KPC-producing *Pseudomonas aeruginosa* from the United States: What is Next?

P1005

LM DESHPANDE, K SIMPSON, RN JONES, M CASTANHEIRA
JMI Laboratories, North Liberty, IA, USA

ECCMID 2014
JMI Laboratories
North Liberty, IA, USA
www.jmilabs.com
319.665.3370, fax 319.665.3371
mariana-castanheira@jmilabs.com

AMENDED ABSTRACT

Objectives: To evaluate carbapenem-resistant *P. aeruginosa* isolates from United States (USA) hospitals for the presence of serine-carbapenemase and metallo-beta-lactamase encoding genes and to analyze genetic elements carrying these resistance determinants. KPC serine-carbapenemases are frequently detected among Enterobacteriaceae isolates in USA and although KPC-producing *P. aeruginosa* have been reported in other countries, these strains have been described only once in the USA.

Methods: *P. aeruginosa* collected as part of the SENTRY Antimicrobial Surveillance Program (2010-2011) were susceptibility tested using CLSI reference methods and 91 carbapenem-resistant isolates were further analyzed. Isolates were screened for bla_{KPC}, bla_{NDM}, bla_{VIM} and bla_{IMP} by PCR methods. Amplicons were sequenced on both strands. Tn*4401* element was analyzed by PCR, restriction digests and sequencing. Genetic location of bla_{KPC} was investigated by S1 nuclease and ICeuI hybridizations. Clonality was assessed by PFGE and MLST.

Results: Carbapenem-resistant *P. aeruginosa* were collected in 20 different states, being 16.6% from Texas, 13.2% from Kentucky and 12.1% from New York. These isolates displayed high level resistance (% non-susceptible according to CLSI/ EUCAST breakpoints) to ceftazidime (100.0/100.0), cefepime (96.7/96.7), piperacillin/tazobactam (98.9/98.9), aztreonam (98.9/100.0) and levofloxacin (81.3/89.0). Gentamicin (54.9% susceptible for both criteria), tobramycin (61.5%) and amikacin (85.7/69.2%) retained moderate activity and colistin (98.9/100.0% S) was the only compound displaying good in vitro activity against these isolates. Two *P. aeruginosa* (imipenem and meropenem MIC values, ≥32 mg/L) from one New York City hospital (2010) were positive for bla_{KPC-2}. Isolates were recovered from tracheal aspirate and sputum samples. No other carbapenemases were detected. Although recovered within one month, KPC-producing *P. aeruginosa* were genetically distinct by PFGE and MLST (ST244 and ST654). bla_{KPC} was located on a copy of Tn*4401* element; however a 134-bp deletion upstream of bla_{KPC} was observed in one isolate (ST244). Probe hybridizations of S1 nuclease and ICeuI digested DNA indicated that bla_{KPC-2} was carried in the chromosome of one isolate and in a 45-Kb plasmid in the other strain (ST654). Carbapenem-resistant Enterobacteriaceae and *Acinetobacter* spp. from the same hospital where the KPC-producing *P. aeruginosa* were detected were screened for the presence of bla_{KPC} and seven *K. pneumoniae* isolates carried this gene, but different isoforms of Tn*4401* were observed in these Enterobacteriaceae strains.

Conclusions: Two isolates from the same hospital carried bla_{KPC-2} in distinct genetic environments, which indicate separate acquisition events and sources. Additionally, the only two samples detected were collected in a hospital with high incidence of KPC-producing Enterobacteriaceae that could be the reservoir for these genes. Among all antimicrobial agents tested, colistin retained greatest activity against the KPC-producing *P. aeruginosa*.

INTRODUCTION

Carbapenem resistance in *Pseudomonas aeruginosa* is usually caused by a combination of intrinsic resistance mechanisms including de-repression of the chromosomal cephalosporinase AmpC, up-regulation of resistance-nodulation-division (RND) efflux systems and loss of the outer membrane channel OprD. Although these mechanisms are the most common in carbapenem-resistant *P. aeruginosa*, the presence of carbapenemase-encoding genes has been reported among these organisms worldwide. Metallo-β-lactamases of the IMP and VIM families and more recently NDM-1 have been described in *P. aeruginosa*, most commonly in geographic areas in which these genes are prevalent. Furthermore, SPM-1-producing *P. aeruginosa* isolates are endemic in Brazil and have been reported in Argentina in a case related to travel to Brazil.

