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Mechanisms of Tigecycline Resistance among *Klebsiella pneumoniae* Clinical Isolates

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Of 26 tigecycline-nonsusceptible *Klebsiella pneumoniae* (TNSKP) clinical isolates, 25 had nonsynonymous mutations in *ramR* and/or *acrR* (23 in *ramR* and 10 in *acrR*). Eight TNSKP isolates possessed overexpression of *ramA*, *acrB*, *rarA*, and *oqxB* simultaneously, while 8 and 1 TNSKP strains had upregulation of *ramA* and *acrB* and of *rarA* and *oqxB*, respectively. Thus, resistance mechanisms of 9 TNSKP isolates cannot be explained by the present pathways. This study underscores the role of RamA in TNSKP and suggests the presence of novel tigecycline resistance mechanisms.

Tigecycline, the first member of glycylcyclines, can overcome the two main resistance mechanisms of tetracycline (ribosomal protection and activity of efflux pumps) due to its long side chain and high affinity to ribosome (1). However, it is intrinsically resistant to *Pseudomonas aeruginosa* due to efflux. Although tigecycline resistance is not yet common in *Enterobacteriaceae* (except in species with intrinsic resistance), it has been described in several species, including *Escherichia coli* (2), *Klebsiella pneumoniae* (3), *Enterobacter* spp. (4), and *Salmonella enterica* (5) because of AcrAB efflux pump overexpression.

The AcrAB efflux pump is regulated by its local transcriptional repressor, AcrR, and a global transcriptional activator, RamA, in tigecycline-nonsusceptible *K. pneumoniae* (TNSKP) isolates (6). High-level expression of *acrAB* can result from mutation in *acrR* and upregulation of *ramA*. The latter can be caused by a mutation in *ramR*, which encodes a local transcriptional repressor of *ramA*. Moreover, overexpression of RarA, functioning as a transcriptional activator of the efflux pump OqxAB, can confer low-level resistance to tigecycline in *K. pneumoniae* as well (7). In summary, the RamA and AcrAB pathway and RarA together with the AcrAB and OqxAB pathways have been implicated mainly in tigecycline resistance in *K. pneumoniae*.

To date, studies on tigecycline resistance mechanisms in *K. pneumoniae* are limited and involve only a small number of isolates. In this study, we investigated the tigecycline resistance mechanisms in 26 unique TNSKP clinical isolates, including 3 isolates that were highly resistant to tigecycline, with an MIC of 16 μg/ml.

Screening of TNSKP clinical isolates. Tigecycline-nonsusceptible isolates were screened from 2,605 consecutive nonduplicate *Enterobacteriaceae* isolates collected at our hospital between January 2012 and January 2013. The MIC of tigecycline (Pfizer Inc.) was determined by the broth microdilution methodology as described previously (8). Tigecycline MICs were tested in triplicate for isolates with reduced susceptibility to tigecycline. The results were interpreted according to the U.S. Food and Drug Administration breakpoints for tigecycline (≤2.0 μg/ml, susceptible; 4.0 μg/ml, intermediate; ≥8.0 μg/ml, resistant) (9).

Of the 2,605 *Enterobacteriaceae* isolates, 141 (5.4%) had a tigecycline MIC of ≥4 μg/ml (Table 1). Twenty-six TNSKP isolates were obtained, a tigecycline nonsusceptibility rate of 2.3% (26/1,116) for *K. pneumoniae* (Table 1), which was similar to the rates observed in the Asia-Western Pacific region and Latin America (10, 11).

Identification of mutations in *ramR* and *acrR*. The presence of mutations in the *ramR* and *acrR* genes was assessed by PCR. Primers for the full length of *ramR* (F-AGTCGTCAAGACGATTTTCAATTTT and R-AGTGTTTCCGGCGTCATTAG) were designed in this study, and published primers were used for *acrR* (6). PCR products were sequenced and analyzed.

Of the 26 TNSKP isolates, 23 (88.5%) had mutations in *ramR*. The remaining 3 isolates that had no *ramR* mutation had a tigecycline MIC of 4 μg/ml, and 2 of these contained a mutation in *acrR*. Only one isolate lacked any mutation in either *ramR* or *acrR*. The various types of mutation are summarized in Table 2; of these, only A19V in *ramR* was reported previously (6). Hentschke et al. identified several mutations in *ramR* that were associated with increased tigecycline MICs in *K. pneumoniae* (12), and a similar observation was also made in *Salmonella enterica* (13).

Mutations in *acrR* were identified in 10 (38.5%) of the 26 TNSKP isolates (Table 2). The most common change resulting from the *acrR* mutation was a transposase insertion after V94 ($n = 7$), followed by substitutions Y114F and V165I ($n = 2$) and substitution M109I ($n = 1$). Among these mutations in *acrR* leading to amino acid substitutions, none has been reported previously.

