively. These concentrations of vancomycin in the serum of rats were very close to the peak and trough vancomycin concentrations previously reported in the same experimental model and in humans.[26,34]

### *Experimental endocarditis*

Figure 2 depicts the natural outcome of valve infection and vancomycin therapy in rats inoculated with the susceptible parent M1V2. The median vegetation bacterial density in untreated control animals was 8.3 $\log_{10}$ cfu/g at treatment onset and increased progressively to 11.3 $\log_{10}$ cfu/g (left panel of Figure 2). In treated animals the outcome of valve infection was treatment success in 14 of 26 (54%) animals and treatment failures in 12 of 26 (46%) animals (right panel of Figure 2). PFGE showed the M1V2 strain used for challenge and the vegetation isolates were indistinguishable, confirming that they had the same origin (data not presented).

### *Population analysis profiles of vegetation homogenates plated directly or indirectly on vancomycin-containing agar*

Figures 3 and 4 depict the crude data of these analyses. No subpopulations with intermediate resistance to vancomycin (determined by the absence of growth on agar plates containing $\geq$4 mg/L vancomycin) were detected in vegetations plated

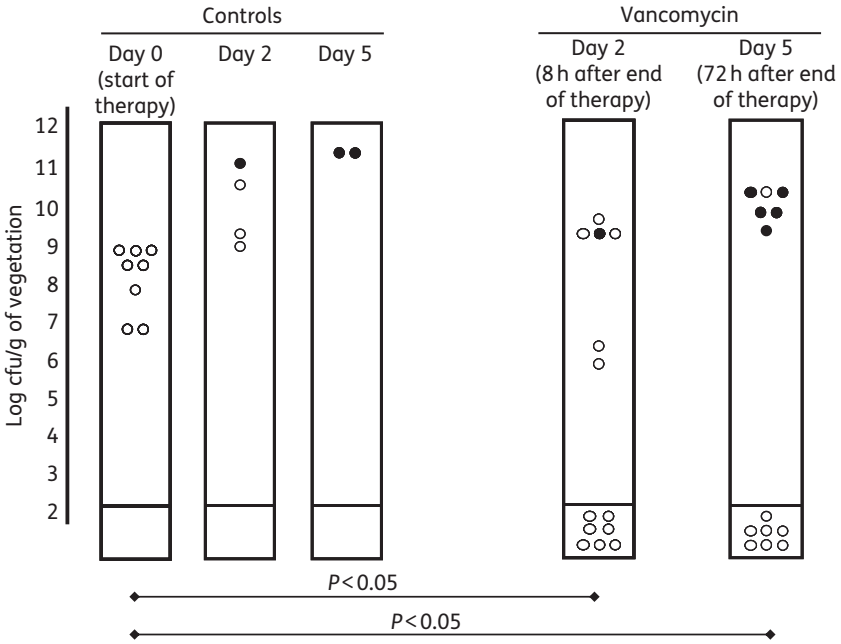


**Figure 2.** Therapeutic results for experimental endocarditis due to vancomycin-susceptible MRSA M1V2. Vancomycin therapy was started 18 h after bacterial challenge and lasted for 48 h. Control animals were sacrificed at the onset of therapy (day 0). Vancomycin-treated animals were sacrificed 8 and 72 h after the end of therapy (days 2 and 5, respectively). Each circle above the bars at 2 log cfu/g represents the bacterial density in the vegetation of a single animal. Circles under the bars represent culture-negative vegetations. Filled circles indicate vegetations that grew staphylococcal colonies on plates containing ≥4 mg/L vancomycin. $P < 0.05$ by the Fisher's exact test.

directly at treatment onset, when vegetation bacterial densities were $<9$ $\log_{10}$ cfu/g (data not shown). On the other hand, few colonies growing on increased vancomycin concentrations (i.e. 4 and 8 mg/L) appeared in untreated control animals (one of four rats killed after 2 days of infection and two of two rats killed after 5 days of infection; marked in black in Figure 2), when their vegetation counts reached $\geq 11$ $\log_{10}$ cfu/g, corresponding to resistance rates of $\sim 10^{-5}$ to $10^{-7}$ (Figure 3a and b). In contrast, when population analyses were repeated after overnight regrowth in drug-free broth, no intermediate-resistant subpopulations were detected (Figure 3c and d). Thus, putative hVISA were already detected in the vegetations of untreated rats, provided that they contained large bacterial densities ($\sim 10^{11}$ cfu/g) and that they were plated directly on vancomycin-containing agar without prior growth in drug-free medium.

