Tigecycline Susceptibility and the Role of Efflux Pumps in Tigecycline Resistance in KPC-Producing *Klebsiella pneumoniae*

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Abstract

*KPC*-producing *Klebsiella pneumoniae* isolates have emerged as important pathogens of nosocomial infections, and tigecycline is one of the antibiotics recommended for severe infections caused by *KPC*-producing *K. pneumoniae*. To identify the susceptibility profile of *KPC*-producing *K. pneumoniae* to tigecycline and investigate the role of efflux pumps in tigecycline resistance, a total of 215 *KPC*-producing *K. pneumoniae* isolates were collected. The minimum inhibitory concentration (MIC) of tigecycline was determined by standard broth microdilution tests. Isolates showing resistance to tigecycline underwent susceptibility test with efflux pump inhibitors. Expression levels of efflux pump genes (*acrB* and *oqxB*) and their regulators (*ramA*, *marA*, *soxS* and *rarA*) were examined by real-time PCR, and the correlation between tigecycline MICs and gene expression levels were analysed. Our results show that the tigecycline resistance rate in these isolates was 11.2%. Exposure of the tigecycline-resistant isolates to the efflux pump inhibitor NMP resulted in an obvious decrease in MICs and restored susceptibility to tigecycline in 91.7% of the isolates. A statistically significant association between *acrB* expression and tigecycline MICs was observed, and overexpression of *ramA* was found in three tigecycline-resistant isolates, further analysis confirmed *ramR* mutations existed in these isolates. Transformation of one mutant with wild-type *ramR* restored susceptibility to tigecycline and repressed overexpression of *ramA* and *acrB*. These data indicate that efflux pump AcrAB, which can be up-regulated by *ramR* mutations and subsequent *ramA* activation, contributed to tigecycline resistance in *K. pneumoniae* clinical isolates.

Introduction

*Klebsiella pneumoniae* has emerged worldwide as an important pathogen of nosocomial infections that causes a variety of infections, including pneumonia, liver abscesses, urinary-tract infections and bacteraemia. Carbapenems are often the last resort for treating infections due to the emergence...
of multidrug-resistant \textit{K. pneumoniae} [1]. However, the acquisition of carbapenemase has contributed to resistance to all \(\beta\)-lactams including carbapenem antibiotics. Carbapenem-hydrolysing \textit{Klebsiella pneumoniae} carbapenemase (KPC)-type enzymes have been identified mostly in \textit{K. pneumoniae}. In fact, most KPC carbapenemase-producing \textit{K. pneumoniae} show resistance to almost all antibiotics except colistin and tigecycline.

Tigecycline, one type of glycylcycline, is a novel expanded-spectrum antibiotic. It is a derivative of minocycline, which inhibits the initial codon recognition step of tRNA accommodation and prevents rescue by the tetracycline resistance protein TetM [2, 3]. Tigecycline is effective against most carbapenemase-producing bacteria including \textit{K. pneumoniae} and has been approved for clinical use in China during recent years. \textit{K. pneumoniae} has previously been reported to be non-susceptible to tigecycline in other countries [4, 5]. The resistance rate to tigecycline in multidrug-resistant \textit{K. pneumoniae} in the USA was approximately 9.2\% (MIC\(\geq\)8 mg/L, FDA) [6], while the resistance rate in ESBL-producing isolates was approximately 33.3\% in Spain (MIC >2 mg/L, EUCAST) [7].

The mechanism of tigecycline resistance has not yet been clearly elucidated. It has been reported that the increased expression of efflux pumps such as AcrAB and OqxAB is one of the possible mechanisms [4, 8, 9]. Expression of the \textit{acrAB} operon is controlled by its local repressor AcrR [10]. Several global transcriptional regulators of the AraC family, RamA, MarA, SoxS, and RarA, may participate in tigecycline resistance via efflux pump activation [5, 11, 12]. \textit{ramR}, \textit{marR} and \textit{soxR} are repressors of \textit{ramA}, \textit{marA} and \textit{soxS}, respectively. RamA is also regulated by the Lon protease [13]. Mutation in these genes might be responsible for \textit{ramA}, \textit{marA} and \textit{soxS} overexpression that subsequently leads to upregulation of the efflux pumps [14–16].

