Original Report

Emergence of vancomycin resistance during therapy against methicillin-resistant Staphylococcus aureus in a burn patient—importance of low-level resistance to vancomycin

Isao Haraga,(1,2) Shuichi Nomura,(3) Shigeru Fukamachi,(4) Hiroyuki Ohjimi,(2) Hideaki Hanaki,(5) Keiichi Hiramatsu(5) and Ariaki Nagayama(1)

Objectives: Staphylococcus aureus with low-level resistance to vancomycin (VLSA) which could develop into vancomycin-resistant S. aureus (VRSA) is most important. However, VLSA is difficult to detect by standard laboratory methods. We describe here improved methods to detect VLSA.

Methods: Three methicillin-resistant S. aureus (MRSA) strains, designated Fu6, Fu10, and Fu18, were sequentially isolated from the burn wound site of a patient, during vancomycin therapy. The properties of these strains were compared with those of reference strains Mu3 and Mu50 (previous resistant isolates from other patients).

Results: The isolated strains, Fu10 and Fu18, had identical phenotypes and genotypes. The vancomycin resistance of Fu10 was equivalent to that of strain Mu3, whereas Fu18 had much higher vancomycin resistance than Fu10 and Mu3, although reaching the level of Mu50. Fu18 showed similar growth to Mu50 on gradient gels and on Mu3 medium.

Conclusions: Our data indicate that the VLSA developed vancomycin resistance during exposure to vancomycin in vivo. The population analysis of tested VLSA and vancomycin intermediately resistant S. aureus (VISA) indicates that a penem at relatively low concentrations induced a significant increase in the number of vancomycin-resistant subpopulations. Furthermore, we confirmed that gradient gel analysis and Mu3 medium are simple and useful methods for the detection of VLSA judged as VSSA by its conventional MIC alone.

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INTRODUCTION

The increase in methicillin-resistant Staphylococcus aureus (MRSA) infections in hospitals is of global concern.1-3 In Japan, vancomycin, which is believed to be the most effective antibiotic against MRSA, has been widely used for the treatment of MRSA infections since 1991. However, vancomycin therapeutic failures have occasionally occurred in spite of the low MIC values of vancomycin for the isolated MRSA strains. The first strains of vancomycin-resistant S. aureus (VRSA), Mu50, and heterogeneous VRSA (hetero-VRSA), Mu3, were reported in 1997.1,4 Thereafter, isolates of VRSA-like Mu50 were reported from different geographic areas, including the USA and France.5-10 Vancomycin-susceptible strains (VSSA) have MICs of 2 μg/mL or less, and are completely inhibited by 4 μg/mL of vancomycin.1 VRSA was defined as S. aureus with an MIC for vancomycin of 8 μg/mL or above by the NCCLS method.1,11 It is equivalent to VISA (vancomycin intermediately resistant S. aureus)5,6,11 or GISA (glycopeptide intermediately resistant S. aureus).12 Hetero-VRSA was defined as S. aureus that gave 10^8 or more colonies in the presence of 8 μg/mL of vancomycin by Hiramatsu and co-workers;1,11 that is, hetero-VRSA is synonymous with an S. aureus strain with low-level resistance to vancomycin (VLSA).12 Therefore, we use this term instead of hetero-VRSA in this paper. In the case of extensive burn patients, MRSA wound infection can often not be eliminated, despite intensive vancomycin therapy. Vancomycin therapeutic failure in MRSA wound infections has been largely attributed to the poor status of such patients (defect of skin, malnutrition, loss and imbalance of electrolytes, decreased neutrophil number, etc.). Furthermore, some VLSA isolates from a patient that are judged to be VSSA according to the NCCLS criteria may cause vancomycin therapeutic failure. During vancomycin therapy of a burn patient with an MRSA wound infection, we recovered sequentially three MRSA wound isolates.
We describe here the properties of these MRSA isolates, showing increases in their vancomycin resistance in vivo on exposure to vancomycin, and we would like to emphasize the importance of VLSA judged as VSSA by conventional MIC alone.

