

# Vancomycin-resistant *Staphylococcus aureus*: a new model of antibiotic resistance

Keiichi Hiramatsu

Vancomycin has been the most reliable therapeutic agent against infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). However, in 1996 the first MRSA to acquire resistance to vancomycin, was isolated from a Japanese patient. The patient had contracted a post-operative wound infection that was refractory to long-term vancomycin therapy. Subsequent isolation of several vancomycin resistant *S aureus* (VRSA) strains from USA, France, Korea, South Africa, and Brazil has confirmed that emergence of vancomycin resistance in *S aureus* is a global issue. A certain group of *S aureus*, designated hetero-VRSA, frequently generate VRSA upon exposure to vancomycin, and are associated with infections that are potentially refractory to vancomycin therapy. Presence of hetero-VRSA may be an important indicator of the insidious decline of the clinical effectiveness of vancomycin in the hospitals. Vancomycin resistance is acquired by mutation and thickening of cell wall due to accumulation of excess amounts of peptidoglycan. This seems to be a common resistance mechanism for all VRSA strains isolated in the world so far.

*Lancet Infectious Diseases* 2001; **1**: 147–155

Methicillin-resistant *Staphylococcus aureus* (MRSA) has occurred in many countries since its discovery in 1961.<sup>1</sup> However, in recent years, clinicians have been concerned by the increased frequency of MRSA infections.<sup>2</sup> This resurging MRSA problem seems to be based on the lack of potent therapeutic agents having an unequivocal cell-killing effect, and thus capable of eliminating MRSA from the patient's body. Increased use of vancomycin—a drug with rather weak cell-killing potency against prevailing MRSA—seems to have set a basis for the selection of vancomycin resistance in MRSA. In 1997, we reported the first MRSA strains with reduced susceptibility to vancomycin, which were isolated from patients in whom vancomycin therapy was ineffective.<sup>3,4</sup>

We reported two classes of vancomycin-resistant strains: vancomycin-resistant *S aureus* (VRSA) that has a vancomycin minimum inhibitory concentration (MIC) of 8 mg/L, and hetero-VRSA that spontaneously generates VRSA within the cell population. The nomenclature is based on the MIC breakpoints of the British Society for Antimicrobial Chemotherapy who define the MIC of 8 mg/L as “resistant.” However according to the National Committee for Clinical Laboratory Standards (NCCLS) breakpoint, these strains are called vancomycin-

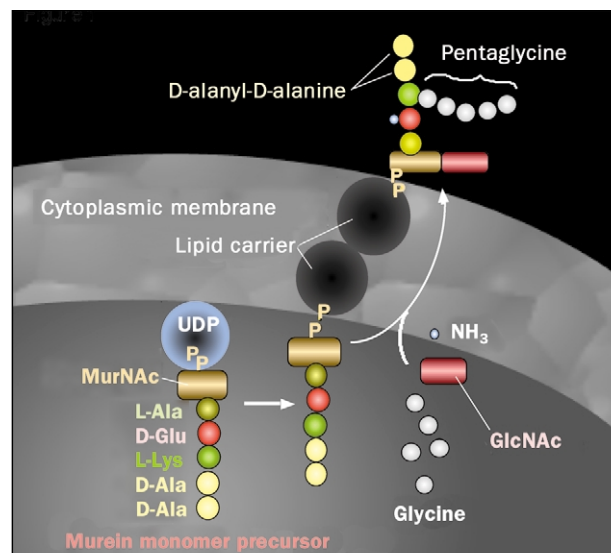


Figure 1. Synthesis of murein monomer (monomeric component of peptidoglycan). Murein monomer is composed of two amino sugars (N-acetyl muramic acid [MurNAc] and N-acetyl glucosamine [GlcNAc]) and ten amino acids. Murein monomer precursor is composed of MurNAc and stem peptides (L-alanine, D-glutamic acid, L-lysine, and two D-alanines). It is synthesised in the cytoplasm and attaches to a lipid carrier in the cytoplasmic membrane. Then, during its transfer to the outer surface of the cytoplasmic membrane, GlcNAc and five glycines are added, and its isoglutaric acid is amidated to become mature murein monomer.

intermediate *S aureus* (VISA) or glycopeptide-intermediate *S aureus* (GISA) in the USA.<sup>5</sup> Although hetero-VRSA is categorised as “susceptible” to vancomycin based on current MIC breakpoints, it generates VRSA cells at a high frequency within its cell population.

To date, as well as Japan, VRSA strains have been isolated from USA, France, Korea, South Africa, Brazil, and Scotland.<sup>6–11</sup> In addition hetero-VRSA strains have been reported from many more countries, indicating that the problem is a global one.<sup>12–16</sup> In this review, the mechanism of glycopeptide resistance in *S aureus* primarily based on the analyses of clinical strains will be summarised. The viewpoint that hetero-VRSA constitutes a precursor stage to vancomycin resistance, and that the emergence of VRSA is an outcome of the prevalence of hetero-VRSA will be explained.

**Correspondence:** Professor Keiichi Hiramatsu, Department of Bacteriology, Juntendo University, 2-1-1 Bunkyo-ku, Tokyo, Japan 113-8421. Tel +81 3 5802 1040; fax +81 3 5684 7830; email: hiram@med.juntendo.ac.jp

## The mechanism of vancomycin resistance

### Cell-wall peptidoglycan synthesis

Both beta-lactam and glycopeptide (including vancomycin and teicoplanin) antibiotics exert their antimicrobial effects by inhibiting the cell-wall synthesis of *S aureus*. The cell has a high osmotic pressure ( $10\cdot13\text{--}20\cdot26 \times 10^5$  Pa). For *S aureus* cells to multiply in an environment with a lower external pressure, they must keep synthesising a strong extracellular structure called peptidoglycan (or murein) to prevent the cells from rupturing. To produce peptidoglycan, its monomeric component (murein monomer) must be synthesised inside the cell, and transferred to the outside by lipid carriers present in the cytoplasmic membrane (figure 1).

Two enzymes located in the cytoplasmic membrane, glycosyltransferase and transpeptidase, assemble the murein monomer into a gigantic structure of peptidoglycan (figure 2). Glycosyltransferase polymerises murein monomers between their amino-sugar moieties to produce nascent peptidoglycan chains. Then, transpeptidase, also known as penicillin-binding protein (PBP), links the newly formed nascent peptidoglycan chains to pre-existing peptidoglycan layers of the *S aureus* cells. In this step, PBP recognises D-alanyl-D-alanine residues of murein monomer, and cuts in between the two D-alanines and ligates penultimate D-alanine to the tip of a pentaglycine chain protruding from pre-existing peptidoglycan layers (figure 2). When the inter-peptide bridge is formed, the terminal D-alanine of the murein monomer is lost from the completed peptidoglycan. However, it is known that about 20% of D-alanyl-D-alanine residues remain unprocessed by PBPs. As a result, as many as  $6 \times 10^6$  unprocessed D-alanyl-D-alanine residues remain in the cell wall of a single *S aureus* cell.<sup>17</sup>

PBP is the target of beta-lactam antibiotics such as penicillin. Beta-lactam is a structural analogue of D-alanyl-D-alanine, and it covalently binds to the *S aureus* PBP (depicted in red in figure 3) at its D-alanyl-D-alanine-binding pocket. This inactivates the PBP and inhibits the

