Correlation of the expression of \textit{acrB} and the regulatory genes \textit{marA}, \textit{soxS} and \textit{ramA} with antimicrobial resistance in clinical isolates of \textit{Klebsiella pneumoniae} endemic to New York City

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\textbf{Objectives}: Nosocomial isolates of \textit{Klebsiella pneumoniae} resistant to all commonly used antimicrobial agents have emerged in many regions of the world. It is unknown if efflux systems contribute to the multidrug resistance phenotype.

\textbf{Methods}: The expression of genes encoding the efflux pump AcrAB and the global regulators MarA, SoxS and RamA were examined and correlated with antimicrobial resistance.

\textbf{Results}: Twenty isolates belonged to the two important clones representing KPC-possessing strains endemic to our region. Virtually all of these isolates had negligible or absent expression of the genes, and resistance to fluoroquinolones and aminoglycosides could be explained by alternative mechanisms. All of these isolates were susceptible to tigecycline. A group of 14 heterogeneous isolates was also examined. There was a correlation between expression of \textit{marA} with expression of \textit{soxS}. Only expression of \textit{soxS} was significantly correlated with expression of \textit{acrB}. With a background substitution in GyrA, increased expression of \textit{acrB} and \textit{marA} appeared to contribute to fluoroquinolone resistance in some isolates. A correlation was noted between expression of \textit{soxS} and \textit{ramA} (but not \textit{marA} and \textit{acrB}) and tigecycline MICs. Following \textit{in vitro} exposure to tigecycline, resistance occurred in association with a marked increase in \textit{marA} and \textit{acrB} expression in isolates lacking expression of \textit{soxS} and \textit{ramA}.

\textbf{Conclusions}: While laboratory-derived tigecycline resistance was associated with increased \textit{acrB} expression, the variation in tigecycline MICs in clinical isolates was associated only with selected regulator genes. It appears that other mechanisms beyond activation of the \textit{acrAB} system mediate tigecycline resistance.

Keywords: efflux, tigecycline, multidrug-resistant

\textbf{Introduction}

\textit{Klebsiella pneumoniae} resistant to all commonly used antimicrobial agents has emerged as a serious nosocomial pathogen in the north-eastern USA.\textsuperscript{1} The acquisition of the carbapenemase KPC has contributed to resistance to all \(\beta\)-lactams. These strains are also frequently resistant to aminoglycosides and fluoroquinolones, and occasional isolates are resistant to the polymyxins and tigecycline.\textsuperscript{1} It is unknown if antibiotic efflux plays a role in the multidrug resistance phenotype.

The AcrAB efflux system, present in most Enterobacteriaceae, belongs to the resistance-nodulation-division (RND) family of transporters and utilizes the outer membrane protein TolC. Substrates for this efflux system have included fluoroquinolones, macrolides, chloramphenicol, trimethoprim and tetracycline.\textsuperscript{2,3} AcrAB-TolC has also been implicated as the mediator for reduced susceptibility to tigecycline in several Gram-negative pathogens, including Escherichia coli, \textit{K. pneumoniae}, Morganella morgani, Proteus mirabilis and Enterobacter cloacae.\textsuperscript{4–8} Several regulators have been demonstrated to influence expression of the AcrAB-TolC system. Mutations involving the repressor \textit{acrR} can affect expression of \textit{acrAB}.\textsuperscript{9,10} In addition, several other mediators have been reported to influence \textit{acrAB} expression. MarA belongs to the XylS/AraC family of transcriptional activators and is repressed by MarR.\textsuperscript{11,12} MarA is a global activator and, in \textit{E. coli}, affects the expression of \textit{acrAB} and the
porin OmpF (via expression of the antisense RNA micF), resulting in reduced susceptibility to a wide range of antimicrobial agents.\textsuperscript{4,11–13} The primary function of SoxS appears to be protection from reactive oxygen species.\textsuperscript{14} Increased expression of soxS in \textit{E. coli}, via the marRAB locus, has also been associated with increased \textit{acrAB} expression and reduced OmpF production.\textsuperscript{3,11} While mutations in the repressors \textit{acrR}, \textit{marR} or \textit{soxR} may be found in clinical isolates overexpressing \textit{acrAB}, it is evident that: (i) other factors can affect the expression of this efflux system; and (ii) increased expression of \textit{soxS} or \textit{marA} does not necessarily dictate increased \textit{acrAB} expression.\textsuperscript{5,15}