**MATERIALS AND METHODS**

**Patients**

A 2-year-old male with a body weight of 12 kg was admitted to our Intensive Care Unit on 18 January 1998 after sustaining flame burns to his face, chest, abdomen, back, and left upper extremities (20% of his total body surface area; Burn Index 15). On hospital day 1, wound cultures revealed no MRSA, but to prevent infection, the patient was given intravenous piperacillin. On hospital day 10, cultures from the wound were positive for MRSA (designated as Fu6). The patient’s temperature was 40°C, and the C-reactive protein (CRP) level was 25.6 mg/dL. The patient was treated with gentamicin (0.8 mg/kg daily). On hospital day 16, fever continued (39°C), but the CRP had declined to 3.4 mg/dL. On hospital day 19, gentamicin was discontinued in favor of vancomycin (20.8 mg/kg, IV, every 12 h). On hospital day 21, the fever continued (41°C), and the CRP had risen to 14 mg/dL. Therefore, on hospital day 22, the treatment was changed to a combination of vancomycin (20.8 mg/kg, IV, every 12 h) and imipenem (62.5 mg/kg, IV, every 12 h). On hospital day 27, cultures from the wound were positive for gentamicin-resistant, arbekacin-susceptible and vancomycin-susceptible MRSA (designated as Fu10), as judged from the sensitivity disk test. On hospital day 30, although fever continued (38.5°C), the CRP declined to 2.3 mg/dL. On hospital day 35, as the patient’s temperature again rose to 40°C, vancomycin and imipenem were discontinued in favor of arbekacin (4.2 mg/kg, IV, every 24 h) and sulbactam–ampicillin (62.5 mg/kg, IV, every 12 h). After this, there was a downward trend in the fever for 9 days; however, on hospital day 44, it rose again to 39°C, and wound cultures were positive for MRSA (designated as Fu18) again. Therefore, therapy with arbekacin and sulbactam–ampicillin was changed to every 12 h. On hospital day 51, the patient’s fever had subsided (36.5°C), and the CRP declined to 0.3 mg/dL. Finally, MRSA disappeared, and the patient was cured and discharged. The case summary is shown in Figure 1.

**Bacterial strains**

Fu6, Fu10 and Fu18 were wound isolates of MRSA obtained on hospital days 10, 27 and 44, respectively. Mu50 and Mu3 have been described previously.1,4 FDA209P (ATCC6538P), a reference strain of S. aureus, was purchased from the Japanese National Institute of Health and Disease Prevention.

**Detection of vancomycin-resistant S. aureus on Mu3 medium**

Mu3 medium13,14 (Nippon Becton Dickinson Co., Ltd, Tokyo, Japan), and modified brain heart infusion agar medium supplemented with 4 mg vancomycin/mL, were inoculated with tested strains. After several disks impregnated with β-lactams (imipenem 0.01, 0.1, 1.0 and 10 μg/mL) had been placed on the agar, the plates were incubated at 37°C for 24 h.

**Genotyping**

The comparison of genetic traits of clinical isolates Fu6, Fu10 and Fu18 with those of Mu3, Mu50 and FDA209P (MSSA) was done by pulsed-field gel electrophoresis (PFGE).15 PFGE patterns were interpreted according to the established criteria.16

**Phenotyping and susceptibility tests**

The biotypes were determined by use of Api Staph Trac from BioMerieux, Tokyo, Japan. The coagulase typing, enterotoxin typing and test for toxic shock syndrome toxin-1 (TSST-1) production were performed with commercially available kits from Dennka Seiken, Tokyo, Japan.

MICs were determined by use of BHI agar plates (Difco, Becton Dickinson Co., Sparks, MD, USA) and 5mL spot inoculum of cell suspension (107 CFU/mL) according to the NCCLS method. Unless otherwise stated, the MIC was defined as the minimum concentration of antibiotic needed to inhibit the growth of 5×104 CFU after 16 h of incubation at 37°C. Methicillin (Sigma Aldrich Japan, Co., Tokyo, Japan), vancomycin (Sigma Aldrich Japan), teicoplanin (Hoechst-Marion-Roussel Ltd, Tokyo, Japan), arbekacin (Meiji Seika Ltd, Tokyo, Japan), gentamicin (Sigma Aldrich Japan), imipenem (Banyu Pharmaceutical Co., Ltd, Tokyo, Japan), cefpirome (Hoechst-Marion-Roussel), sulbactam–ampicillin (Pfizer Pharmaceutical Inc., Tokyo, Japan), piperacillin (Toyama Chemical Co., Ltd, Tokyo, Japan) and oxacillin (Sigma Aldrich Japan) were tested.

**Gradient gel analysis**

Fu10, Fu18, Mu3, Mu50 and FDA209P were grown in BHI broth at 37°C with shaking. A suspension of 2.5×10⁸ cells/mL of an overnight culture was streaked onto defined BHI agar plates containing gradients of vancomycin.18

**Population analysis**

Population analysis was done according to the method described by Berger-Bachi et al. Briefly, 100 mL of the starting cell suspension (prepared by diluting overnight cultures to an OD₅₇₈ of 0.3) and its serial diluents were spread over BHI agar plates containing various concen-
RESULTS

Emergence of vancomycin-resistant *S. aureus* during vancomycin treatment as detected by use of Mu3 medium

The course of medical treatment and growth patterns of the six tested strains on Mu3 medium are shown in Figure 1. FDA209P and Fu6 obtained before vancomycin treatment showed essentially no growth at all on Mu3 medium. Fu18 isolated after a total of 16 days of vancomycin treatment and then during the course of arbekacin and sulbactam–ampicillin treatment grew all over the surface on Mu3 medium. This growth pattern was almost the same as that of Mu50.

