International Journal of Antimicrobial Agents 50 (2017) 523–528

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

In vivo development of tigecycline resistance in *Klebsiella pneumoniae* owing to deletion of the *ramR* ribosomal binding site

Meiping Ye [a,1], Baixing Ding [a,1], Hongliang Qian [b], Qingqing Xu [a], Jianping Jiang [c], Jinwei Huang [d], Hongyu Ou [b], Fupin Hu [a,*], Minggui Wang [a,*]

[a] *Institute of Antibiotics, Huashan Hospital, Fudan University, 12 M. Wulumuqi Road, Shanghai 200040, China*
[b] *State Key Laboratory of Microbial Metabolism, Shanghai Jiaotong University, Shanghai, China*
[c] *Department of Bioinformatics, SJTU–Yale Joint Center for Biostatistics, Shanghai Jiaotong University, Shanghai, China*
[d] *Fifth Affiliated Hospital of Wenzhou Medical University, Lishui, China*

ARTICLE INFO

Article history:
Received 11 January 2017
Accepted 27 April 2017

Keywords:
Klebsiella pneumoniae
Tigecycline resistance
Ribosomal binding site
Deletion
RamR translation

ABSTRACT

Tigecycline resistance is emerging among *Klebsiella pneumoniae*, but knowledge regarding in vivo development of tigecycline resistance is limited. Here we report a new mechanism of tigecycline resistance in *K. pneumoniae* that evolved during tigecycline therapy. *Klebsiella pneumoniae* isolates were consecutively obtained from urine samples of a patient with scrotal abscess and urinary tract infection before and during tigecycline treatment. Two tigecycline-resistant *K. pneumoniae* strains (KP-3R and KP-4R; MIC = 8 μg/mL) were isolated after 41 days and 47 days of tigecycline therapy. These isolates had the same sequence type (ST11) and PFGE patterns as tigecycline-susceptible strains (KP-1S and KP-2S; MIC = 2 μg/mL) initially isolated from the patient. Compared with KP-1S and KP-2S, KP-3R and KP-4R exhibited higher expression of efflux pump AcrAB. Sequence comparison of the repressor gene *ramR* did not find any mutation within the open-reading frame that exist frequently in tigecycline-resistant *K. pneumoniae*. Instead, a 12-bp deletion of *ramR* upstream region including the ribosomal binding site (RBS) TGAGG was observed in KP-3R and KP-4R. qRT-PCR and immunoblotting analyses showed that KP-3R and KP-4R did not have impaired *ramR* transcription but had abolished RamR protein production. Furthermore, *xylE* reporter assay demonstrated that KP-3R and KP-4R had a defect in RamR translation caused by the 12-bp deletion. Complementing KP-3R and KP-4R with functional *ramR* suppressed expression of *acrAB* and consequently restored tigecycline susceptibility. This is the first report identifying deletion of the *ramR* RBS as a mechanism of in vivo tigecycline resistance in *K. pneumoniae* developing during tigecycline therapy.

1. Introduction

The emergence of multidrug-resistant and extensively drug-resistant pathogenic micro-organisms, especially carbapenemase-producing *Klebsiella pneumoniae* and *Acinetobacter baumannii* [1,2], is one of the most problematic issues in the current management of bacterial infections. Tigecycline, a first-in-class broad-spectrum glycylcycline antibiotic, together with polymyxins constitute the few therapeutic choices for the treatment of infections caused by these multidrug-resistant pathogens [3]. In mainland China, polymyxins are not available for human use, and tigecycline is the last line of defence against these lethal infections. Unfortunately, resistance to tigecycline has been increasingly reported [4].

