Cell wall thickening is associated with adaptive resistance to amikacin in methicillin-resistant Staphylococcus aureus clinical isolates

Wenchang Yuan1, Qiwen Hu1, Hang Cheng1, Weilong Shang1, Nan Liu2, Ziyu Hua2, Junmin Zhu1, Zhen Hu1, Jizhen Yuan1, Xia Zhang1, Shu Li1, Zhijin Chen1, Xiaomei Hu1, Jianfeng Fu3† and Xiancai Rao1*†

1Department of Microbiology, College of Basic Medical Sciences, Third Military Medical University, Chongqing, China; 2Department of Laboratory Medicine, Children’s Hospital of Chongqing Medical University, Chongqing, China; 3Department of Laboratory Medicine, The Military General Hospital of Urumchi People’s Liberation Army, Xinjiang, China

*Corresponding author. Tel: +86-23-68752240; E-mail: raoxiancai@126.com
†Drs Rao and Fu made an equal contribution to this study.

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Objectives: Methicillin-resistant Staphylococcus aureus (MRSA) infection is increasing and causing global concern. The mechanism of MRSA resistance to amikacin is poorly understood. We report on the first matched-pair study to reveal that the phenotypic cell wall thickening of MRSA is associated with adaptive resistance to amikacin.

Methods: Two MRSA strains (CY001 and CY002) were isolated from blood and synovial fluid samples, respectively, from a 12-year-old male patient with osteomyelitis. The strains were subjected to a matched-pair study, including antimicrobial agent susceptibility determination, molecular typing, morphological observation and in vitro resistance induction.

Results: Both strains are Panton–Valentine leucocidin-positive, multilocus sequence type 59, staphylococcal cassette chromosome mec type IV and spa type 437 MRSA with identical PFGE profiles. The drug susceptibility spectra of the two isolates are similar. However, CY001 is resistant to amikacin (CY001-AMI R; MIC = 64 mg/L), contrary to the susceptible CY002 (CY002-AMI S; MIC = 8 mg/L). CY001-AMI R may have developed adaptive resistance, because it lacks aminoglycoside-modifying enzymes and has an altered growth curve. Interestingly, CY001-AMI R has a thicker cell wall (36.43 ± 4.25 nm) than CY002-AMI S (18.15 ± 3.74 nm) in the presence of amikacin at its MIC. The thickened cell wall can also be observed in an in vitro-induced strain (CY002-AMI R) in the presence of amikacin at its MIC (36.78 ± 3.41 nm); this strain was obtained by gradually increasing the amount of amikacin. However, the cell wall-thickened strains cultured in the presence of amikacin are still susceptible to vancomycin.

Conclusions: Cell wall thickening is associated with adaptive resistance in MRSA and alternative antibiotics can be used to treat patients when adaptive resistance to amikacin has developed.

Keywords: osteomyelitis, matched-pair study, aminoglycosides, penicillin-binding protein 2

Introduction

Staphylococcus aureus, especially methicillin-resistant S. aureus (MRSA), is a crucial human pathogen that causes both healthcare- and community-acquired infections. MRSA was first discovered in 1961 and the prevalence of MRSA strains has reached 80% worldwide and it has become a major global public health concern. The severity of the disease caused by MRSA varies from mild to debilitating pathogenic infections (such as skin and soft tissue infections) to severe life-threatening diseases (such as pneumonia, septic arthritis, endocarditis, osteomyelitis and sepsis). Aminoglycosides are among the most commonly used broad-spectrum antibiotics in the anti-infective armamentarium. The in vitro antimicrobial spectrum of the aminoglycosides includes a broad range of aerobic Gram-negative bacilli, staphylococci and certain mycobacteria. Aminoglycosides exert their bactericidal effects by irreversibly binding to the 30S ribosomal subunit of the susceptible bacteria, inhibiting protein synthesis. MRSA developed resistance to aminoglycosides. The most common mechanism for clinical resistance to these antibiotics is the structural modification of aminoglycosides by aminoglycoside-modifying enzymes (AMEs). Three classes of AMEs, namely, aminoglycoside acetyltransferases (AACs), aminoglycoside phosphotransferases...
(APHs) and aminoglycoside adenylyltransferases (ANTs), are responsible for the structural modification of aminoglycosides.\textsuperscript{6} The bifunctional enzyme AAC(6′)/APH(2′′) \textsuperscript{a} [encoded by the gene aac(6′)/aph(2′′)] and ANT(4′)-I \textsuperscript{b} [encoded by ant(4′)-Ia] are the most frequently encountered AMEs in MRSA.\textsuperscript{6–8} Amikacin, a broad-spectrum semi-synthetic derivative of kanamycin, is a poor substrate of many AMEs. Furthermore, even if amikacin is modified by AMEs, the modified amikacin can still bind to the 30S ribosomal subunit. Thus, amikacin becomes especially considered when patients are treated empirically for suspected sepsis.\textsuperscript{5} However, amikacin-resistant MRSA strains can still be isolated from clinics and the resistance mechanism is ambiguous.

