Klebsiella pneumoniae belonging to ST258 from a New York City Hospital Carrying KPC-2- and VIM-4-Encoding Genes: Report from the SENTRY Antimicrobial Surveillance Program

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AMENDED ABSTRACT

Background: KPC-producing Enterobacteriaceae (ENT) are prevalent in the USA; however, isolates producing metallo-β-lactamases (MBLs) are still very uncommon. We describe a ST258 *K. pneumoniae* (KPN) recovered in a New York (NY) city hospital that carried bla_{KPC-2} and bla_{VIM-4}.

Methods: ENT collected during 2013 in USA hospitals were susceptibility (S) tested and carbapenem (CARB)-resistant (R) isolates were screened for bla_{KPC}, bla_{NDM}, bla_{VIM} and bla_{IMP} by PCR/sequencing. Genetic context and location of CARBase genes and plasmid incompatibility (Inc) types were determined. MLST, screening for ESBL and transferable AmpC genes were performed as well. Expression of AcrAB-TolC and outer membrane proteins (OMP)-encoding genes was assessed by quantitative RT-PCR. OMP genes were sequenced.

Results: Among 139 CARB-R ENT detected in the USA during 2013, 69 (49.6% of CARB-R) carried bla_{KPC} and 34 were collected in hospitals located in NY State. Among 10 KPC-producing KPN detected in a NY City hospital, one isolate was also positive for bla_{VIM}. This isolate, obtained from urinary tract specimen of a 23 year-old female, was R to all β-lactams, aminoglycosides and quinolones, being S only to polymyxins and tigecycline. Sequencing confirmed that the isolate carried bla_{KPC-2} and bla_{VIM-4} and this isolate belonged to ST258 by MLST. bla_{KPC-2} was located on *Tn4401* element with a 99-bp deletion upstream of KPC gene. bla_{VIM-4} was embedded in a class 1 integron and this gene was located in the first position followed by *aacA7, dhfrA1* and *aadA1*. bla_{KPC-2} and bla_{VIM-4} were located on distinct plasmids (~120-Kb and~190-Kb) belonging to Inc types A/C or FII. The isolate also carried bla_{CMY-4}, bla_{TEM-1} and bla_{SHV-11}. Elevated expression of *acrA* (15.3X) and significantly reduced expression *ompK35* and *ompK37* (0.16X and 0.081X, respectively) were observed compared to control. Mutations in OMP genes included two insertions in *ompK37.*

Conclusions: Isolates carrying two CARBase genes have been reported in other countries, but not in the USA. Furthermore, KPC-2 and VIM-4-producing KPN have been detected in Greece in a different ST type (ST383) compared to this reported isolate. The presence of two CARBases in KPN ST258 is of great concern due to the ability of this organism to disseminate.

INTRODUCTION

The first reported KPC-producing isolate was a *Klebsiella pneumoniae* collected in 1996 from a patient hospitalized in North Carolina. Over the past decade, KPC variants became the most clinically significant β-lactamase among Class A derivatives and KPC-producing Enterobacteriaceae have been reported worldwide. Within the USA, there are differences in prevalence of KPC-producing isolates which have been reported in 36 USA states, Washington DC and Puerto Rico; however, these strains seem to be endemic in the New York City area.

Overall, the dissemination of bla_{KPC} has been associated with clonally-related strains and the majority of bla_{KPC}-carrying *K. pneumoniae* belong to multilocus sequence type (MLST) 258 and double-locus variants, which may have contributed to the worldwide dissemination of KPC-producing strains. Moreover, there is mounting evidence that bla_{KPC} genes are also consistently associated with a specific genetic element (i.e. transposon Tn*4401*). Tn*4401* is a Tn*3*-like transposon that has been identified in distinct plasmids carried by KPC-producing Enterobacteriaceae and *Pseudomonas aeruginosa* isolates from various geographic areas.

