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Full Length Research Paper

Genetic similarity and antimicrobial susceptibility of *Klebsiella pneumoniae* – producing carbapenemase (KPC-2) isolated in different clinical specimens received from University Hospitals in Northeastern Poland

Paweł T. Sacha[1]*, Dominika Ojdana[1], Piotr Wieczorek[1], Wioletta Kłosowska[2], Małgorzata Krawczyk[2], Sławomir Czaban[3], Elżbieta Ołdak[4] and Elżbieta Tryniszewska[1, 2]

[1]Department of Microbiological Diagnostics and Infectious Immunology, Medical University of Bialystok, Waszyngtona Str. 15A,15-269 Bialystok, Poland.
[2]Department of Microbiological Diagnostics and Infectious Immunology, University Hospital of Bialystok, Poland.
[3]Department of Anesthesiology and Intensive Therapy, Medical University of Bialystok, Poland.
[4]Department of Pediatric Infectious Diseases, Medical University of Bialystok, Poland.

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Worldwide scientific data show that there is an increasing level of resistance to carbapenems among *Enterobacteriaceae* rods. We examined the genetic similarity and antimicrobial susceptibility of four KPC-2-positive *Klebsiella pneumoniae* (with gene *bla*$_{KPC}$) which were isolated (for the first time) in two University Hospitals in northeastern Poland. Isolates were obtained from March 2011 to May 2011. The minimum inhibitory concentrations (MICs) of thirteen antibiotics were determined for each isolate. Three of *K. pneumoniae* isolates (USK1/1, USK2/1 and USK4/1) were intermediate to imipenem (MICs 4 mg/L), and only one isolate (DSK1/1) was resistant to imipenem (MIC >32 mg/L). In addition they were intermediate or susceptible to meropenem (MICs 1-8 mg/L) and doripenem (MICs 2-4 mg/L). Genotyping and dendrogram analysis of four KPC-producing isolates were performed using the repetitive sequence-based polymerase chain reaction (rep-PCR) in the DiversiLab™ Microbial Typing System. Two rep-PCR clusters (A and B) were determined in University Hospitals in the same city (Bialystok, Poland). Among KPC-2-producing isolates, three isolates (USK1/1, USK2/1, USK4/1) with a similarity of >98% belonged to cluster A, and one (DSK1/1) was dissimilar (<95%) to all the other isolates.

Key words: *Klebsiella pneumoniae*, KPC-2, genetic similarity, antimicrobial susceptibility, northeastern Poland.

INTRODUCTION

Resistance to third-generation cephalosporins and carbapenems is a major health concern, with fears expressed that we will shortly run out of these antibiotics among *Enterobacteriaceae* and non-fermentative Gram-negative rods (Giske et al., 2009; Nordmann and Poirel, 2002; Pfeifer et al., 2010).

The emergence of *Klebsiella pneumoniae carbapenemase* (KPC) in strains of *Enterobacteriaceae* is attracting significant attention. KPC enzymes efficiently hydrolyze β-lactam antibiotics, conferring various levels of resistance to all β-lactam compounds, including carbapenems (Queenan and Bush, 2007; Sacha et al., 2009).

These enzymes are an international clinical and public health concern. In current surveys, *K. pneumoniae* is the most common pathogen harboring *bla*$_{KPC}$ genes. Additionally, *bla*$_{KPC}$-containing *K. pneumoniae* isolates are becoming endemic in certain hospitals and are

*Corresponding author. E-mail: sachpt@umb.edu.pl.

responsible for increasing numbers of outbreaks in several health-care facilities located in Europe and many countries of the world (Cai et al., 2008; Giakoupi et al., 2009; Hirsch and Tam, 2010; Monteiro et al., 2009; Toth et al., 2010).

KPC was first reported in the United States in 2001 (Yigit et al., 2001). At least eleven different KPC enzymes have been reported in many species of bacteria: http://www.lahey.org/studies/other.asp#table1. The aim of this study was analysis of clonal relatedness, identification of genes encoding KPC enzymes, and susceptibility to antibiotics among *K. pneumoniae* isolates that are KPC-positive, isolated for the first time in two University Hospitals in northeastern Poland.

MATERIALS AND METHODS

Bacterial isolates and susceptibility testing

We analyzed four *K. pneumoniae* KPC-positive isolates detected in specimens received from different departments (neurology, intensive therapy, and pediatric hematology/oncology) of two University Hospitals in Bialystok (Poland). Isolates were detected in nasal swabs, urine, bronchial secretions, and anus swabs. Clinical specimens were obtained from March 2011 to May 2011 at University Hospital (USK, three isolates) and Children's University Hospital (DSK, one isolate). *K. pneumoniae* isolates were identified and tested for susceptibility in an automated VITEK 2 System (bioMérieux, France). Identification was performed using Gram-negative cards (VITEK 2, bioMérieux) and susceptibility test using AST-GN60 (bioMérieux) and/or AST-N93 card (bioMérieux).