Adaptive resistance is a phenomenon in which certain environmental cues, such as subinhibitory concentrations of antimicrobials, can transiently induce resistance to lethal doses of antimicrobial agents.\textsuperscript{9} Aminoglycosides have concentration-dependent antibacterial activity, indicating that the eradication of bacteria is only a function of the drug concentration. Based on a series of pharmacodynamic studies of aminoglycosides, once-daily aminoglycoside dosing regimens have been established and optimal antibacterial activity is achieved when the serum aminoglycoside peak concentration is 8–10× greater than the MIC. However, even at the higher amikacin doses applied for patients with severe sepsis and septic shock, the peak concentrations remained below the therapeutic target levels in about one-third of patients.\textsuperscript{10} A low dose is usually applied to children and adolescents; thus, the peak concentration might fall further below the therapeutic target levels. A low dosage will lead to adaptive resistance, resulting in the clinical failure of aminoglycoside administration. In addition, the adaptive resistance of \textit{Pseudomonas aeruginosa} to aminoglycosides has been discovered in animal models and in cystic fibrosis patients.\textsuperscript{9} However, whether MRSA can develop adaptive resistance to amikacin because of improper dosage is unknown.

In this study, we surprisingly isolated both an amikacin-resistant and an amikacin-susceptible MRSA strain from one patient. A matched-pair study was performed to reveal that improper amikacin dosage can induce adaptive resistance and that cell wall thickening is associated with adaptive resistance to amikacin in MRSA.

**Methods**

**Patient and treatment summary**

A 12-year-old male adolescent, with 11 days of high fever, chills, sharp pains in the right hip and difficulty in ambulating, was hospitalized. Magnetic resonance imaging results showed the presence of fluid collection in his right hip after 7 days of empirical treatment with amikacin at a rural clinic. He recovered after 48 days of treatment and the relationship of the white blood cell (WBC) count and C-reactive protein to the therapy regimen by treatment day is shown in Table 1.

**Strain isolation, characterization and typing**

A blood sample obtained 1 day after admission and a synovial fluid sample obtained 13 days after admission were subjected to bacterial isolation (Table 1). Two bacterial strains, CY001 and CY002, were isolated from the blood and synovial fluid samples, respectively, and characterized as staphylococcal strains using a Microscan Walkaway-40 Automatic Bacteria Analyzer (Dade Behring, USA). The samples obtained 24 days after admission tested negative for bacteria. The 16S rRNA, mecA, \textit{femB} and \textit{pvl} genes were detected using a multiplex PCR assay as previously described.\textsuperscript{7} MRSA strain JCSC 4744 served as a control.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Days after & Therapy regimen & WBC count (normal, & C-reactive protein & Samples for \\
admission & & 4000–10000/mm\textsuperscript{3})/\% & (normal, 0.0–5.0 mg/L) & bacteria isolation \\
& & neutrophils & & \\
\hline
0 & 1.5 g of intravenous amoxicillin/sulbactam sodium every 12 h; 0.5 g of intravenous ceftriaxone every 12 h; 2.0 g of intravenous vitamin C daily & 20560/88 & 160 & blood \\
1 & & 11080/83 & 160 & \\
5 & 0.5 g of intravenous ceftriaxone every 12 h; 1.0 g of intravenous vitamin C daily & & & synovial fluid \\
9 & & & & blood \\
13 & & & & synovial fluid \\
24 & & & & \\
27 & & & & \\
32 & 0.5 g of intravenous ceftriaxone every 12 h & 5580/61 & 23 & \\
33 & & & 19 & \\
34 & & & 16 & \\
43 & & & & \\
47 & & & & \\
48 & resolution & & & \\
\hline
\end{tabular}
\caption{Relationship of WBC count and C-reactive protein to therapy regimen by treatment day}
\end{table}
Adaptive resistance to amikacin in MRSA

The typing methods, including PFGE,11 multilocus sequence typing,12 staphylococcal cassette chromosome (SCC) mec typing13 and staphylococcal protein A (spa) gene typing,15 were applied to the MRSA strains of interest.