In this study, a total of 215 KPC-producing \textit{K. pneumoniae} were collected from four hospitals in three provinces in China. We identified the tigecycline susceptibility profiles of these isolates. Furthermore, we investigated the role of efflux pumps and the function of regulators in tigecycline resistance.

**Material and Methods**

**Bacterial isolates**

A total of 215 KPC-producing \textit{K. pneumoniae} isolates were collected between Jan. 2010 and Dec. 2013 from the following centres in China: First Affiliated Hospital, School of Medicine, Zhejiang University (ZJF); Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University (ZJS); The First Affiliated Hospital of Kunming Medical University (KM); The First Affiliated Hospital of Zhengzhou University (ZZ). All isolates were identified using the VITEK 2 system (bioMérieux, France). The \textit{bla}_{KPC} gene was amplified to confirm the KPC-producing \textit{K. pneumoniae} isolates [17].

**Antimicrobial susceptibility test**

The MIC of tigecycline was determined using standard broth microdilution tests with fresh (<12 h) ISO-Sensitest broth (Oxoid LTD, Basingstoke, Hampshire, England). MIC results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (for tigecycline, \(\leq\)1.0 mg/L is susceptible, 2.0 mg/L is intermediate, and \(>\)2.0 mg/L is resistant) [18]. \textit{Escherichia coli} ATCC 25922 was used for quality control in the susceptibility assays. Isolates that showed resistance to tigecycline also underwent susceptibility testing for tigecycline by adding efflux pump inhibitors 1-(1-naphthylmethyl)-piperazine (NMP), phenylalanine arginine \(\beta\)-naphthylamide (PA\(\beta\)N) or carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) to the medium [19]. The MICs of other antimicrobial agents were determined using the agar dilution method or Etest method.
**PFGE analysis**

Genomic DNA was digested with restriction enzyme XbaI (TaKaRa, Dalian, China), and DNA fragments were separated by electrophoresis in 1% agarose III (Sangon, Shanghai, China) in 0.5x TBE (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA; pH 8.0) buffer with a CHEF apparatus (CHEF Mapper XA, Bio-Rad, USA) at 14°C and 6 V/cm and with alternating pulses at a 120° angle in a 6 to 36 s pulse time gradient for 22 h. The results of PFGE were analysed using BioNumerics 7.0 (Applied Maths, Austin, TX, USA) software.

**Real-time PCR**

mRNA expression levels of efflux pump genes (acrB and oqxB) and regulators (ramA, marA, soxS and rarA) were examined by real-time PCR. Overnight bacterial cultures were diluted 1/100 in LB broth (Sangon, Shanghai, China) and grown to log phase at 37°C with vigorous shaking (230 rpm). RNase-free DNase (TaKaRa, Dalian, China)-treated RNA was harvested using the Purelink RNA Mini Kit (Ambion, Carlsbad, USA). The yield and quality of RNA were determined using a Nanodrop 2000C (Thermo, USA). Two micrograms of total RNA were reverse transcribed into cDNA using the PrimeScript RT Reagent kit (TaKaRa, Dalian, China). Real-time quantitative RT-PCR was run on a LightCycler 480 II (Roche, Germany) with 40 cycles of 5 s at 95°C, 30 s at 54°C, and 30 s at 72°C. SYBR Premix Ex Taq (TaKaRa, Dalian, China) was used to quantify the expression of the target gene. The reactions were performed in a volume of 20 μL. All experiments were performed in triplicate. The primers used in these experiments are listed in Table 1. Expression of each gene was normalised to that of a housekeeping gene (rpoB). Relative expression of each target gene was then calibrated against the corresponding expression of a tigecycline-susceptible isolate K134 (expression = 1), which served as the control. Data were analysed by using the 2−ΔΔCT method.