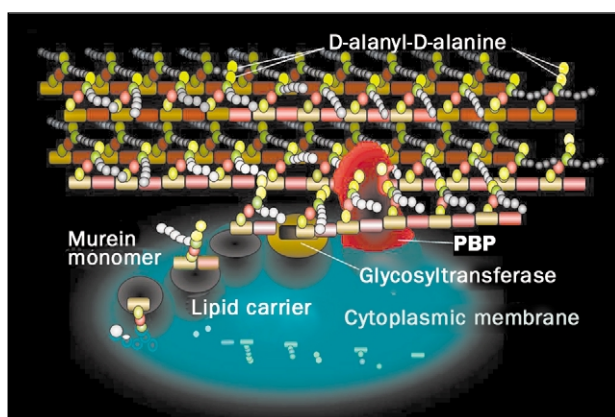


Figure 2. Assembly of peptidoglycan viewed from outside of the cell. In blue is the cytoplasmic membrane. Glycosyltransferase polymerises the murein monomer to produce a nascent peptidoglycan single chain. Penicillin-binding protein (PBP) grasps at the D-alanyl-D-alanine residues of stem peptide and cleaves in between the residues to ligate the penultimate D-alanine to the pentaglycine of the neighbouring peptidoglycan chain. The twisting of peptidoglycan chains is omitted from the illustration for visual simplicity.

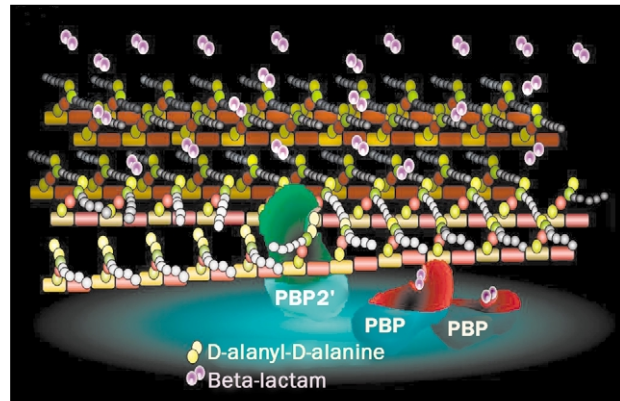


Figure 3. Action of beta-lactam: Beta-lactam (purple double cubes) is a structural analogue of D-alanyl-D-alanine residues. It inactivates *S aureus* PBPs (in red), but cannot bind to PBP2' (in green; MRSA-specific PBP) with high affinity. Therefore, MRSA can continue peptidoglycan synthesis in the presence of beta-lactams whereas methicillin-susceptible *S aureus* cannot.

cross-bridge formation step of peptidoglycan synthesis, causing the cell to rupture from the peptidoglycan mesh. However, MRSA produces a unique PBP, designated PBP2' (or PBP2A; in green in figure 3), which has an extremely low binding affinity to beta-lactam antibiotics.<sup>18–20</sup> As a result, the PBP2' can keep on synthesising the peptidoglycan even in the presence of beta-lactam antibiotics. This is the basis of beta-lactam resistance of MRSA. The unique PBP2' is the product of the exogenous gene called *mecA* carried by a mobile genetic element, *SCCmec*, which *S aureus* has acquired from an as yet unknown bacterial species by lateral gene transfer.<sup>21</sup>

### Glycopeptides inhibition of transpeptidation and nascent peptidoglycan synthesis

By contrast with beta-lactams, glycopeptides bind to D-alanyl-D-alanine residues of the murein monomer (figure 4). There are two classes of binding targets in the *S aureus* cell: firstly, D-alanyl-D-alanine residues in the completed peptidoglycan layers or on the nascent peptidoglycan chain; and secondly, the murein monomers located in the cytoplasmic membrane that serves as the substrates for glycosyltransferase (figure 4). The binding of glycopeptides to the former targets does not inhibit nascent peptidoglycan synthesis, though it may interfere with cross-bridge formation mediated by PBPs. This may be the reason why teicoplanin is synergistic with beta-lactam antibiotics. If glycopeptides bind to murein monomers in the cytoplasmic membrane, peptidoglycan synthesis is completely inhibited, and the cells cease to multiply. However, for the glycopeptide molecules to bind to such targets, they have to pass through about 20 peptidoglycan layers (only two layers are drawn in figures 2–4) without being trapped by the first targets. Since there are many D-alanyl-D-alanine targets in the peptidoglycan layers, many glycopeptide molecules are trapped in the peptidoglycan layers. This compromises the therapeutic effectiveness of glycopeptides. For example, if high numbers of *S aureus* cells are present in the infected tissue of the patient, many glycopeptide molecules will be adsorbed to their cell walls, and tissue concentrations will be

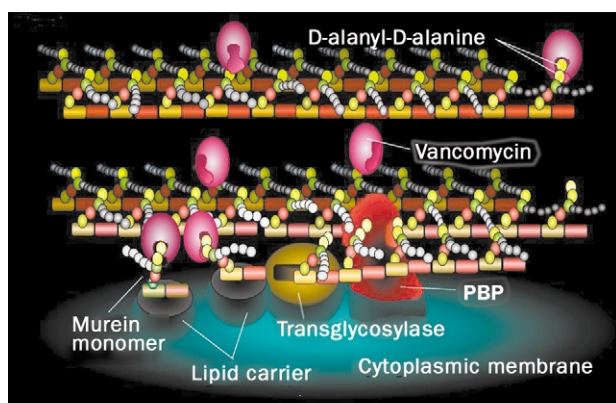


Figure 4. Action of vancomycin and teicoplanin. Drug binds to D-alanyl-D-alanine residues of murein monomer. The murein monomer bound by vancomycin does not serve as a substrate for glycosyltransferase.

lower than the required therapeutic threshold. Therefore, measures to decrease bacterial cell numbers in the patient's body by surgical elimination of an abscess or by drainage of pus would frequently be required to make glycopeptide therapy more effective. For the same reason, accurate susceptibility testing of glycopeptides are much more difficult to do than with other antibiotics, because variations of inoculum size (cell number) added to the broth or the agar plates containing glycopeptides can affect the free drug concentration, resulting in variations of MIC values.

#### Cell-wall thickness is a major contributor to vancomycin resistance

Mechanism of vancomycin resistance has been extensively studied with the first clinical VRSA strain, Mu50.<sup>22–24</sup> Biochemical and transmission electron microscopy (TEM) examination of the Mu50 cell, suggested that it produces increased amounts of peptidoglycan. More murein monomers and more layers (probably 30–40 layers as judged by cell-wall thickness observed with TEM) of peptidoglycan are considered to be present in the cell wall (figure 5; only three layers are drawn). As a result, more vancomycin molecules are trapped in the peptidoglycan layers before reaching the cytoplasmic membrane where peptidoglycan synthesis occurs. Moreover, a higher concentration of vancomycin would be required to saturate all the murein monomers that are supplied at an increased rate in Mu50 (figure 5). Besides the vancomycin-trapping mechanism, designated “affinity trapping,”<sup>12,13,17</sup> our recent experiments suggest that the mesh structure of the outer layers of thickened peptidoglycan is destroyed by the trapped vancomycin molecules themselves. This prevents further penetration of vancomycin molecules into the inner part of cell-wall layers (otherwise known as the “clogging” phenomenon).<sup>24</sup>

A thickened cell wall, presumably due to the accumulation of increased amounts of peptidoglycan, is the cardinal feature of all the VRSA clinical strains isolated so far from various countries. The cell walls of 16 VRSA strains isolated from seven countries are significantly thicker (mean 31.3 nm, SD 2.6 nm) than the average of vancomycin-susceptible *S. aureus* (VSSA) strains (mean 23.4 nm, SD