**Growth curve determination**

The growth curves of CY001 and CY002 were obtained by measuring the optical density (OD) at 600 nm at 1 h intervals for 24 h at 37°C. To achieve the same initial inoculation, the overnight culture of each strain was 1:100 diluted with fresh bacterial medium and subjected to growth curve determination.

**PCR analysis for amikacin resistance genes**

The isolates were screened for the presence of the ant(4′)-Ia and aac(6′)/aph(2′) genes that encode the ANT(4′)-I and AAC(6′)/APH(2′) enzymes, respectively, which are involved in mediating amikacin resistance.7,8 MRSA N315 and MRSA NF65, a vancomycin-intermediate resistant S. aureus (VISA) isolate with a vancomycin MIC of 8 mg/L, were used as controls.

**Quantitative real-time PCR (qRT–PCR)**

Total RNA was extracted from CY001 and CY002 cultures supplemented with amikacin using the TriPure isolation reagent (Roche Applied Science, Germany). The bacteria were lysed using lysostaphin (Sigma Life Science, USA) prior to RNA extraction. The cDNA was synthesized using a PrimeScript RT reagent kit (TaKaRa, China) from 500 ng of total RNA with random primers. qRT–PCR was performed to quantify the mRNA expression of the pppB gene using SYBR Premix Ex Taq II (TaKaRa, China). The primers used are listed in Table S1 (available as Supplementary data at JAC Online).

**Transmission electron microscopy (TEM)**

The isolates CY001 and CY002 were cultured in tryptic soy broth with a suitable concentration of amikacin (CY001, 64 or 128 mg/l; and CY002, 8 mg/l) for 24 h at 37°C with shaking. Strains cultured without antibiotics served as controls. The samples were prepared as previously described15 and observed under a TECNAI 10 transmission electron microscope (Philips, The Netherlands). The cell wall thickness was determined in four separate quadrants of each cell and >30 cells of each strain with nearly equatorial cut surfaces were chosen for determination. The results are expressed as means ± SD and the data were evaluated using Student’s t-test, where a P value <0.05 was considered statistically significant. All statistical tests were two-tailed.

**In vitro screening of amikacin-resistant clone**

An overnight CY002 culture, grown from a single colony, was diluted 1:500 in brain heart infusion (BHI) broth supplemented with gradually increasing concentrations of amikacin (from 2 to 64 mg/l) and grown at 37°C with shaking. Daily-culture individual isolates were obtained by plating the diluted culture onto a BHI agar plate containing various concentrations of amikacin. A single colony in the presence of the highest concentration of amikacin was designated as CY002-AMI(R).

**Results**

**Isolation of two MRSA strains from a patient**

Two staphylococcal strains (CY001 and CY002) were isolated from the same patient. Given the high prevalence of MRSA strains in sepsis, we examined whether these two strains were MRSA isolates. PCR amplification with the set of 16S rRNA-specific primers yielded a product of 756 bp for both strains. Their sequences were determined by direct DNA sequencing and confirmed that these two strains were S. aureus. Furthermore, both strains harboured mecA and femB genes (Table 2). Thus, we conclude that MRSA strains caused the sepsis of the patient. Molecular typing methods confirmed that both strains were Panton–Valentine leucocidin-positive, SCCmec type IV, multilocus sequence type 59 and spa type 437 MRSA. In addition, the PFGE profiles of the two MRSA isolates were identical (data not shown), suggesting that CY001 and CY002 were generated from the same parental strain.

**CY001-AMI(R) is resistant to amikacin, contrary to amikacin-susceptible CY002-AMI(S)**

The patient was treated with amikacin for 7 days at a rural clinic, but failed to eradicate the MRSA strains. Given the multidrug resistance of MRSA strains, we measured the amikacin susceptibility of the two strains and expected to see that both strains were resistant to amikacin. However, surprisingly, CY001 was resistant to amikacin (CY001-AMI(R)), with an expected MIC value of 64 mg/l, whereas CY002, isolated from synovial fluid, was susceptible to amikacin (CY002-AMI(S)), with an MIC value of 8 mg/l (Figure 1a).

**CY001-AMI(R) does not have AMEs**

The most common mechanism for clinical resistance to aminoglycosides is the structural modification by AMEs. CY001-AMI(R) and CY002-AMI(S), with similar molecular types, prompted us to examine whether AMEs are responsible for the resistance of CY001 to amikacin. The aac(6′)/aph(2′) and ant(4′)-Ia genes are the most frequently identified in MRSA isolates.6–8 As shown in Table 2, both CY001-AMI(R) and CY002-AMI(S) strains do not carry either of these genes, as detected by PCR, indicating that the most frequently encountered AMEs in clinical isolates are not responsible for the amikacin resistance in CY001-AMI(R).