**Statistical analysis**

The association between MICs and gene expression levels was analysed using the SPSS Statistics 17.0 software. According to the normality test and homogeneity of variances test, expression levels of soxS, marA, rarA and oqxB appear to be a normal distribution with equal variance, so an analysis of variance (ANOVA) statistical test was adopted. The expression levels of acrB and ramA appear to be a normal distribution with unequal variance, so a Kruskal-Wallis Test was adopted. Statistical significance was established by using a conventional level of P < 0.05.

**Mutation analysis of acrR, ramR, marR, soxR and lon**

acrR, ramR, marR, soxR and lon, were amplified and sequenced to identify mutations within these genes. The primers designed for these studies are listed in Table 1.

**ramR plasmid construction and transformation**

A DNA fragment carrying the wild-type ramR gene was amplified from a tigecycline-susceptible isolate (K134) with the primers listed in Table 1. After amplification, the amplimer was cloned into pCR-Blunt II -TOPO. The mutant strain S21 (kanamycin-susceptible) was used for transformation. The influence of the ramR mutation on the tigecycline MIC and transcriptional expression levels of ramA and acrB was examined using standard broth microdilution tests and real-time RT-PCR.
Results

Tigecycline resistance and MICs distribution

Of the 215 KPC-producing K. pneumoniae isolates, 24 isolates were resistant to tigecycline (MIC > 2 mg/L). The MIC distribution is presented in Fig. 1. The tigecycline resistance rate for these strains was 11.2% (MIC > 2 mg/L, EUCAST). The range of tigecycline MICs was 0.25 – 8 mg/L. The MIC50 and the MIC90 were 1 and 4 mg/L, respectively.

The results of antimicrobial susceptibility testing of tigecycline-resistant isolates are presented in Table 2. All isolates were resistant to multiple antimicrobial agents. Exposure of tigecycline-resistant isolates to the efflux pump inhibitor NMP resulted in an obvious decrease (4 to 16-fold) in the MICs of tigecycline and restored susceptibility for all except two strains (S21 and K23), while susceptible isolate MICs declined slightly from 0.75 to 0.125 mg/L. The effects of PAβN and CCCP were not significant (Table 2).

PFGE analysis

Three groups of isolates were selected for PFGE analysis; the 24 tigecycline-resistant isolates were designated as group one, a random selection of 24 isolates (equal to the number of tigecycline-resistant isolates) with a MIC = 1 mg/L isolates were designated as group two, and seven total isolates with a MIC = 0.25 mg/L were designated as group three. PFGE analysis revealed that isolates in group one could be divided into nine clonal groups (Fig. 2), group two isolates could be divided into 13 clonal groups (S1 Fig.), and group three isolates could be divided into seven clonal groups (S2 Fig.).
Among the tigecycline-resistant isolates, all five isolates from ZJF hospital are clonally distinct (group A—E). One isolate from ZJS hospital belongs to an individual clonal group (group F), and the isolate from KM hospital also belongs to an individual clonal group (group G). Clone dissemination was observed in ZZ hospital isolates. Sixteen of 17 isolates were clustered together with similar patterns belonging to clone group H, and the other isolate belonged to an individual clonal group (group I).

**Gene expression analysis and relationship with tigecycline MICs**

Clonally distinct isolates from each MIC group were selected for gene expression analysis. The gene expression levels of different MIC groups are presented in Table 3. According to the results of the Kruskal-Wallis Test (Table 4), there was a statistically significant association between \acrB expression and tigecycline MICs ($P<0.05$). The Kruskal-Wallis Test indicates that \acrB expression level in isolates with MICs of $\geq 4$ mg/L is statistically significantly different from those with MICs of 1 mg/L and 0.25 mg/L and that \acrB expression in isolates with MICs of 1 mg/L is also statistically significantly different from MICs of 0.25 mg/L. From these results, we found a trend for higher \acrB expression as the tigecycline MIC increases (Fig. 3).