In several members of Enterobacteriaceae (but not \textit{E. coli}), the regulator RamA can also induce \textit{acrAB} expression and reduce antimicrobial susceptibility.\textsuperscript{5,7,16,17} While RamA may mediate a multidrug resistance phenotype via induction of the \textit{marRAB} operon, it is also evident that RamA may increase expression of \textit{acrAB} and decrease porin expression independent of the \textit{marRAB} locus.\textsuperscript{5,10,16}

In this report, we examine the expression and contribution of the \textit{acrAB} efflux system and the three regulatory genes \textit{marA}, \textit{soxS} and \textit{ramA} in: (i) two important clones that include multidrug-resistant \textit{K. pneumoniae}; and (ii) a heterogeneous collection of clinical isolates of \textit{K. pneumoniae}.

Materials and methods

Bacterial isolates and susceptibility testing

Thirty-four clinical isolates of \textit{K. pneumoniae} obtained from prior surveillance studies underwent genetic fingerprinting by ribotyping, rep-PCR and PFGE;\textsuperscript{1} isolates that were similar by all three methods were considered to belong in the same clonal group (data not shown). Twenty isolates were chosen because they represent important \textit{bla}\textsubscript{KPC}-possessing strains endemic to our region;\textsuperscript{18} 11 isolates belonged to clonal group A and nine to group B. The remaining 14 isolates were selected based on their varying susceptibility to β-lactams, fluoroquinolones, aminoglycosides and tigecycline. Susceptibility testing was performed using the Etest method (AB Biodisk, Solna, Sweden). Isolates also underwent susceptibility testing for tigecycline by the microdilution method using fresh Mueller-Hinton broth with and without 1-((1-naphthylmethyl)-piperazine (NMP), an inhibitor of RND-type efflux pumps in Enterobacteriaceae.\textsuperscript{19}

DNA amplification studies

To identify conserved regions, genetic sequences for the target genes were amplified and sequenced for representative isolates of clones A and B, and for all of the isolates in the heterogeneous group. Primers for these studies are listed in Table 1. DNA sequences for the quinolone-resistance determining region of \textit{gyrA} and \textit{parC} were also determined.\textsuperscript{10} Isolates were screened by PCR for the presence of \textit{qnrA}, \textit{qnrB} and \textit{qnrS} using previously established primers and conditions.\textsuperscript{20} DNA sequencing was performed using the automated fluorescent dye terminator sequencing system (Applied Biosystems, Foster City, CA, USA) and analysed using the NCBI BLAST program. Detection of genes coding for common aminoglycoside-modifying enzymes (\textit{aac(6′)-Ib-cr}, \textit{aadA1}, \textit{aadB}, \textit{aphA6} and \textit{aacC2}) was also performed using previously described primers and PCR conditions.\textsuperscript{21} In addition, select isolates were screened for the presence of \textit{aac(6′)-Ib-cr} by PCR and DNA sequencing using previously identified primers and conditions.\textsuperscript{22}

