Decreased susceptibility to tigecycline in Acinetobacter baumannii mediated by a mutation in trm encoding SAM-dependent methyltransferase

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Objectives: Acinetobacter baumannii is an important opportunistic pathogen and multidrug-resistant isolate. Although tigecycline is a potent antibiotic for treating infections with multidrug-resistant isolates, resistance is becoming a problem. This study aimed to explore the mechanism of tigecycline resistance in A. baumannii.

Methods: A serial passage experiment was performed to collect isolates selected by tigecycline. The expression of efflux pumps was quantified in the final selected isolate, 19606-T8. The whole genome of 19606-T8 was sequenced and the putative mutations were confirmed using PCR and Sanger sequencing. A complementation experiment was performed to evaluate the contribution of the mutations to decreased susceptibility to tigecycline. The significance of a deletion mutation was further investigated in terms of growth rate and antibiotic susceptibilities.

Results: We collected serial isolates by selective pressure of tigecycline, and designated them 19606-T1 to 19606-T8. The efflux pumps AdeABC, AdeFGH and AdeIJK were not overexpressed in 19606-T8, which had decreased susceptibility to tigecycline. Isolate 19606-T8 carried one deletion mutation in trm and three non-synonymous substitutions in msbA (A84V), lolA (P91L) and filC (N168K). The deletion mutation in trm (encoding S-adenosyl-l-methionine-dependent methyltransferase) resulted in decreased susceptibility to tigecycline as well as to minocycline and doxycycline. In complementation experiments, the MICs of tigecycline, minocycline and doxycycline in a tigecycline-resistant isolate were restored by complementation with wild-type trm.

Conclusions: Given that the deletion mutation in trm was associated with decreased susceptibility to tigecycline and that a wild-type trm plasmid could restore the susceptibility, trm is considered to play an important role in decreased susceptibility to tigecycline in A. baumannii.

Keywords: serial passage experiments, whole genome comparisons, efflux pumps

Introduction

Acinetobacter baumannii, a non-fermentative Gram-negative opportunistic pathogen, is responsible for healthcare-associated infections such as pneumonia, urinary tract infections, bloodstream infections, meningitis, and skin and soft tissue infections, among others.1,2 With the extensive use of broad-spectrum antibiotics, especially the carbapenems and third-generation cephalosporins, multidrug-resistant A. baumannii has become a global problem for which the treatment options are limited, mainly to colistin and tigecycline.3 Multidrug-resistant A. baumannii is mostly tigecycline susceptible, according to an evaluation of susceptibility to tigecycline in the Asia–Western Pacific region.3

Tigecycline, the first glycylicycline antibiotic, is a derivate of minocycline and shows broad-spectrum activity against pathogenic microorganisms.4 Its adverse reactions do not include nephrotoxicity, in contrast with colistin. The US FDA has approved its use for complicated skin and skin-structure infections, complicated intra-abdominal infections and community-acquired bacterial pneumonia.5,6 Recently, several tigecycline-resistant A. baumannii strains were isolated during tigecycline therapy,7–11 and the resistance-nodulation-cell division (RND) efflux pump system (including AdeABC, AdeFGH and AdeIJK) was confirmed to mediate tigecycline resistance.12 However, Yoon et al.13 observed no correlation between the tigecycline MIC and the level of AdeABC expression, and suggested the presence of other
mechanism for tigecycline resistance. It is known that the mechanisms of antibiotic resistance are diverse and complicated, and include decreased permeability of the outer membrane, modification of the antibiotic target site, resistance determinants carried by transposons, modification enzymes encoded by the chromosome and overexpression of multidrug efflux pumps. New resistance mechanisms for tigecycline hence require investigation, besides the efflux pumps previously described.

Whole genome comparison provides an attractive and effective method of showing genetic changes in the process of bacterial evolution and detecting gene mutations associated with the rapid development of antibiotic resistance.11,14 In this study, the genomic differences of a pair of isolates were studied in order to detect genetic changes during the process of selective pressure by tigecycline and to explore the possible mechanism responsible for decreased susceptibility to tigecycline.