The relationship between \oqxB expression and tigecycline MICs was not evident (Table 5). High expression of \oqxB was found both in tigecycline resistant and susceptible isolates (Table 3). The role of OqxAB pump in tigecycline resistance was uncertain.
Table 2. Susceptibilities of 24 tigecycline-resistant isolates to 12 antimicrobial agents and MIC values of tigecycline in the presence of efflux pump inhibitors NMP, PAβN or CCCP.

| Isolate | Clonal group | Hospital | TZP | CAZ | FEP | SCF | IPM | MEM | AKC | CIP | TS | MC | CO | TGC | TGC +NMP | TGC +PAβN | TGC +CCCP |
|---------|--------------|---------|-----|-----|-----|-----|-----|-----|-----|-----|----|----|----|-----|-------|--------|----------|
| K22     | A            | ZJF     | >256| 128 | 64  | >256| 32  | 16  | >256| >32 | >32 | 8  | 0.25| 4   | 0.25 | 1      | 1      |
| K23     | B            | ZJF     | 256 | >256| 32  | 64  | 16  | 8   | >256| >32 | >32 | 8  | 0.25| 4   | 0.25 | 1      | 1      |
| K83     | C            | ZJF     | >256| 128 | 128 | >256| 128 | 64  | 0.25| >32 | >32 | 32 | 0.5 | 4   | 1    | 4      | 2      |
| Y13     | D            | ZJF     | >256| >256| >256| >256| 128 | 256 | >256| >32 | >32 | 6  | 0.5 | 4   | 1    | 2      | 4      |
| Y17     | E            | ZJF     | >256| >256| >256| >256| >256| >256| >32 | 1   | 8   | 0.5 | 4   | 1    | 2    | 4      |
| S21     | F            | ZJS     | >256| 256 | 64  | >256| 8   | 128 | 1.5 | >32 | >32 | 256| 0.5 | 8   | 2    | 4      | 8      |
| H65     | G            | KM      | >256| 256 | 32  | >256| 64  | 128 | >256| >32 | >32 | 1  | 8   | 0.5 | 8    | 1      | 2      | 8      |
| Q4      | H            | ZZ      | >256| >256| 64  | >256| 64  | 128 | >256| >32 | >32 | 4  | 0.5 | 4   | 1    | 2      | 4      |
| Q5      | H            | ZZ      | >256| >256| >256| >256| >256| >256| >256| >32 | >32 | 4  | 0.5 | 4   | 1    | 2      | 4      |
| Q6      | H            | ZZ      | >256| >256| >256| >256| 64  | 128 | >256| >32 | >32 | 4  | 0.5 | 4   | 0.5  | 4      | 2      |
| Q8      | H            | ZZ      | >256| >256| >256| >256| 64  | 128 | >256| >32 | >32 | 8  | 0.5 | 4   | 1    | 2      | 2      |
| Q10     | H            | ZZ      | 256 | >256| >256| >256| >256| >256| >256| >32 | >32 | 8  | 0.5 | 4   | 1    | 4      | 2      |
| Q11     | H            | ZZ      | >256| >256| >256| >256| 64  | 128 | >256| >32 | >32 | 4  | 0.5 | 4   | 0.5  | 2      | 2      |
| Q12     | H            | ZZ      | >256| >256| >256| >256| 64  | 128 | >256| >32 | >32 | 8  | 0.5 | 8   | 0.5  | 4      | 4      |
| Q14     | H            | ZZ      | >256| >256| >256| >256| 64  | 128 | >256| >32 | >32 | 8  | 0.5 | 8   | 0.5  | 4      | 4      |
| Q15     | H            | ZZ      | >256| >256| >256| >256| 64  | 128 | >256| >32 | >32 | 4  | 0.5 | 4   | 0.5  | 4      | 2      |
| Q17     | H            | ZZ      | >256| >256| >256| >256| 64  | >256| 0.5 | >32 | >32 | 4  | 0.5 | 4   | 0.5  | 4      | 2      |
| Q20     | H            | ZZ      | >256| >256| >256| >256| 64  | 128 | >256| >32 | >32 | 4  | 0.5 | 8   | 1    | 4      | 4      |
| Q22     | H            | ZZ      | >256| >256| >256| >256| 64  | 128 | >256| >32 | >32 | 4  | 0.5 | 4   | 0.5  | 2      | 2      |
| Q28     | I            |ZZ       | >256| 64  | 256 | >256| 128 | 256 | 1   | >32 | >32 | 8  | 0.5 | 8   | 0.5  | 4      | 2      |
| Q30     | H            | ZZ      | >256| >256| 64  | >256| 128 | 256 | >256| >32 | >32 | 4  | 0.5 | 4   | 0.5  | 4      | 4      |
| Q38     | H            | ZZ      | >256| >256| >256| >256| 128 | 256 | >256| >32 | >32 | 4  | 0.5 | 8   | 0.5  | 4      | 4      |
| Q39     | H            | ZZ      | >256| >256| >256| >256| 128 | 256 | >256| >32 | >32 | 4  | 0.5 | 8   | 0.5  | 4      | 4      |
| Q40     | H            | ZZ      | >256| >256| >256| >256| 128 | 256 | 0.5 | >32 | >32 | 4  | 0.5 | 4   | 0.5  | 4      | 2      |