After 2 days of vancomycin treatment the frequency of such intermediate-resistant subpopulations had increased ~100-fold (corresponding to rates of $10^{-3}$ to $10^{-5}$) when vegetation homogenates were plated directly (Figure 4a and b). Moreover, these intermediate-resistant subpopulations persisted after treatment arrest (Figure 4b). On the other hand, population analysis profiles returned to a vancomycin-susceptible profile when homogenates were regrown overnight in drug-free medium before testing (Figure 4c and d).

### *In vitro versus in vivo persistence of vancomycin intermediate-resistant subpopulations*

The observations described above indicate that the vancomycin intermediate-resistant subpopulations could persist *in vivo* in the absence of vancomycin pressure, but were unstable *in vitro* after one passage in drug-free broth. To study this *in vitro*/*in vivo* discrepancy further, the vancomycin-susceptible M1V2 and its VISA derivative M1V8 (Figure 1) were inoculated *in vitro* and *in vivo* in drug-free conditions and the bacterial resistance phenotypes were followed over time.

For *in vitro* experiments, $10^6$ cfu of both M1V2 and VISA M1V8 were inoculated in 10 mL of drug-free BHI and incubated with shaking at 37°C for 15 days. At several timepoints, samples of the cultures were serially diluted and plated on both plain agar and plates containing 4 or 8 mg/L vancomycin. Figure 5(a) shows that bacterial counts on plain agar were stable over the entire period of time. In contrast, in the VISA M1V8 the proportion of bacteria able to grow on 4 or 8 mg/L vancomycin declined progressively by up to 4 $\log_{10}$ cfu.

For *in vivo* tests, rats with sterile aortic vegetations were inoculated with 10 times the $ID_{90}$ of either strain and were killed at various times over a follow-up period of 7 days. Their vegetations were processed as described and plated directly on plain agar and on plates containing 4 or 8 mg/L vancomycin. As for *in vitro* cultures, the entire populations persisted when assessed on plain agar. On the other hand, the proportion of intermediate-resistant colonies of VISA M1V8 remained stable through the 7 days of follow up (Figure 5b). These experiments further suggest that some *in vivo* conditions may promote the stability and persistence of hVISA and VISA subpopulations.

## Discussion

The capacity of vancomycin therapy to select for hVISA and their detection were investigated in rats with experimental endocarditis due to a vancomycin-susceptible MRSA. Several interesting