Although serine-carbapenemased production in *P. aeruginosa* is rare; KPC-producing isolates of this species have been described since 2009 from Colombia and more recently from Argentina, Brazil, Puerto Rico, Trinidad and Tobago and China. Additionally, there has been one report of KPC-producing *P. aeruginosa* from the United States (USA). This isolate was recovered in south Florida in 2009 from a patient with no history of previous hospitalization or travel to other countries.

In this study, we evaluated 91 carbapenem-resistant *P. aeruginosa* isolates, collected as part of the SENTRY Program in USA hospitals during 2010 and 2011, for the presence of bla_{KPC} and other common carbapenemases. The KPC-2-producing *P. aeruginosa* isolates were characterized for the genetic support of bla_{KPC} and other β-lactam resistance mechanisms. Additionally, we evaluated carbapenem-resistant Enterobacteriaceae and *Acinetobacter* spp. isolates from the same hospital where the KPC-producing *P. aeruginosa* were detected since a correlation of the presence of these genes in Enterobacteriaceae and other species has been previously observed.

MATERIALS AND METHODS

Bacterial strains. Ninety-one carbapenem-resistant *P. aeruginosa* isolates collected from USA hospitals were susceptibility tested using broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines. Categorical interpretations for all antimicrobials were those found in the CLSI document 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.

Screening for acquired carbapenemases. Isolates were screened for the presence of bla_{KPC}, bla_{NDM}, bla_{VIM} and bla_{IMP} using a multiplex PCR approach. Amplicons generated were sequenced on both strands; nucleotide and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, Wisconsin, USA). Amino acid sequences were compared using NCBI/BLAST searching tools.

Molecular typing. KPC-producing *P. aeruginosa* were epidemiologically typed by pulsed-field gel electrophoresis (PFGE) using genomic DNA prepared in agarose blocks, digested with SpeI (New England, Beverly, Massachusetts, USA) and resolved in the CHEF-DR II apparatus. Results were analyzed by GelCompar II software (Applied Math, Kortrijk, Belgium). Percent similarities were identified on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.2% and 0.5%, respectively. Multilocus sequence typing (MLST) was also performed for *P. aeruginosa* isolates according to instructions on the website http://pubmlst.org/paeruginosa/.

MATERIALS AND METHODS

Genetic location of KPC. 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}-specific probe. Plasmid sizes were estimated using concatenated Lambda DNA ladder.

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.

Conjugative transfer of plasmids. Conjugative transfer of bla_{KPC-2} was attempted by filter mating using *E. coli* J53 azide-resistant strain as recipient. Selection of transconjugants was performed using Nutrient agar plates containing ceftazidime (2 mg/L) and sodium azide (150 mg/L).

Genotypic detection of extended spectrum β-lactamases (ESBL). KPC-producing *P. aeruginosa* were additionally screened for presence of bla_{CTX-M}, bla_{GES}, bla_{VEB}, bla_{PER}, bla_{PSE} and oxacillinases with ESBL spectrum (bla_{OXA-2}-, bla_{OXA-10}- and bla_{OXA-30}-group, bla_{OXA-18} and bla_{OXA-45}) by PCR, as previously described.

Expression analysis of the chromosomally encoded AmpC and efflux pumps. The expression of *ampC*, *mexA* (MexAB-OprM), *mexC* (MexCD-OprJ), *mexE* (MexEF-OprN) and *mexX* (MexXY-OprM) 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) >8 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 Power SYBR Green RNA-to-CT kit (Life Technologies) and custom designed primers showing efficiency >98.0%. Transcription levels were considered significantly different if at least 5- or 10-fold difference was noted compared with *P. aeruginosa* PAO1 for efflux pumps and AmpC, respectively.

Porin Detection. The phenotypic detection of OprD was performed as described by Carlone et al. Outer membrane proteins were purified in the FastPrep-24 instrument (MP Biomedicals, Solon, Ohio, USA), according to the manufacturer's instructions. Normalized concentrations of purified outer membrane proteins were separated by electrophoresis and transferred onto PVDF membranes. Western blots were probed with an affinity-purified polyclonal antibody raised in rabbits using the synthetic OprD peptide N'-SDKTGTGNLPVMNDGKPPD-C' (ThermoFisher Scientific, Rockford, Illinois, USA) and developed with the WesternBreeze chromogenic kit (Life Technologies). *P. aeruginosa* PAO1 and two OprD down regulated laboratory constructs were used as positive and negative controls for comparative analysis.