*Abbreviations: TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; SCF, cefoperazone/subbactam; IPM, imipenem; MEM, meropenem; AK, amikacin; CIP, ciprofloxacin; TS, trimethoprim/sulfamethoxazole; MC, minocycline; CO, colistin; TGC, tigecycline; TGC+NMP, tigecycline with NMP; TGC+PAβN, tigecycline with PAβN, TGC+CCCP, tigecycline with CCCP

*Tested by agar dilution method

*Tested by Etest method

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Overexpression of ramA were found in tigecycline resistant isolates (Y17, H65, S21) which also with high expression level of acrB (Table 3). Overexpression of ramA could contribute to the up-regulation of acrB in these isolates. The difference of marA, soxS, and rarA expression between tigecycline resistant isolates and susceptible isolates were not significant.

Mutation analysis of acrR, ramR, marR, soxR and lon

The sequences of the lon, marR and soxR regions of tigecycline-resistant isolates are identical to those of the susceptible isolates, and the sequences also align with the reference sequence of tigecycline-susceptible isolate K. pneumoniae subsp. pneumoniae MGH 78578 (GenBank accession no. CP000647) [15].

Mutations in the acrR gene were observed in 14 isolates (Table 3), and 12 isolates have IS5 insertion element into the nucleotide position 276–277 of the gene and two isolates harbourled
point mutations. The expression level of the \textit{acrB} gene in the isolates harboring mutant \textit{acrR} genes were higher than the control strain K134 which with a wild-type \textit{acrR} gene, but not significant.

Mutations in the \textit{ramR} gene were observed in three resistant isolates (Y17, H65, S21). One isolate (S21) harboured a point mutation leading to a premature stop codon, which resulted in a predicted truncated RamR protein that was most likely non-functional, and two isolates (Y17, H65) harboured point mutations leading to amino acid exchanges in the coding region.
Table 3. Expression of acrB, ramA, soxS, marA, rarA, oqxB and mutation of acrR, ramR in different MIC groups of K. pneumoniae clinical isolates.