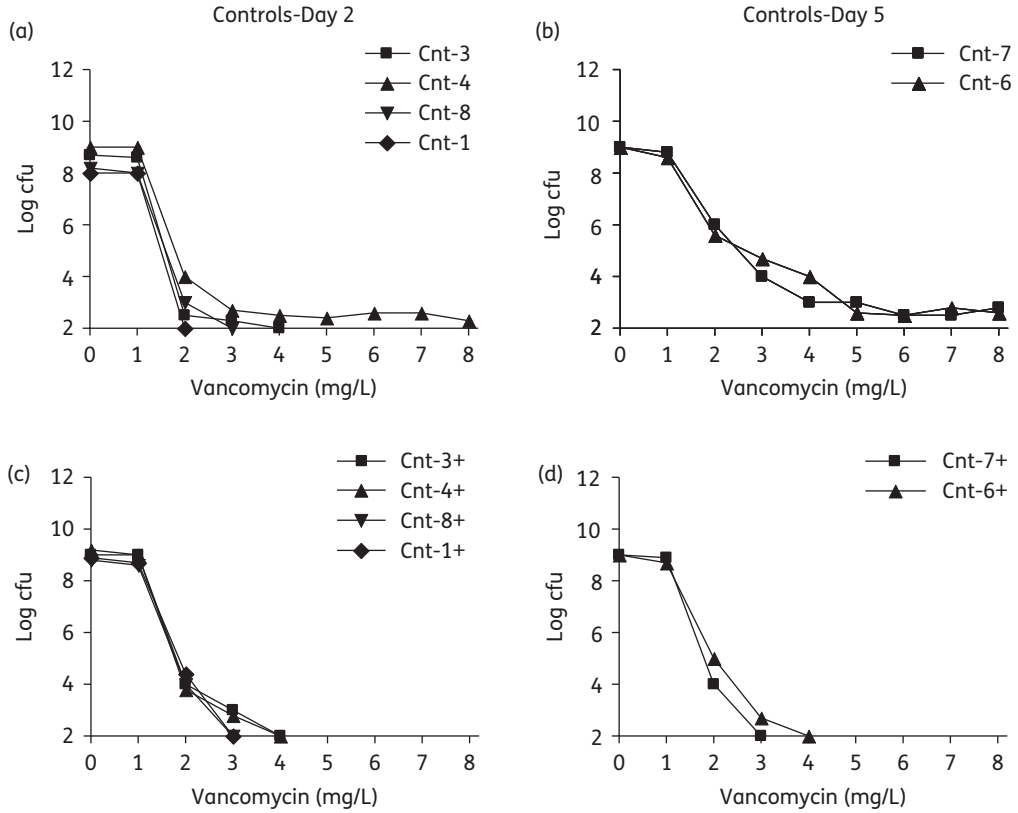


**Figure 3.** *Ex vivo* population analysis performed with vegetation homogenates of animals infected with the vancomycin-susceptible MRSA M1V2 strain and left untreated for 2 or 5 days (also see Figure 1). Population analysis was performed with vegetation homogenates both directly (a and b) and after one subculture of the homogenate on vancomycin-free broth (c and d), in order to mimic laboratory conditions when susceptibility tests are performed. When population analysis was done directly, hVISA emerged from vegetation homogenates of each group of animals. However, after a single passage on drug-free medium the hVISA phenotype reverted to the susceptible pattern of the parent. Cnt, control. Cnt-<number> indicates animal vegetation homogenate plated directly on vancomycin-containing agar plates. Cnt-<number>+ indicates animal vegetation homogenate plated on vancomycin-containing agar plates after one passage in drug-free medium.

observations were made. First, using population analysis as the screening method, we demonstrated that treatment of vancomycin-susceptible MRSA with vancomycin at doses simulating human kinetics resulted in the selection of hVISA in animals with infective endocarditis. This occurred in spite of the fact that serum concentrations of vancomycin (mean peak and trough concentrations of vancomycin in the serum of 42.2 and 8.8 mg/L, respectively) were constantly above the MIC for the infecting organism (no growth above 4 mg/L in population analysis). Moreover, the selected hVISA were associated with vancomycin treatment failure. Second, hVISA could be detected in infected vegetations only if population analysis was performed directly from the homogenate. Population analysis done after a single passage of the tissue in drug-free broth, simulating the conditions in the laboratory when processing clinical specimens, resulted in the disappearance of the hVISA phenotype. This could have occurred by spontaneous reversion of the phenotype to that of the susceptible parent strain by a dilution effect upon subculture in drug-free medium, as observed previously.[37] When hVISA colonies from infected vegetations were picked from plates containing 8 mg/L vancomycin, regrown in drug-free broth and retested for their susceptibility to vancomycin, the MIC of the drug for the test organism was 2 mg/L, thus formally considered as susceptible (data not shown). This further indicates that even the confirmation as hVISA of *S. aureus* colonies growing on plates supplemented with vancomycin could be problematic if standard susceptibility techniques requiring dilution are used.

