Effect of drug treatment options on the mobility and expression of blaKPC

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Objectives: Both transposition and increases in gene expression have been implicated in the success of KPC-producing pathogens, but the stimulus required for these phenomena are unknown. It is possible that exposure to antimicrobials during patient treatment increases blaKPC expression or induces Tn4401 transposition. The purpose of this study was to determine if exposure to carbapenems or other antimicrobial drug classes could stimulate expression of blaKPC or the in vitro transposition of Tn4401.

Methods: Five KPC-producing clinical isolates were evaluated in this study. Gene expression of RNA from each isolate exposed to subinhibitory, MIC or suprainhibitory levels of antibiotics was evaluated using real-time RT–PCR. Southern blots were performed on plasmids from isolates exposed to subinhibitory levels of antibiotics.

Results: There were subtle changes in blaKPC RNA expression following antibiotic exposure that were both strain and drug dependent. Multiple plasmids ranging from ≏ 8 to ≏ 200 kb were observed for the Enterobacteriaceae isolates, whereas the Pseudomonas aeruginosa isolate had one ≏ 55 kb plasmid. No changes in hybridization patterns or binding intensity for the blaKPC probe were observed after antibiotic exposure.

Conclusions: While the changes in blaKPC RNA expression are subtle, the different responses observed suggest both strain- and genera-specific variations in response to different antibiotic treatments.

Keywords: KPC, Tn4401, collateral damage

Introduction

Klebsiella pneumoniae carbapenemase (KPC)-producing pathogens have become a major threat to patient health. Patients infected with KPC-producing pathogens have a higher incidence of morbidity and mortality.1–3 In addition, these pathogens are a challenge for infection control in healthcare facilities due to the inability to detect all KPC-producing isolates using current guidelines for susceptibility testing.3 Most commonly found in K. pneumoniae, KPC enzymes have been identified in a variety of Gram-negative organisms, including Escherichia coli, Enterobacter species, Pseudomonas aeruginosa and Acinetobacter baumannii. Organisms that produce the KPC β-lactamase have been associated with multiple β-lactam resistance, including carbapenem resistance.4,5 This, in addition to the lack of novel antimicrobials with action against resistant Gram-negative pathogens, forces physicians to treat infections caused by KPC-producing organisms with potentially toxic antibiotics, such as the aminoglycosides and polymyxins.6 The rapid spread of blaKPC into multiple genera of Gram-negative pathogens, the lack of good treatment regimens and the inability of clinical laboratories to identify all KPC-producing pathogens underscore the need to understand the selective pressures driving the rapid emergence and spread of KPC-producing organisms.

Some studies suggest that higher levels of blaKPC RNA expression are associated with increases in carbapenem MICs.7,8 However, the majority of these studies have been carried out using clinical strains producing multiple types of β-lactamases; therefore, it is difficult to assess the contribution of blaKPC in β-lactam susceptibility profiles. A recent study by our laboratory evaluated the effect of KPC production in the absence of other resistance mechanisms in four genera of Gram-negative pathogens and compared them with KPC-producing clinical isolates of the same genera.9 Our findings demonstrated that the levels of blaKPC expression by the KPC-producing clinical isolates were not at the level observed for the KPC-producing transformants, yet the clinical isolates had higher carbapenem MICs. Clearly, other mechanisms, such as porin down-regulation and as yet unidentified mechanisms, contribute to the complexity of the carbapenem susceptibility patterns observed for KPC-producing pathogens.
One explanation as to why the identification of KPC-producing isolates is difficult in the clinical laboratory could be that the stimulus required for β-lactamase production is present during the course of treatment in vivo, but not during in vitro susceptibility testing. It is possible that certain antimicrobial drug classes used during empiric therapy, combination therapy or to treat multiple infections within the patient could provide a stimulus to either increase the amount of bla\text{KPC} expression or stimulate the mobility of transposon Tn4401 carrying bla\text{KPC}. Therefore, the possibility of ‘collateral damage’ associated with other drug classes used in the treatment of patients infected with KPC-producing isolates exists. In this study, we tested drugs from multiple antibiotic classes, including β-lactams, macrolides, aminoglycosides and fluoroquinolones, for their ability to act as a stimulus to increase bla\text{KPC} expression and/or contribute to the mobilization of Tn4401 carrying bla\text{KPC} in four different genera of Gram-negative pathogens.