| Isolate | MIC(mg/L) c | Relative expression a | Mutation |
|---------|-------------|-----------------------|----------|
|         | acrB | ramA | soxS | marA | rarA | oqxB | acrR | ramR |
| K134    | 0.25 | 1    | 1    | 1    | 1    | 1    | -    | -    |
| K27     | 0.25 | ±0.09 | 1.56±0.66 | 2.00±1.08 | 4.50±1.36 | 1.65±0.56 | 2.04±1.20 | -    | -    |
| K32     | 0.25 | ±0.16 | 1.53±0.42 | 1.83±0.86 | 5.05±1.15 | 1.58±1.02 | 2.29±1.18 | -    | -    |
| K72     | 0.25 | 1.12±0.23 | 0.97±0.37 | 1.65±0.37 | 3.28±1.80 | 1.58±0.51 | 2.47±0.97 | -    | -    |
| K82     | 0.25 | ±0.07 | 1.00±0.08 | 1.13±0.33 | 3.43±0.69 | 1.05±0.14 | 1.23±0.34 | -    | -    |
| K135    | 0.25 | ±0.12 | 0.71±0.27 | 0.90±0.44 | 2.79±0.46 | 0.75±0.22 | 0.73±0.33 | -    | -    |
| K148    | 0.25 | ±0.10 | 1.23±0.10 | 1.92±0.76 | 5.62±1.66 | 1.26±0.16 | 3.55±2.89 | -    | -    |
| K16     | 1    | ±0.39 | 1.33±0.60 | 1.70±0.76 | 6.01±3.39 | 0.96±0.26 | 1.77±0.76 | -    | -    |
| K25     | 1    | ±0.18 | 1.77±0.94 | 1.64±1.02 | 4.98±4.09 | 0.92±0.55 | 1.63±1.10 | -    | -    |
| K29     | 1    | ±0.19 | 1.06±0.50 | 1.36±0.74 | 4.21±1.87 | 1.28±0.92 | 1.54±1.10 | -    | -    |
| K49     | 1    | ±0.19 | 0.73±0.41 | 0.79±0.50 | 2.64±1.38 | 0.62±0.23 | ND b IS5 d | -    | -    |
| K76     | 1    | ±0.17 | 1.49±0.49 | 1.74±0.58 | 5.51±1.95 | 1.30±0.33 | 2.50±0.19 | -    | -    |
| K101    | 1    | ±0.21 | 0.98±0.51 | 1.09±0.67 | 3.38±1.87 | 0.98±0.43 | 1.17±0.56 | IS5 d | -    |
| K128    | 1    | ±0.28 | ND b IS5 d | 1.52±0.95 | 4.99±3.83 | 0.98±0.28 | 1.68±0.80 | -    | -    |
| K155    | 1    | ±0.34 | 0.96±0.57 | 1.12±0.59 | 3.93±2.33 | 0.68±0.27 | ND b IS5 d | -    | -    |
| Y8      | 1    | ±0.25 | ND b IS5 d | 1.50±1.31 | 4.62±4.25 | 0.98±0.52 | ND b IS5 d | -    | -    |
| H33     | 1    | ±0.42 | 1.14±0.68 | 1.34±0.72 | 4.23±2.51 | 0.87±0.42 | ND b IS5 d | -    | -    |
| S7      | 1    | ±0.15 | 2.07±0.18 | 2.33±0.11 | 6.86±0.84 | 1.51±0.03 | 2.91±0.34 | IS5 d | -    |
| S10     | 1    | ±0.16 | 1.38±0.41 | 1.53±0.50 | 4.39±1.96 | 1.30±0.20 | ND b IS5 d | -    | -    |
| S17     | 1    | ±0.18 | ND b IS5 d | 1.00±0.42 | 3.24±1.43 | 0.95±0.22 | ND b IS5 d | -    | -    |
| K22     | ≥4   | ±0.21 | 1.10±0.39 | 0.99±0.43 | 3.54±1.11 | 0.76±0.11 | 1.00±0.28 | A20D - | -    |
| K23     | ≥4   | ±0.40 | 0.29±0.12 | 0.17±0.06 | 1.02±0.13 | 0.37±0.11 | 3.04±0.87 | A20D - | -    |
| K83     | ≥4   | ±0.15 | 2.71±0.41 | 2.24±0.64 | 11.48±1.76 | 1.56±0.35 | 3.35±0.29 | -    | -    |
| Y13     | ≥4   | ±0.30 | 1.93±0.50 | 1.02±0.21 | 3.89±0.98 | 0.82±0.07 | ND b IS5 d | -    | -    |
| Y17     | ≥4   | ±0.64 | 9.43±3.89 | 0.67±0.48 | 2.11±1.08 | 0.70±0.22 | 1.24±0.48 | IS5 d E113K | -    |
| H65     | ≥4   | ±1.06 | 7.82±2.17 | 1.17±0.24 | 4.49±1.76 | 1.06±0.08 | ND b IS5 d E113K | -    | -    |
| S21     | ≥4   | ±0.49 | 13.77±2.90 | 1.94±0.74 | 7.19±2.25 | 1.88±0.71 | 2.11±0.94 | Q122Stop | -    |
| Q28     | ≥4   | ±0.18 | 0.73±0.08 | 1.01±0.22 | 2.30±0.68 | 0.67±0.09 | ND b IS5 d | -    | -    |
| Q38     | ≥4   | ±0.43 | 1.67±0.42 | 1.84±0.66 | 5.27±1.67 | 1.79±1.17 | 2.84±1.73 | IS5 d | -    |