*S. aureus* colonies growing on plates containing 8 mg/L vancomycin were also recovered from some vegetations of animals left untreated throughout the experiment (2–5 days), provided that they had high (~11 $\log_{10}$ cfu/g) vegetation bacterial titres. The emergence of glycopeptide-resistant subpopulations in the absence of antibiotic pressure has been reported in a rat model of chronic tissue cage infection with *S. aureus*, when high bacterial densities were reached at the infected site,[38] as well in humans.[39] This 'inoculum' effect was also apparent in population analysis performed *in vitro*, when bacterial densities of strain M1V2 reached 11 $\log_{10}$ cfu/mL. In contrast, the proportion of hVISA was greatly increased in vancomycin-treated animals, and resistant variants grew readily from vegetations containing much lower bacterial densities (9–10 $\log_{10}$ cfu/g) and at ~100-fold higher frequency. This suggests that rare *S. aureus* cells expressing the heterogeneous vancomycin resistance trait pre-existed at very low, and thus undetectable, frequencies before antibiotic treatment, and were readily selected by vancomycin therapy.

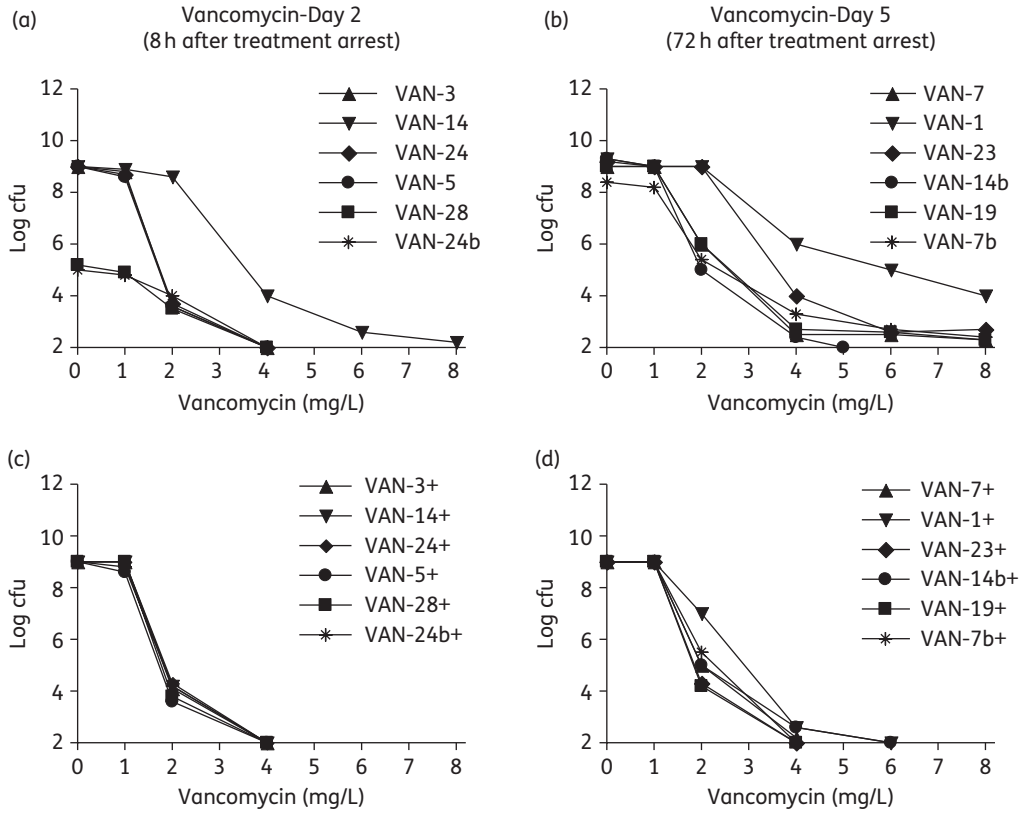


**Figure 4.** *Ex vivo* population analysis performed with vegetation homogenates of animals infected with the vancomycin-susceptible MRSA M1V2 strain, treated with vancomycin for 2 days and sacrificed 8 h (day 2) or 72 h (day 5) after treatment arrest (also see Figure 1). Population analysis was performed with vegetation homogenates both directly (a and b) and after one subculture of the homogenate on vancomycin-free broth (c and d), in order to mimic laboratory conditions when susceptibility tests are performed. When population analysis was done directly, hVISA emerged from vegetation homogenates of each group of animals. However, after a single passage on drug-free medium the hVISA phenotype reverted to the susceptible pattern of the parent. VAN, vancomycin. VAN-<number> indicates animal vegetation homogenate plated directly on vancomycin-containing agar plates. VAN-<number>+ indicates animal vegetation homogenate plated on vancomycin-containing agar plates after one passage in drug-free medium.

It has been proposed that inoculation of vancomycin-containing plates with heavy bacterial densities (11 $\log_{10}$ cfu) could result in vancomycin absorption by bacterial cell walls, thus allowing the growth of colonies that were not genuinely resistant to the drug.