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Characterization of KPC-producing *Klebsiella pneumoniae* Isolates with Decreased Cefepime MIC Values: Negative Correlation with Clonal Complex 258

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AMENDED ABSTRACT

Background: Cefepime (FEP) MICs among KPC-producers can vary remarkably and in some instances these isolates are categorized as FEP-susceptible. We characterized eight KPC *K. pneumoniae* (KPN) displaying variable FEP MICs for presence of other β-lactam (βL) resistance mechanisms, genetic context of KPC gene, MLST, expression of bla_{KPC} and copy number of this gene.

Methods: Four KPC-KPN displaying FEP MICs from 2 to 16 µg/mL and four KPN producing the same bla_{KPC} variant from the same hospital with FEP MIC results at >16 µg/mL were analyzed. MIC testing was performed by broth microdilution in triplicate for 10 βLs. βLases were screened by PCR/sequencing. Expression of bla_{KPC}, *ompK35*, *ompK36*, *ompK37* and *acrA* was determined using high quality RNA in triplicate reactions by relative quantitative RT-PCR. S1 nuclease and ICeuI followed by Southern blot and probe hybridization and MLST were also performed. Tn*4401* was amplified and sequenced.

Results: MIC results for FEP and other βLs were reproducibly lower for four KPC-KPN when compared to control KPC-KPN with higher FEP MICs. Isolates with lower MICs had similar bla_{KPC} expression when compared to KPC-KPN controls, with the exception of one pair, for which the isolate with greater FEP MIC had 5X greater expression. Four isolates from both groups had additional βLases that include OXA-1/-30, OXA-9, OXA-18/-45, TEM-1 and CTX-M-2, but no correlation with greater or lower FEP MIC was noted. 2/8 isolates had expression decrease of one or more porins: One had reduced *ompK35* and *ompK37*, and another reduced *ompK36*, but porin decreased expression was not consistent with differences in FEP MIC. No isolates hyper-expressed AcrAB-TolC. Three of four isolates displaying FEP MIC >16 µg/mL belonged to clonal complex (CC) 258 and the one that was not (ST1116) had 5X greater bla_{KPC} expression. All KPN-KPC with lower FEP MICs did not belong to CC258. All isolates carried bla_{KPC} in plasmids and two isolates had two copies of this gene (one in two plasmids and one had a chromosomal bla_{KPC} copy). Two of eight isolates did not carry bla_{KPC} in Tn*4401b*.

Conclusions: No KPN-KPC with decreased FEP MICs belonged to CC258, whereas three of four control KPC-KPN displaying higher FEP and other βLs MICs belonged to this lineage, implying that this strain-type could provide a more appropriate environment for KPC activity. Increased bla_{KPC} expression was observed in only one strain, not CC258.

INTRODUCTION

Klebsiella pneumoniae carbapenemase (KPC)-producing bacteria have been detected worldwide and this carbapenem hydrolyzing β-lactamase is commonly identified in *Klebsiella* spp. clinical isolates, but it has also been found among other Enterobacteriaceae, *Pseudomonas* spp. and *Acinetobacter* spp. KPC-producing isolates are usually highly resistant to all β-lactam agents, but cefepime MIC values can be variable and some isolates are considered intermediate or susceptible against cefepime using the current Clinical and Laboratory Standards Institute (CLSI) breakpoint criteria. In a recently published study, we demonstrated that 14.4% of a worldwide collection of KPC-producing Enterobacteriaceae, including *K. pneumoniae*, were categorized as susceptible to cefepime. In a survey of 10 hospitals located in the New York City area, it was observed that 40.0% of 96 KPC-producing *K. pneumoniae* isolates collected were susceptible to cefepime. Furthermore, susceptibility rates among KPC-producing isolates can be higher if a non-reference method, such as the Vitek system or Etest® strips were used.

We characterized eight KPC-producing *K. pneumoniae* isolates; four cefepime-susceptible or -intermediate and four control isolates from the same hospital that demonstrated resistance against this cephalosporin. We analyzed for the presence of other β-lactamases and β-lactam resistance mechanisms, their genetic location and surroundings of bla_{KPC}, the genetic relatedness of the isolates and the relative expression of bla_{KPC} in the two subsets.