During the processing of 2013 isolates for the SENTRY Antimicrobial Surveillance Program, we detected one *K. pneumoniae* isolate producing KPC-2 and VIM-4 and in this study we report the characterization of this strain.

MATERIALS AND METHODS

Bacterial strains. During 2013, a total of 139 Enterobacteriaceae isolates with elevated imipenem or meropenem MIC values (≥2 μg/ml) were collected from USA medical centers as part of SENTRY Antimicrobial Surveillance Program. Isolates were susceptibility tested using broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI). Categorical interpretations for all antimicrobials were those found in M100-S24 and the EUCAST website and quality control (QC) was performed using *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. All QC results were within specified ranges as published in CLSI documents (2014).

Screening for acquired carbapenemases. Isolates were screened for the presence of bla_{KPC}, bla_{NDM}, bla_{VIM} and bla_{IMP} in a multiplex polymerase chain reaction (PCR). All PCR experiments included reactions containing target DNA templates for each screening primer set utilized. Amplicons generated were sequenced on both strands; nucleotide and deduced amino acid sequences were analysed using the Lasergene software package (DNASTAR, Madison, Wisconsin, USA). Amino acid sequences were compared with those available via internet sources (http://www.ncbi.nlm.nih.gov/blast/).

METHODS-CONTINUED

Genetic location of the carbapenem-encoding genes. Agarose embedded chromosomal DNA was subjected to ICeuI digestion and partial digestion with S1 nuclease. DNA digests were resolved by electrophoresis on CHEF DRII (BioRad, Richmond, California) followed by Southern blotting and hybridization with digoxigenin labeled (Roche Diagnostics GmbH, Mannheim, Germany) bla_{KPC}- and bla_{VIM}-specific probes. Plasmid sizes were estimated using concatenated Lambda DNA ladder. Plasmid incompatibility types was determined by multiplex PCR tests described elsewhere.

The Tn*4401* bla_{KPC}-carrying element was amplified with primers targeting the surrounding structures and the carbapenemase-encoding gene. PCR products were digested with EagI and RFLP patterns were compared to reference Tn*4401* bla_{KPC}-carrying elements previously sequenced. The region directly upstream of bla_{KPC} was sequenced.

Primers annealing to the 5' and 3' conserved sequence (CS) regions of class 1 integron were used in combination with the bla_{VIM} primers to determine the size and structure of the integron. Additional primers targeting the genes detected in the integron were used to complete sequencing. Sequencing was analyzed as described above. Integron sequence was deposited in GenBank.

Molecular typing. Multilocus sequence typing (MLST) was performed for the *K. pneumoniae* isolate according to instructions on the website http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html.

Expression analysis of the chromosomally encoded resistance mechanisms. The expression of *acrAB-TolC, ompK35, ompK36* and *ompK37* was determined by quantitative real-time PCR (qRT-PCR) using 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 was 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) ≥7 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 (*rpsL*) on the StepOne Plus instrument (Life Technologies, Carlsbad, California, USA) using custom designed primers showing >93.0% efficiency. Transcription levels were considered significantly different if at least a 5-fold difference was noted compared with *K. pneumoniae* ATCC 13883.

Sequence analysis of outer membrane proteins. Outer membrane proteins of *K. pneumoniae* were sequenced in full using specific primers. Amplicons were sequenced on both strands; nucleotide and deduced amino acid sequences were analyzed as described above.