Genotype

PFGE banding patterns of Fu6, Fu10, Fu18, Mu3, Mu50 and FDA209P are shown in Figure 2. Fu10 and Fu18 had identical PFGE banding patterns. The pattern was similar to that of Mu3 and Mu50, representative of the MRSA clonotype II-A, with two band differences (arrows). By contrast, these strains had banding patterns different from those of the other strains (Fu6 and FDA209P).
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Figure 2. Pulsed-field gel electrophoresis banding patterns of Fu 6, Fu10, Fu18, Mu3, Mu50, and FDA209P. MWM is the molecular weight marker (concatemer of lambda-phage DNA).

**Phenotype and MICs**

The properties of these strains are shown in Table 1. Whereas strain Fu6 showed phenotypic differences in biotype and enterotoxin type, the other four strains (Fu10, Fu18, Mu3, Mu50) were identical by conventional phenotyping assays, including biotype, coagulase type, TSST-1 production, and enterotoxin type. MICs of vancomycin for Fu6 and Fu10 remained the same as that of Mu3 (≥ 28 μg/mL). The observed MIC difference between Fu10 and Fu18 was increased vancomycin resistance in the latter, although not to the level of Mu50. In addition to vancomycin resistance, Fu18 had increased resistance to telocoplanin and arbekacin.

**Gradient gel analysis**

Figure 3 shows a comparison of the gradient gel analysis of serial isolates Fu10 and Fu18 described above with that of Mu3, Mu50, and FDA209P. The vancomycin resistance of Fu10 was equivalent to that of Mu3. Fu18 showed greater vancomycin resistance than Fu10 and Mu3, almost the same as Mu50. The growth patterns of these strains on gradient gel represent vancomycin resistance. Although Mu3, Fu10 and Fu18 contained small resistant populations that grew in the presence of 4 μg/mL of vancomycin, MICs of these strains were 2–4 μg/mL. Therefore, VLSA strains cannot be discriminated from VSSA strains by MIC values alone. From this point of view, population analysis and gradient gel analysis are useful for detecting VLSA.

**Table 1. Properties of MRSA strains**

<table>
<thead>
<tr>
<th>Coagulase type</th>
<th>Enterotoxin type</th>
<th>Minimum Inhibitory Concentrations (MICs, μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fu6</td>
<td>6336153</td>
<td>2</td>
</tr>
<tr>
<td>Fu10</td>
<td>6736153</td>
<td>2</td>
</tr>
<tr>
<td>Fu18</td>
<td>6736153</td>
<td>4</td>
</tr>
<tr>
<td>Mu3</td>
<td>6736153</td>
<td>2</td>
</tr>
<tr>
<td>Mu50</td>
<td>6736153</td>
<td>2</td>
</tr>
<tr>
<td>FDA209P</td>
<td>ND</td>
<td>2</td>
</tr>
</tbody>
</table>

ND: Not determined.
MICS: inoculum size was 3 × 10^9 CFU/mL; spot size was 3 μL/spot with 1 × 10^9 CFU/mL.
Effects of combinations of β-lactam and vancomycin on vancomycin-resistant MRSA

Sieradzki et al showed that the combinations of β-lactam antibiotics and vancomycin were highly effective against their isolate of VRSA, and noted that it should be determined whether this combination of antibiotics is effective against other VRSA isolates. Thereafter, Climo et al described the evidence that combinations of vancomycin and β-lactams were synergistic in vitro and also in an in vivo endocarditis model against vancomycin-resistant S. aureus. We have often observed a growth zone around the β-lactam disks on Mu3 medium plates containing vancomycin at 4 μg/mL. On the basis of these findings, we have already reported the effectiveness of vancomycin against VLSA strains in combination with various concentrations of β-lactams. Furthermore, we here found similar effects on VLSA with a combination of vancomycin and a penem. Figure 4 shows the effects of a combination of vancomycin and imipenem on Mu3.
FulO, Fu18, and Mu50 respectively. The population analysis indicates that imipenem at a relatively high concentration of 2 μg/mL, which is 1/8 to 1/64 of the MIC values, induced a reduction in the number of subpopulations resistant to vancomycin. However, at relatively lower imipenem concentrations of 0.05–0.2 μg/mL (data not shown), the number of surviving colonies increased as expected; for example, with 4 μg/mL of vancomycin together with 0.05 μg/mL of imipenem, surviving colonies of Fu18 were about one hundred times those found with the use of vancomycin alone (Figure 4C, arrow).