MATERIALS AND METHODS

Bacterial isolates and susceptibility testing. Four KPC-producing *K. pneumoniae* isolates displaying cefepime MIC results from 2 to 16 µg/mL (susceptible or intermediate) and four cefepime-resistant *K. pneumoniae* (MIC ≥16 µg/mL) collected in the same hospital were analyzed. Isolates were susceptibility tested in triplicate against 10 β-lactam agents by CLSI reference broth microdilution method. Quality control (QC) was performed by concurrent testing of *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

Genotypic detection of β-lactamase-encoding genes. KPC-producing isolates were also screened for other β-lactamase-encoding genes including bla_{TEM}, bla_{SHV}, ESBLs (bla_{CTX-M}, bla_{OXA-2}, bla_{OXA-10}, $bla_{OXA-18/45}$, bla_{OXA-30}, bla_{PER}, bla_{VEB}, bla_{GES}, bla_{PSE} and bla_{BEL-1}), plasmid-mediated AmpC genes (bla_{MOX}, bla_{ACT}, bla_{MIR-1}, bla_{DHA}, bla_{ACC}, bla_{CMY} and bla_{FOX}) using multiplex PCR approaches. Amplicons were sequenced on both strands and the nucleotide sequences and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, Wisconsin, USA). Sequences were compared to others available via internet sources (http://www.ncbi.nlm.nih.gov/blast/).

Molecular typing. KPC-producing *K. pneumoniae* isolates were subjected to multilocus sequence typing (MLST) according to the instructions on the website http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html.

Genetic location of the KPC-encoding gene. Total cellular DNA embedded in 1% agarose plugs was subjected to partial digestion with S1 nuclease. Plasmids were resolved by electrophoresis performed on the CHEF-DR II (BioRad), with the following conditions: 0.5 x TBE, 1% agarose, 13°C, 200V, for 6 hours with switch time ramping from 5 to 25 seconds and another 10 hours with the switch time from 30 - 45 seconds. ICeuI digested genomic DNA was also resolved on PFGE. DNA gels were transferred to nylon membranes by Southern blotting and hybridized with a digoxigenin labeled bla_{KPC}-specific probe (Roche Diagnostics GmbH, Mannheim, Germany).

The bla_{KPC}-carrying element (*Tn*4401) upstream of KPC gene was amplified with specific primers and PCR products were sequenced. Sequences were compared within the pairs of cefepime-susceptible and -resistant isolates.

Expression analysis of KPC, efflux pump AcrAB-TolC and porins. The expression of *acrA*, *ompK35*, *ompK36*, *ompK37* and bla_{KPC} was determined by quantitative real-time PCR (qRT-PCR) using high quality DNA-free RNA preparations. Total RNA was extracted from mid-log-phase bacterial cultures (cell density at OD_{600} of 0.3-0.5) using RNA Protect Reagent and RNeasy Mini Kit (Qiagen, Hilden, Germany) in the Qiacube workstation (Qiagen) and residual DNA was eliminated with RNase-free DNase (Promega, Wisconsin, USA). Quantification of mRNA and sample quality were assessed using the RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) according to manufacturer instructions. Only preparations with RNA integrity number (RIN) >6.5 that showed no visual degradation were used for experiments. Relative quantification of target genes was performed in triplicate by normalization to an endogenous reference gene (*gyrA*) on the StepOne Plus instrument (Life Technologies, Carlsbad, California, USA) using custom designed primers showing >98.0% efficiency. Transcription levels were considered significantly different if at least a ±5-fold difference was noted compared with *K. pneumoniae* ATCC 13883.

RESULTS

- Four isolates selected due to lower cefepime MIC values had results reproducibly low against this cephalosporin (2 or 4 µg/mL). Isolates were also susceptible or intermediate to ceftazidime (MIC, 2-8 µg/mL), but carbapenems and ceftriaxone MIC results were elevated into the resistant range. Cefepime-resistant strains were resistant to all β-lactams tested, see **Table 1**.
- All but one isolate carried KPC-2 and six isolates carried this gene in a copy of the *Tn4401b* structure. The remaining cefepime-resistant strain carried bla_{KPC-3} and a 68-bp deletion on the transposon structure and another had a 188-bp deletion in *Tn4401* (**Table 1**).
- Four isolates carried additional β-lactamase-encoding genes, including some enzymes known to have elevated cefepime hydrolysis rates. These enzymes were OXA-1/-30, OXA-2, OXA-9, OXA-18/-45, TEM-1, SHV-30 and CTX-M-2 and they were present in cefepime-resistant or susceptible/intermediate strains (**Table 1**).
- Three of four cefepime-resistant isolates belonged to clonal complex (CC) 258. The remaining cefepime-resistant strain belonged to a new sequence type (ST1116). Susceptible isolates belonged to four different non-CC258 STs (**Table 1**).
- All isolates carried bla_{KPC} in plasmids. One cefepime-resistant strain had two plasmid copies and another had an additional chromosomal copy of the carbapenemase gene (**Table 1**).
- The expression of bla_{KPC} was generally similar, regardless of the cefepime MIC value. For one pair, the cefepime-resistant isolate expressed bla_{KPC} at a level >5X greater than that of its cefepime-susceptible counterpart.
- Only two isolates (from the same hospital) had decreased expression of outer membrane proteins (OMPs): a cefepime-susceptible strain had reduced *ompK35* and *ompK37* expression, and the cefepime-resistant strains had reduced *ompK36* expression (**Table 1** and **Figure 1**).
- None of the isolates had hyperexpression of AcrAB-TolC efflux pump when compared to the ATCC control strain.