RESULTS

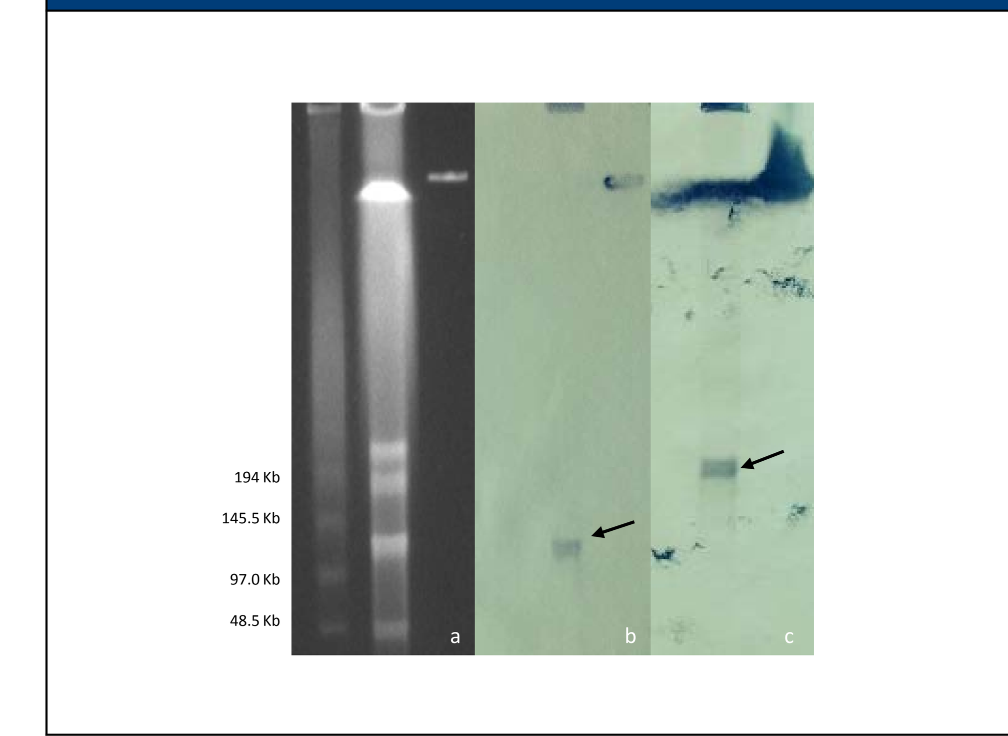
- Among 139 carbapenem-resistant Enterobacteriaceae isolates detected in the USA during 2013, 123 were *K. pneumoniae.* A total of 69 (49.6%) Enterobacteriaceae isolates carried bla_{KPC} and among those, 34 (49.3%) were collected in hospitals located in the state of New York.
- Ten KPC-producing *K. pneumoniae* were recovered in one hospital and among those one was also positive for bla_{VIM}. This isolate was recovered from a nephrostomy urine specimen of a 23 year-old female. At the time of culture, the patient was 2 years post allogeneic stem cell transplant and developed hemorrhagic cystitis for which a nephrostomy tube was placed. The patient had a remote history of bacteremia with *K. pneumoniae,* for which she had received meropenem.
- This isolate producing two carbapenemases was resistant to all β-lactams including aztreonam, aminoglycosides (gentamicin and tobramycin), quinolones and trimethoprim-sulfamethoxazole, but was susceptible to polymyxin B, colistin and tigecycline (**Table 1**).
- MLST analysis revealed that the isolate belonged to ST258 and sequencing of amplicons confirmed that the isolate carried bla_{KPC-2} and bla_{VIM-4}.
- bla_{KPC-2} was located on *Tn4401* element with a 99-bp deletion upstream of this carbapenemase gene and bla_{VIM-4} was embedded in a 4.5-Kb class 1 integron. This metallo-β-lactamase-encoding gene was located in the first position of the class 1 integron followed by *aacA7, dhfrA1, aadA1*-like gene and *qacEΔ1/sul1*. The integron sequence has been deposited in GenBank under accession number KJ551510.
- Genes encoding KPC-2 and VIM-4 were located on distinct plasmids of approximately 120-Kb and 190-Kb, respectively (**Figure 1**). Incompatibility (Inc) types for these plasmids were A/C and FII, respectively.
- Additional screening for β-lactamase-encoding genes demonstrated that the KPC-2- VIM-4-producing *K. pneumoniae* also carried bla_{CMY-4}, bla_{TEM-1} and bla_{SHV-11}.
- This isolate had elevated expression of the efflux pump AcrAB-TolC (15.3X) and reduced expression of *ompK35* and *ompK37* (0.16X and 0.081X, respectively) when compared to control *K. pneumoniae* strain ATCC 13883 (**Table 1**; **Figure 3**).
- Multiple mutations and insertions have likely disrupted the function of OmpK37 (**Table 1**).