RT-PCR studies

All isolates were examined by real-time RT-PCR for expression of \textit{acrB} and the regulators of the \textit{acrAB} operon (\textit{soxS}, \textit{marA} and \textit{ramA}). DNase-treated RNA was obtained from late log-phase cultures using the RNeasy Kit (Qiagen, Inc., Valencia, CA, USA) and 25 ng of RNA was used for each sample. Controls without reverse transcriptase confirmed the absence of contaminating DNA in the samples. The Brilliant\textsuperscript{15} II QRT-PCR Master Mix (Stratagene, Cedar Creek, TX, USA) was used for the RT-PCR studies. Primer and probe concentrations were adjusted to achieve efficiencies of 90%–110% and all experiments were performed in triplicate. The primers and probes used in these experiments are given in Table 1. Expression of each gene was normalized to that of a ribosomal housekeeping gene (\textit{rfrAs}). Relative expression of each target gene was then calibrated against the corresponding expression by \textit{K. pneumoniae} ATCC 11296 (expression=1), which served as the control.

The effect of tigecycline on \textit{acrAB} and the regulatory genes was also analysed in selected isolates. Spontaneous mutants were selected by inoculating ~3×10\textsuperscript{5} cfu/mL onto LB agar plates containing 8 mg/L tigecycline.\textsuperscript{5} For the RT-PCR experiments, RNA from the resistant isolates was obtained from cultures grown in the presence of 4 mg/L tigecycline and gene expression was then compared with the susceptible parent.

Comparison of tigecycline MICs was performed by Student’s \textit{t}-test. Correlation between expression of the target genes and levofloxacin or tigecycline MICs was made by ANOVA for bivariate analysis and by multivariate regression analysis (Microsoft Excel 2002). A two-tailed \textit{P} value of ≤0.05 was considered significant.

Results

Isolates belonging to \textit{bla}\textsubscript{KPC}-producing multidrug-resistant strains endemic to New York City

Eleven isolates belonged to clonal group A, a strain that accounted for 88% of KPC-possessing \textit{K. pneumoniae} in New York City.\textsuperscript{18} Six of the 11 possessed \textit{bla}\textsubscript{KPC-2} and all were resistant to fluoroquinolones. Compared with the susceptible \textit{K. pneumoniae} ATCC 11296 control strain, relative expression (mean±SD) of \textit{acrB} (0.19±0.19), \textit{marA} (0.12±0.19), \textit{soxS} (0.38±0.75) and \textit{ramA} (0.28±0.41) was diminished or negligible for virtually all of the isolates in this group. All 11 isolates possessed substitutions in \textit{gyrA} (Ser\textsubscript{83}→Ile) and in \textit{ParC} (Ser\textsubscript{106}→Ile) to account for fluoroquinolone resistance. Amikacin and tobramycin resistance was explained by the presence of \textit{aacA4}. Although several isolates were also resistant to gentamicin, this resistance did not appear to be mediated by \textit{acrAB} activity. All isolates remained susceptible to tigecycline. Similarly, nine isolates (five harbouring \textit{bla}\textsubscript{KPC-2} and all resistant to fluoroquinolones) belonging to clone B had reduced expression of \textit{acrB} (0.75±0.47) and \textit{marA} (0.81±0.48). While the \textit{soxS} gene was detected by PCR in the isolates, expression of this gene was absent by RT-PCR. None of the 11 clone B isolates had evidence of \textit{ramA} when screened by PCR and, as expected, none had evidence of expression by RT-PCR. All of these isolates possessed substitutions in \textit{gyrA} (Ser\textsubscript{83}→Phe) and
ParC (Ser80→Ile) to account for fluoroquinolone resistance and had aacA4 and/or aacC2 to account for aminoglycoside resistance. All of these isolates also remained susceptible to tigecycline. Although the isolates in clonal group B had no amplicon when screened for ramA and had absent expression of soxS, when compared with clonal group A, the MICs of tigecycline were actually higher (0.96±0.38 versus 0.49±0.20 mg/L, P = 0.006). The tigecycline MICs fell to ≤0.125 mg/L in the presence of 200 mg/L NMP, which represented 0.25–0.5 times the MICs for all of the isolates, suggesting that efflux was being mediated by mechanisms other than AcrAB in these strains.