Materials and methods

Bacterial strains

Five previously characterized KPC-producing clinical isolates were evaluated: E. coli 233, K. pneumoniae isolates UMM3 and HUH40, Enterobacter sp. isolate 01MGH049 and P. aeruginosa PSS.15,16 E. coli ATCC 25922, P. aeruginosa ATCC 27853 and Staphylococcus aureus ATCC 29213 were used as quality control strains for susceptibility testing.

PCR mapping and sequencing of Tn4401

Template DNA for PCR was prepared from overnight cultures of E. coli, K. pneumoniae, Enterobacter sp. and P. aeruginosa using 1.5 mL of culture as previously described.15 Prior to lysis, a final concentration of 400 mg/L proteinase K was added to P. aeruginosa supernatant to prevent nucleic acid degradation by P. aeruginosa nucleases. Alternatively, DNA fragments were generated using Genome Walker (Clontech, Mountain View, CA, USA) according to the manufacturer’s protocols.

PCR mapping of the KPC-bearing 10.5 kb transposon, Tn4401, was performed using transposon- or adapter-specific primers for each clinical isolate evaluated in this study (Table S1, available as Supplementary data at JAC Online). Amplicons were generated using the proof-reading enzyme Platinum Taq DNA Polymerase High Fidelity, purified and sequenced as previously described.15 Sequence analysis was performed using the DNA Baser version 2.75 software program (Heracle Software, Lilienthal, Germany).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using broth microdilution according to CLSI recommendations and/or Etest (AB Biodisk, Solna, Sweden). The results were interpreted using CLSI criteria.3,16,19 Antimicrobial agents were obtained from the following suppliers: ceftazidime, meropenem, gentamicin, pipercillin, tazobactam, azithromycin and ciprofloxacin were from Sigma Chemical Co. (St Louis, MO, USA); ticarcycline was from Pfizer (New York, NY, USA); cefepime was from Bristol-Myers Squibb (Prince- ton, NJ, USA); doripenem was from Johnson & Johnson (La Jolla, CA, USA); and imipenem and ertapenem were from Merck & Co. (Rahway, NJ, USA).

Antibiotic exposure

Antibiotics were added to inoculated flasks of each isolate grown to early logarithmic phase [∼0.4 optical density at 600 nm (OD_{600})] at 37 °C with shaking at the concentration indicated for each experiment. The subinhibitory level of antibiotics used in these experiments was 0.25× MIC of that antibiotic observed for each bacterial isolate. There are no breakpoints for macrolides with respect to Enterobacteriaceae, so the trough concentration in epithelial lung fluid of 0.8 mg/L was used for the subinhibitory concentration.17 If the MIC of an antibiotic exceeded the upper limit of our antimicrobial susceptibility assay, the next concentration above the highest concentration tested was used for suprainhibitory exposure experiments of that antibiotic.

Following the addition of antibiotic, the cultures were incubated with shaking for an additional 15 min to an OD_{600} of 0.5 before removing samples for RNA isolation. Cultures were incubated for an additional 6–8 h before plasmid isolation. For ciprofloxacin- and ticarcycline-treated cultures, plate counts were performed to determine the viability of the culture after drug treatment, due to a slight decrease in the OD_{600} observed for these treatments. This ensured that the number of cells used to extract the plasmid DNA was equivalent to that of the other drug-treated cultures.

Plasmid isolation and Southern blot analysis

Plasmid isolation and Southern blot analysis was performed as previously described.18

RNA isolation and RT–PCR

RNA from each strain was isolated from mid-logarithmic-phase cultures using TRIzol Max (Invitrogen) as previously described.15 Contaminating DNA was removed by treating 8 μg of RNA with 16 U of RQ1 RNase-free DNase (Promega, Madison, WI, USA) for 2.5 h at 37 °C. Real-time RT–PCR was performed as previously described using the QuantiTect® SYBR Green RT–PCR Kit.15 Expression of the genus-specific single-copy ampD gene for isolates of Enterobacteriaceae or the rpsL gene for P. aeruginosa was used to normalize the expression data for the Tn4401 genes of interest (Table S1, available as Supplementary data at JAC Online). Expression studies were performed in triplicate (coefficient of variation ≤10%) and the average Ct for each transcript was calculated. Relative expression data were determined using the calculation \( RQ = 2^{-\Delta\Delta Ct} \).