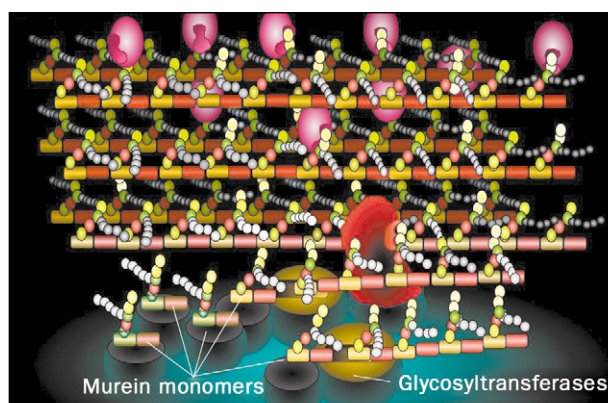


Figure 5. Thickened cell wall of Mu50. Affinity trapping mechanism of resistance Mu50 has 30–40 layers of peptidoglycan. Supply of murein monomer is increased and more monomers are incorporated into nascent peptidoglycan chains. Increased D-alanyl-D-alanine residues are present in the completed peptidoglycan layers. More vancomycin molecules are trapped in the peptidoglycan layers and less reach the cytoplasmic membrane than usual.

1.9 nm) as measured by TEM (L Cui, Department of Bacteriology, Juntendo University, Tokyo, Japan, personal communication). Revertant strains susceptible to vancomycin (MIC < 4 mg/L) were obtained from these VRSA strains. They all had decreased cell-wall thicknesses that were indistinguishable from those of VSSA strains. Furthermore, vancomycin strains that were again made resistant by vancomycin selection of susceptible revertant strains regained a thickened cell wall (L Cui, personal communication). Therefore, thickening of cell wall and vancomycin resistance are well correlated in all the VRSA strains tested, further supporting the view that thickening of the cell wall is a major contributor to vancomycin resistance.

A report maintains that strain PC-3 isolated in New York has a VRSA “normal” cell-wall thickness in spite of its vancomycin resistance.<sup>25</sup> However, the authors of this report did not measure the cell-wall thickness quantitatively with appropriate control strains.<sup>25</sup>

Theoretically, there are two different ways to thicken the cell-wall peptidoglycan layers. One is to produce excess amounts of peptidoglycan, as seen in Mu50. The other is to reduce peptidoglycan turnover. New peptidoglycan layers are always produced on the surface of the cytoplasmic membrane; they displace the older layers outwards so that they are eventually cast off from the cell surface. Autolytic enzymes (peptidoglycan hydrolysing enzymes) are involved in these shedding processes. A VRSA strain isolated from Michigan, USA, has a remarkably reduced autolytic activity that returns to normal with the loss of vancomycin resistance and reduction in the cell wall thickness (L Cui, personal communication). Therefore, the Michigan strain and Mu50 seem to employ a different strategy to achieve the same goal—ie, thickening of the cell wall.

Other factors are also known to contribute to vancomycin resistance in Mu50, though to a lesser degree than the cell-wall thickness. Enhanced supply of murein monomers in Mu50 cells is associated with a decrease in the intracellular glutamate level. Glutamine is consumed by the increased activity of one of the key enzymes (glucosamine



6-phosphate synthetase) of the murein monomer synthesis pathway.<sup>24</sup> This results in the increased synthesis of structurally altered murein monomers (the non-amidated form) that are inefficient substrates for cross-bridge formation by PBPs.<sup>23</sup> The final outcome of this sequential event is a raised proportion of D-alanyl-D-alanine residues in the peptidoglycan layers. In fact, about 2–4 times the amount of D-alanyl-D-alanine residues are found in a unit weight of purified peptidoglycan of Mu50 compared with VSSA strains.<sup>23</sup> This means that a single cell of Mu50, with its 1.5 times thickened cell wall, can trap as many as 3–6 times more vancomycin molecules than a VSSA cell. Reduced cross-linkage of peptidoglycan has also been shown in a VRSA strain obtained *in vitro*.<sup>26</sup> In this study a drastic decrease in peptidoglycan cross-linkage due to mutational inactivation of the PBP genes (PBP2' and PBP4) is associated with vancomycin resistance in a VRSA strain generated *in vitro*, called VM.<sup>26</sup> However, both the mutant strain VM and MRSA strain COL, from which the former mutant was derived, have an unusually thickened cell wall as far as we can judge from the published electron microscopy.<sup>27</sup> In our experiments, reduction of peptidoglycan cross-linking alone does not cause glycopeptide resistance.<sup>24</sup> Its contribution is effective only when the strain has a thickened cell wall. Cell wall thickening is considered the prerequisite for vancomycin resistance.

We also found that the non-amidated murein monomer has an increased binding affinity for vancomycin compared with the normal murein monomer.<sup>23</sup> Therefore, the production of the abnormal murein monomers also contributes to the vancomycin resistance of Mu50 by enhancing the affinity-trapping, and clogging the peptidoglycan mesh.<sup>24</sup>

The genetic basis for vancomycin resistance has not been elucidated yet. My research group has identified some novel genes whose expression is either increased or decreased in Mu3 and/or Mu50, compared with vancomycin-susceptible strains.<sup>28</sup> More information will become available by comparing the whole genome sequences of Mu50 and N315, (the latter is a vancomycin-susceptible Japanese MRSA strain), since the strains are closely related and only different in a few phenotypes, including vancomycin resistance.<sup>29</sup> One thing now apparent is that the *SCCmec* element carrying the *mecA* gene is not required for vancomycin resistance. The precise deletion of the element from Mu50, Mu3, and other Japanese hetero-VRSA strains did not alter the level and patterns of vancomycin resistance.<sup>22</sup> Recent isolation of a vancomycin-resistant, meticillin-susceptible strain further indicates that vancomycin resistance is not necessarily confined to MRSA.<sup>30</sup>

### Teicoplanin resistance

Teicoplanin and vancomycin belong to the glycopeptide class of antibiotics. Both exert antimicrobial activity by binding to the D-alanyl-D-alanine residue of murein monomer. Therefore, a common resistance mechanism for the two antibiotics is to be expected. In fact, all the VRSA strains analysed possess teicoplanin resistance (defined by MIC  $\geq 8$  mg/L). Cell-wall thickness also contributes to

teicoplanin resistance as expressed by the VRSA strains (MIC 8–32 mg/L) and resistance decreases when cell-wall thickness decreases. However, about half of the vancomycin-susceptible revertants of VRSA strains still maintain intermediate levels of teicoplanin resistance (MIC 8 or 16 mg/L). This finding suggests that there may be other mechanisms than cell-wall thickness for teicoplanin resistance.

In support of this suggestion is the historical overview of glycopeptide resistance in *S. aureus*.<sup>17</sup> Historically, *S. aureus* acquired teicoplanin resistance before it acquired vancomycin resistance.<sup>31,32</sup> There are quite a few MRSA strains that are resistant to teicoplanin but are still “susceptible” to vancomycin as judged by MIC values. However, acquisition of teicoplanin resistance is frequently accompanied by a small increase in vancomycin resistance; in fact, hetero-VRSA strains belong to this category of strains (see below).