a Relative expression compared with K134 (expression = 1). Results are means of 3 runs ± standard deviation.
b ND, not determined. Mutations in primer region or gene deletion may have affected expression.
c MIC of tigecycline.
d IS5 insertion element into the nucleotide position 276–277 of the acrR gene.

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of ramR (Table 3). Overexpressing of ramA could be due to the mutations of ramR in these strains.

ramR mutations contribute to tigecycline resistance in clinically isolated Klebsiella pneumoniae

Transformation of S21 with wild-type ramR K134 (S21/ramR K134) lowered the MIC for tigecycline from 8 mg/L to 1 mg/L. The influence of ramR mutations on the transcript expression levels of ramA and acrB in S21 was analysed by real-time PCR. Transformation of wild-type ramR (from K134) into S21 (S21/ramR K134) resulted in strongly repressed ramA expression.
(14.20-fold), and acrB expression was also downregulated (4.73-fold) (Table 6). No change was noted when transformed with the empty pCR-BluntII-TOPO vector (S21/pCR-BluntII-TOPO). These data indicate that ramR mutations via ramA activation subsequently resulted in the up-regulation of efflux pump acrAB contribute to tigecycline resistance in K. pneumoniae.

Discussion

KPC-producing K. pneumoniae isolates have emerged as important pathogens of nosocomial infections. These strains often show resistance to almost all antibiotics, and their worldwide spread usually causes a great threat to public health. Tigecycline is one of the antibiotics recommended for severe infections caused by KPC-producing K. pneumoniae [20]. In this study, we identified the susceptibility profile of tigecycline in KPC-producing K. pneumoniae in China. The MIC range and MIC90 in these isolates are identical to previous findings in the USA in
which the activity of tigecycline was determined in multidrug-resistant *K. pneumoniae* [6]. According to US FDA criteria, the resistance rate in these isolates was 4.7% (MIC ≥8 mg/L), and the susceptibility rate was 88.8% (MIC ≤2 mg/L). It appears that tigecycline retains good activity against KPC-producing *K. pneumoniae* in vitro.

Tigecycline resistance occurring in *K. pneumoniae* during therapy has recently been reported in many cases [21–24]. The mechanism of resistance is not yet clear. However, it is becoming apparent that the development of resistance to tigecycline is rather complicated, and more than one mechanism may be involved. In this study, 24 isolates belong to 9 clonal groups show resistance to tigecycline. Exposure of these isolates to the efflux pump inhibitor NMP resulted in an obvious decrease in the MICs of tigecycline and restored susceptibility to tigecycline in 91.7% of the isolates. These data suggest that the efflux pumps are involved in decreased tigecycline susceptibility. However, the effects of PAβN and CCCP were not significant. Kern WV et al. deem that different antibiotics may have different binding sites on the pump with which the EPIs might interfere in a variable manner [25]. The different effect of the three EPIs on tigecycline MICs might be due to the different action mode of the EPIs and the particular

