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Identification and Characterization of KPC-Producing *Klebsiella Pneumoniae* in Clinical Isolates

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Abstract

The dissemination of carbapenemase-producing *Klebsiella pneumoniae* (KPC) strains is an increasing problem worldwide. KPC ß-lactamases are Ambler class A enzymes mostly plasmid-encoded; their global spread represents a threat to clinical patients care and public health. Between September-December 2012, have been isolated 60 *Klebsiella pneumoniae* strains that showed a decrease of susceptibility to carbapenems, collected from patients at the Provincial Laboratory of Clinical Microbiology 'S. Agostino-Estense' Hospital (Modena, Italy). Species identification and antimicrobial susceptibility testing were carried out using the Vitek 2 and studied for carbapenemases production using phenotypic confirmatory tests. The presence of carbapenemase genes were confirmed by PCR and sequencing. The localization of beta-lactamase genes was established by conjugation experiments. We reported the emergence of KPC-2-producing *Klebsiella pneumoniae* isolates PCR and sequencing experiments revealed the presence of bla_{KPC-2} gene in 56/60 isolates. The KPC-encoding plasmid was analysed by transconjugation experiments and DNA sequencing. The findings indicate the need to supplement surveillance systems based on susceptibility data with the surveillance of resistance mechanisms.

Keywords KPC, *Klebsiella pneumoniae*, Molecular Genetics, Antibiotic-Resistance

1. Introduction

Carbapenems such as imipenem and meropenem are recommended as first-line therapy for severe infections caused by *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs) (1). The emergence of carbapenem-resistant enterobacteria is therefore worrisome, since consequently antimicrobial treatment options are very restricted. Carbapenemases are beta-lactamases and are divided into two major molecular groups, differentiated by the hydrolytic mechanism in the active site. The first group contains at least one zinc atom at the active site, establishing them as metalloenzymes, represented mainly by Verona integron-encoded metallo-beta-lactamase (VIM) and Imipenemase metallo-beta-lactamase (IMP). The second group utilizes serine at the active site and its main representatives are *Klebsiella pneumoniae* carbapenemase (KPC) type enzymes belonging to the Bush group 2f (1).

The recent emergence and spread of acquired carbapenem resistance are of great concern. However, *Klebsiella pneumoniae* carbapenemase (KPC) type β-lactamases capable of hydrolyzing penicillins, cephalosporins, aztreonam, and carbapenems are becoming increasingly common in the northeastern United States (2). They are categorized as Ambler class A and Bush functional group 2f, and they differ from metallo-β-lactamases, which require divalent cations as metal cofactors for activity (3, 4). KPC producers have recently undergone an important dissemination in the United States, Israel, and Greece and have been detected elsewhere in Europe, Latin America, and Asia (5, 6, 7, 8, 9). For this purpose, 60 *K. pneumoniae* isolates were analyzed to detect and characterize the production of KPC and the ability of horizontal transfer of the genetic determinants of resistance.

2. Materials and Methods

2.1. Bacterial Strains

We have analyzed a total of 60 consecutive non-duplicate strains of *K. pneumoniae* isolated at the Provincial Laboratory of Clinical Microbiology of "S. Agostino-Estense" Hospital (Modena, Italy) during September-December 2012 and fulfilled the following criteria: imipenem (IPM), meropenem (MEM),ertapenem (ERT) Minimum inhibitory concentration (MIC) => 2 mg/L. Sites of isolation included urine (42%), body fluids (17%), wounds (15%), catheter tips (12%), blood (8%), and

respiratory tracts (7%).

2.2. Antimicrobial Susceptibility Determination And Carbapenemases Assays

Species identification and antimicrobial susceptibility testing were carried out using the Vitek2 system and the ASTGN201 cards (bioMérieux Italia S.p.A). MIC was determined by the broth microdilution according to Clinical Laboratory Standards Institute guidelines 2012 (8), against using Imipenem, Meropenem, Ertapenem Peperacillina/tazobactam, Ceftazidime, Cefotaxime, Trimethoprim-sulfamethoxazole, Fluoroquinolones, Tetracycline, Tigecycline, Amikacin, Gentamicin. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines were used for the susceptibility categorization (10).

Screening for the presence of a carbapenemase was performed with the modified Hodge test (12) using *Eschericha coli* ATCC 25922 as the indicator strain and 10 mg imipenem (IPM) discs.

The isolates were further investigated by combined disk test with IPM and IPM plus phenylboronic acid (PBA) or ethylenediaminetetraacetic acid (EDTA) as inhibitors of KPC or MBLs, respectively (13).

2.3. PCR Amplification and DNA Sequencing

Bacterial isolates which were confirmed for their capacity to produce of carbapenemase were further analyzed by PCR. DNA was extracted by a standard heat lysis protocol (14). Thereafter, specific primers were used to search for bla_{KPC} (15), bla_{IMP} (16), bla_{VIM} (17), bla_{OXA} (15) and are shown in Table 2. In all reactions, previously characterized isolates from our collection carrying all types of β-lactamases tested were used as positive controls.