Shlaes and colleagues<sup>33</sup> demonstrated that PBP2 is overproduced in a teicoplanin-resistant *S. aureus* mutant strain (MIC 16 mg/L) compared with its parent clinical strain.<sup>33</sup> Over-production of PBP2 is also observed in Mu50 and the hetero-VRSA strain Mu3—both are resistant to teicoplanin.<sup>22</sup> We demonstrated that experimental over-expression of PBP2' in a VSSA strain causes the vancomycin MIC to increase by 1 mg/L (from 1 to 2 mg/L), whereas that of teicoplanin increased significantly from 2 to 8 mg/L.<sup>22</sup> In agreement with its marginal contribution to vancomycin resistance, over-expressed PBP2' alone does not lead to cell-wall thickening. On the other hand, it increases the rate of cross-linking of cell-wall peptidoglycan (K Hiramatsu, unpublished observation). This finding highlights again the difference between the two glycopeptides. It may be that teicoplanin is more prone to inhibiting transpeptidation than vancomycin, and vancomycin more inclined to inhibit transglycosylation.

### Hetero-VRSA

#### Clinical significance of hetero-VRSA

Mutant strains having vancomycin MIC of 8 mg/L are not obtainable *in vitro* by one-step selection of vancomycin in VSSA strain. However, some Japanese clinical MRSA strains having susceptible vancomycin MIC values (<8 mg/L) generate VRSA at a resistance frequency of  $10^{-6}$  or greater.<sup>4</sup> These strains, represented by strain Mu3, are considered as precursor strains for VRSA. When strain Mu3 is grown overnight in drug-free medium to  $10^7$  cells/mL, several hundred cells are found growing in samples plated on agar plates containing 4 mg/L of vancomycin, implying that the MIC values of these cells are equal to or greater than 8 mg/L. Mu3 also contains a subpopulation of cells that are resistant to various other concentrations of vancomycin as illustrated in figure 6. Therefore, Mu3 has a “heterogeneous” population of cells with different levels of vancomycin susceptibility including vancomycin-resistant cells (MIC  $\geq 8$  mg/L). Thus, the Mu3 strain is designated hetero-VRSA.<sup>4</sup> Population analysis is the standard method for identifying hetero-VRSA.<sup>34</sup> It analyses as many as  $10^{7-9}$  CFU (colony-forming units) by contrast with about  $10^4$  CFU in standardised methods used today. Standard MIC

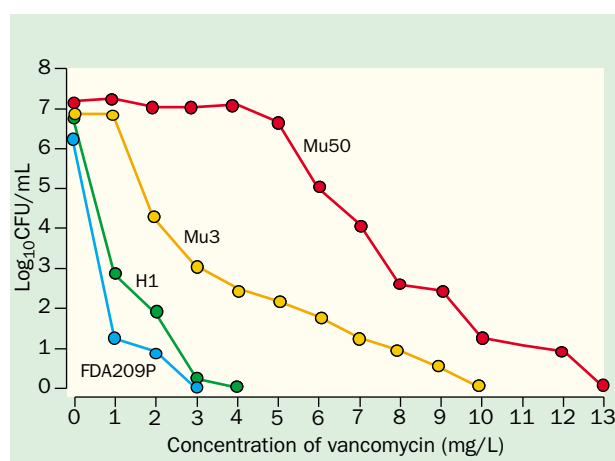


Figure 6. Population analysis of hetero-VRSA and VRSA. The population analysis shows how many cells in a fixed number of cells (usually about  $10^7$  CFU) of the strain are resistant to each of the various concentrations of antibiotics. H1 and FDA209P are vancomycin-susceptible MRSA and MSSA, respectively (both are VSSA strains). Mu50 is a VRSA strain, and 100% of the population grow in 4 mg/L of vancomycin. Mu3 is a hetero-VRSA,  $10^7$  cells/mL of which contain about 200 cells resistant to 4 mg/L of vancomycin.

methods cannot quantitatively detect the resistant cell sub-population present in hetero-VRSA strains, which constitutes only  $1/10^{5-6}$  of the entire population due to the low inoculum density used.

Conventional susceptibility tests (MIC, disk diffusion tests, &c) cannot discriminate between VSSA and hetero-VRSA.<sup>17</sup> However, the two may behave in a significantly different manner in response to vancomycin therapy. Figure 7 illustrates a test tube experiment comparing Mu3 with a VSSA strain 87/20. Mu3 has a slightly higher vancomycin

MIC value (2 mg/L) than the MRSA strain 87/20 (MIC=1 mg/L). In spite of this minor difference in MIC value, 10 mg/L of vancomycin is required to completely suppress the growth of about  $2 \times 10^6$  cells/mL of Mu3, whereas 2 mg/L was sufficient to suppress an equivalent number of cells of strain 87/20. This difference is due to a sub-population of cells in Mu3 that are resistant to vancomycin (figure 7). It is also important to observe that Mu3 cell number decreases in the initial 72 h of exposure to 5 mg/L of vancomycin, but increases substantially thereafter. This correlates with the unique clinical case from whom Mu3 was isolated, where the patient's pneumonia initially responded favorably to vancomycin therapy, but became exacerbated after the 9th day of vancomycin therapy.<sup>35</sup>

Resistance heterogeneity is not confined to vancomycin but is also well described for other antibiotics such as beta-lactams, and aminoglycosides.<sup>17</sup> In the era of antibiotic resistance, the concept of hetero-resistance is important not only for the prediction of clinical effectiveness of therapy for individual patients, but also for the detection of trends of emerging resistance. Because hetero-resistance escapes detection by conventional susceptibility tests, is not an acceptable reason to neglect its importance from a clinical and epidemiological viewpoint.

#### Biological significance of hetero-VRSA

VRSA is obtained through two-step selection of vancomycin resistance in Japanese MRSA strains (figure 8). The hetero-VRSA strains obtained by in-vitro selection with 1 mg/L of vancomycin maintained the hetero-resistance phenotype for a week's serial passage in drug-free media. However, the strains tend to lose the resistant subpopulations by the second week. This observation contrasts with the stability of