Nucleotide sequence accession numbers

GenBank accession numbers for Tn4401 in isolates UMM3, 233 and 01MGH049 are JX500679, JX500680 and JX500681, respectively.

Results

PCR and sequencing results

To ensure that the five KPC-producing isolates carried the required genes for transposition, the 10.5 kb Tn4401 element was sequenced in each isolate. PCR mapping with different primer sets showed that isolates K. pneumoniae UMM3, E. coli 233 and Enterobacter sp. 01MGH049 had the full-length isofrom b of Tn4401 identical to sequences in the GenBank database (EU176012 and FJ223605). We were able to obtain sequence data for Tn4401 in all the isolates except for K. pneumoniae HUH40 and P. aeruginosa PSS. In these two isolates, there were two regions within the trnaR and istA–istB regions from which clean sequence reads could not be obtained. Using GenBank accession number EU176012, the unreadable sequences for HUH40 were located between 2496–2583 and 6565–7254 nt; for strain PSS, the unreadable sequences were between 1797–1818 and 6547–6560 nt.

Antimicrobial susceptibility testing results

The MICs of the tested drugs observed in the clinical isolates were interpreted according to 2010 CLSI breakpoints (Table 1) with the...
exception of tigecycline, whose MICs were interpreted according to
FDA-established breakpoints for Enterobacteriaceae (susceptible if
≤2 mg/L). The MICs of imipenem, meropenem, ertapenem, cef-
tazidime and ceftepime have been previously reported for these iso-
lates.3

The MICs of piperacillin/tazobactam, ceftazidime, imipenem,
meropenem, ertapenem and doripenem for all five clinical isolates
indicated resistance. The MICs of ceftazidime, gentamicin and cipro-
ofloxacin were also at or above the resistance breakpoint for four out
of five clinical isolates; susceptible MICs were observed for these
drugs for isolate UMM3. All four Enterobacteriaceae isolates
demonstrated susceptibility to tigecycline. The MICs of azithromycin
ranged from 16 to 256 mg/L for these isolates.

Localization of the blakpc gene in five KPC-producing isolates
Plasmid profiles and blakpc Southern analysis of all five isolates are
shown in Figure 1a and b). Multiple plasmids ranging from 8 to
>200 kb were observed for the Enterobacteriaceae. K. pneu-
moniae UMM3 possessed two large molecular weight plasmids of
~55 and ~165 kb in size, with the blakpc gene located on the
~55 kb plasmid. K. pneumoniae HUH40 had four plasmids,
ranging from ~20 to ~95 kb, and the blakpc gene was located on
both the ~55 and ~95 kb plasmids. E. coli 233 had three plasmids
of ~120, ~95 and ~30 kb in size. DNA isolation from E. coli 233 was
difficult due to the strain’s production of a DNA nuclease that con-
sistently resulted in different fragmentation patterns during DNA
isolation. However, the ~30 kb plasmid consistently hybridized
with the blakpc probe. Enterobacter sp. isolate 01MGH049 had
two plasmids that were ~70 and ~10 kb in size, but only the
~70 kb plasmid hybridized with the KPC probe. P. aeruginosa P55
had one ~55 kb plasmid, which also hybridized with the KPC
probe. tnpA and trpR were also located on the same plasmids as
blakpc in each isolate evaluated (data not shown).

Evaluation of blakpc mobilization upon subinhibitory antibiotic challenge
Subinhibitory concentrations of drugs were used to simulate the
effect suboptimal drug concentrations may have within an
infected patient (Table 2). However, upon exposure to subinhibitory
levels of the selected antibiotics, no changes in the hybridization
patterns or binding intensity for the blakpc probe were observed
for any of the organisms evaluated (Figures 2 and 3, data not
shown for P. aeruginosa P55). As a control, hybridization patterns
using the trpR and tnpA probes from all five bacterial isolates
treated with the carbapenems were also unchanged (data not
shown).

