Nosocomial spread of meticillin-resistant Staphylococcus aureus with \( \beta \)-lactam-inducible arbekacin resistance

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A meticillin-resistant Staphylococcus aureus (MRSA) strain with additional \( \beta \)-lactam-inducible aminoglycoside resistance was previously reported by a group at the Kitasato University in Japan. In addition to gentamicin, the ‘Kitasato strain’ was resistant to arbekacin (ABK), which is primarily used as an anti-MRSA aminoglycoside. No further studies regarding the spread of MRSA strains with the newly identified resistance mechanism have been reported to date. To obtain epidemiological data on MRSA strains with the antagonistic resistance and to analyse their genetic features, we examined the emergence of \( \beta \)-lactam-inducible ABK-resistant MRSA strains at our university hospital using longitudinal analysis. Among the 396 isolates, 35 (8.8 %) were found to be ABK-resistant MRSA strains (the resistance being induced by \( \beta \)-lactams). Moreover, based on the pulsed-field gel electrophoresis profiles, the clonality of those MRSA strains changed at different time periods. In the Kitasato strain, the antagonistic mechanism was clearly demonstrated by the integration of transposable elements; a Tn4001-IS257 hybrid structure that contained an aminoglycoside resistance gene cointegrated into a region downstream of the \( \beta \)-lactamase gene. In most of the MRSA strains detected in our study, the antagonistic interaction was explained by the same mechanism as that found in the Kitasato strain. Interestingly, sequence analysis showed that all of our strains carried IS257 insertion sites which were different from those of the Kitasato strain. This study shows that MRSA strains with the additional antagonistic resistance are not uncommon and have been increasingly disseminating in clinical settings.

INTRODUCTION

Meticillin-resistant Staphylococcus aureus (MRSA), which is now frequently detected among S. aureus isolates worldwide, has become a problematic pathogen for nosocomial infections (Liu et al., 2011). Vancomycin and linezolid are major antibiotics used against MRSA infections. However, the resistance to these antibiotics is constantly increasing (Liu et al., 2011). Arbekacin (ABK), which belongs to the aminoglycoside family, is used as an anti-MRSA agent, particularly in Japan. Recently, the strong activity of ABK against MRSA has also been recognized in other countries (Hamilton-Miller & Shah, 1995; Lee et al., 2003; Hwang et al., 2012). ABK is often used in combination with \( \beta \)-lactam agents in cases of serious and/or polymicrobial infections, since ABK is a strong aminoglycoside agent highly active against Gram-negative bacteria (Watanabe et al., 1997; Hamada et al., 2011).

Approximately 10 years ago, the antibiotic resistance of MRSA to ABK was reported to be induced by the antagonistic action of aminoglycosides and \( \beta \)-lactams (Ida et al., 2002). The MRSA strain was isolated as a monoclonal strain at the Kitasato University Hospital in Japan. Genetic analysis of that strain demonstrated the transposable element-related mechanism that the Tn4001-IS257 hybrid structure generated through IS257-mediated cointegration is related to the \( \beta \)-lactam-induced expression of an aminoglycoside-modifying enzyme. No subsequent studies...
regarding this strain have been reported to date. Therefore, the emergence of those strains may be extremely rare. We examined the emergence of β-lactam-inducible ABK-resistant MRSA strains over a 6 year period at our hospital.

**METHODS**

**Samples.** The interaction between meropenem (MEPM) and non-β-lactam anti-MRSA antibiotics was examined using the chequerboard technique with the isolated MRSA strains from our previous study (Tsuchimochi et al., 2007). Antagonistic resistance was detected only with the combined use of MEPM and ABK (data not shown). The present study was conducted at Kyushu University Hospital from 2003 to 2008. The study samples were 396 strains of MRSA for which resistance to MEPM was confirmed based on the MICs. The number of blood, sputum and urine samples collected was 68, 236 and 92, respectively. When multiple samples were obtained from the same patient, only the first isolate from each patient was included in the analysis.

**Antibiotics and susceptibility testing.** Different antibiotics of known potency were used: MEPM (Dainippon Sumitomo Pharma) and ABK (Meiji Seika Kaisha). The MICs were determined by the agar plate dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2006). MRSA was detected based on the criteria recommended by the CLSI. Strains with ABK MICs of ≥4 μg ml⁻¹ were assessed as ABK-resistant, based on the criteria recommended by the Japanese Society of Chemotherapy (Saito, 1995). Strains with MEPM MICs of ≥16 μg ml⁻¹ were assessed as MEPM-resistant, based on the CLSI criteria. Among the 396 MRSA isolates, the MIC range of ABK was 0.25–16 and ≤0.125–16 alone and in combination with MEPM, respectively. Among the 35 isolates with antagonistic interaction, the MIC range of ABK was 0.5–2 and 4–16 alone and in combination with MEP, respectively.