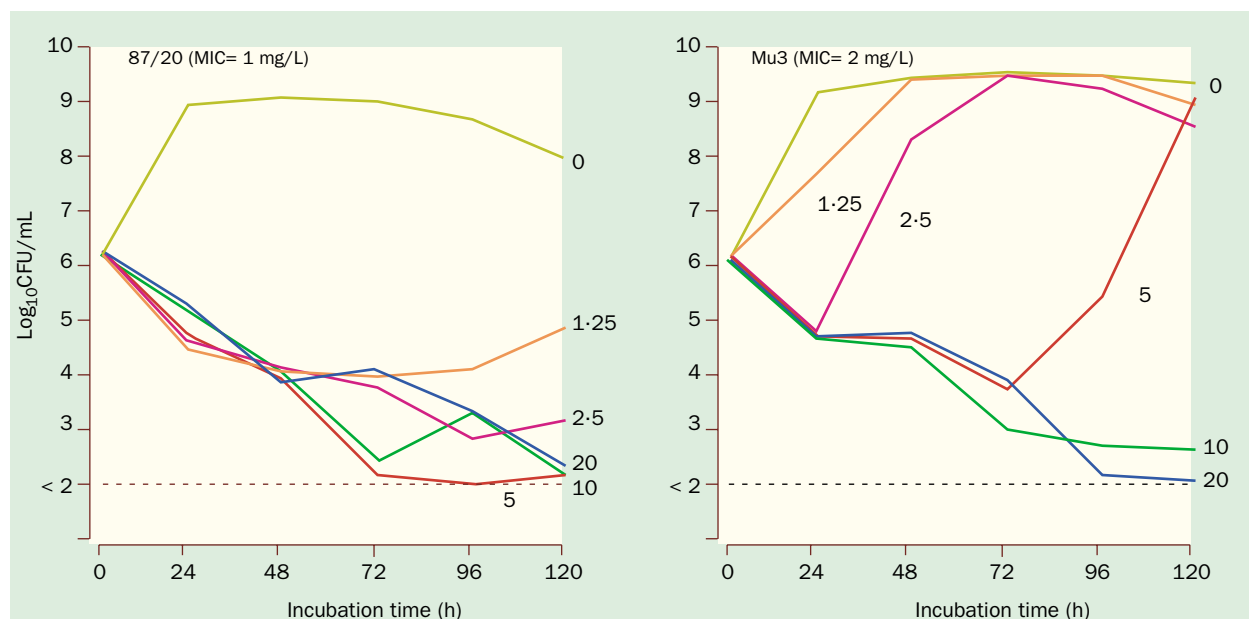


Figure 7. Hetero-VRSA contains vancomycin-resistant cell sub-populations. MRSA strain 87/20 (vancomycin MIC, 1 mg/L) and hetero-VRSA strain Mu3, were inoculated in the Mueller-Hinton broth containing various concentrations (0, 1.25, 2.5, 5, 10, and 20 mg/L) of vancomycin. The test tubes were incubated at 37°C with gentle shaking. Viable cell counts were done periodically, and plotted on the graph. Note that Mu3 starts to grow after 72 h incubation in the 2.5 times MIC (5 mg/L) of vancomycin.

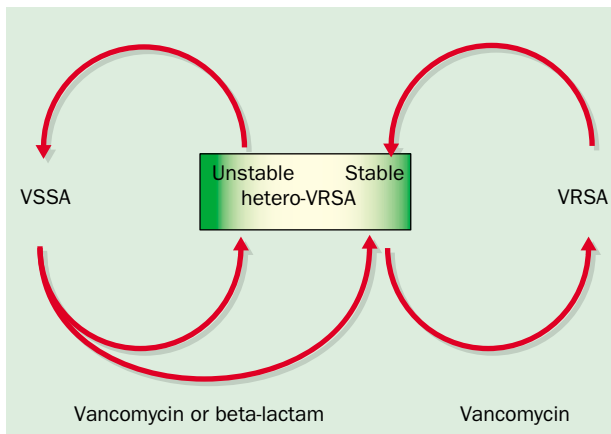


Figure 8. Hetero-VRSA as the “protagonist” of staphylococcal glycopeptide resistance. Hetero-VRSA is a preferable evolutionary status for *S. aureus* to survive the attack of glycopeptides. It is situated in between VSSA and VRSA. It produces VRSA cells at a high frequency within the cell population to secure its survival as a strain. VRSA cells that have survived the vancomycin pressure may return to hetero-VRSA status when the pressure is lifted. There are cycles of selection and reversion between VSSA and hetero-VRSA as well.

the hetero-resistance of Mu3, which is stably expressed even after 80 days' serial passage in a drug-free medium. Therefore, it suggests that there are two types of hetero-VRSA: stable and unstable (figure 8). The stable variant of hetero-VRSA may be established either by cycles of exposure to vancomycin or by one-step acquisition of a single but stable genetic alteration. In-vitro experiments predict that many unstable hetero-VRSA strains are produced in the clinical setting. These strains express hetero-resistance directly after isolation from patients who undergo glycopeptide therapy, but the phenotype tends to be lost during strain storage in drug-free media, contributing to the underestimation of the prevalence of hetero-VRSA. However, it would still be important to detect stable hetero-VRSA strains, because they would prevail more easily in the hospital environment eventually causing more vancomycin therapeutic failures by generating VRSA at high frequency during the therapy.

Vancomycin-resistance in the VRSA phenotype also tends to revert.<sup>36</sup> Experiments with 16 VRSA strains from different parts of the world showed that the vancomycin MIC returned to “susceptible” levels (2 mg/L) after 10 to 84 days' of serial passages. Therefore, the stability of VRSA phenotype can vary significantly. A notable observation, was that the “susceptible” revertants obtained from 15 of the 16 VRSA strains still expressed heterogeneous vancomycin resistance similar to that of Mu3—one strain returned to a susceptibility level with a VSSA-type population curve characteristic (see the population curve of FDA209P in figure 6). Exposure of these revertants to 4 mg/L of vancomycin, however, selected VRSA strains at very high frequencies of about  $10^{-4}$ – $10^{-5}$  (L Cui, unpublished observation). This finding indicates that, though the VRSA strain may not disseminate itself as a stable resistance phenotype that tends to return to the hetero-VRSA status, it can readily revert to VRSA when exposed to vancomycin (figure 8).

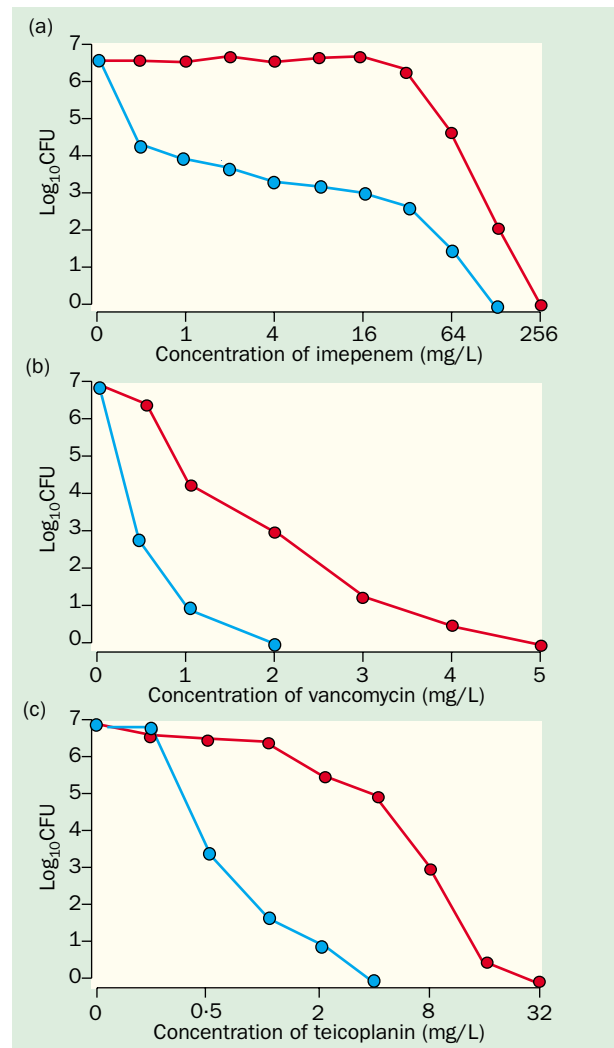


Figure 9. Hetero-VRSA can be selected by beta-lactams. Population analysis of hetero-MRSA strain N315ΔIP (in blue) and homo-MRSA strain N315ΔIP14 (in red). The latter was obtained by selecting N315ΔIP with 8 mg/L of imipenem. N315ΔIP14 expresses not only homo-resistance to imipenem (a) but also hetero-resistance to vancomycin (b) and resistance to teicoplanin compared to vancomycin (c).