An increase in transposition events would most likely require an
increase in the production of the transposase and resolvase asso-
ciated with Tn4401. To confirm the lack of mobility of Tn4401 in the
presence of antibiotic observed using Southern analysis, we tested
the ability of imipenem, meropenem or ertapenem to increase
the RNA expression of these genes. There was no change in the
level of transposase or resolvase RNA upon carbapenem treatment
compared with the untreated controls. Therefore, these data
support the lack of transposition observed using Southern analysis
described above. They also support the observation that antibiotic
treatment at suboptimal concentrations of the tested drugs under
the experimental parameters tested did not stimulate the trans-
position of Tn4401.

To ensure that the cell number used for each drug treatment did
not skew the results, optical densities (OD600) were measured and
viability counts of cultures treated with ciprofloxacin and
tigecycline were evaluated, as a slight decrease in the OD600 was
observed. A decrease of no more than 1 log (data not shown) for
four of the five strains was observed in the presence of ciprofloxacin
or tigecycline. This log decrease, however, did not influence the
ability of imipenem, meropenem or ertapenem to increase
the RNA expression of these genes. There was no change in the
presence of antibiotic observed using Southern analysis, we tested
the RNA expression after exposure to antibiotics
Southern analysis did not indicate an increase in blakpc mobility
when isolates were exposed to subinhibitory levels of antibiotics.
However, it was possible that these antibiotic drug classes could
stimulate the expression of blakpc. Therefore, blakpc gene expres-
sion was evaluated using 11 different antibiotics from five different
drug classes at concentrations that were subinhibitory, at the MIC
or suprainhibitory for the organisms (Table 2). A summary of the
RNA data is presented in Table 3.

Table 1. MICs for KPC-producing isolates

<table>
<thead>
<tr>
<th>Genus</th>
<th>Strain</th>
<th>KPC allele</th>
<th>CAZa</th>
<th>FEPa</th>
<th>IPMa</th>
<th>ETPa</th>
<th>DORA</th>
<th>MEMa</th>
<th>TGCa</th>
<th>TZPa</th>
<th>GENa</th>
<th>CPIb</th>
<th>AZMa</th>
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<td>233</td>
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<td>32</td>
<td>16</td>
<td>8</td>
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CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; ETP, ertapenem; DOR, doripenem; MEM, meropenem; TGC, tigecycline; TZP, piperacillin/tazobactam; GEN, gentamicin; CIP, ciprofloxacin; AZM, azithromycin.
aMICs were determined using broth microdilution methodology.
bMICs were determined using agar dilution methodology.
Figure 1. Plasmid profiles and $bla_{KPC}$ Southern analysis in five KPC-producing organisms. (a) Electrophoretic profiles of plasmids in strains $K. pneumoniae$ UMM3, $K. pneumoniae$ HUH40, $E. coli$ 233, Enterobacter sp. 01MGH049 and $P. aeruginosa$ PS5 and a BAC Tracker Supercoil Ladder. (b) Hybridization with a $bla_{KPC-2}$-specific probe. Chr, chromosomal DNA.

Table 2. $\beta$-Lactam concentrations used in RNA expression analyses$^a$

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<th>GEN</th>
<th>TGC</th>
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IPM, imipenem; MEM, meropenem; ETP, ertapenem; DOR, doripenem; TZP, piperacillin/tazobactam; CAZ, ceftazidine; FEP, cefepime; TGC, tigecycline; GEN, gentamicin; CIP, ciprofloxacin; AZM, azithromycin.