**CY001-AMI(R) developed adaptive resistance to amikacin**

The lack of AMEs prompted us to examine whether CY001-AMI(R) developed adaptive resistance to amikacin. We measured the growth rates of CY001-AMI(R) and CY002-AMI(S) with or without amikacin treatment. Without amikacin treatment, the growth rate of CY001-AMI(R) is slower than that of CY002-AMI(S) (Figure 1b).

<table>
<thead>
<tr>
<th>Strain</th>
<th>16S rRNA</th>
<th>mecA</th>
<th>femB</th>
<th>pvl</th>
<th>ant(4′)-Ia</th>
<th>aac(6′)/aph(2′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY001</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CY002</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>JCSC 4744</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>N315</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>+</td>
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</tr>
<tr>
<td>NF65</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.
The altered growth pattern in the presence of amikacin indicates that CY001-AMIR developed adaptive resistance to amikacin.

**CY001-AMIR** has a thickened cell wall and increased expression of the pbpB gene

VISA strains exhibit characteristic cell wall thickening that is believed to confer vancomycin resistance. A thickened cell wall may decrease the permeability of amikacin. Whether CY001-AMIR also has a thickened cell wall was determined. We used TEM to observe the phenotypic changes of the strains. Without amikacin treatment, the cell wall thickness of CY001-AMIR is similar to that of CY002-AMIS. However, in the presence of amikacin at its MIC, CY001-AMIR has a thicker cell wall (36.43 ± 4.25 nm) than that of CY002-AMIS (18.15 ± 3.74 nm). In the presence of amikacin at 2× its MIC, the cell wall thickness is further increased (70.97 ± 12.50 nm) (Figure 2a and b). The biosynthesis of the MRSA cell wall is complex and >20 enzymes are involved in the catalysis of peptidoglycan synthesis. The S. aureus pbpB gene encodes a two-domain protein, penicillin-binding protein 2 (PBP2), which plays an important role in the late stages of peptidoglycan assembly. We measured the expression of the pbpB gene in CY001-AMIR after treatment with 64 mg/L amikacin and the results showed that the expression of pbpB increased by >6-fold, whereas no change in CY002-AMIS was found (Figure 2c). Thus, the amikacin-induced increased expression of pbpB and thickened cell wall may confer adaptive resistance to amikacin in CY001-AMIR.

**Induced amikacin-resistant strain CY002-AMIR** shows a thickened cell wall phenotype

CY001-AMIR and CY002-AMIS were isolated from the same patient, but they show different amikacin susceptibilities and cell wall thicknesses. We reasoned that the amikacin concentration in the blood is higher than that in the synovial fluid. Seven days of exposure to amikacin at therapeutic levels may cause CY001-AMIR to develop resistance. If this inference is true, we would obtain an amikacin-resistant strain from CY002-AMIS by in vitro induction. This inference was tested by treating strain CY002-AMIS with gradually increasing concentrations of amikacin. As expected, a resistant strain (CY002-AMIR) with an MIC of 64 mg/L was obtained. We also measured the cell wall thickness of CY002-AMIR using TEM. As shown in
Figure 3, the cell wall thickness of CY002-AMI\textsuperscript{R} in the presence of amikacin at its MIC is similar to that of CY001-AMI\textsuperscript{R}. These results suggest that the amikacin resistance of both CY001-AMI\textsuperscript{R} and CY002-AMI\textsuperscript{R} is adaptive and that cell wall thickening may have greatly contributed to the resistance.

\textbf{CY001-AMI}\textsuperscript{R} and CY002-AMI\textsuperscript{R} are susceptible to vancomycin and several other antibiotics

Cell wall thickening is a characteristic of VISA.\textsuperscript{16} Whether cell wall-thickened CY001-AMI\textsuperscript{R} and CY002-AMI\textsuperscript{R} post-amikacin treatment are also resistant to vancomycin was determined. It
was found that CY001-AMI\textsuperscript{R} and CY002-AMI\textsuperscript{R} cultured in the presence of amikacin are still susceptible to vancomycin, with MIC values of 1 mg/L. This condition suggests that the construction of the thickened cell wall post-amikacin and vancomycin treatments may be different. CY001-AMI\textsuperscript{R} and CY002-AMI\textsuperscript{R} are also susceptible to several other tested antibiotics, such as ceftizoxime, chloramphenicol, ciprofloxacin, gentamicin, rifampicin, tetracycline, teicoplanin and linezolid, suggesting that clinicians can use alternative antibiotics to treat patients when adaptive resistance to amikacin has developed.