RESULTS

- Among 91 carbapenem-resistant *P. aeruginosa* collected in 28 USA hospitals located in 20 states, a significant number of isolates were from Texas (n=16; 16.6%), Kentucky (n=12; 13.2%) and New York (n=11; 12.1%).
- Overall, isolates displayed high level resistance to various antimicrobial agents tested, including all β-lactams (0.0 to 3.3% susceptible) and levofloxacin (18.7/11.0% CLSI/EUCAST criteria). Aminoglycosides displayed activity against 54.9 to 85.7% of the isolates according to CLSI criteria and 54.9 to 69.2% according to EUCAST breakpoints. Colistin (98.9/100.0% susceptible) was the only compound displaying good activity against these isolates (**Table 1**).
- Two *P. aeruginosa* (2/91; 2.2%) collected from a single New York City hospital in 2010 were positive for bla_{KPC-2}. These isolates were recovered from tracheal aspirate and sputum samples from two patients and displayed imipenem and meropenem MIC values of ≥32 mg/L. No other carbapenemase genes were detected in this study.
- KPC-producing *P. aeruginosa* isolates exhibited distinct PFGE patterns and unrelated MLST (ST244 and ST654; **Table 2** and **Figure 1**), although they were recovered within one month interval.
- One isolate displayed a 134-bp deletion in *Tn4401* upstream of bla_{KPC-2} that was carried in the chromosome. On the remaining strain, bla_{KPC-2} was located on Tn*4401b* and this isolate carried two copies of this gene, one chromosomal and another located in a 45-Kb plasmid.
- These isolates carried no additional β-lactamase genes, but one strain hyperexpressed the chromosomal AmpC gene. Both strains had loss of OprD and expression of at least two efflux pumps was elevated in each strain. One isolate had elevated AmpC expression, MexAB-OprM and MexXY-OprM (204-, 6- and 23-fold greater than PAO1 reference strain, respectively; **Table 2**) whereas the other isolate had elevated expression of MexAB-OprM, MexEF-OprN and MexXY-OprM (7-, 46-, 16-fold greater than control strains; **Table 2**).
- Despite multiple attempts, bla_{KPC} could not be transferred to an *E. coli* recipient strain by conjugation using the *P. aeruginosa* strain carrying this gene in a plasmid.
- Among 19 other carbapenem-resistant Gram-negative organisms from the same hospital, seven *K. pneumoniae* isolates carried bla_{KPC-2}. Differently from the *P. aeruginosa* strains, five *K. pneumoniae* carried this gene in a copy of Tn*4401a* and another two strains had a 68-bp deletion (data not shown).
- bla_{KPC} was carried in plasmids of various sizes in the *K. pneumoniae* isolates displaying higher molecular weight compared to the one carried by *P. aeruginosa*.

Table 1. Summary of antimicrobial susceptibility testing of 91 carbapenem-resistant *P. aeruginosa* isolates.

Antimicrobial agent	MIC in mg/L:			CLSI[a]	EUCAST[a]
	50%	90%	Range	%S/ %I/ %R	%S/ %I/ %R
Imipenem	>8	>8	8- >8	0.0 / 0.0 / 100.0	0.0 / 29.7 / 70.3
Meropenem	8	>8	8- >8	0.0 / 0.0 / 100.0	0.0 / 62.6 / 37.4
Ceftazidime	32	>32	32- >32	0.0 / 0.0 / 100.0	0.0 / 0.0 / 100.0
Cefepime	16	>16	8- >16	3.3 / 56.0 / 40.7	3.3 / 0.0 / 96.7
Aztreonam	>16	>16	8- >16	1.1 / 17.6 / 81.3	0.0 / 18.7 / 81.3
Piperacillin/tazobactam	>64	>64	16- >64	1.1 / 13.2 / 85.7	1.1 / 0.0 / 98.9
Amikacin	8	32	1- >32	85.7 / 7.7 / 6.6	69.2 / 16.5 / 14.3
Gentamicin	4	>8	≤2- >8	54.9 / 6.6 / 38.5	54.9 / 0.0 / 45.1
Tobramycin	1	>16	0.25- >16	61.5 / 2.2 / 36.3	61.5 / 0.0 / 38.5
Levofloxacin	>4	>4	≤0.5/ >4	18.7 / 7.7 / 73.6	11.0 / 7.7 / 81.3
Colistin	1	2	≤0.5- 4	98.9 / 1.1 / 0.0	100.0 / 0.0 / 0.0