$^a$Units for drug amounts are mg/L.
$^b$Subinhibitory (sub), MIC and suprainhibitory (supra) concentrations based on MICs for the organisms listed in Table 1.
$^c$MICs for all strains were $>128/4$; MIC and suprainhibitory experiments were not performed.
$^d$0.8 mg/L is considered to be a trough level of AZM in epithelial lining fluids and was used as the subinhibitory level for AZM.
$^e$Experiments with suprainhibitory levels of antibiotics were not performed for this isolate.
Although changes in the expression of \textit{bla}\textsubscript{KPC} for each isolate tested were not dramatic, there were modifications in \textit{bla}\textsubscript{KPC} expression upon exposure to some antibiotics. Evaluation of the data shows that changes in \textit{bla}\textsubscript{KPC} expression were strain, genus and antibiotic specific. For example, when both strains of \textit{K. pneumoniae} were compared, no change in and antibiotic specific. For example, when both strains of \textit{K. pneumoniae} were compared, no change in expression was observed for doripenem at any concentration tested for strain UMM3, but up to a 2-fold increase was observed for strain HUH40 at MIC and subinhibitory concentrations. Other differences between these two strains were observed for the subinhibitory concentrations of imipenem, meropenem, ertapenem, doripenem; TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin; GEN, gentamicin; TGC, tigecycline; AZM, azithromycin; Chr, chromosomal DNA.

![Figure 2. \textit{bla}\textsubscript{KPC} Southern analysis of KPC-producing \textit{K. pneumoniae} strains. Localization of the \textit{bla}\textsubscript{KPC-2} gene in (a) \textit{K. pneumoniae} HUH40 and (b) \textit{K. pneumoniae} UMM3 treated with subinhibitory levels of the following: IPM, imipenem; MEM, meropenem; ETP, ertapenem; DOR, doripenem; TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin; GEN, gentamicin; TGC, tigecycline; AZM, azithromycin; Chr, chromosomal DNA.](image1.png)

Figure 2. \textit{bla}\textsubscript{KPC} Southern analysis of KPC-producing \textit{K. pneumoniae} strains. Localization of the \textit{bla}\textsubscript{KPC-2} gene in (a) \textit{K. pneumoniae} HUH40 and (b) \textit{K. pneumoniae} UMM3 treated with subinhibitory levels of the following: IPM, imipenem; MEM, meropenem; ETP, ertapenem; DOR, doripenem; TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin; GEN, gentamicin; TGC, tigecycline; AZM, azithromycin; Chr, chromosomal DNA.

when treated with suprainhibitory concentrations of ciprofloxacin. Other interesting differences observed with the \textit{Enterobacter} strain were increases in expression when treated with subinhibitory concentrations of imipenem, meropenem, ertapenem, piperacillin/tazobactam and ceftazidime. \textit{P. aeruginosa} strain PS5 was difficult to evaluate given the high MICs of most of the drugs tested. However, treatment with suboptimal concentrations of meropenem, doripenem and cefepime all resulted in a 2- to 3-fold decrease in \textit{bla}\textsubscript{KPC} expression. Gentamicin also resulted in 2.5- to 3.5-fold decreases in \textit{bla}\textsubscript{KPC} expression when using concentrations equal to or exceeding the MIC. With respect to doripenem, the \textit{E. coli}, \textit{Enterobacter} sp. and \textit{P. aeruginosa} isolates had similar responses in \textit{bla}\textsubscript{KPC} expression when treated with subinhibitory concentrations.

![Figure 3. \textit{bla}\textsubscript{KPC} Southern analysis of KPC-producing \textit{E. coli} and \textit{Enterobacter} strains. Localization of the \textit{bla}\textsubscript{KPC-2} gene in (a) \textit{E. coli} 233 and (b) \textit{Enterobacter} sp. 01MGH049 treated with subinhibitory levels of the following: IPM, imipenem; MEM, meropenem; ETP, ertapenem; DOR, doripenem; TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin; GEN, gentamicin; TGC, tigecycline; AZM, azithromycin; Chr, chromosomal DNA.](image2.png)

Figure 3. \textit{bla}\textsubscript{KPC} Southern analysis of KPC-producing \textit{E. coli} and \textit{Enterobacter} strains. Localization of the \textit{bla}\textsubscript{KPC-2} gene in (a) \textit{E. coli} 233 and (b) \textit{Enterobacter} sp. 01MGH049 treated with subinhibitory levels of the following: IPM, imipenem; MEM, meropenem; ETP, ertapenem; DOR, doripenem; TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin; GEN, gentamicin; TGC, tigecycline; AZM, azithromycin; Chr, chromosomal DNA.