Figure 1. Relative expression of genes encoding *K. pneumoniae* porins and AcrAB-TolC pump compared to *K. pneumoniae* ATCC 13883. Expression >10X the control for *acrA* was considered significant. For the OMP-encoding genes a decrease of 10X was considered significant. Thresholds are represented by doted lines.

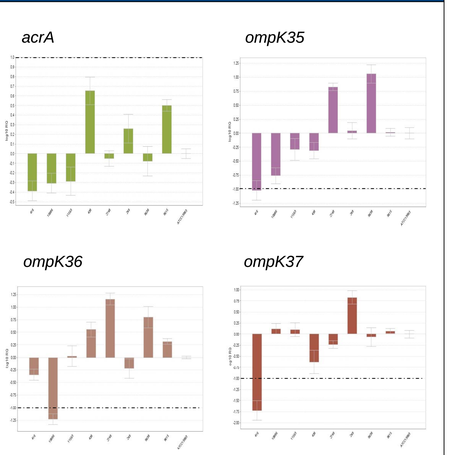

Table 1. Characteristics of KPC-producing *K. pneumoniae* isolates displaying non-susceptible cefepime MIC values and cefepime-resistant controls from the same hospitals.

Isolate[a]	MIC (µg/mL):[b] FEP	IMI	CRO	CAZ	bla_{KPC}-variant	Other β-lactamases	MLST	bla_{KPC} genetic element	KPC location[c]	Relative expression of:[d] bla_{KPC}	*acrA*	*ompK35*	*ompK36*	*ompK37*
416A	2	4	>8	8	KPC-2	-	ST14	Tn*4401b*	P 48-kb	1	0.410	0.085	0.451	0.019
10886A	>16	>8	>8	>32	KPC-2	-	ST258 (CC258)	Tn*4401b*	P 97-kb	2.364	0.493	0.174	0.0584	1.325
11563A	4	8	8	2	KPC-2	TEM-1, OXA-30, SHV-30	ST45	Tn*4401b*	P 48-kb	1	0.517	0.512	1.071	1.264
438A	>16	>8	>8	>32	KPC-2	-	ST1116	188 bp deletion	P 45-kb	5.304	4.498	0.487	3.586	0.235
2748A	2	>8	>8	8	KPC-2	-	ST234	Tn*4401b*	P 72-kb	1	0.891	6.689	14.480	0.583
249A	>16	>8	>8	>32	KPC-3	OXA-9, OXA-18/-45	ST258 (CC258)	68 bp deletion	P 40- and 142-kb	0.332	1.821	1.115	0.596	6.749
9638A	4	4	>8	4	KPC-2	CTX-M-8, OXA-9, OXA-18/-45	ST45	Tn*4401b*	P 48-kb + C	1	0.833	11.466	6.319	0.871
9615A	>16	>8	>8	>32	KPC-2	CTX-M-2, OXA-2	ST11 (CC258)	Tn*4401b*	P 48-kb	1.203	3.176	1.040	2.076	1.187

a. Isolates from the same hospital were grouped together.
b. FEP= cefepime; IMI= imipenem; CRO= ceftriaxone; CAZ= ceftazidime. Non-resistant MIC values are in red.
c. P= plasmid; C= chromosome.
d. Expression of bla_{KPC} among cefepime-resistant isolates was relative to the more sensitive isolate in the pair. Expression of the remaining isolates was relative to *K. pneumoniae* ATCC 13883. Expression >5X or >10X compared to the control for bla_{KPC} and *acrA*, respectively was considered significant (in red). For the OMP-encoding genes a decrease in 10X was considered significant (in red).

CONCLUSIONS

- Cefepime MIC values seemed not affected by the presence of resistance mechanisms other than KPC, nor the genetic location of the bla_{KPC} gene. However, in three of four groups analyzed, the cefepime-resistant isolate belonged to CC258.
- CC258 *K. pneumoniae* has been encountered carrying bla_{KPC} in various countries and seems to be associated with the dissemination of these isolates. This successful lineage seems to be a particularly good background for the expression and activity of KPC enzymes.
- The findings of strains having KPC enzymes and low cefepime or ceftazidime MIC results requires further study to discern the level predicting *in vivo* responses to these cephalosporins.

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