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Real-Time PCR and Statistical Analyses of *acrAB* and *ramA* Expression in Clinical Isolates of *Klebsiella pneumoniae*▽

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Clinical isolates of *Klebsiella pneumoniae* were tested for a correlation between tigecycline MIC and expression of *ramA* by using real-time PCR. At MICs of 4 and 8 μg/ml, the expression of *ramA* was statistically significantly different from MICs of 2 μg/ml or less, supporting the tigecycline susceptibility breakpoint of ≤2 μg/ml for *K. pneumoniae*.

Tigecycline is a novel expanded-broad-spectrum glycylcycline antibiotic that is not affected by classical tetracycline resistance mechanisms, including ribosomal protection and efflux by tetracycline-specific pumps (8). Decreased tigecycline susceptibility in gram-negative bacteria is associated with constitutive overexpression of multidrug efflux pumps such as MexXY, AcrAB, and AdeABC (3–5, 9, 10, 12).

Although *Klebsiella pneumoniae* is generally susceptible to tigecycline, a few clinical strains with decreased tigecycline susceptibility have been isolated. Decreased susceptibility to tigecycline in *K. pneumoniae* is associated with RamA, a transcriptional activator that is involved in the upregulation of the AcrAB multidrug efflux pump (11). The additional possibility that RamA might affect tigecycline susceptibility by regulating efflux pumps other than AcrAB was not ruled out (11). The aim of this study was to further investigate the role of RamA and AcrAB in decreased susceptibility to tigecycline in *K. pneumoniae* by studying the relationship between *acrAB* and *ramA* expression and tigecycline MIC in a large collection of *K. pneumoniae* clinical isolates in order to assess the appropriateness of MIC breakpoints for distinguishing between susceptible and resistant organisms.

The study included a total of 72 strains collected from a diverse group of patients that represented various regions and infection sites and were enrolled in phase 3 clinical trials for tigecycline. Tigecycline MICs for this set of isolates ranged from 0.25 μg/ml to 8 μg/ml; the distribution of MICs is shown in Table 1. All available *K. pneumoniae* isolates with tigecycline MICs of 4 and 8 μg/ml were included in the study, and multisusceptible strains were included for comparison. The strains were propagated at 37°C in Luria-Bertani broth or agar. The MIC of tigecycline was determined by a standard broth microdilution test (1, 2). Tests for tigecycline susceptibility were performed using fresh Mueller-Hinton broth (<12 h old).

Preparation of RNA templates and TaqMan quantitative real-time PCR (RT-PCR) analysis of gene expression were done as described previously (10). Oligonucleotide primers and probes used for RT-PCR are shown in Table 2. RNA templates were used at final concentrations of 4 μg/ml for *acrA* and *ramA* and 0.0004 μg/ml for *rrsE* expression analyses; these concentrations produced the optimal amplification efficiencies. Each sample was run in duplicate. Cycle threshold (C_T) values were generated by iCycler iQ5 software. In the previous studies, which involved only a few strains, relative quantification of the target gene expression was performed by using a normalized expression analysis method, where the 16S rRNA gene served as a reference gene and one of the susceptible strains served as a reference condition (5, 10, 11). As this study involved a large population of clinical isolates, relative quantification of gene expression was performed by calculating delta C_T values for each strain and each target gene, implying that no single strain was used as a reference condition in order to avoid biases. To adjust for the differences in concentration between the target genes (*acrA* and *ramA*) and the housekeeper gene (*rrsE*), a value of $\log_2$ 10,000 (10,000 is the ratio between 4 μg/ml and 0.0004 μg/ml) was subtracted from each *rrsE* C_T value. Adjusted *rrsE* C_T values were then subtracted from the corresponding C_T values for the target genes, resulting in the delta C_T values, which were used for the statistical analyses. Because there is an inverse correlation between delta C_T and gene expression level, the lower delta C_T value implies that gene expression is increased.

The association between MIC and expression level was addressed by two statistical methods, analysis of variance (ANOVA) and linear regression. For ANOVA, mean delta C_T values were calculated for each MIC and pairwise comparisons of mean expression levels were made between MICs. The pairwise comparisons were summarized using the least-signif-

TABLE 1. Distribution of tigecycline MICs for *K. pneumoniae* isolates used in this study

MIC (μg/ml)	*n*[a]
0.25	9
0.5	14
1	16
2	14
4	10
8	8

[a] Number of strains.

