Letter to the Editor

Genotype Analysis of Methicillin-Resistant *Staphylococcus aureus* With and Without Reduced Susceptibility to Vancomycin Using Pulsed-Field Gel Electrophoresis

To the Editor:

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most frequently isolated bacterial pathogens in nosocomial infections. MRSA strains recently have emerged that show reduced susceptibility to vancomycin, including vancomycin-resistant *S aureus* (VRSA), vancomycin-intermediate *S aureus* (VISA), and *S aureus* heterogeneously resistant to vancomycin (heterogeneously resistant VRSA or hetero-VRSA). Such strains, identified in Tokyo,[1] Michigan, and New Jersey,[2] are associated with failure of vancomycin therapy. While the frequency of isolation of these strains appears to be increasing, the strains have not been investigated previously at our university hospital. We therefore used pulsed-field gel electrophoresis (PFGE) to perform molecular epidemiological analysis of MRSA isolates with and without reduced susceptibility to vancomycin.

Seventy-eight *S aureus* isolates that were isolated from many kinds of clinical samples at Sapporo Medical University Hospital from October to November 1998 were analyzed. All isolates were from different patients. MRSA was defined as *S aureus* showing a minimum inhibitory concentration (MIC) for oxacillin of ≥4 μg/mL using a Microscan Walkaway 96 system (Dade Behring, Tokyo, Japan) with a microdilution method (National Committee for Clinical Laboratory Standards methods). The N315 strain (MRSA clonotype II-A),[3] the Mu3 strain (hetero-VRSA), and the Mu50 strain (VRSA)[1,4] were generously provided by the Department of Bacteriology, Juntendo University, Tokyo, Japan. MU3 agar (Becton-Dickinson, Tokyo, Japan) was used for detection of MRSA with reduced susceptibility to vancomycin.[4,5] The cultures were incubated at 37°C on sheep blood agar. Bacterial suspensions were adjusted to an optical density of 0.3 at 578 nm and smeared on MU3 agar on a base of brain-heart infusion agar containing vancomycin at 4.0 μg/mL and MU3 additive (Becton-Dickinson) at 1.0 mg/mL. To induce vancomycin resistance,[6] three disks were placed on the MU3 agar prior to incubation at 35°C; one each contained cefminox, cefixime, and aztreonam (Sensidisk, Becton-Dickinson). Bacterial growth was inspected at 24 and 48 hours.

S aureus was defined as vancomycin-sensitive if it revealed no growth on MU3 agar at either time point. Hetero-VRSA status also was assigned if the strain produced a subclone with a vancomycin MIC of ≥8 μg/mL upon selection with vancomycin, provided that the strain retained these properties after 9 days in drug-free medium. VSRA was identified by confluent growth on MU3 agar, confirmed by a vancomycin MIC of ≥8 μg/mL. Coagulase type, staphylococcal enterotoxin type, and production of toxic shock syndrome toxin-1 (TSST-1) also were determined using an antiserum kit for detection of *S aureus* coagulase types, a SET-RPLA kit for *S aureus* enterotoxins, and a TST-RPLA kit for detection of *S aureus* TSST-1, respectively (Denkaseiken, Tokyo, Japan). PFGE was performed using the GenePath strain typing system (Bio-Rad, Tokyo, Japan). DNA from the isolates was prepared according to the manufacturer's protocol, and all reagents were supplied in the GenePath Reagents Kit 1. Samples of 150 μL from overnight cultures grown in 3 mL of brain-heart infusion broth were harvested by centrifugation at 12,000*g* for 1 minute, resuspended in 150 μL of cell suspension buffer, and

TABLE 1
CLASSIFICATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* USING COAGULASE TYPE, STAPHYLOCOCCAL ENTEROTOXIN TYPE, AND PRODUCTION OF TOXIC SHOCK SYNDROME TOXIN-1