**Chequerboard technique and double-disc diffusion test.** The combined effect of MEPM and ABK was measured by a chequerboard titration assay using the agar dilution method and was evaluated on the basis of the fractional inhibitory concentration (FIC) index values. The antibiotic interaction was considered to be antagonistic if the FIC index was >2. The chequerboard technique was performed in our previous study (Tsuchimochi et al., 2007) for the 207 samples isolated from 2003 to 2005, and was conducted in the present study for the 189 samples isolated from 2006 to 2008. The FIC index ranged from 0.28 to 16.25 in the 396 MRSA strains examined, of which 35 isolates displayed antagonistic interaction (FIC range: 2.13–16.25). The double-disc method was also conducted to confirm the interpretation of the chequerboard assay.

**Pulsed-field gel electrophoresis.** PFGE of DNA digested with Smal (Takara Bio) was conducted using CHEF Genomic DNA plug kits (Bio-Rad) according to the manufacturer’s instructions. The resulting dendrogram was calculated using the unweighted pair group method with arithmetic averages using Molecular Analyst Software Fingerprinting DST (Bio-Rad) (Uchida et al., 2010). A similarity cut-off point of ≥80 % was used for cluster definition (Tenover et al., 1995; McDougal et al., 2003).

**PCR and sequencing.** PCR primers were synthesized on the basis of the reported DNA sequences (Ida et al., 2002) and were as follows: forward primer (P1), 5′-AATCCCTGGAAAGAAGGTTAG-3′ and reverse primer (A2), 5′-ATGGCAAGCTTAGGATTAC-3′. Total DNA from the cultured bacteria was purified using a Wizard Genomic DNA Purification Kit (Promega Corporation). PCR amplification was performed with a DNA thermal cycler (Applied Biosystems). The cycling program was repeated for 30 cycles and included a denaturing step at 94°C for 1 min, an annealing step at 57°C for 2 min and an extension step at 72°C for 3 min. The reaction products were analysed by electrophoresis on 1.5% agarose gels. PCR products were detected by ethidium bromide staining, followed by UV illumination. Sequencing was performed using synthetic PCR primers and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer’s instructions. Sequence analysis was performed using an ABI Prism 3100 genetic analyser (Applied Biosystems).

**Nucleotide sequence accession number.** The DNA sequences of the PCR fragments of pattern I, II, and III were determined and deposited in the GenBank/EMBL/DDBJ nucleotide sequence databases.

**RESULTS AND DISCUSSION**

Among the 396 MRSA isolates detected from 2003 to 2008, 35 (8.8 %) displayed the antagonistic interaction between MEPM and ABK (Table 1). Those MRSA isolates were isolated from various sites. With particular regard to those from blood samples, approximately 20 % of the isolates showed the antagonistic interaction. Longitudinal analysis showed 20 (9.7 %) and 15 (7.9 %) strains collected during the periods 2003–2005 and 2006–2008, respectively, were characterized by the interaction, indicating the constant presence of these MRSA strains.

The genetic relatedness of the MRSA isolates carrying the antagonistic interaction was examined using the PFGE profiles (Fig. 1). Based on an 80 % similarity cut-off value, a total of 15 distinct PFGE clusters were found among the 35 isolates. Tenover’s criteria are often used to interpret the genetic relatedness of bacterial strains by studying PFGE profiles (Tenover et al., 1995; Blanc et al., 2001; McDougal et al., 2003). Based on those criteria, PFGE profiles suggested that the 15 distinct clusters could be further divided into two clonally unrelated groups with a similarity of <40 %. Clusters A–H and I–O were detected approximately from 2003 to 2005 and from 2006 to 2008, respectively. Thus, those clonally unrelated MRSA strains emerged in succession.

Staphylococcal strains, *S. aureus* in particular, carrying a Tn4001-IS257 hybrid structure have been previously reported (Byrne et al., 1990; Berg et al., 1998; Lange et al., 2003; Kozitskaya et al., 2004). The Kitasato University group (Ida et al., 2002), which initially reported the existence of clinical MRSA strains carrying the mechanism of β-lactam-inducible ABK resistance, demonstrated that a Tn4001-IS257 hybrid structure was integrated downstream of a *bla*Z gene carried on Tn552 (Rowland & Dyke, 1990) (Fig. 2). In the Tn4001-IS257 hybrid structure, a 105 bp inverted repeat, which is usually located in the 5′-end region of IS256 on Tn4001 (Rouch et al., 1987), was truncated by the IS257 insertion. The inverted repeat region is involved in preventing the overexpression of the transposase gene of IS256 in response to a foreign upstream promoter (Ida et al., 2002). Since penicillinase induces the expression of the *aac(6′)-Ie-aph(2′)-Ia* gene carried on
Tn4001, which encodes an aminoglycoside-modifying enzyme, the Kitasato group attributed the antagonistic mechanism to the β-lactam-inducible transcription of penicillinase. We confirmed the expression of the aac(6′)-le-aph(2′)-Ia gene using PCR in all of the MRSA isolates with antagonistic function (data not shown). Therefore, we presumed that the antagonistic mechanism detected in our isolates might be similar to that demonstrated for the Kitasato strain.