Table 1. Antimicrobial susceptibility and molecular characterization of KPC-2- and VIM-4-producing *K. pneumoniae* recovered in a New York City hospital.

Test/Characteristic	Results for *K. pneumoniae* isolate
Antimicrobial Susceptibility testing	**MIC (μg/ml; categorical interpretation[a]):**
Imipenem	>8 (R)
Meropenem	>8 (R)
Ceftazidime	>32 (R)
Cefepime	>16 (R)
Aztreonam	>16 (R)
Piperacillin-tazobactam	>64 (R)
Ciprofloxacin	>4 (R)
Gentamicin	>8 (R)
Tobramycin	>16 (R)
Trimethoprim-sulfamethoxazole	>4 (R)
Tigecycline	1 (S)
Colistin	0.5 (S)
Polymyxin B	0.5 (S)
Genetic location of carbapenemase genes	**Molecular size/Inc type/Tn*4401* type:**
bla_{KPC-2} plasmid size	120-Kb
bla_{VIM-4} plasmid size	190-Kb
Plasmid Inc types	IncA/C and IncFII
Tn*4401* isoform (bla_{KPC-2})	Tn*4401a* (99-base pair deletion upstream of bla_{KPC})
Gene expression	**Relative expression[b,c]:**
acrAB	<u>15.3</u>
ompK35	<u>0.16</u>
ompK36	6.6
ompK37	<u>0.08</u>
OMP mutations	**Amino acid alterations[d,e]:**
OmpK35	G211S, V241I
OmpK36	R345H
OmpK37	R239K, <u>237-TERY-238 insertion</u>, E244D, N274S, D275T, <u>275-SSTNGG-276 insertion</u>, V277I, V295G, D350G

a. S= susceptible; I= intermediate; R= resistant. Criteria as published by the CLSI [2014] and EUCAST [2014] or tigecycline product insert [Tygacil Product Insert, 2012].
b. *K. pneumoniae* ATCC13883 was used as baseline for the analysis of the expression results.
c. Underlined values are considered significant.
d. Amino acid sequences were compared to *K. pneumoniae* 342 and ATCC 13883 sequences available over the internet.
e. Underlined alterations are considered significant.

Figure 1. (a) S1 nuclease digested DNA and southern blot membranes hybridized with (b) bla_{KPC}- and (c) bla_{VIM}-specific probes labeled with digoxigenin. Lambda DNA ladder was used to estimate plasmid molecular weight and arrows point to the location of the hybridization.



CONCLUSIONS

- *K. pneumoniae* isolates co-harboring KPC and VIM have been described from Greece, Germany and recently in Colombia, but have not been described so far from the USA. Interestingly, *K. pneumoniae* isolates carrying bla_{KPC-2}, bla_{VIM-4} and bla_{CMY-4} have been reported from Athens, Greece in 2010, but in a strain with a different sequence type (ST383) compared to the isolate reported in this study.
- The KPC-2- and VIM-4-producing *K. pneumoniae* detected in this study harbored multiple β-lactam resistance mechanisms, including a transferable cephalosporinase, elevated expression of a tripartite efflux pump system and reduced expression and/or disruptions of at least two outer membrane proteins. These results suggest that *K. pneumoniae* isolates are able to accumulate various resistance mechanisms contributing to the success of this pathogen.
- The presence of two carbapenemases in *K. pneumoniae* ST258 is of great concern due to the ability of this organism to disseminate.

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