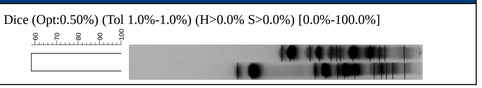
a. Criteria as published by the CLSI (2014) and EUCAST (2014).

Table 2. Results for the two KPC-producing *P. aeruginosa* isolated in a New York City hospital.

Test/Characteristic	Isolate A	Isolate B
Date of isolation	Jan-2010	Feb-2010
PFGE pattern	PSA15B	PSA15C
MLST	ST244	ST654
bla_{KPC-2} location	Chromosomal	Chromosomal and 45-Kb plasmid
Tn*4401* isoform	134-bp deletion upstream of bla_{KPC}	Tn*4401b*
Gene expression[a,b]		
ampC	204.3	1.5
mexA	6.6	7.6
mexC	1.9	0.42
mexE	0.00 (0.62)	0.03 (46.0)
mexX	23.4	16.6
OprD loss	pos	pos

a. Underlined values are considered significant.
b. *P. aeruginosa* PAO1 was used as baseline for the analysis of the results. Due to PAO1 high expression levels of MexEF-OprN, the expression of this efflux system was also analyzed using another *P. aeruginosa* wild type laboratory strain, results are given in parenthesis.

Figure 1. PFGE comparison of KPC-2-producing *P. aeruginosa* isolates detected in one New York City hospital.



CONCLUSIONS

- Two KPC-2 carrying *P. aeruginosa* strains were detected in a single hospital in a short time period (January and February/2010); however, the distinct genetic backgrounds of bla_{KPC-2} indicate separate acquisition events and sources.
- These isolates were recovered in a hospital with high incidence of KPC-producing Enterobacteriaceae that could be the reservoir for these genes, as previously observed in different institutions; but the evidence obtained did not corroborate this hypothesis.
- Although production of serine-carbapenemases is not prevalent among *P. aeruginosa* and 97.8% of the isolates tested did not harbour this resistance mechanism, these isolates may harbor these genes and remain undetected due to the challenges involved in carbapenemase detection among non-Enterobacteriaceae species.

REFERENCES

1. Clinical and Laboratory Standards Institute (2012). *M07-A9. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard: ninth edition*. Wayne, PA: CLSI.
2. Clinical and Laboratory Standards Institute (2014). *M100-S24. Performance standards for antimicrobial susceptibility testing; 24th informational supplement*. Wayne, PA: CLSI.
3. European Committee on Antimicrobial Susceptibility Testing (2014). Breakpoint tables for interpretation of MICs and zone diameters. Version 4.0, January 2014. Available at: http://www.eucast.org/clinical_breakpoints/. Accessed January 1, 2014.
4. Garcia Ramirez D, Nicola F, Zarate S, Relloso S, Smayevsky J, Arduino S (2013). Emergence of *Pseudomonas aeruginosa* with KPC-type carbapenemase in a teaching hospital: an 8-year study. *J Med Microbiol* 62: 1565-1570.
5. Hagihara M, Crandon JL, Urban CM, Nicolau DP (2013). KPC presence in *Pseudomonas aeruginosa* has minimal impact on the *in vivo* efficacy of carbapenem therapy. *Antimicrob Agents Chemother* 57: 1086-1088.
6. Landman D, Babu E, Shah N, Kelly P, Olawole O, Backer M, Bratu S, Quale J (2012). Transmission of carbapenem-resistant pathogens in New York City hospitals: progress and frustration. *J Antimicrob Chemother* 67: 1427-1431.
7. Poirel L, Nordmann P, Lagrutta E, Cleary T, Munoz-Price LS (2010). Emergence of KPC-producing *Pseudomonas aeruginosa* in the United States. *Antimicrob Agents Chemother* 54: 3072.