Of 15 TNSKP isolates without mutations in *acrR* but with mutations in *ramR*, 7 (46.7%) had tigecycline MICs of ≥8 μg/ml. Of 8 TNSKP isolates with mutations in both *ramR* and *acrR*, 5 (62.5%) had tigecycline MICs of ≥8 μg/ml. Taken together, these results further support the main role of *ramR* mutation in tigecycline resistance in *K. pneumoniae* and also the potential role of *acrR* in augmenting the level of resistance conferred by *ramR* mutation.

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TABLE 1 Tigecycline-nonsusceptible clinical isolates in *Enterobacteriaceae*

Bacterium	Total no. of isolates	No. of nonsusceptible isolates with indicate tigecycline MIC (μg/ml)			No. (%) of nonsusceptible isolates
		4	8	16	
Klebsiella spp.	1,152	15	8	3	26 (2.3)
K. pneumoniae	1,116	15	8	3	26 (2.3)
Others	36	0	0	0	0 (0)
Escherichia coli	832	1	1	0	2 (0.2)
Serratia spp.	164	4	1	0	5 (3.0)
S. marcescens	161	4	1	0	5 (3.1)
S. liquefaciens	3	0	0	0	0 (0)
Enterobacter spp.	171	2	3	0	5 (2.9)
E. cloacae	93	2	2	0	4 (4.3)
E. aerogenes	73	0	1	0	1 (1.4)
Others	5	0	0	0	0 (0)
Citrobacter spp.	72	3	1	0	4 (5.6)
C. koseri	40	0	1	0	1 (2.5)
C. freundii	23	2	0	0	2 (8.7)
C. amalonaticus	2	1	0	0	1 (50.0)
Others	5	0	0	0	0 (0)
Proteus spp.	137	40	39	2	81 (59.1)
P. mirabilis	121	39	37	2	78 (64.5)
P. vulgaris	15	1	2	0	3 (20.0)
P. penneri	1	0	0	0	0 (0)
Providencia spp.	35	14	2	0	16 (45.7)
P. stuartii	30	12	2	0	14 (46.7)
P. rettgeri	4	2	0	0	2 (50.0)
P. alcalifaciens	1	0	0	0	0 (0)
Morganella morganii	42	1	1	0	2 (4.8)
Total	2,605	80	56	5	141 (5.4)

qRT-PCR analysis. Quantitative real-time PCR (qRT-PCR) was used to assess the transcriptional expression level of efflux pump genes (*acrB* and *oqxB*) and their regulatory genes (*ramA* and *rarA*) in TNSKP isolates. Previously described primers were used for *acrB* and an endogenous reference gene, *rrsE* (3, 12), and new primers were designed for *ramA* (F-ATTTCCGCTCAGGTGATT and R-GTTGCAGATGCCATTTCG), *rarA* (F-ATTGCCCTCGGCTTTGAC and R-AACAGAGCGGCTGATACTCC), and *oqxB* (F-TCATTGGCGGCGTGAAGA and R-CGGCGTGTTGGTGAACTGC) in this study. Total RNA was prepared as previously described (3), and qRT-PCR was performed using SYBR Premix *Ex Taq* (TaKaRa) on the model 7500 real-time PCR system (Applied Biosystems). Reactions were repeated in triplicate, and the fold changes in expression of these genes were calculated as previously described (3). A tigecycline-susceptible *K. pneumoniae* clinical isolate (TSKP1; MIC, 0.5 μg/ml) was used as a reference isolate for the gene expression analysis.

Of the 26 TNSKP isolates, 8 (TNSKP1 to -8) had uniformly high expression levels of the 4 genes, namely *ramA*, *acrB*, *rarA*, and *oqxB* (Fig. 1A). Eight (TNSKP9 to -16) TNSKP isolates had elevated expression levels of *ramA* and *acrB* but not of *rarA* and *oqxB* (Fig. 1B). One isolate (TNSKP17) had increased expression levels of *rarA*, *oqxB*, and *acrB* but a baseline expression level of *ramA* (0.5-fold) (Fig. 1B). These data indicate that tigecycline nonsusceptibility in these 17 isolates may have been caused by the upregulation of RamA and/or RarA through the AcrAB and/or OqxAB efflux pumps, respectively.

Five (TNSKP18 to -22) of the 26 isolates had upregulation of *ramA* (3 of them also with upregulation of *oqxB*) but exhibited baseline expression of *acrB* and *rarA* (Fig. 1C). In addition, the remaining 4 TNSKP isolates (TNSKP23 to -26) exhibited baseline expression of these 4 efflux-related genes (Fig. 1C). Taken together, the reported regulatory pathways of tigecycline resistance were partially and completely absent in 5 and 4 TNSKP isolates, respectively, which indicated that tigecycline resistance mechanisms were not limited to the upregulation of RamA or RarA and that alternative regulatory pathways may exist.