From December 1998 to January 1999, an outbreak of VRSA was recorded in a Brazilian hospital (G A Oliveira, General Hospital of Vila Pentead and School of Pharmaceutical Sciences, University of Sao Paulo, Sao Paulo, Brazil, personal communication). The incidence indicated that VRSA can transmit from patient to patient if vancomycin is used liberally for burn-unit patients housed in the same room. Aside from this special case, it would be the hetero-VRSA that would disseminate in the hospital and contribute to the incidence of vancomycin-refractory infection. In fact, many hetero-VRSA strains were found even in the Brazilian hospital case besides the multiple VRSA isolates. The emergence of clinically significant VRSA could be the tip of the iceberg, and a sign of the more widespread and insidious prevalence of hetero-VRSA in the hospital.

From a biological point of view, the hetero-VRSA status seems to be a successful ecological achievement of *S. aureus* for the procurement of survival against vancomycin

pressure. Although vancomycin suppresses the growth of 99·9% of the population of hetero-VRSA, the rest of the population survives and grows in the presence of 4 mg/L of vancomycin, which is almost the upper limit of vancomycin concentration achieved in most infected tissues. Some subpopulation of cells can grow even in 9 mg/L of vancomycin (figure 6). Those VRSA cells spend much energy to produce thickened cell walls, to survive the vancomycin pressure. Once the vancomycin pressure is alleviated, the VRSA cells return to the hetero-VRSA status to conserve energy. *S aureus* seems to have chosen an effective survival mechanism to prevail as a successful parasite of people. *S aureus* may not need to acquire the *van* genes from vancomycin-resistant enterococci (VRE), since associated with selective pressure are limited tissue concentrations and the limited cytotoxic activity of vancomycin.

A significant increase of MRSA was noticed in the early 1980s in Japan<sup>37</sup> when the third-generation cephalosporins were used widely throughout Japan. Based on a prediction from a plausible mathematical model on the prevalence of resistant bacteria in hospitals,<sup>38</sup> unrestricted use of the broad-spectrum cephalosporins with weak antimicrobial activity against MRSA might have promoted this quick rise in MRSA.<sup>37</sup> Since, vancomycin and arbekacin (an aminoglycoside antibiotic approved in 1990 for MRSA infection) were not available in Japan until 1991, it became general practice in Japan to treat MRSA infection with a class of beta-lactam antibiotic, such as imipenem, flomoxef, and cefmetazole, which have good MIC values against hetero-MRSA.<sup>39</sup> From MIC data, these beta-lactams appeared effective since MRSA strains in the early 1980s were heterogeneously resistant to methicillin (hetero-MRSA).<sup>37</sup> Towards the end of the 1980s, this practice led to a clone having a high methicillin resistance (homo-MRSA clone designated clonotype II-A),<sup>40</sup> which became the dominant clone in Japan in the 1990s.<sup>37</sup>

In-vitro selection of hetero-MRSA strains of clonotype II-A with imipenem, cefmetazole, or flomoxef, yields homo-converted mutants at a high frequency of  $10^{-4}$  to  $10^{-5}$ .<sup>41</sup> This observation agrees with the historical rise of clonotype-II-A MRSA in Japan.<sup>37</sup> Unexpectedly about 5–10% of the homo-MRSA mutants are hetero-VRSA (figure 9) which indicates that hetero-VRSA might have emerged in Japan in the late 1980s from the MRSA strains exposed to the beta-lactams used at the time. Indeed, several hetero-VRSA strains were found in the late 1980s in Japan before the introduction of vancomycin. There are several genetic mechanisms underlying hetero-to-homo conversion of methicillin resistance.<sup>41</sup> Evidently, there exists some common genetic mechanisms that confer both the hetero-to-homo conversion of methicillin resistance and VSSA to hetero-VRSA conversion.

With regard to the relationship between methicillin and vancomycin resistance, Sieradzki and Tomasz reported a phenomenon in which raised vancomycin resistance is associated with reduced methicillin resistance.<sup>27</sup> This phenomenon is observed with an in-vitro VRSA strain produced in the laboratory, which is based on the inactivation of PBP activities and a drastic decrease in peptidoglycan cross-linkage. In this condition, it is understandable that the cells become very vulnerable to the

action of beta-lactam antibiotics. But, in the case of 16 clinical VRSA strains, the level of methicillin resistance remains high (oxacillin MICs  $\geq 64$  mg/L) except for one strain PC-3 isolated in New York, whose oxacillin MIC is 8 mg/L (L Cui, personal communication).

The glycopeptide-resistance expression of hetero-VRSA is also influenced by the exposure to beta-lactams. Practically all beta-lactam antibiotics when used at an optimal concentration, increases vancomycin resistance of the hetero-VRSA strain, Mu3.<sup>42</sup> This antagonism which can be demonstrated in vitro, poses a potential problem in the use of combination regimens of beta-lactam and vancomycin against VRSA. It is surprising that there is no antagonism shown between beta-lactams and teicoplanin.<sup>43</sup> The reason for this difference is unknown, but may be correlated with the difference in the resistance mechanism for the two glycopeptides. It would be worthwhile to further explore this difference, in an effort to optimise a combined use of teicoplanin and beta-lactams in the treatment of MRSA infections.

### Strategies to counter VRSA infection

The nature of the resistance mechanism of VRSA—production and accumulation of excess amounts of cell-wall peptidoglycan—indicates that VRSA would not be prevalent in an environment where the glycopeptide selective pressure is not strong. If a hospital reduces the consumption of glycopeptides, VRSA should not prevail in the hospital. However, this action does not solve the problem completely, because hetero-VRSA may be capable of dissemination without glycopeptide pressure. Therefore, it is necessary to expand antibiotic prescription policy to include beta-lactam antibiotics as well. If we reduce consumption of broad-spectrum cephalosporins (which are ineffective against MRSA), and, this measure combined with effective infection control,<sup>44</sup> the number of MRSA in the hospital would decrease according to a mathematical model developed by Lipsitch et al.<sup>38</sup> Reducing the total number of MRSA is the most effective measure for preventing emergence of VRSA and hetero-VRSA. Recently, successful reduction of MRSA was achieved in a Japanese hospital by cutting the total use of broad-spectrum cephalosporins by half without compromising infection outcome.<sup>45</sup>

It may also be possible to reduce the selection of vancomycin resistance in MRSA isolates in the hospital by substituting cephalosporins and carbapenems with penicillins that have a relatively strong anti-MRSA activity among beta-lactam antibiotics.<sup>39,46</sup> In our hospital, use of ampicillin/sulbactam was encouraged as a substitute for broad-spectrum cephalosporins and carbapenems for surgical prophylaxis after the emergence of VRSA in 1996.<sup>3</sup> As a result, penicillinase-producing MRSA increased from 47% in 1996–1997 to 96% in 1999–2000 (note that both Mu3 and Mu50 are characteristically non-producers of penicillinase). At the same time, MRSA strains having vancomycin MICs of 4 mg/L or above decreased from 0·43% to 0·08% of total MRSA isolates during this period (T Oguri, and J Igari, Clinical Laboratory, Juntendo University, Tokyo, Japan, personal communication). Evidently, this is only a preliminary observation. A well-designed clinical



study would be required to confirm this anecdotal decrease in vancomycin-resistance among MRSA isolates. In any such clinical study in this direction, however, it would be important to monitor the prevalence of hetero-VRSA by alternative methods that can detect subtle changes in glycopeptide susceptibility of a high number of MRSA isolates.

### Treatment of VRSA infection

New agents are being developed against MRSA. Some of them are expected to have considerable activity against hetero-VRSA and VRSA strains as well. Synercid has potent activity against hetero-VRSA and VRSA strains.<sup>17</sup> A new quinolone antibiotic, DU-6859a, has MICs of 0.5 and 1 mg/L against Mu3 and Mu50 which are resistant to other quinolones such as levofloxacin, ciprofloxacin, sparfloxacin, and tosufloxacin.<sup>47</sup> Linezolid also has good activity against MRSA, and is expected to be useful for cases where vancomycin therapy fails. However, linezolid does not have a cytotoxic effect against MRSA.