### Table 5. ANOVA of *marA*, *soxS*, *rarA* and *oqxB* expression on the tigecycline MICs.

| Gene | Number of clonally distinct isolates | MIC x ± s | F | P |
|------|------------------------------------|----------|---|---|
| *marA* | 7 | 0.25 | 3.67±1.56 | 0.495 | 0.615 |
|       | 13 | 1 | 4.54±1.16 | 0.530 | 0.530 |
|       | ≥4 | 4.59±3.17 | 0.930 | 0.407 |
| *soxS* | 7 | 0.25 | 1.49±0.47 | 0.650 | 0.530 |
|       | 13 | 1 | 1.44±0.39 | 0.382 | 0.688 |
|       | ≥4 | 1.23±0.66 | 0.382 | 0.688 |
| *rarA* | 7 | 0.25 | 1.27±0.35 | 0.930 | 0.407 |
|       | 13 | 1 | 1.03±0.26 | 0.382 | 0.688 |
|       | ≥4 | 1.07±0.54 | 0.382 | 0.688 |
| *oqxB* | 7 | 0.25 | 1.90±0.99 | 0.382 | 0.688 |
|       | ≥4 | 2.26±0.98 | 0.382 | 0.688 |

* Five isolates were not determined.
* Three isolates were not determined.

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### Table 6. Tigecycline MIC and relative expressions of *ramA* and *acrB* when complemented with wild-type *ramR* in S21.

| Isolates       | MIC (mg/L) | ramR mutations | Relative expression |
|----------------|------------|----------------|---------------------|
|                |            | ramA           | acrB                |
| S21            | 8          | Q122Stop       | 13.77±2.90          | 3.97±0.49 |
| S21/ramR<sub>K134</sub> | 1          | 0.97±0.22      | 0.84±0.14           |
| S21/pcCR-Blunt -TOPO | 8          | 12.91±1.77     | 3.55±0.41           |
| K134           | 0.25       | 1              | 1                   |

* Relative expression compared with K134 (expression = 1). Results are means of 3 runs ± standard deviation.
* MIC of tigecycline.

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binding sites of tigecycline. AcrAB-TolC, an RND-type efflux pump, has been linked to the non-susceptibility of tigecycline in a variety of Enterobacteriaceae [4, 8, 15, 16, 26]. Our results support the hypothesis that increased expression of the AcrAB pump is associated with increased MICs of tigecycline in K. pneumoniae. However, in two strains (K22, K83), the expression level of acrB was relatively low, suggesting that efflux pumps other than AcrAB may take effect in these strains. We also examined the expression level of oqxAB in this study, but the role of OqxAB pump in tigecycline resistance was uncertain.

Transcriptional activators RamA, MarA, SoxS, and RarA have been linked to efflux pump-mediated resistance to tigecycline [5, 11, 12, 16]. However, the difference of marA, soxS, and rarA expression between tigecycline resistant and susceptible isolates was not significant, which indicates marA, soxS, and rarA may not be in the domination position in tigecycline resistance of K. pneumoniae.

Mutations in the acrR gene were observed in some isolates. The expression level of the acrB gene in the isolates harboring mutant acrR genes were higher than those with a wild-type acrR gene, but not significant. The acrR gene mutations may partly contribute to AcrAB pump-mediated tigecycline resistance in K. pneumoniae. Mutations in the ramR gene were observed in three tigecycline resistant isolates (Y17, H65, S21). The high expression of acrB in isolate S21 could be due to mutations in ramR which led to the up-regulation of ramA. Moreover, isolates Y17 and H65 both have acrR mutation and ramR mutation, and the two mutations may together contribute to the overexpression of acrB.

For the one tigecycline-resistant strain (K23) that is insensitive to efflux pump inhibitors and had low expression of all genes examined in this study, it is likely that other mechanisms are involved in the development of tigecycline resistance.

Supporting Information

S1 Fig. Phylogenetic clone analysis of 24 tigecycline MIC = 1 mg/L isolates. These isolates were divided into 13 clonal groups. (TIF)

S2 Fig. Phylogenetic clone analysis of seven tigecycline MIC = 0.25 mg/L isolates. These isolates were divided into 7 clonal groups. (TIF)

Author Contributions

Conceived and designed the experiments: YSY. Performed the experiments: FH YF QC. Analyzed the data: ZR XTH. Contributed reagents/materials/analysis tools: HZ ZR. Wrote the paper: FH ZR YSY.

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