Therefore, for the two important multidrug-resistant (and blaKPC-possessing) strains of K. pneumoniae in our region, AcrAB-associated efflux activity and the corresponding regulatory genes do not appear to be important mediators for antimicrobial resistance.

Three isolates from clone B were further analysed by generating spontaneous mutants resistant to tigecycline. For the mutants, the MICs of tigecycline were 8–16 mg/L (compared with 0.75–1 mg/L in the parents). Ribotyping confirmed the genetic relatedness of the resistant mutants and parents. Gene expression studies of the resistant mutants revealed a marked increase in both acrB (4.4–12.7 times that of the susceptible parent) and marA (18.3–31.9 times that of the parent). As with the susceptible parent, there was absent expression of soxS and ramA. For one isolate, the MIC of tigecycline fell from 8 to 0.25 mg/L in the presence of NMP, confirming the role of increased efflux activity in the mutant. Therefore, efflux-mediated tigecycline resistance was still achievable in this clone, despite the absent expression of the two regulatory genes.

**Isolates representing a heterogeneous collection of strains**

There were nine different clonal groups identified among the 14 remaining isolates. Isolates with increased expression of one regulatory gene often had increased expression of other regulatory genes and/or acrB. There was an association between expression of the regulatory genes marA and soxS (P = 0.02) and marA and ramA (P = 0.07), and between soxS and the efflux-related gene acrB (P = 0.02). By regression analysis, the relationship between acrB and soxS expression remained a significant association (P < 0.01).

For the 14 isolates, aminoglycoside resistance was predicted by the presence of genes encoding modifying enzymes (aacA4 and/or aadB; data not shown). Four isolates possessed either qnr genes or mutations in parC and were excluded from further

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**Table 1. Primers and probes used for the PCR amplification and real-time RT-PCR studies**

| Primers and probes for the RT-PCR studies | Reference |
|------------------------------------------|-----------|
| rrsAfor                                  | GAAGAAACACCGGCGTAACTC | this study |
| rrsArev                                  | CACATCCGACCTGACAGACC | |
| rrsAprobe                                | Fam-TGCCACGACCCCGCGTAATA-Tamra |
| marAfor                                  | ATGTACTGGCGCAGGAAT | this study |
| marArev                                  | AGCGTTCGCAGTACTCTAAG | |
| marAprobe                                | Fam-CTTCTAAACATAGTTGAGGAG-Tamra |
| soxSfor                                  | GCATCCGGTACGGGAACAT | this study |
| soxSrev                                  | AGTCGCCGAAATCGGAGGTAG | |
| soxSprobe                                | Fam-CCGCTGCACTCCACTTGAAT-ACTGAGGGAT |
| ramAfor                                  | ATCGTGCAATGGATCTGATGA | this study |
| ramArev                                  | AGATGCCATTTCCGATATACC | |
| ramAprobe                                | Fam-AAGCTTGCAATCCGGACTGCG-Tamra |
| acrBfor                                  | CAATACGGGAGGTGTTGAG | this study |
| acrBrev                                  | CAGACGAACCTGGGATACC | |
| acrBprobe                                | Fam-TCCTGGTTTACCTCAGATG-Tamra |
analysis regarding fluoroquinolone resistance. Among the remaining 10 isolates (Table 2), 4 lacked gyrA mutations and 1 possessed a Ser83→Tyr change in GyrA that did not appear to affect fluoroquinolone susceptibility, which is in agreement with another report.10 These isolates remained exquisitely susceptible to fluoroquinolones, regardless of acrB or regulatory gene expression. Among the five remaining isolates, all possessed a Ser83→Phe change in GyrA and aac(6’)-Ib-cr, although none had the Trp 102→Arg and Asp 179→Tyr changes that typify aac(6’)-1b-cr. For these isolates, there appeared to be a relationship between the levofloxacin MICs and expression of marA (P=0.06) and acrB (P=0.07).