While we do not have any single cytotoxic anti-MRSA antibiotic that exceeds the potency of vancomycin, it is important for us to explore several antibiotic combination therapies. Ampicillin/sulbactam in this regard would be a good partner for vancomycin; even the agent alone has good anti-microbial activity against VRSA in an experimental infection model.<sup>48</sup> Linezolid has either a synergistic or additive effect on hetero-VRSA and VRSA strains when combined with ampicillin/sulbactam.<sup>49</sup> This combination therapy, as well as arbekacin and ampicillin/sulbactam,<sup>17,35</sup> takes advantage of ampicillin's high affinity to MRSA PBP2'.<sup>50,39</sup> When we use vancomycin, it is evident from its mechanism of action and mechanism of resistance that we have to try and reduce the bacterial burden from the patient's body with such procedures as surgical drainage, debridement, and removal of contaminated lines, foreign bodies, or prosthetic materials. When vancomycin therapy is still unsuccessful with these procedures, we use triple therapy of vancomycin, rifampicin (oral) and cotrimoxazole (oral).

### Future perspectives

This year, the whole genome sequences of VRSA strain Mu50 and MRSA strain N315 were published.<sup>29</sup> The latter strain, susceptible to vancomycin, is closely related to Mu50; 96% of the nucleotide sequences are identical between the two. By comparing the sequences of N315 and Mu50, we should be able to identify the genetic basis for glycopeptide resistance. These genome data will serve as an invaluable source of information for the future development of novel antibiotics as well as an *S aureus* vaccine. However, the information inscribed in the Mu50 and N315 genomes also showed that *S aureus* is an extremely flexible pathogen. It is capable of acquiring any useful genes for survival across species barriers, and updating its armamentarium by multiplying toxin genes by successive gene duplication to attack any people of diverse immune history and genetic backgrounds.<sup>29</sup> We may never be free from the threat of *S aureus* since part of the benign natural flora when we are healthy becomes a formidable intruder when our body

### Search strategy and selection criteria

I primarily cite articles that I am aware of through exchange of information among the international research community in the past 4 years. Additional articles were identified by a Medline search using the combination of terms aureus, vancomycin, and resistance, and from the references of relevant publications. For more basic research articles, the term mechanism was added to the search. English language papers and relevant non-English language paper were included.

defences become compromised. The best strategy is to maintain a healthy ecological balance with effective intervention strategies to reduce the organisms need to acquire resistance to the best antibiotics. Decreasing the total consumption of antibiotics both in the hospitals and community, and, using them with good rationale based on accurate diagnosis and susceptibility testing information may help preserve mankind's precious drug.

### Acknowledgments

I thank M Lipsitch, C T Bergstrom, and B R Levin, for their lively discussion on the mathematical model for antibiotic resistance. I am also grateful to Yuh Morimoto for her assistance in the preparation of computer graphics. This work was supported by the Core University System Exchange Programme under the Japan Society for the Promotion of Science, co-ordinated by the University of Tokyo, Graduate School of Medicine and Mahidol University. The work was also partly supported by a Grant for International Health Cooperation Research (11C-4) from the Ministry of Health and Welfare.

### References

- 1 Jevons MP. "Celbenin"-resistant staphylococci. *BMJ* 1961; 1: 124-25.
- 2 Chambers HF. The changing epidemiology of *Staphylococcus aureus*. *Emerg Infect Dis* 2001; 7: 178-82.
- 3 Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *J Antimicrob Chemother* 1997; 40: 135-36.
- 4 Hiramatsu K, Aritaka N, Hanaki H, et al. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet* 1997; 350: 1668-71.
- 5 Tenover FC, Lancaster MV, Hill BC, et al. Characterization of staphylococci with reduced susceptibility to vancomycin and other glycopeptide. *J Clin Microbiol* 1998; 36: 1020-27.
- 6 Ploy MC, Grelaud C, Martin C, de Lumley L, Denis F. First clinical isolate of vancomycin-intermediate *Staphylococcus aureus* in a French hospital. *Lancet* 1998; 351: 1212.
- 7 Kim M-N, Pai CH, Woo JH, Ryu JS, Hiramatsu K. Vancomycin-intermediate *Staphylococcus aureus* in Korea. *J Clin Microbiol* 2000; 38: 3879-81.
- 8 Smith TL, Pearson ML, Wilcox KR, et al. Emergence of vancomycin resistance in *Staphylococcus aureus*. *N Engl J Med* 1999; 340: 493-501.
- 9 Ferraz V, Duse AG, Kassel M, Black AD, Ito T, Hiramatsu K. Vancomycin-resistant *Staphylococcus aureus* occurs in South Africa. *S Afr Med J* 2000; 90: 1113.
- 10 Chesneau O, Morvan A, El Solh N. Retrospective screening for heterogeneous vancomycin resistance in diverse *Staphylococcus aureus* clones disseminated in French hospitals. *J Antimicrob Chemother* 2000; 45: 887-90.
- 11 Hood J, Edwards GFS, Cosgrove B, Curran E, Morrison D, Gemmell CG. Vancomycin-intermediate *Staphylococcus aureus* at a Scottish hospital. *J Infect* 2000; 40: A11.
- 12 Marchese A, Balistreri G, Tonoli E, Debbia EA, Schito GC. Heterogeneous vancomycin resistance in methicillin-resistant *Staphylococcus aureus* strains isolated in a large Italian hospital. *J Clin Microbiol* 2000; 38: 866-69.
- 13 Rotun SS, McMath V, Schoonmaker DJ, et al. *Staphylococcus aureus*