Phenotype	Coagulase Type	SE Type	Production of TSST-1	No. of Samples (%)
I	II	C	+	62† (87.2)
II	II	BC	+	7 (9.0)
III	II	C	−	2 (2.6)
IV	NT	C	+	1 (1.3)
V	II	AC	+	1‡ (1.3)
VI	III	A	−	1 (1.3)
VII	II	B	−	1 (1.3)
VIII	II	NT*	−	1 (1.3)
IX	VII	NT*	−	1 (1.3)
X	NT	NT*	−	1 (1.3)
Total				78

Abbreviations: +, positive; −, negative; NT, not typeable; SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin-1; VRSA, vancomycin-resistant *Staphylococcus aureus*.
* No toxin (non-producing).
† Five hetero-VRSA isolates are contained.
‡ This isolate is a hetero-VRSA.

maintained at 55°C. Electrophoresis was performed in 1% agarose gels using CHEF-DRIII (Bio-Rad) for staphylococcal species, using parameters preprogrammed with the Bio-Rad GenePath system. Electrophoretic patterns were analyzed according to criteria described by Tenover et al.[7] *S aureus* National Collection of Type Cultures 8325 was used as a control for PFGE.

Seventy-eight MRSA isolates were cultured on MU3 agar for detection of MRSA with reduced susceptibility to vancomycin. Of 6 isolates that grew on MU3 agar, 1 isolate showed a VRSA pattern and 5 showed a hetero-VRSA pattern. One isolate that showed a VRSA pattern on MU3 agar was sensitive to vancomycin at ≤4 μg/mL. Another five isolates that showed a hetero-VRSA pattern on MU3 agar also were sensitive to vancomycin at ≤4 μg/mL. All six isolates were sensitive to teicoplanin at ≤8 μg/mL. Accordingly, these 6 (7.7%) among 78 MRSA isolates were determined to be hetero-VRSA, considering the growth pattern on MU3 agar together with the MIC for vancomycin.

Seventy-eight MRSA isolates were classified into nine major types (designated A to I; Figure 1 available on request) using PFGE analysis following *Sma*I digestion. Seventy of 78 isolates exhibited variations on this (type A) theme with two to six restriction-fragment differences from the epidemic pattern. PFGE type A was further classified from A1 to A27. Isolates from A1 to A12 showed closely related patterns, and those from A13 to A27 showed possibly related patterns. Three isolates of hetero-VRSA showed the same pattern (A1), and the remaining three isolates showed patterns closely related to A1 (A2, A7, and A18).

The 10 phenotypes (types I to X in Table 1) and 9 genotypes (PFGE types A to I) were compared (Table 2). MRSA strains showed complete correspondence between phenotype VI and genotype E; phenotype VII and genotype I; phenotype VIII and genotype C; and phenotype IX and genotype F. However, phenotypes I and II both were represented among strains with genotype A. Genotypes B, D, G, and H all showed phenotype I.

We assessed molecular epidemiological methods in characterizing MRSA with and without reduced susceptibility to vancomycin. Analysis of the 9 genotypes and 27 subtypes by PFGE facilitated clear epidemiological tracing. Moreover, three isolates among six hetero-VRSA had the same pattern (A1) by PFGE, whereas the other three isolates showed genetic patterns closely related to A1 (A2, A7, and A18). Genotyping could not specifically identify these six isolates. However, from a practical standpoint, a PFGE type of A1 indicates that an isolate may represent hetero-VRSA and be likely to result in vancomycin treatment failure.