[40] This was not likely to be the case in the present study, and should alert investigators against the reassuring impression of rare false-positive resistance phenotypes when using large inocula. It is more likely that these rare phenotypes represent genuine heteroresistant variants that are hard to isolate, especially when regrown in drug-free medium during the isolation process, because they become overgrown by susceptible populations. These data, however, may potentially be influenced by different variables. First, the bacterium used in these experiments had an *agr* type II background, which has been shown to be more prone to develop glycopeptide heteroresistance than other *agr* types.[35,36] Whether such pre-existing cells are also present in other *agr* groups remains to be clarified. Second, the presence of a heterogeneous subpopulation with intermediate resistance to vancomycin is more likely in MRSA with vancomycin MIC of 2 mg/L than in MRSA with vancomycin MIC of $\leq$1 mg/L.[41]

Finally, another important observation of the present experiments was that the hVISA subpopulations selected during vancomycin therapy persisted *in vivo* in the absence of antibiotic pressure. Indeed, while hVISA disappeared after one passage on drug-free media, they could readily persist in the vegetations, in the absence of vancomycin selection, for at least 3 days after the arrest of therapy. The contrast between the instability of the hVISA phenotype in prolonged liquid culture *in vitro* and their persistence *in vivo* is unexplained. After inoculation, the organisms grew to the stationary phase in both conditions (Figure 5). Thereafter, *in vitro* cultures tended to lose viable counts and hVISA decreased more rapidly than susceptible cells, whereas *in vivo* cultures remained stable and kept their original proportion of total cfu and hVISA subpopulations. A major characteristic of VISA strains is cell wall thickening, which is related to an activated cell wall biosynthetic pathway and nutrient transport system.[42] It has been suggested that cell wall thickness in hVISA depends on the concentrations of nutrients in the environment.[42] Therefore, one can hypothesize that the progressive disappearance of hVISA *in vitro* is associated with deprivation of the culture medium of metabolites necessary for cell wall synthesis. In contrast, *in vivo*, nutrients would always be available to construct a thick cell wall, thus maintaining the expression of the hVISA phenotype. Alternatively, hVISA could persist *in vivo* using the same strategy as that used by small colony variants