- with reduced susceptibility to vancomycin isolated from a patient with fatal bacteraemia. *Emerg Infect Dis* 1999; 5: 147–9.
- 14 Geisel R, Schmitz FJ, Thomas L, et al. Emergence of heterogeneous intermediate vancomycin resistance in *Staphylococcus aureus* isolates in the Dusseldorf area. *J Antimicrob Chemother* 1999; 43: 846–48.
  - 15 Bierbaum G, Fuchs K, Lenz W, Szekat C, Sahl HG. Presence of *Staphylococcus aureus* with reduced susceptibility to vancomycin in Germany. *Eur J Clin Microbiol Infect Dis* 1999; 18: 691–96.
  - 16 Trakulsomboon S, Danchaivijitr S, Rongrungruang Y, et al. The first report on methicillin-resistant *Staphylococcus aureus* with reduced susceptibility to vancomycin in Thailand. *J Clin Microbiol* 2001; 39: 591–595.
  - 17 Hiramatsu K. Vancomycin resistance in staphylococci. *Drug Resistance Updates* 1998; 1: 135–50.
  - 18 Reynolds PE, Brown DFJ. Penicillin-binding proteins of beta-lactam-resistant strains of *Staphylococcus aureus*. *FEBS Lett* 1985; 192: 28–32.
  - 19 Utsui Y, Yokota T. Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1985; 28: 397–403.
  - 20 Hartman BJ, Tomasz A. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *J Bacteriol* 1984; 158: 513–16.
  - 21 Katayama Y, Ito T, Hiramatsu K. A new class of genetic element, *Staphylococcus* Cassette Chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2000; 44: 1549–55.
  - 22 Hanaki H, Kuwahara-Arai K, Boyle-Vavra S, Daum RS, Labischinski H, Hiramatsu K. Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50. *Antimicrob Chemother* 1998; 42: 199–209.
  - 23 Hanaki H, Labischinski H, Inaba Y, Kondo N, Murakami H, Hiramatsu K. Increase in glutamine-non-amidated muropeptides in the peptidoglycan of vancomycin-resistant *Staphylococcus aureus* strain Mu50. *J Antimicrob Chemother* 1998; 42: 315–20.
  - 24 Cui L, Murakami H, Kuwahara-Arai K, Hanaki H, Hiramatsu K. Contribution of a thickened cell wall and its glutamine non-amidated component to the vancomycin resistance expressed by *Staphylococcus aureus* Mu50. *Antimicrob Agents Chemother* 2000; 44: 2276–285.
  - 25 Sieradzki K, Roberts RB, Haber SW, Tomasz A. The development of vancomycin resistance in a patient with methicillin-resistant *Staphylococcus aureus* infection. *N Engl J Med* 1999; 340: 517–23.
  - 26 Sieradzki K, Tomasz A. Gradual alterations in cell wall structure and metabolism in vancomycin-resistant mutants of *Staphylococcus aureus*. *J Bacteriol* 1999; 181: 7566–70.
  - 27 Sieradzki K, Tomasz A. Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant of *Staphylococcus aureus*. *J. Bacteriol* 1997; 179: 2557–66.
  - 28 Kuroda M, Kuwahara-Arai K, Hiramatsu K. Identification of the up- and down-regulated genes in vancomycin-resistant *Staphylococcus aureus* strains Mu3 and Mu50 by cDNA differential hybridization method. *Biochem Biophys Res Commun* 2000; 269: 485–90.
  - 29 Kuroda M, Ohta T, Uchiyama I, et al. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 2001; 357: 1225–40.
  - 30 Bobin-Dubreux S, Reverdy ME, Nervi C, et al. Clinical isolate of vancomycin-hetero intermediate *Staphylococcus aureus* susceptible to methicillin and in vitro selection of a vancomycin-resistant derivative. *Antimicrob Agents Chemother* 2001; 45 (1): 349–52.
  - 31 Kaatz GW, Seo SM, Dorman NJ, Lerner SA. Emergence of teicoplanin resistance during therapy of *Staphylococcus aureus* endocarditis. *J Infect Dis* 1990; 162: 103–08.
  - 32 Brunet G, Vedal G, Dreyfus F, et al. Failure of teicoplanin therapy in two neutropenic patients with staphylococcal septicaemia who recovered after administration of vancomycin. *Eur J Clin Microb Infect Dis* 1990; 9: 145–47.
  - 33 Shlaes DM, Shlaes JH, Vincent S, Etter L, Fey PD, Goering RV. Teicoplanin-resistant *Staphylococcus aureus* expresses a novel membrane protein and increases expression of penicillin-binding protein 2 complex. *Antimicrob Agents Chemother* 1993; 37: 2432–437.
  - 34 Hanaki H, Hiramatsu K. Detection methods of glycopeptide-resistant *Staphylococcus aureus* I: Susceptibility testing. In: Gillespie SH, ed. Antibiotic resistance methods and protocol Totowa, New Jersey: Humana Press, 2001: 85–92.
  - 35 Hiramatsu K. The emergence of *Staphylococcus aureus* with reduced susceptibility to vancomycin in Japan. *Am J Med* 1998; 104: 7S–10S.
  - 36 Boyle-Vavra S, Berke SK, Lee JC, Daum RS. Reversion of the glycopeptide resistance phenotype in *Staphylococcus aureus* clinical isolates. *Antimicrob Agents Chemother* 2000; 44: 272–77.
  - 37 Tanaka T, Okuzumi K, Iwamoto A, Hiramatsu K. A retrospective study on methicillin-resistant *Staphylococcus aureus* clinical strains in Tokyo University Hospital. *J Infect Chemother*. 1995; 1: 40–49.
  - 38 Lipsitch M, Bergstrom CT, Levin BR. The epidemiology of antibiotic resistance in hospitals: paradoxes and prescriptions. *Proc Natl Acad Sci U S A* 2000; 97: 1938–43.
  - 39 Asada K, Inaba Y, Tateda-Suzuki E, Kuwahara-Arai K, Ito T, Hiramatsu K. Evolution and resistance expression of MRSA; evaluation of beta-lactam antibiotics against a set of isogenic strains with different types of phenotypic expression. *Acta Biochim Pol* 1995; 42: 517–24.
  - 40 Hiramatsu K, Ito T, Hanaki H. Evolution of methicillin and glycopeptide resistance in *Staphylococcus aureus* In: Finch RG, Williams RJ, eds. Bailliere's Clinical Infectious Disease. London: Bailliere Tindall, 1999: 221–42.
  - 41 Kondo N, Kuwahara-Arai K, Kuroda-Murakami H, Tateda-Suzuki E, Hiramatsu K. Eagle-type methicillin resistance: new phenotype of high methicillin resistance under *mec* regulator gene control. *Antimicrob Agents Chemother* 2001; 45: 815–24.
  - 42 Aritaka N, Hanaki H, Cui L, Hiramatsu K. The combination effect of vancomycin and beta-lactams against *Staphylococcus aureus* strain Mu3 with heterogeneous resistance to vancomycin. *Antimicrob Agents Chemother* 2001; 45: 1292–94.
  - 43 Hanaki H, Hiramatsu K. Combination effect of teicoplanin and various antibiotics against hetero-VRSA and VRSA. *Kansenshogaku Zasshi* 1999; 73: 1048–53.
  - 44 Pittet D, Hugonnet S, Harbarth S, Mourouga P, Sauvan V, Touveneau S. Effectiveness of a hospital-wide programme to improve compliance with hand hygiene. *Lancet* 2000; 356: 1307–12.
  - 45 Kuramoto S, Kizu J, Yamamoto K. Appropriate and restricting antibiotics prescribing to combat VRE and VRSA. *Antibiot Chemother* 1999; 15: 91–97.
  - 46 Chambers HF, Kartalija M, Sande M. Ampicillin, sulbactam, and rifampin combination treatment of experimental methicillin-resistant *Staphylococcus aureus* endocarditis in rabbits. *J Infect Dis* 1995; 171: 897–902.
  - 47 Tanaka M, Wada N, Mori-Kurosaka S, Chiba M, Sato K, Hiramatsu K. In-vitro activity of DU-6859a against methicillin-resistant *Staphylococcus aureus* isolates with reduced susceptibilities to vancomycin. *J Antimicrob Chemother* 1998; 42: 552–53.
  - 48 Backo M, Gaenger E, Burkart A, Chai YL, Bayer AS. Treatment of experimental staphylococcal endocarditis due to a strain with reduced susceptibility in vitro to vancomycin: efficacy of ampicillin-sulbactam. *Antimicrob Agents Chemother* 1999; 43: 2565–68.
  - 49 Kato D, Ito T, Imai D, Hiramatsu K. In vitro synergy between linezolid and sulbactam/ampicillin against methicillin-resistant *Staphylococcus aureus* clinical isolates including those with reduced susceptibility to vancomycin. 40th Interscience Conference on Antimicrobial Agents and Chemotherapy 2000; Abstract no. 2297.
  - 50 Chambers HF, Sachdeva M. Binding of beta-lactam antibiotics to penicillin-binding proteins in methicillin-resistant *Staphylococcus aureus*. *J Infect Dis* 1990; 161: 1170–76.