### Discussion

Severe illness and prior exposure to antibiotics, particularly third-generation cephalosporins and fluoroquinolones, have been identified as risk factors for infection with KPC-producing organisms, although whether it is exposure to specific antibiotics or a cumulative effect of multiple exposures is unknown.\cite{1,2,20}

It has been established that exposure to antibiotics can result in the selection of resistant mutants, changes in bacterial
metabolism and/or colonization of the infected patient. All of these outcomes with antibiotic exposure can be considered unwanted ‘collateral damage’ during the treatment of the patient.21,22,23 Non-antibiotic drug treatments required to treat the patient’s underlying condition(s) could also act as a stimulus for modifications in antibiotic drug treatments required to treat the patient’s underlying condition(s). At the present time, there are no data available that have examined the potential for these and other ‘collateral damage’ effects in the treatment of patients infected with KPC-producing organisms. This study looked beyond the relationship between bla<sub>KPC</sub> expression and its influence on β-lactam susceptibilities. We hypothesized that unintended consequences of antibiotic treatment could include changes in bla<sub>KPC</sub> gene expression and/or the mobility of its associated mobile genetic element, Tn4401. Stimulation of transposition through the exposure of cells to antibiotics is not a new concept. The role antibiotics play in the transposition of mobile genetic elements has been explored in Gram-positive organisms. Subinhibitory concentrations of erythromycin have been shown to induce the transposition of Tn917 in Streptococcus faecalis.24 More recently, ciprofloxacin and vancomycin were shown to increase the frequency of IS256 transposition in S. aureus.25,26 Subinhibitory concentrations of antibiotic have been identified as contributors to the emergence of a resistance phenotype.27,28 Therefore, we reasoned that increased transposition and/or modifications of bla<sub>KPC</sub> gene expression may take place when antibiotic treatment in the patient is suboptimal.

Table 3. bla<sub>KPC</sub> RNA expression fold changes following exposure to subinhibitory, MIC and suprainhibitory levels of antibiotic

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<th>TZR</th>
<th>CAZ</th>
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IPM, imipenem; MEM, meropenem; ETP, ertapenem; DOR, doripenem; TZR, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin; GEN, gentamicin; TGC, tigecycline; AZM, azithromycin; ND, not determined.

*Refers to the amount of drug used; subinhibitory (sub), MIC and suprainhibitory (supra) concentrations based on the MICs for the organisms listed in Table 1.

RNA expression; data are expressed as fold differences as compared with the untreated control, which is set to 1; — represents no change in expression compared with the untreated control and up or down arrows indicate an increase or decrease in expression, respectively. Three independent RNA experiments were performed and the Ct values gave a coefficient of variation ≤10%.

However, using Southern analysis, exposure to subinhibitory concentrations of the drugs tested did not influence the transposition of Tn4401 or duplication of bla<sub>KPC</sub> in any of the strains analysed. However, changes in bla<sub>KPC</sub> gene expression were noted after treatment of some antibiotic drug classes. Although the fold differences in bla<sub>KPC</sub> gene expression were subtle, these differences should not be ignored. Of particular interest, some non-β-lactam drugs, such as tigecycline, had a greater influence on bla<sub>KPC</sub> gene expression than β-lactam drugs, but strain and genera variation was also observed. This variation may represent differences in metabolic processes or other compensatory mechanisms that may have been selected for by the organism during drug treatment of the infected patient.

Experimentation evaluating mechanisms required for KPC production within the cell has been minimal, with few answers revealed.7,8,26 Every experimental approach has limitations and this study was no exception. One limitation of the present study was the small number of clinical isolates used. In addition, we were limited on the number of drug concentrations we could evaluate using Southern blot analysis and gene expression assays. It is possible that evaluating gene copy number changes using real-time PCR would be more sensitive and enable the analysis of low-frequency events that Southern analysis would not be able to detect. The evaluation of additional isolates based on the bla<sub>KPC</sub> gene expression data is warranted to establish a connection between exposure to specific antibiotics and modifications in bla<sub>KPC</sub> gene expression.
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References