The most commonly reported mechanism of tigecycline resistance in *K. pneumoniae* is overexpression of the resistance–nodulation–cell division (RND) family efflux pump encoded by *acrAB* [5,6]. Constitutive activation of *acrAB* is often caused by mutations within its regulator genes, mostly in the repressor gene *ramR*, which further lead to increased expression of the activator gene *ramA* [7,8]. Mutations in *rpsJ* encoding ribosomal protein S10, the target site of the agent, have also been associated with tigecycline resistance [9,10]. In addition, another efflux pump (KpgABC) was also recently implicated in tigecycline resistance in *K. pneumoniae* [11].

Understanding the mechanisms underlying the de novo development of tigecycline resistance in *K. pneumoniae* in patients during treatment is important for optimising treatment of infection while avoiding the emergence of resistance. In this regard, longitudinal monitoring of tigecycline susceptibility before and throughout tigecycline therapy is critical. In this study, we consecutively collected and analysed *K. pneumoniae* isolates from a single patient for tigecycline susceptibility during 48 days of tigecycline treatment. It was observed that tigecycline-resistant isolates emerged after 6 weeks of therapy. A new mechanism of *ramR* inactivation that

* Corresponding author. Institute of Antibiotics, Huashan Hospital, Fudan University, 12 M. Wulumuqi Road, Shanghai 200040, China.
E-mail addresses: hufupin@163.com (F. Hu); mgwang@fudan.edu.cn (M. Wang).
[1] Co-first authors.

https://doi.org/10.1016/j.ijantimicag.2017.04.024
0924-8579/

converts tigecycline-susceptible *K. pneumoniae* to resistant was further uncovered, deepening our understanding of how *K. pneumoniae* may develop resistance in vivo in response to selective pressure.

2. Materials and methods

2.1. Isolation of Klebsiella pneumoniae strains

Four *K. pneumoniae* isolates were obtained before and throughout tigecycline treatment from the urine samples of a patient with left-sided scrotal abscess and urinary tract infection (UTI) at Huashan Hospital in Shanghai, China. The strains were identified by VITEK®2 Compact System (bioMérieux, Lyon, France).

2.2. Antimicrobial susceptibility testing and ramR sequence comparison of Klebsiella pneumoniae isolates

Susceptibility to amikacin, gentamicin, ciprofloxacin, norfloxacin, sulfamethoxazole, nitrofurantoin, piperacillin, piperacillin/tazobactam (TZP), cefazolin, cefuroxime, cefotaxime, ceftazidime, cefepime, cefoperazone/sulbactam, cefmetazole, imipenem, meropenem and polymyxin B was determined at the Clinical Microbiology Laboratory of the hospital by the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [12]. Minimum inhibitory concentrations (MICs) of tigecycline were determined by broth microdilution according to CLSI guidelines, and breakpoints were interpreted according to the US Food and Drug Administration (FDA), as follows: susceptible, 2.0 μg/mL; intermediate, 4.0 μg/mL; resistant, 8.0 μg/mL. Among the four *K. pneumoniae* isolates, two (KP-1S and KP-2S) were tigecycline-susceptible with an MIC of 2 μg/mL, and two (KP-3R and KP-4R) were tigecycline-resistant with an MIC of 8 μg/mL. Sequence comparison of *ramR* and its flanking regions among the *K. pneumoniae* isolates was performed by PCR and sequencing using primers F-TCCACCTGGCTAAGCTGTGCC and R-CGGTAAACGGGTAGGTCAGGG.

2.3. Molecular typing

The phylogenetic distance among *K. pneumoniae* isolates was analysed by multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) as described previously [13]. MLST was performed by PCR amplification and subsequent sequencing of seven *K. pneumoniae* housekeeping genes according to protocols provided by the MLST website for *K. pneumoniae* (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). PFGE was performed to determine genetic relatedness of the isolates. Genomic DNA was digested with *Xba*I for 4 h at 37 °C. Digested DNA was then separated on a 1% agarose gel using a CHEF-DRIII system (Bio-Rad Laboratories, Hercules, CA), with a linear pulse time ramped from 2 s to 70 s for 24 h at 14 °C and a constant voltage of 6 V/cm. Similarity between PFGE patterns was determined according to the criteria proposed by Tenover et al, in which strains sharing restriction patterns of less than or equal to three band differences were considered to be a closely related strain type [14].