Of TNSKP isolates with *ramA* and/or *acrB* overexpression, isolates with higher tigecycline MICs (8 or 16 μg/ml) had higher expression levels of *ramA* and *acrB* than did isolates with MICs of 4 μg/ml (Fig. 1D). Similarly, among TNSKP isolates with *rarA* and/or *oqxB* upregulation, expression of *rarA* and *oqxB* in isolates with tigecycline MICs of 8 or 16 μg/ml were higher than those in isolates with lower tigecycline MICs (4 μg/ml) (Fig. 1E). These results together suggested that expression levels of efflux genes (*acrB* and *oqxB*) as well as their regulator genes (*ramA* and *rarA*) were generally in agreement with the tigecycline MICs in the TNSKP isolates in this study.

Three isolates (TNSKP18, -19, and -21) had remarkable expression levels of *ramA* and *oqxB* but baseline expression levels of *acrB* and *rarA* (Fig. 1C), suggesting that RamA likely upregulated the OqxAB efflux pump directly. In addition, increased *ramA* expression has been associated with upregulation of *rarA* and *oqxA*

TABLE 2 Mutations of negative regulatory genes *ramR* and *acrR* in the 26 TNSKP isolates

Mutation type[a]	*ramR* mutations ($n = 23$)	*acrR* mutation(s) ($n = 10$)
Insertion	A transposase and an integrase insertion ($n = 2$)	A transposase insertion ($n = 7$)
Frameshift mutation	Deletion of a sequence of ACAAAGCGAT ($n = 4$), deletion of a sequence of CTCGACGTCGGCCAT, deletion of a sequence of CACAAAGCGAT, insertion of a sequence of GC, deletion of G	
Missense mutation	K5E, A16D, T43M, G96D, I88N, T162I, A19V, A19V+R3P, A19V+A183D	M109I, Y114F+V165I ($n = 2$)
Nonsense mutation	E53Stop, W89Stop, R108Stop	
Missense mutation + nonsense mutation	A19V+Q122Stop	

[a] Of 26 TNSKP isolates, 8 harbored *ramR* and *acrR* mutations simultaneously, and only one had no mutation in these two genes.