**Discussion**

In this study, we isolated two clinical \textit{S. aureus} strains from one patient. The matched-pair study showed that cell wall thickening is associated with adaptive resistance to amikacin in \textit{S. aureus}. This condition was supported by several observations. First, both strains belong to the same molecular type and the most frequently encountered genes that encode AMEs were not discovered. Second, CY001-AMI\textsuperscript{R} has an altered growth curve when treated with amikacin, consistent with adaptive resistance characteristics. Third, CY002-AMI\textsuperscript{R} has a thickened cell wall and increased expression of \textit{pbpB} when treated with amikacin, indicating that cell wall thickening is associated with adaptive resistance to amikacin. Finally, the CY002-AMI\textsuperscript{R} induced by increased amounts of amikacin also showed the thickened cell wall phenotype in the presence of amikacin at its MIC. Thus, our work reveals for the first time that cell wall thickening is associated with adaptive resistance to amikacin in \textit{S. aureus}. The development of adaptive resistance to aminoglycosides in \textit{S. aureus} has also been suggested to be responsible for the aminoglycoside resistance of clinical isolates lacking AMEs. In this study, we report that the adaptive resistance of \textit{S. aureus} can be observed in patients. Thus, bacterial adaptive resistance is conserved and may be widely involved in drug resistance.

The mechanisms involved in adaptive resistance to aminoglycosides are still not completely understood. The efflux pump (MexXY-OprM) and genes involved in the anaerobic respiratory pathway or energy metabolism are necessary for adaptive resistance to aminoglycosides in \textit{P. aeruginosa}.

Adaptive resistance is thought to be an important mechanism of the clinical failure of aminoglycoside administration. After low-dose exposure to aminoglycosides, \textit{P. aeruginosa} cells were able to withstand concentrations 128-fold greater than the original MIC for the strain. The exposure of cells to a higher antibiotic dose produces a greater and longer adaptive response. In the present study, the boy infected with \textit{S. aureus} was first treated with amikacin for 7 days in a rural clinical hospital. During the treatment, CY001-AMI\textsuperscript{S} developed adaptive resistance, contrary to CY002-AMI\textsuperscript{S}, possibly because the drug concentration in the hip fluid was below the minimum bactericidal concentration (MBC) that can kill the bacterial invader. Thus, both strains were not eradicated. Arbekacin, another aminoglycoside antibiotic, showed concentration-dependent bactericidal activity with a post-antibiotic effect of 2.3–3.8 h against MRSA strain 1936. It induced marked morphological changes at 0.5× MIC and the changes remained for 2 h after removal of the agent. With these characteristics, aminoglycosides, especially arbekacin and amikacin, have been widely used to treat \textit{S. aureus} infections. In the present study, we found that CY001-AMI\textsuperscript{S} grows slower in the presence of amikacin, reflecting characteristics similar to those observed with arbekacin treatment. Thus, treatment of patients with higher doses and longer dose intervals of aminoglycosides will be more effective. Our clinical study further emphasizes that longer dose intervals are important and may possibly revert the adaptive resistance state to the original state, thus facilitating the clearance of bacteria.

Adaptive resistance to aminoglycosides usually does not lead to cross-resistance to other antibiotics. In this study,
we found that the thickened cell walls of CY001-AMI\textsuperscript{8} and the in vitro-induced CY002-AMI\textsuperscript{8} in the presence of amikacin at its MIC are associated with adaptive resistance to amikacin. However, both strains are still susceptible to ceftizoxime, chloramphenicol, ciprofloxacin, gentamicin, rifampicin, tetracycline, teicoplanin, linezolid and vancomycin. Although the exact molecular mechanisms underlying the cell wall thickening observed during amikacin treatment are not yet determined, we strongly suggest that clinicians use alternative antibiotics to treat patients when adaptive resistance to amikacin develops. In our case, the sick boy recovered after 48 days of treatment with amoxicillin/subbactam sodium and ceftizoxime.

In conclusion, this work revealed that the thickened cell wall associated with the adaptive resistance of \textit{S. aureus} to amikacin. Our work also has several implications for clinical practice. First, given that adaptive resistance can be widely developed in bacteria, we should maintain the concentration of the therapeutic drug at least above the MBC. Second, given that the adaptive resistance is inducible and can be reverted to the original state, we can use longer dose intervals to fully eradicate the bacteria. Finally, we may use different strategies to treat different infection sites, because different adaptive resistances may develop at these infection sites.

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Transparency declarations

None to declare.

Supplementary data

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

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