2.4. Quantitative reverse transcription PCR (qRT-PCR)

RNA samples were extracted from mid-log phase bacterial cultures using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocols, and cDNA was synthesised using the RT Reagent Kit with gDNA Eraser (TaKaRa, Shiga, Japan). qRT-PCR was performed using SYBR® Premix ExTaq™ (TaKaRa) on a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Reactions were repeated in triplicate and the fold change in gene expression was calculated as previously described [15]. Tigecycline-susceptible *K. pneumoniae* KP-1S was used as the reference strain for the gene expression analysis. PCR primers for *acrB*, *ramA* and the endogenous reference gene *rrsE* were as previously described [5,11]. The primers F-GGCTCGTCCAAAGAGTGAAG and R-CATCCTTGGTGGCGAAATAG were used for PCR of the *ramR* gene.

2.5. Purification of recombinant RamR and generation of rabbit polyclonal anti-RamR antibody

To obtain the recombination protein RamR-6xHis, the full-length *ramR* was PCR-amplified from KP-1S genomic DNA using the primer pair ramR1-F (GGGCCATGGCTCGTCCAAAGAGTGAAGATAAA)/ramR1-R(GGGCTCGAGGGCGTCCGCCTCATGCA) and was cloned into pET-28a to generate pMY39. The RamR-6xHis fusion protein was expressed in *Escherichia coli* BL21(DE3) and was purified as described previously [16]. Rabbit polyclonal antibody against the purified RamR was generated as previously described [17].

2.6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

SDS-PAGE and immunoblot analysis were performed as described previously [18]. Briefly, bacterial cells were harvested and were washed three times with phosphate-buffered saline at 4 °C. Pellets were suspended in SDS buffer. Cell lysates were separated by 12% SDS-PAGE and were transferred to nitrocellulose membranes. Membranes were blotted with polyclonal antibodies against RamR with 1:500 dilutions, and then with goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:1000). Horseradish peroxidase activity was determined by the enhanced chemiluminescence method and subsequently by exposure to X-ray film.

2.7. xylE reporter gene assay

Approximately 250 bp upstream of *ramR* was PCR-amplified from genomic DNA of tigecycline-susceptible strain KP-1S and resistant strain KP-3R using the primer pair pramR-F (GCAGATCTGTGACACG GTTCATATCCTGAC)/pramR-R (CGCCATGGCTTGCTTTTTATCTTCACT CTTTGG). The resulting amplicons and plasmid pxylE harbouring the promoterless gene *xylE* were digested using *Nco*I and *Bgl*II restriction enzymes and were ligated to generate pMY41 and pMY42, respectively. pMY41 and pMY42 were transformed into *E. coli* DH10B and were selected on lysogeny broth agar plates containing 50 μg/mL kanamycin (see Table 1 for details). XylE activity was determined in colonies on agar plates after spraying with catechol [19].

2.8. ramR overexpression

The full-length *ramR* gene was PCR-amplified from KP-1S genomic DNA using the primer pair ramR2-F (CGCTGCAGGGTTTGTTTAAACCTG CGTGAG)/ramR2-R (CGAAGCTTTTAGGCGTCCGCCTCATGC) and was cloned into an arabinose-inducible vector (pBAD33). The resulting plasmid was designed pMY43. KP-3R and KP-4R were then transformed with pMY43 by electroporation and were selected on lysogeny broth agar plates containing 50 μg/mL apramycin. The final strains (KP-3R/pMY43 and KP-4R/pMY43) were confirmed by qRT-PCR.

3. Results

3.1. Patient information

A 50-year-old man was admitted to Huashan Hospital on 10 September 2015 with pain in the left side of the scrotum accompanied