PCR-positive amplicons were purified with the QIAquick PCR Purification Kit (Qiagen, Italia S.p.A) and directly sequenced using amplification primers on the 3130 Genetic Analyzer (Applied Biosystems). Purification and sequencing were carried out by Genex CZ, s.r.o. Sequence alignment and analysis were performed online using the BLAST program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Table 1. Antimicrobial susceptibilities of 60 strains of *Klebsiella pneumoniae*

Antibiotic	MIC (μg/mL)		
	MIC_{50}	MIC_{90}	Range
Imipenem	>32	>32	>32
Meropenem	>32	>32	>32
Ertapenem	>32	>32	>32
Piperacillin/tazobactam	>256	>256	>256
Ceftazidime	>256	>256	>256
Cefotaxime	>256	>256	>256
Trimethoprim-sulfamethoxazole	>32	>256	>256
Fluoroquinolones[a]	>32	>32	>32
Tetracycline	32	64	8-96
Tigecycline	1	3	1-16
Amikacin	>256	>256	>256
Gentamicin	2	2	0.25-2.0
Colistin	0.25	0.38	0.19-0.038

MIC, minimum inibitory concentration; MIC 50/90, MIC for 50% and 90% of the organisms

[a]Fluoroquinolones include ofloxacin and ciprofloxacin

Table 2. Primers used for assay

Target gene	Forward sequence	Reverse sequence	Lenght	Reference
KPC	CATTCAAGGGCTTTCTTGCTGC	ACGACGGCATAGTCATTTGC	538	(15)
IMP	GGAATAGAGTGGCTTAATTCTC	GTGATGCGTCYCCAAYTTCACT	500	(16)
VIM	CAGATTGCCGATGGTGTTTGG	AGGTGGGCCATTCAGCCAGA	450	(17)
OXA	GCTTGATCGCCCTCGATT	GATTTGCTCCGTGGCCGAAA	281	(15)

2.4. Transfer of Resistance

Conjugation transfer assay was performed in broth culture (Luria broth, (bioMérieux Italia S.p.A,)) where the *Klebsiella pneumoniae* (imi^R) were the donors and *Escherichia coli* J53-2 (met-, pro-, rif^R) were the recipient. Donor and recipient cells were mixed at a ratio of 1:1. Transconjugants were selected on MacConkey agar (bioMérieux Italia S.p.A) containing imipenem (0.5 mg/l) plus rifampicin (100 mg/l). Plasmid DNA extraction from donors and transconjugants was performed using a plasmid midi prep kit (Qiagen, Italia S.p.A) according to manufacturer's instructions. The sizes of plasmids were estimated by electrophoresis on 0.7% agarose gels using plasmids from *Escherichia coli* V517 as the standard markers (18). Conjugation frequency per recipient was expressed by division the number of transconjugants by the initial number of recipients.

Transconjugants were subjected to antibiotic-susceptibility testing and PCR to determine the presence of bla_{KPC}.

3. Results and Discussion

Minimum inhibitory concentration (MIC) results for the tested isolates are given in Table 1. All 60 isolates were resistant to carbapenems (MIC > 32 µg/mL) and fluoroquinolones (ciprofloxacin and ofloxacin >32 µg/mL). Two of them were also resistant to tigecycline (MIC= 8 µg/mL and 16 µg/mL). All strains were sensitive to colistin (MIC= 0.19-0.38 µg/mL) and gentamicin (MIC= 0.25 µg/mL) and 58 also sensitive to tigecycline (MIC= 1-2 µg/mL).

Fifty-six/60 (94%) isolates were positive for the combined disk with boronic acid, suggesting the production of KPC-type enzyme. Synergy-test with EDTA was positive for 4/60 (6.7%) strains, suggesting the production of MBL-type enzyme.

PCR analysis of the $bla_{KPC,}$ bla_{VIM}, bla_{IMP} and bla_{OXA} genes showed that 56 strains were KPC- positive and four isolates were MBL- positive (2= VIM and 2=IMP), while all were negative for bla_{OXA} gene.

Genotyping of $bla_{KPC,}$ bla_{VIM} and bla_{IMP} and PCR products of all 60 isolates was performed by nucleotide sequencing analysis and were identified by BLAST program NCBI web site as bla_{KPC-2} in 56/60 strains and bla_{VIM-1} and bla_{IMP-1} gene in 2/60 and 2/60 cases, respectively.

Carbapenem resistance was transferred from *K. pneumoniae* to *E. coli* J53 after conjugation. Conjugation experiments successfully transferred a plasmid from both clinical isolates to the recipient *E. coli* J53 with a conjugation frequency of 2.3×10^{-7} for recipient cell.

In Figure 1 is shown an example of the plasmid profiles of the original strains and the respective transconjugants. In all the original strains the plasmids with different molecular weights were present: one large plasmid with different-sized from 48 to 52 kb and other plasmids of low molecular weight plasmids (from 4.5 to 17 kb). The transconjugants presented only the large plasmid unlike the parental strains; PCR and sequencing confirmed that the transconjugants carried the bla_{KPC-2} gene as reported by other authors (19, 20, 21, 22, 23).