Materials and methods

Bacteria and MICs

ATCC 19606 was the parental isolate. All isolates mentioned in this study (Table 1) were cultured in Luria–Bertani broth (Sangon Biotech, Shanghai, China) and Mueller–Hinton agar plates (Oxoid, Hampshire, UK) at 37°C overnight. The MIC of tigecycline was determined using the broth microdilution method with Iso-SensitestTM broth (Oxoid), following the manufacturer’s instructions. Other antibiotic MICs were determined using the Etest: tetracycline, minocycline, doxycycline, ciprofloxacin, norfloxacin, gentamicin, aztreonam, ceftazidime, cefotaxime, imipenem and chloramphenicol.

Selection for reduced susceptibility to tigecycline in vitro

A serial passage experiment was performed as previously described.9,15 In brief, a single clone of the parental isolate ATCC 19606 was inoculated in Luria–Bertani broth and the overnight cultures were subjected to serial exposure to tigecycline, the selective concentration of which began with 0.1 mg/L and doubled every 24 h. The protocol was repeated until there was no further bacterial growth; the final selective concentration of tigecycline was 12.8 mg/L. The overnight cultures of the serial passage experiment were stored at −80°C and designated 19606-T1 to 19606-T8. The MICs of tigecycline in all selected isolates were determined simultaneously in all selected isolates using the broth microdilution method. The stability of the tigecycline MIC for 19606-T8 was tested by 10 passages without selective pressure, and the colony diameter was used to evaluate the growth kinetics of the isolate.

RNA analysis of efflux pumps

The expression of efflux pumps of the RND system AdeABC (forward, 5′-AATGGAATAGCCACCAACA-3′ and reverse, 5′-ACAAAGGATTACACCAACG-3′), AdeFGH (forward, 5′-ACCCGAAAGGCAAACA-3′ and reverse, 5′-CGAGGACATCCACCAAC-3′) and AdeLJK (forward, 5′-ATCGGATTCGGGTATTAC-3′ and reverse, 5′-CAGTTAGCTGCTGATGTT-3′) was quantified. The total RNA of 19606-T8 and its parental isolate was extracted from the cells in exponential phase. The relative expression of the efflux pumps was determined using real-time PCR using SYBR® Premix Ex Taq™ (Takara, Japan) on the LightCycler 2.0 Real-Time PCR system. Reactions were repeated in triplicate and the fold changes in expression of these genes were calculated relative to rpoB (forward, 5′-TGGCGTATGCTGTCTCC-3′ and reverse, 5′-CTTTGGTTCAAGCCAGTTAT-3′) using the comparative Ct method (2−ΔΔCT method).9

Genomic DNA sequencing and analysis

The genomic DNA of 19606-T8 was extracted using a QIAamp DNA MiniKit (Qiagen, Valencia, CA, USA) following the manufacturer’s recommendations, and was sequenced using Illumina HiSeq™ 2000 (Illumina Inc., San Diego, CA, USA) following a pair-end 2×100 bp protocol. The crude DNA sequences of 19606-T8 were deposited in the NCBI SRA database (SRR833591) and the reads were mapped against the reference genome (gi: 260159508 in the NCBI database) using the Bowtie2 program,16 and

Table 1. Bacterial isolates and plasmids used in this study

<table>
<thead>
<tr>
<th>Isolate or plasmid or source</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 19606</td>
<td>parental isolate</td>
<td>this study</td>
</tr>
<tr>
<td>19606-T2</td>
<td>selected isolate carrying inactivated Trm</td>
<td>this study</td>
</tr>
<tr>
<td>19606-T8</td>
<td>selected isolate carrying four mutations</td>
<td>this study</td>
</tr>
<tr>
<td>pWH1266 plasmid</td>
<td>E. coli–A. baumannii shuttle plasmid, TICr, TETr</td>
<td>this study</td>
</tr>
<tr>
<td>pWH-trm plasmid</td>
<td>plasmid with wild-type trm</td>
<td>Hunger et al.18</td>
</tr>
<tr>
<td>pWH-msbA plasmid</td>
<td>plasmid with wild-type msbA</td>
<td>this study</td>
</tr>
<tr>
<td>pWH-lolA plasmid</td>
<td>plasmid with wild-type lolA</td>
<td>this study</td>
</tr>
<tr>
<td>pWH-filC plasmid</td>
<td>plasmid with wild-type filC</td>
<td>this study</td>
</tr>
<tr>
<td>Trans-T8-pwh-trm isolate</td>
<td>19606-T8 transformed with pWH-trm plasmid</td>
<td>this study</td>
</tr>
<tr>
<td>Trans-T8-pwh-msbA isolate</td>
<td>19606-T8 transformed with pWH-msbA plasmid</td>
<td>this study</td>
</tr>
<tr>
<td>Trans-T8-pwh-lolA isolate</td>
<td>19606-T8 transformed with pWH-lolA plasmid</td>
<td>this study</td>
</tr>
<tr>
<td>Trans-T8-pwh-filC isolate</td>
<td>19606-T8 transformed with pWH-filC plasmid</td>
<td>this study</td>
</tr>
<tr>
<td>Trans-T8-pwh isolate</td>
<td>19606-T8 transformed with shuttle plasmid as a control</td>
<td>this study</td>
</tr>
<tr>
<td>Trans-T2-pwh-trm isolate</td>
<td>19606-T2 transformed with pWH-trm plasmid</td>
<td>this study</td>
</tr>
<tr>
<td>Trans-T2-pwh isolate</td>
<td>19606-T2 transformed with shuttle plasmid pWH1266 as a control</td>
<td>this study</td>
</tr>
</tbody>
</table>