The MICs of tigecycline for the 14 isolates ranged from 0.19–3 mg/L (Table 3); all of the MICs fell to <0.125–0.25 mg/L in the presence of NMP. When correlated with the tigecycline MIC, a significant association was observed with expression of soxS (P=0.025) and a trend with expression of ramA (P=0.06). However, there were no significant associations by regression analysis. There was also considerable overlap and isolates clearly overexpressing soxS (e.g. DM152 and ME12) and ramA (isolate KB417) remained susceptible to tigecycline. Conversely, the two isolates (CI855 and BD503) with increased MICs of tigecycline had disparate levels of expression of soxS and ramA.

Table 2. Expression of acrB, marA, soxS and ramA, fluoroquinolone susceptibility and changes involving GyrA in 10 clinical isolates of K. pneumoniae

| Isolate | Clonal group | Relative expression<sup>a</sup> | MIC (mg/L) | GyrA mutations | aac(6’)-Ib-cr |
|---------|--------------|-------------------------------|------------|----------------|---------------|
| KB351   | U            | 0.34 0.05 0.15 0.11           | 0.125 0.064 | Ser83→Tyr     | no            |
| ME12    | V            | 2.85 4.92 15.6 6.93           | 0.032 0.064 |                | no            |
| MA340   | H            | 0.24 0.06 0.15 0.36           | 0.094 0.064 |                | no            |
| ME54    | X            | 1.5 0.99 4.3 1.95            | 0.064 0.064 |                | no            |
| ME5     | W            | 2.65 ND<sup>b</sup> ND<sup>b</sup> 3.1 | 0.047 0.094 |                | no            |
| KB370   | C            | 0.07 0.01 0.19 <0.01          | 0.38 0.38  | Ser83→Phe     | no            |
| VA302   | C            | 0.21 0.13 0.17 0.31          | 0.5 1.5    | Ser83→Phe     | no            |
| LU107   | D            | 0.45 0.25 4.23 3.1           | 3 3        | Ser83→Phe     | no            |
| CI302   | D            | 1.6 1.33 4.26 26.6           | 8 8        | Ser83→Phe     | no            |
| BD503   | D            | 6.01 0.91 21.3 63.5          | 12 8       | Ser83→Phe     | no            |

<sup>a</sup>Relative expression compared with K. pneumoniae ATCC 11296 (expression = 1).

<sup>b</sup>Not determined; mutations in primer/probe region may have affected expression.

Table 3. Expression of acrB, marA, soxS and ramA and tigecycline susceptibility in 14 clinical isolates of K. pneumoniae

| Isolate | Clonal group | Relative expression<sup>a</sup> | MIC of tigecycline (mg/L) |
|---------|--------------|-------------------------------|--------------------------|
| KB351   | U            | 0.34 0.05 0.15 0.11           | 0.19                     |
| KB370   | C            | 0.07 0.01 0.19 <0.01          | 0.25                     |
| MA340   | H            | 0.24 0.06 0.15 0.36           | 0.25                     |
| WH307   | C            | 0.38 0.02 0.19 0.38           | 0.25                     |
| ME54    | X            | 1.5 0.99 4.3 1.95            | 0.25                     |
| KB417   | J            | 1.24 0.9 6 52.3              | 0.38                     |
| ME5     | W            | 2.65 ND<sup>b</sup> ND<sup>b</sup> 3.1 | 0.5                     |
| LU107   | D            | 0.45 0.25 4.23 3.1           | 0.5                     |
| DM152   | I            | 1.71 1.87 13.2 5.43          | 0.5                     |
| ME12    | V            | 2.85 4.92 15.6 6.93          | 0.5                     |
| VA302   | C            | 0.21 0.13 0.17 0.31          | 0.75                     |
| CI302   | D            | 1.6 1.33 4.26 26.6           | 1                        |
| CI855   | C            | 0.17 0.23 1.68 2.67          | 3                        |
| BD503   | D            | 6.01 0.91 21.3 63.5          | 3                        |

<sup>a</sup>Relative expression compared with K. pneumoniae ATCC 11296 (expression = 1).