The Centers for Disease Control and Prevention recently emphasized that hospital laboratories need to be able to identify strains with reduced susceptibility; because intermediate resistance is difficult to detect by standard laboratory methods, discovery of spread and outbreaks may be delayed.[8] Indeed, in Japanese hospitals, hetero-VRSA may represent 1.3% to 20% of MRSA, including a reported 9.3% in one university hospital.[1] Moreover, the risk of VRSA clones arising from hetero-VRSA has been emphasized.[1] In our own university hospital, the detection rate of strains of hetero-VRSA amounted to 7.7% of MRSA isolates (6/78). In emergence of VRSA from hetero-VRSA, underlying genomic changes in the

TABLE 2
CORRELATION BETWEEN GENOTYPE AND PHENOTYPE

PFGE Genotype		Interpretation of PFGE	Phenotype	
A	(17)*	Outbreak strain (17)	I	(14)
			II	(3)
A1	(7)		I	(7)†
A2	(6)		I	(5)‡
			II	(1)
A3	(2)		I	(2)
A4	(2)		II	(2)
A5	(1)		I	(1)
A6	(1)	Closely related (34)	X	(1)
A7	(1)		I	(1)‡
A8	(14)		I	(14)
A9	(1)		II	(1)
A10	(1)		I	(1)
A11	(1)		I	(1)
A12	(1)		I	(1)
A13	(1)		I	(1)
A14	(1)		I	(1)
A15	(1)		I	(1)
A16	(1)		I	(1)
A17	(1)		I	(1)
A18	(1)		V	(1)‡
A19	(1)		I	(1)
A20	(1)	Possibly related (19)	I	(1)
A21	(1)		I	(1)
A22	(1)		IV	(1)
A23	(1)		III	(1)
A24	(1)		I	(1)
A25	(1)		I	(1)
A26	(1)		III	(1)
A27	(1)		I	(1)
B	(1)		I	(1)
C	(1)		VIII	(1)
D	(1)		I	(1)
E	(1)		VI	(1)
F	(1)	Different (8)	IX	(1)
G	(1)		I	(1)
H	(1)		VII	(1)
I	(1)		VI	(1)
Total	78			78

Abbreviations: PFGE, pulsed-field gel electrophoresis; VRSA, vancomycin-resistant *Staphylococcus aureus*.
* Indicates isolate numbers.
† Includes three hetero-VRSA isolates.
‡ Includes one hetero-VRSA isolate.

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strains have not yet been characterized. Such genetic change in hetero-VRSA strains could not be investigated in this study, because VRSA was not isolated at our hospital.

Pulsed-field gel electrophoresis patterns of hetero-VRSA isolates (A1, A2, A7, and A18) were compared with those of MRSA strains with reduced susceptibility to vancomycin isolated elsewhere in the world. PFGE patterns of our hetero-VRSA differed by five to nine bands from those of the Juntendo strains (N315, Mu3, and Mu50), the first to be reported. Hiramatsu et al reported that Mu3 and Mu50 were genetically the same by PFGE analysis and were genetically closely related to N315 (MRSA clonotype II-A), which represented 70% of Japanese MRSA isolates.[1] These authors therefore concluded that Mu3 and Mu50 originated from MRSA clonotype II-A, and that this MRSA clonotype had the ability to give rise to VRSA more rapidly than other strains.[1] However, our hetero-VRSA may be of a different type from N315, Mu3, and Mu50. Our strain therefore may include new clones with the ability to give rise to hetero-VRSA in Japan. Furthermore, we compared PFGE patterns in our strains with those in the Michigan strain (VISA, the first reported US case) and the New Jersey strain (VISA, the second reported US case), based on the PFGE pattern of molecular weight marker.[2] The Michigan strain (1-3 bands) and the New Jersey strain (2-5 bands) were different from our hetero-VRSA strains, although our PFGE patterns for A1 and A7 genetically resemble the Michigan strain and the New Jersey strain. Smith et al reported that the Michigan strain and another MRSA isolate from the same patient showed an identical PFGE pattern.[2] In our study, the A1 genotype was present in three hetero-VRSA and four vancomycin-sensitive MRSA isolates, whereas genotype A2 included one hetero-VRSA and six vancomycin-sensitive MRSA. Notably, all of our strains were isolated from different patients. These results suggest that certain MRSA clones that can give rise to VRSA, VISA, and hetero-VRSA may exist in various geographic areas.

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