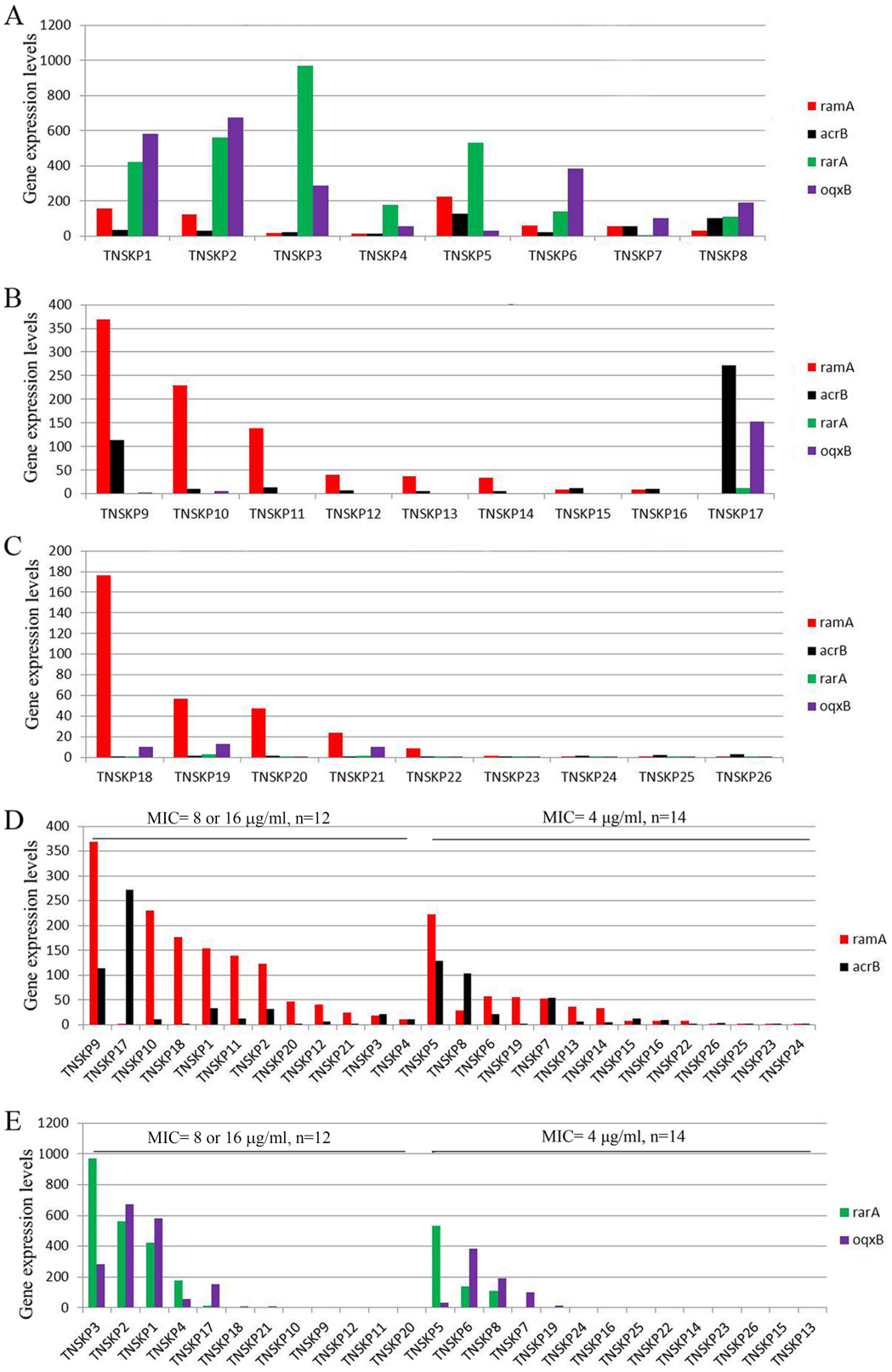

FIG 1 Expression of *ramA*, *acrB*, *rarA*, and *oqxB* and relationship between gene upregulation and tigecycline MICs in 26 TNSKP isolates. (A) Overexpression of *ramA* (10.8- to 222.4-fold) and *acrB* (11.6- to 128.2-fold) and of *rarA* (5.2- to 968.8-fold) and *oqxB* (31.6- to 672.7-fold) in 8 TNSKP isolates. (B) Upregulation of *ramA* (8.6- to 368.4-fold) and *acrB* (5.4- to 111.4-fold) but not of *rarA* or *oqxB* in 8 isolates (TNSKP9 to -16). TNSKP17 had increased expression levels of *rarA* and *oqxB*, as well as *acrB*, but a baseline expression level of *ramA* (0.5-fold). (C) Of 9 TNSKP isolates, 5 had overexpression of *ramA* (3 of them also with upregulation of *oqxB*) but baseline expression levels of *acrB* and *rarA*, and 4 had baseline expression levels of the 4 genes. (D) Among TNSKP isolates with *ramA* and/or *acrB* overexpression, isolates with higher tigecycline MICs (8 or 16 μg/ml) possessed higher expression levels of *ramA* (10.8- to 229.7-fold) and *acrB* (10.9- to 272.7-fold) than did isolates with tigecycline MICs of 4 μg/ml (*ramA*, 8.6- to 222.4-fold; *acrB*, 5.4- to 128.2-fold). (E) Of TNSKP isolates with *rarA* and/or *oqxB* upregulation, expression levels of *rarA* (421.5- to 968.8-fold) and *oqxB* (10- to 581.9-fold) in isolates with higher MICs (8 or 16 μg/ml) were higher than those (*rarA*, 5.2- to 532.7-fold; *oqxB*, 13.2- to 382-fold) in isolates with lower tigecycline MICs (4 μg/ml).

in *Enterobacter cloacae* (4). Nonetheless, whether RamA has an activator effect on the OqxAB efflux pump is still uncertain. Therefore, further research is needed to confirm the relationship between RamA and OqxAB, which will help clarify the regulatory networks involved in tigecycline resistance in *K. pneumoniae* and other *Enterobacteriaceae*.

Exclusion of other resistance mechanisms. Although *tetX* and its orthologous genes have been reported to confer tigecycline resistance in *Enterobacteriaceae* and *Acinetobacter baumannii* (14, 15), they were not found in TNSKP isolates in this study. Recently, a mutation in *rpsJ*, coding for ribosomal protein S10, was reported to mediate tigecycline resistance in *K. pneumoniae* (16); however, no mutation in *rpsJ* was detected in the 26 TNSKP isolates. In addition, tigecycline MICs were not significantly inhibited by the efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN) in any of the TNSKP isolates in this study.

In conclusion, this study underscores the key role RamA plays in TNSKP. However, the reported modulation of regulatory pathways was absent in 9 of the 26 TNSKP isolates, which suggests that novel mechanisms mediating tigecycline resistance exist. Therefore, further studies are needed to elucidate the tigecycline resistance mechanisms of TNSKP isolates.

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