<sup>b</sup>Not determined; mutations in primer/probe region may have affected expression.
Discussion

Many medical centres in the north-eastern USA have been troubled by isolates of K. pneumoniae harbouring the carbapenemase KPC; most of these isolates are resistant to other classes of antimicrobial agents and treatment options are very limited. Analysis of the strains belonging to the two clonal groups (A and B) that represent the great majority of multidrug-resistant isolates in our region suggests that the AcrAB efflux system and the regulatory proteins MarA, SoxS and RamA are not important mediators of fluoroquinolone and aminoglycoside resistance in the clinical setting. It appears that, due to the pressures in the hospital environment, the acquisition of other mechanisms leading to fluoroquinolone and aminoglycoside (and β-lactam) resistance has offset the need for these systems and they have been down-regulated. Therefore, investigational inhibitors against the acrAB efflux system (or the purported regulatory systems) do not appear likely to be effective agents to restore antimicrobial activity. It is notable that isolates belonging to these clonal groups remained susceptible to tigecycline and had reduced expression of marA and acrB with negligible or absent expression of ramA and soxS. However, tigecycline resistance could still be induced in vitro in these isolates. Therefore, these isolates still have the potential to become resistant to this agent in the clinical setting.

It is becoming increasingly apparent that the development of resistance to tigecycline is a complex process. Reduced susceptibility to tigecycline has been linked to the AcrAB-ToIC efflux system in a variety of Entrobacteriaceae.1-8 Increased expression of acrAB has been reported in clinical isolates of E. coli and E. cloacae with reduced susceptibility to tigecycline and transposon mutagenesis involving the operon restored susceptibility to tigecycline.4,5 However, an association between tigecycline MICs and acrA expression was not evident in isolates of K. pneumoniae.23 Whether other efflux systems are involved in resistance to this agent is unknown.

Increased expression of marA has also been linked to AcrAB-mediated resistance to tigecycline in E. coli.4 However, it was apparent that genes other than marA (and acrAB) can contribute to tigecycline resistance in E. coli.4 Also, expression of marA has not been linked to tigecycline resistance in other Entrobacteriaceae.5 In K. pneumoniae and E. cloacae, increased expression of ramA has been noted in isolates with reduced susceptibility to tigecycline,5,7 and a correlation was noted between expression of ramA with tigecycline MICs.22 Transposon mutagenesis of the ramA gene restored susceptibility (and also diminished acrA expression) in one isolate of K. pneumoniae.7 The association of tigecycline resistance with soxS or other regulatory genes has not been previously reported.

The results from our heterogeneous collection of isolates are in general agreement with Ruzin et al.,23 in that an association was observed between expression of ramA (and not acrB) and the MICs of tigecycline. However, we found considerably greater overlap in the expression of ramA among the clinical isolates with different MICs of tigecycline. In addition, isolates from one clone not expressing ramA (and soxS) could still be induced in vitro to become resistant to this agent. Taken together, these studies indicate that isolates derived in the clinical setting with moderately elevated MICs of tigecycline have increased expression of one or more of the regulatory genes, and factors other than the AcrAB efflux system may mediate resistance. When clinical isolates develop or laboratory isolates are induced to develop high-level resistance to tigecycline, marked overexpression of one (or more) of the regulatory genes and the acrAB operon occurs and appears critical for resistance.

Given the known interplay between marA, ramA and soxS, it is not surprising that increased expression of one gene was often associated with increased expression of the other regulatory genes. It was also apparent that the absence of expression of some of these regulatory genes (ramA and soxS) does not preclude markedly increased expression of another (marA) and elevation of tigecycline MICs. Because expression of these regulatory genes can have far reaching effects (e.g. influencing porin expression and other efflux systems), it is likely that systems beyond AcrAB-ToIC are involved in the development of tigecycline resistance.

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Transparency declarations

None to declare.

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