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TABLE 2. Primers and fluorescent probes used for RT-PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Fluorescent probe[a] (5′–3′)
acrA	GTCCGGCACGCTGGA	ATAGCGCGTAGCGTGATTGA	CGGATGTCACCGTCGATCAGACCAC
ramA	GCATCAACCGCTGCGTATT	CGTTGCAGATGCCATTTCG	ATCGCTCGCCATGCCGGGTAT
rrsE	TTGACGTTACCCGCAGAAGAA	GCTTGCACCCTCCGTATTACC	TAACTCCGTGCCAGCAGCCG

[a] Labeled with 6-carboxyfluorescein at the 5′ end and with 6-carboxytetramethylrhodamine at the 3′ end.

icant difference approach and also using the Waller-Duncan approach, which adjusts for multiple comparisons. Pairwise comparisons were based on *t* statistics, using the error term from the ANOVA. The error term was also used to calculate appropriate standard errors and 95% confidence limits for the mean expression levels. In the ANOVA, delta C_T was a dependent variable and MIC was an independent, categorical variable. In the regression analysis, MIC was an independent continuous variable and was $\log_2$ transformed for the analysis. Statistical analyses were performed using SAS for Windows, version 9.1, with SAS procedure GLM. Statistical significance was established by using a conventional *P* level of 0.05.

The ANOVA indicated that there was a statistically significant association of *ramA* delta C_T values with the tigecycline MICs ($P < 0.001$). Mean *ramA* expression levels, along with their 95% confidence limits, are shown in Fig. 1. According to the Waller-Duncan method, mean delta C_T values for the MICs of 4 and 8 μg/ml were statistically significantly different from those for MICs of 2 μg/ml or less, which is in agreement with the currently established tigecycline-susceptible breakpoint of ≤ 2 μg/ml for *K. pneumoniae* (Tygacil package insert, available at http://www.fda.gov/cder/foi/label/2005/021821lbl.pdf; Wyeth Pharmaceuticals Inc., Collegeville, PA). The linear regression of *ramA* delta C_T on $\log_2$ MIC was statistically significant, with a *P* value of <0.0001 (data not shown). There was a statistically significant linear trend for a lower *ramA* delta C_T as the tigecycline MIC increased, with a predicted decrease of 6.55 in mean delta C_T (corresponding to a 94-fold increase in expression) between MICs of 0.25 and 8 μg/ml. These results confirm the previously established role of transcriptional activator RamA in decreased tigecycline susceptibility in *K. pneumoniae* (11).

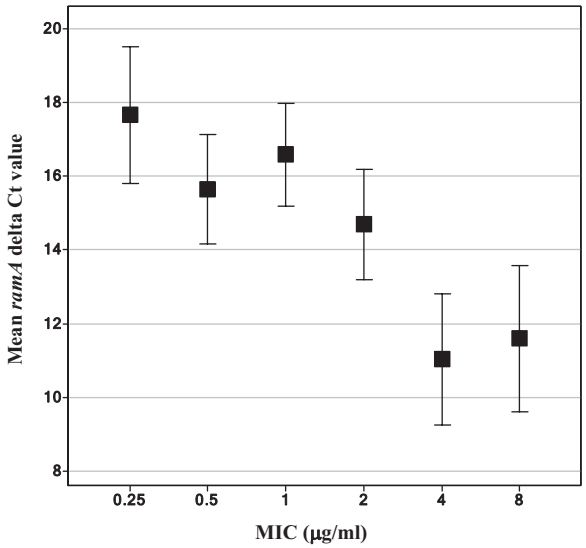

FIG. 1. ANOVA of *ramA* expression on the tigecycline MIC. The expression of *ramA* was analyzed by TaqMan RT-PCR. Mean *ramA* expression levels (delta C_T values) and 95% confidence limits (based on ANOVA) are shown for each MIC from 0.25 to 8 μg/ml.

In contrast to *ramA* expression, although there was a statistically significant linear trend for lower *acrA* delta C_T values as the tigecycline MIC increased, neither ANOVA nor linear regression analysis provided sufficient statistical evidence that mean *acrA* expression levels differ over the range of MICs. As suggested previously, RamA might have AcrAB-independent functions, e.g., regulation of efflux pumps other than AcrA (11). Further experiments are required to identify those additional functions of RamA in *K. pneumoniae*.

The most important implication from this study is an agreement between the results of quantitative analyses and the currently established tigecycline susceptibility breakpoint. An MIC breakpoint can be defined as a discriminating concentration used in the interpretation of results of susceptibility testing to define isolates as susceptible (will probably respond to antibiotic treatment), intermediate (the response is indeterminate or uncertain), or resistant (will probably not respond to antibiotic treatment) (6). According to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS), several features of both antibiotic and bacterial pathogens must be considered when determining susceptibility breakpoints, including in vitro characteristics of the drug, distribution of susceptibilities for at least 500 isolates, pharmacokinetic/pharmacodynamic parameters, and clinical outcome statistics (7). The results of this study indicate that an understanding of the resistance mechanisms coupled with the quantitative methods, such as RT-PCR and statistical analyses, for monitoring the expression of resistance determinants may be used as an additional factor to facilitate the determination or to assess the appropriateness of MIC breakpoints.

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