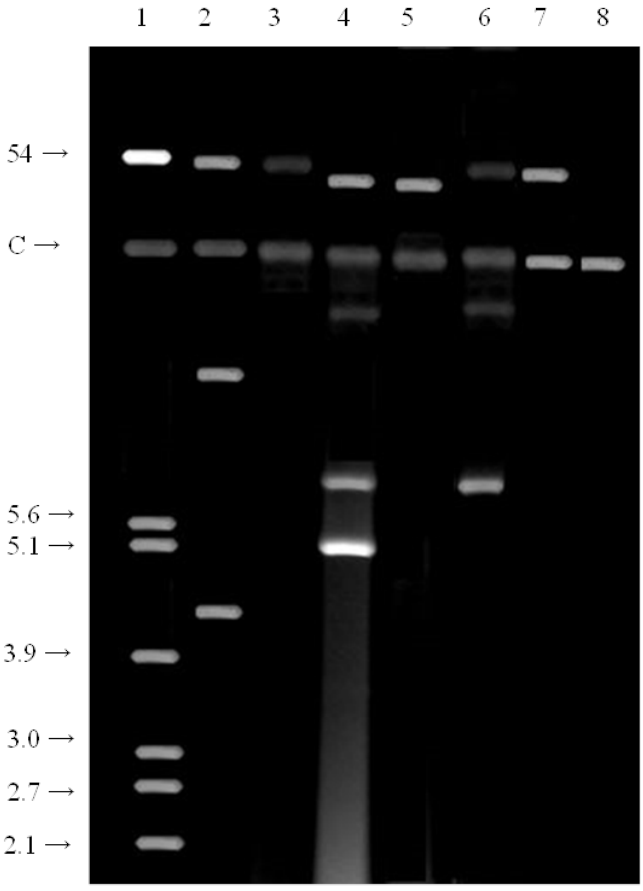

Figure 1. Example of Plasmid profiles of bla_{KPC-2}- carbapenem-resistant *K. pneumoniae* clinical strains and their respective transconiugants Lane 1: molecular size markers prepared from *E. coli* V517 (54, 5.6, 5.1, 3.9, 3.0, 2.7 and 2.1 kb), lanes 2, 4, 6: parental strains, lane 3, 5, 7: transconiugants, 8: *E. coli* J53-2 recipient strain. C, chromosomal.

The transconjugants has shown of reduced susceptibility to all beta-lactams, cephalosporins and carbapenems tested, while remained susceptible to sulfamethoxazole/trimethoprim, aminoglycosides and tigecycline, indicating that KPC-plasmids did not contain additional resistance. In most incidents of isolation of KPC-producing *K. pneumoniae* in European countries, a link could be established to countries where occurrence of KPC had been reported previously. Naas et al. reported that the KPC-2 identified in France likely resulted from an intercontinental transfer of the KPC producer from the United States (24), whereas at least one of the two recently described *K. pneumoniae* isolates with KPC-3 carbapenemase detected in the United Kingdom was probably imported into the United Kingdom from Israel (25). Two KPC-2-producing *K. pneumoniae* isolates were exported from Greece; one to France (24) and the other to Sweden (26). The dissemination of KPC-producing

organisms is no longer restricted to the United States and has emerged as a global concern, aided by the recently described mobile genetic element Tn 4401, which carries the KPC genes (24). Carbapenemase-producing pathogens cause infections that are difficult to treat and have high mortality rates due to their appearance in multidrug-resistant pathogens such as *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter spp* and *E. coli* (27).

4. Conclusions

The dissemination of multidrug resistance plasmids among Gram-negative bacteria is one of the major factors contributing to the spread of antimicrobial resistance (28, 29).

The rapid spread of bla_{KPC} genes can be attributed in part to their mobility and presence on plasmids or transposons (30, 31, 32), in fact the bla_{KPC} genes have usually been identified in large plasmids. Therefore, the horizontal transmission of bla_{KPC} genes highly contributes to the dissemination of strains resistant to several classes of antibiotics leaving a few therapeutic choices.

The ease of horizontal transmission of carbapenem resistance observed in this study has serious public health and epidemiological implications. Additionally, transmission of bla_{KPC} to *Enterobacteriaceae* of reduced or no virulence could lead to the establishment of a reservoir of carbapenem resistance genes in patients and/or in the environment. In conclusion, this report offers of an insight into the current prevalence and molecular types of KPC-producing organisms in Italian hospital, contributing to a better understanding of the epidemiology of these enzymes at local level. A coordinated approach by clinicians and microbiologists in hospital settings is required for their laboratory detection, continuous monitoring of their genetic environment and formulation of proper antimicrobial policy to prevent or at least slow down the horizontal transmission of this drug resistant determinant. Our results provide important information on the genetic environment of bla_{KPC-2} genes identified in clinical isolates in Italy and the significance bla_{KPC-2} in this genetic context warrants further study.

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