DISCUSSION

We have reported here a case of a patient with extensive burns and described the properties of MRSA strains isolated from the burn wound. On hospital day 10, the first MRSA strain, Fu6, sensitive to gentamicin, was isolated; this disappeared after treatment with gentamicin. On hospital day 22, we isolated another MRSA strain, Ful0, which was clearly an MRSA strain different from Fu6 by biotype, genotype, and antibiotic sensitivity. As is shown in Figures 1, 3, and 4, Ful0 was obviously a VLSA strain that had properties similar to those of Mu3 in terms of growth on Mu3 medium, gradient gel pattern, and population analysis. On hospital day 44, 9 days after changing the 16-day vancomycin therapy to arbekacin with sulbactam-ampicillin, MRSA strain Ful0 was isolated. Ful0 was entirely identical to Fu10 in terms of genotype and phenotype, but differed in having greater vancomycin resistance. Ful0 and Ful0 had identical PFGE banding patterns, similar to those of Mu3 and Mu50, with only two additional bands. Based on these banding patterns, Ful0 and Fu18 belong to clonotype II-A, which is the prominent MRSA clonotype in Japan, producing type 2 coagulase and TSST-1 toxin. Ful0 was more resistant to vancomycin than Ful0, as indicated by population analysis. A very small population of Ful18 cells grew in 8 μg/mL of vancomycin, whereas Ful0 and Mu3 did not. The growth of Ful0 on Mu3 medium or on gradient gels also indicated that the resistance of Ful0 was much higher than that of Fu10 (Figures 1 and 3). We assume that Ful0 was selected from Ful0 in vivo, during vancomycin treatment, as a subclone with vancomycin resistance close to that of Mu50. It is surprising that such selection of a resistant subclone occurred within 2 weeks.

The peak tissue concentrations obtained by intravenous vancomycin administration are around 5 μg/mL or less (e.g. 5 μg/mL in abscess, 2.49 μg/mL in sputa), except in the blood of hemodialysis patients or in the peritoneal fluid of peritoneal dialysis patients. Therefore, it is likely that in a large number of respiratory or wound infections, not only VRSA but also VLSA will not be eliminated by vancomycin. From these findings, we confirmed that vancomycin therapeutic failure against VLSA resulted in selection of subclones of VLSA with greater vancomycin resistance which then gave rise to the emergence of VRSA in vivo. Fortunately, in the case of our patient, before the subclone with vancomycin resistance equal to that of Mu50 (MIC 8 μg/mL) was selected, the symptoms were relieved by arbekacin and sulbactam–ampicillin combination therapy.

In our experiments with the combinations of vancomycin and imipenem, such combinations were effective against the VLSA and VISA strains at relatively high concentrations of β-lactams, as reported previously. However, the relatively low concentrations of β-lactams affected the resistance of MRSA to vancomycin. It seems that the activated synthesis and turnover of cell walls in VLSA may be enhanced by relatively low concentrations of β-lactams. When compared with vancomycin alone, the resistant subpopulation does significantly increase with the combination of β-lactams. This is one of the reasons why VLSA showed visible growth around β-lactam disks on Mu3 medium. Such lower concentrations of β-lactams occur in all tissues or plasma during the treatment of patients with antibiotics. According to pharmacokinetic studies of imipenem, its mean half-life is 54–110 min; therefore, the peak tissue or plasma concentrations of imipenem obtained with intravenous administration readily decline with time and become undetectable within 8 h. Similar processes occur for all β-lactams. Therefore, one should be cautious about using combination therapy of vancomycin and β-lactams including penems for a patient infected with VLSA.

The VLSA and VISA strains from our present case were also resistant to teicoplanin: MIC for Ful0, 8 μg/mL; MIC for Mu3, Fu18 and Mu50, 16 μg/mL. In Japan, vancomycin was introduced in 1991, and teicoplanin in 1998. It has been reported that S. aureus produces teicoplanin-resistant mutants more readily than vancomycin-resistant mutants, and the resultant mutants are cross-resistant to vancomycin. Therefore, we would like to stress that in the case of VLSA infection, vancomycin and teicoplanin should be used with caution. As Waldvogel has pointed out, intermediate resistance is difficult to detect by standard laboratory methods, and Tabaqchali has emphasized the need to improve the methods to detect VLSA and to monitor the emergence of VRSA, especially in patients unresponsive to vancomycin or teicoplanin therapy. Therefore, we recommend gradient gel analysis and especially Mu3 medium, which are simple and useful for the detection of MRSA with low-level vancomycin resistance. Finally, as seen in Figure 4, population analysis profiles showed that not only hetero-VRSA (Mu3) but also VISA (Mu50) had heterogeneous resistance to vancomycin. To avoid confusion, we proposed and used the new term vancomycin low-level resistant S. aureus (VLSA), which serves as a precursor for VRSA (VISA); VLSA itself can cause vancomycin therapeutic failure.

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REFERENCES