TIC, ticarcillin; TET, tetracycline; r, resistance.

The wild-type genes (trm, msbA, lolA and filC) were cloned into plasmid pWH1266 at the site of the open reading frame of tet(A), resulting in loss of tetracycline resistance.
the putative single nucleotide polymorphisms (SNPs) and deletion mutations in 19606-T8 were predicted using GATK software, referenced to the original genome of ATCC 19606. The predicted mutations were confirmed using PCR and Sanger sequencing. The DNA isogenicity of 19606-T8 and ATCC 19606 was analysed by PFGE after digestion with ApaI.

**Complementation studies with wild-type plasmids in tigecycline-non-susceptible isolate 19606-T8**

A complementation experiment was performed to evaluate the contribution of the confirmed mutations in 19606-T8 to its decreased susceptibility to tigecycline. The wild-type genes were amplified using the DNA template of the parental isolate ATCC 19606, introducing BamHI and SalI restriction sites. Those PCR products were purified and cloned into the pWH1266 plasmid using T4 DNA ligase. The recombinant plasmids were constructed and transfected into electroporated 19606-T8 cells, and their contribution to decreased susceptibility to tigecycline was investigated. The tigecycline MIC for the transformed isolate was determined and the growth rate of 19606-T8 was evaluated.

**Complementation with plasmid pWH-trm in 19606-T2 harbouring a deletion mutation in trm**

Isolate 19606-T2 was selected during the serial passage experiment and only carried a deletion mutation in trm. The plasmid pWH-trm was transferred into electrocompetent 19606-T2 cells, and its contribution to decreased susceptibility to tigecycline was investigated. The tigecycline MIC for the transformed isolate was determined and the growth rate of 19606-T2 was evaluated.

**Results and discussion**

**Expression of efflux pumps in the selected isolate with decreased susceptibility to tigecycline**

During the process of selection, eight isolates were collected. ATCC 19606 was the parental isolate and was tigecycline susceptible. The final selected isolate during the serial passage experiment was 19606-T8, which had the largest increase in tigecycline MIC; its susceptibility to tigecycline reduced 128-fold after serial tigecycline exposure (Table 2). The rapid emergence of decreased susceptibility to tigecycline in the selection process is supported by reports of resistant clinical isolates during tigecycline therapy.

The susceptibility of 19606-T8 to tigecycline was stable after 10 passages without antibiotic pressure. The efflux pumps of the RND system mediate tigecycline resistance. AdeABC was the first confirmed efflux pump of the RND system in A. baumannii, and extrudes tigecycline, aminoglycosides, β-lactams, fluoroquinolones, macrolides, chloramphenicol, tetracycline and trimethoprim. AdeFGH and AdeIJK are also members of the RND system and their overexpression confers tigecycline and multidrug resistance. We therefore quantified the expression of the RND system efflux pumps in isolate 19606-T8. As the efflux pump genes were organized as the operons and co-transcribed, we detected the expression level of AdeB, AdeG, AdeJ and AdeY. The relative fold changes of AdeB, AdeG, AdeJ and AdeY in 19606-T8 were 0.49 ± 0.27, 0.42 ± 0.63, 1.46 ± 0.30 and 1.25 ± 0.26, respectively, compared with the parental isolate (ATCC 19606), meaning that these efflux pumps were not overexpressed.