To verify this assumption, we conducted PCR amplification on the gene region including the 5′-end region of blaZ, IS257, and the 3′-end region of IS256, by using the same primers that were used by the Kitasato University group (Ida et al., 2002). The length of the PCR fragment in the ‘Kitasato strain’ was 2158 bp. All of the 35 strains examined in this study yielded PCR fragment lengths different from those yielded by the Kitasato strain (data not shown). The PCR fragments obtained were classified into three structural patterns (i.e. I, II and III) depending on the length of the amplicon. The detected numbers of patterns I, II and III were 21, 12 and 2, respectively. To compare the amplified fragments with those of the Kitasato strain, DNA sequencing of the PCR products was performed.

The lengths of the DNA fragments of patterns I, II and III were found to be 1876, 2229 and 2849 bp, respectively (Fig. 2). In the Kitasato strain, the amplified DNA fragment included a portion of blaRI, truncated blaZ, IS257, truncated IS256 and orf132 (Fig. 2). Both the blaRI and blaZ genes are carried on Tn552 (Rowland & Dyke, 1990), while IS256 and orf132 genes are located on Tn4001 (Rouch et al., 1987). Thus, in the Kitasato strain, a Tn4001-IS257 hybrid structure is inserted downstream of the Tn552 gene region.

In the Kitasato strain, the downstream region of the blaZ gene was truncated at the level of nucleotide 405 from the beginning of the gene, and was inserted by the IS257 element (Ida et al., 2002) (Fig. 2). In the sequences of the PCR products belonging to patterns I and II, the blaZ regions were deleted at the level of nucleotides 89 and 448, respectively (Fig. 2). In addition, both of the sequences of patterns I and II contained IS257 and truncated IS256 elements. Therefore, the inverted repeat in the 5′-end region of IS256 was substituted by the IS257 insertion. In the sequence of the PCR products belonging to pattern III, the blaZ gene was intact and followed by IS256 without the insertion of IS257 (Fig. 2). Therefore, the IS256 element region retained the inverted repeat that was found on Tn4001.

Our findings suggest that the antagonistic interaction detected in our MRSA strains could be explained by the mechanism demonstrated by the Kitasato group, although the mechanism in the strains with PCR pattern III has not been elucidated. Interestingly, the insertion sites of the Tn4001-IS257 hybrid structure detected in our strains were different from that of the Kitasato strain, meaning that diverse insertion sites of the hybrid structure are available.

<table>
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<th>Source</th>
<th>Total no. of isolates</th>
<th>No. (%) of isolates with antagonism</th>
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<tr>
<td></td>
<td>2003-2005</td>
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<tr>
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<td>Total</td>
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<td>20 (9.7)</td>
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<tr>
<td>Sputum</td>
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<tr>
<td>Total</td>
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<th>Source</th>
<th>Total no. of isolates</th>
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<tr>
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<tr>
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<td>682</td>
<td>46 (6.8)</td>
</tr>
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**Table 1. Numbers of β-lactam-inducible ABK-resistant MRSA strains isolated from hospitalized patients.**

ABK, Arbekacin; MRSA, meticillin-resistant Staphylococcus aureus.
Fig. 1. Dendrogram of PFGE profiles of Smal-digested DNA from MRSA strains showing the antagonistic interaction between arbekacin and meropenem. These MRSA strains were isolated from in-patients in the Kyushu University Hospital. Strain and cluster designation, PCR patterns and date of isolation are shown on the right-hand side. Using a similarity cut-off point of ≥ 80%, PFGE identified 15 clusters (i.e. A–O).

Fig. 2. Schemes and comparison of transposon-like gene regions including Tn552-like and Tn4001-IS257 hybrid elements. The upper panel shows a scheme of the transposon-like elements identified in the plasmid isolated from the ‘Kitasato strain’. The lower panel shows a comparison between the structures of the 5′-end region of blaZ, IS257, and the 3′-end region of IS256 of the Kitasato strain and the MRSA strains detected in this study.
The multiple copies of IS257 may provide a number of insertion sites for resistance determinants (Byrne et al., 1990). In Japan, the Tn4001 transposon structure was detected at high frequency in the MRSA strains isolated in another hospital (Udou, 2004). The Tn4001-IS257 hybrid structure, as well as Tn4001, may be frequently inserted into various regions involving β-lactamase genes via IS257-mediated cointegration.

The effectiveness of ABK as an anti-MRSA agent is well established in Japan. The use of anti-MRSA agents in combination with β-lactams is clinically common. Genetic analysis suggests that efficient mobile elements carried by MRSA may have accelerated the genetic diversity and the rapid spread of antibiotic resistance genes. Therefore, a surveillance scheme for MRSA strains carrying the additional antagonistic resistance that was detected in our hospital may be needed.

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REFERENCES