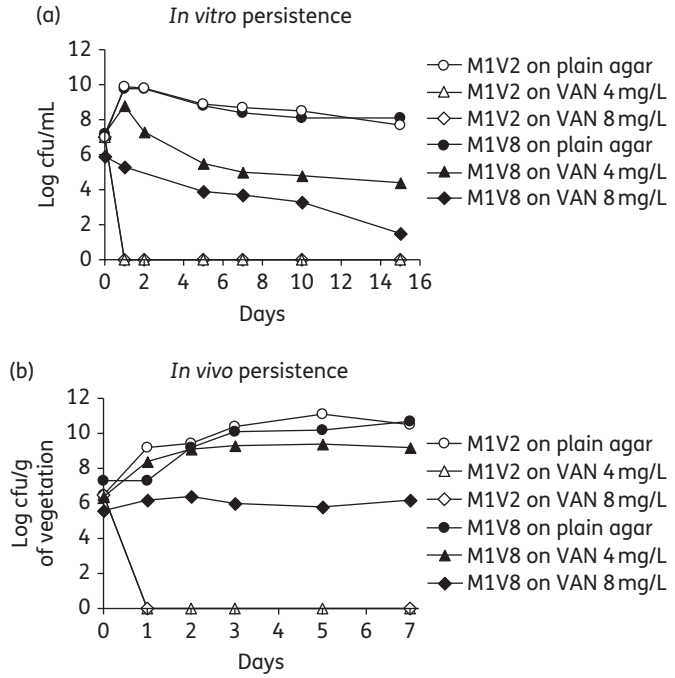


**Figure 5.** *In vitro* (a) and *in vivo* (b) persistence of parent strain M1V2 and its VISA M1V8 mutant in the absence of vancomycin pressure. For the *in vitro* test, 10 mL of drug-free BHI was inoculated with $10^6$ of either the parent M1V2 or the VISA M1V8 mutant and incubated with shaking at 37°C for 15 days. At several timepoints, a sample of each culture was serially diluted and plated either onto plain agar plates or onto agar plates containing vancomycin at 4 or 8 mg/L for colony counts. For the *in vivo* test, rats with sterile aortic vegetations were inoculated with either $10^5$ cfu (parent M1V2) or $10^6$ cfu (VISA M1V8). At different times after challenge, aortic vegetations were removed, serially diluted and plated onto either plain agar or agar plates containing vancomycin at 4 or 8 mg/L for colony counts. Values represent the mean of three or four different animals. *In vitro* (a), the more resistant subpopulations of VISA M1V8 (able to grow on 4 and 8 mg/L vancomycin) progressively disappeared during the culture period. In sharp contrast, in aortic vegetations (b) the subpopulations of VISA M1V8 remained stable for up to 7 days. VAN, vancomycin.

(SCVs) of *S. aureus*. Previous studies have suggested that SCVs of *S. aureus* display slow growth and reduced haemolytic activity.[43] As a consequence, they have a lower ability to induce cell lysis and are able to persist intracellularly, where they are protected from the host's defences.[43,44] Like SCVs, hVISA are characterized by their slow growth and reduced haemolytic activity.[4] Moreover, hVISA may produce increased expression of fibronectin-binding proteins, which are known to promote endothelial uptake.[45,46] Thus, hVISA could easily enter the endothelial cell, which would provide a niche for their survival.

Detection of hVISA is difficult and different methods and protocols have been proposed to recognize them.[4] The present results indicated that vancomycin induced the emergence of hVISA *in vivo*, but also that appropriate culture techniques, i.e. spreading infected organs directly on drug-containing media, should be used to detect them. Thus, we provide an additional explanation for the elusive nature of hVISA strains in the diagnostic laboratory, where the clinical samples, and even the colonies growing on vancomycin-containing agar plates, are usually subcultured in drug-free medium before processing for susceptibility tests. These results suggest that, in humans, therapeutic failures with vancomycin against supposedly susceptible *S. aureus* infection could indeed be the result of hVISA selected during therapy but remaining undiagnosed. Moreover, the present experiments also indicated that the hVISA phenotype is stable *in vivo* in the absence of antibiotic pressure. These findings are clinically relevant because if hVISA are undetected *in vitro* while they persist in the host, continuing vancomycin therapy might promote the selection of more resistant, stable VISA in patients.

The search for potential markers that are specifically expressed by hVISA strains regardless of their genetic background is warranted. In exploratory experiments we tested whether *in situ* imaging or maybe cell size determination would be of any help, using, for instance, transmission electron microscopy of infected vegetations (Figure S1, available as Supplementary data at *JAC* Online). However, although thickening of cell walls was suggested in some micrographs, it was by no means a reliable diagnostic tool due to the heterogeneity of the microbial population. Thus, other markers, which might be either phenotypic or genotypic, must be sought.

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## Transparency declarations

None to declare.

## Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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