**Gene mutations associated with decreased susceptibility to tigecycline**

Isolate 19606-T8 was isogenic with ATCC 19606 and had the largest increase in tigecycline MIC during the serial passage experiment. The genomic differences between 19606-T8 and its parental isolate were therefore compared. Four mutations in 19606-T8 were confirmed by sequencing the PCR amplification region around the putative mutation site.

The first genetic change was a deletion mutation in trm (HMPREF0010_02765, designated tigecycline-related-methyltransferase gene – trm) resulting in a frameshift and truncated protein, and was directly correlated with an increase in the tigecycline MIC. The second mutation occurred in lolA (HMPREF0010_02888, lolA, P91L), without an elevated tigecycline MIC. The third mutation was located in filC (HMPREF0010_01195, filC, N168K) and was associated with an increase in the tigecycline MIC. The last mutation occurred in msbA (HMPREF0010_00803, msbA, A84V), without an increased tigecycline MIC. The four mutations accumulated in 19606-T8.

Whole genome comparison is able to detect multiple genetic changes correlated with antibiotic resistance scattered throughout the whole genome. For example, Hornsey et al. found eight SNPs in an A. baumannii isolate collected from a post-therapy patient treated with tigecycline, and postulated that an ade5 mutation was responsible for tigecycline resistance and that the other SNPs might have been the result of rapid evolution of the clinical isolate during tigecycline exposure. In our study we made two presumptions. Firstly, the multiple genetic changes implicated in antibiotic resistance are random and diverse, and these mutations might not be responsible for antibiotic resistance but only correlate with decreased fitness. We observed that 19606-T8 had a significantly delayed exponential growth phase and that its colony was smaller than that of the parental isolate. Secondly, given that the mechanisms of antibiotic resistance are complicated, there might be another mechanism involved in tigecycline resistance besides the efflux pumps mentioned earlier.

**Contribution of the mutant genes to decreased susceptibility to tigecycline**

Isolate 19606-T8 was transformed with the wild-type plasmids pWH-trm, pWH-msbA, pWH-lolA and pWH-filC, respectively, and the contribution of the mutant genes to the decreased susceptibility to tigecycline was determined in terms of the tigecycline MICs for the transformed isolates. During the complementation experiment, isolates transformed with wild-type trm recovered susceptibility to tigecycline from 8 mg/L to 1 mg/L, but isolates that were transformed with the three other wild-type genes (msbA, lolA and filC) did not have restored tigecycline susceptibility. It is possible that trm was correlated with the decreased susceptibility to tigecycline, but that the other genes might result in decreased fitness, supported by the smaller colony size of 19606-T8.
We do not rule out the possibility that Trm might metabolize the reactions of physiological metabolism, including the metabolism of proteins, lipid, small molecules and secondary metabolites. Since methyltransferase catalyses the reactions of physiological metabolism, including the metabolism of proteins, lipid, small molecules and secondary metabolites, we do not rule out the possibility that Trm might participate in the physiological metabolism of bacteria. We consequently evaluated the growth rate of 19606-T2 carrying the transformed isolate.

The gene trm encodes S-adenosyl-L-methionine-dependent methyltransferase. In bacteria, methyltransferase protects the host genome against foreign DNA and plays an important role in epigenetic regulation as well as in antibiotic resistance. For example, the methyltransferases ArmA, RmtA, NpmA and Cfr methylate the ribosome target site of antibiotics and mediate antimicrobial drug resistance. By contrast, TlyA of Mycobacterium tuberculosis methylates 16S and 23S rRNA, and its mutation causes decreased TlyA-directed methylation and confers resistance to capreomycin and viomycin. In our study, the trm deletion mutation was associated with decreased susceptibility to tigecycline in the selective process, and the wild-type plasmid pWH-trm was able to restore tigecycline susceptibility. The inactivated Trm and the growth curve showed no decreased fitness (Figure 1), implying that the trm mutation is not associated with cellular growth.

Four mutations were detected in 19606-T8, which has decreased susceptibility to tigecycline. Although mutations in msbA, lolA and filC were associated with elevated tigecycline MICs, wild-type plasmids were unable to restore tigecycline susceptibility. The trm deletion mutation was correlated with an increased tigecycline MIC and the wild-type plasmid pWH-trm was able to restore susceptibility to tigecycline as well as to minocycline and doxycycline. We believe that Trm plays an important role in decreased susceptibility to tigecycline.

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Transparency declarations
None to declare.

References