Susceptibility was also confirmed for imipenem, meropenem, ertapenem, doripenem, gentamicin, colistine, tigecycline, cefepime, ceftazidime, cefotaxime, aztreonam, piperacillin/tazobactam, and amoxicillin/clavulanic acid using Etest strips (AB bioMérieux) according to the manufacturer's recommendations. Minimum inhibitory concentrations (MICs) were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints available at the web site http://www.eucast.org/clinicalbreakpoints/. *Escherichia coli* ATCC 25922 was used as control for susceptibility testing.

Plasmid DNA isolation

Plasmid DNA extractions of *K. pneumoniae* isolates were performed with the Plasmid Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) for determination of the bla_{KPC} genes

Detection of bla_{KPC} genes was performed with a specific primer pair: forward, PS-KPCf: 5' – ATGGCCGCTGGCTGGCTTTT – 3'; and reverse, PS-KPCr: 5' – CGGCCTCGCTGTGCTTGTTCA – 3; the primer pair was used to amplify a 785 bp fragment of gene encoding the KPC enzymes. PCR amplification was performed with the LabCycler Gradient (SensoQuest, GmbH, Germany) thermal cycler. The cycling parameters of amplification were as follows: initial denaturation at 94°C for 5 min; denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 60 s repeated for 35 cycles. The final elongation step was at 72°C for 10 min.

Detection of bla_{KPC} genes

Products of PCR were analyzed in Mini-Sub Cell® GT (BIO-RAD, USA) by electrophoresis at 5 V/cm for 60 min in 1.5% agarose gel (Basica LE GQT Agarose, PRONA Marine Research Institute, Spain) containing 0.5 µg/ml ethidium bromide (MP Biomedicals, Poland) in TBE buffer and photographed using a ChemiDoc™ XR System (BIO-RAD, USA). The sizes of the fragments were calculated from their positions relative to the positions of the molecular weight marker and control isolates. *K. pneumoniae* ATCC BAA-1705 (positive bla_{KPC}) and *K. pneumoniae* ATCC BAA-1705 (negative) were used as control isolates in PCR.

Sequencing of PCR product

Amplification products were purified using Clean Up Kit (A&A Biotechnology, Poland). Sequencing was performed by Big Dye Terminators V.1.1 (Applied Biosystems, Foster City, CA, USA), and products were migrated with an automated Genetic Analyzer (Applied Biosystems® 3500). Sequences were aligned and compared using NCBI BLAST program (National Center for Biotechnology Information database: http://www.ncbi.nlm.nih.gov/).

Genotyping analysis using rep-PCR fingerprinting

DNA was extracted from 10 µl loopful of a *K. pneumoniae* colony using an UltraClean™ Microbial DNA isolation kit (Mo Bio Laboratories Inc., Solana Beach, CA, USA) following the manufacturer's instructions. The purity and concentrations of the DNA preparations were measured using a NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA). Fifty nanograms of DNA were amplified using the DiversiLab® Klebsiella Kit (bioMérieux), which contains the rep-PCR primers, 2.5 U AmpliTaq® DNA polymerase, and 10X PCR buffer (Applied Biosystems). PCR was performed on a LabCycler Gradient (SensoQuest, Biomedical Electronics GmbH, Germany). Analyses of the PCR amplicons were performed using a 2100 Bioanalyzer (Agilent Technologies, Germany). This procedure uses a microfluidics chip (LabChip device; Caliper Technologies Inc, Hopkinton, MA, USA) that separates DNA fragments of different sizes resulting in chromatograms with peaks for each amplicon. Analysis was performed with DiversiLab software version 3.4 and using the Pearson correlation coefficient to calculate similarity among all possible pairs of sample fingerprints. The unweighted pair group method of averages (UPGMA) was used to automatically compare the rep-PCR profiles and to create the corresponding dendrograms (Healy et al., 2005). The isolates were considered indistinguishable (no difference in bands on visual inspection, similarity of DNA fragment pattern was 99% or greater), related (one to two bands of difference, similarity was 95 to 98.9%), or distinct (three or more bands of difference, similarity was less than 95%), as recommended by the manufacturer.

RESULTS

Antimicrobial susceptibility

All of the isolates studied were resistant to cefepime (MICs 16-32 mg/L), ceftazidime (MICs 32-128 mg/L), cefotaxime (MICs 16-128 mg/L), piperacillin/tazobactam (MICs >256 mg/L), amoxicillin/clavulanate (MICs >256 mg/L), and aztreonam (MICs >256 mg/L) and ertapenem (MICs 8-16 mg/L). Whilst three *K. pneumoniae* isolates

Table 1. Resistance phenotype of four KPC-2-producing *K. pneumoniae* isolates.

Antibiotics	Neurology[a]				Intensive Therapy Unit A[a]		Pediatric Hematology/ Oncology[b]	
	USK 1/1[c]		USK 4/1[c]		USK 2/1[c]		DSK 1/1[c]	
	MIC	Result[d]	MIC	Result[d]	MIC	Result[d]	MIC	Result[d]
Imipenem	4	I	4	I	4	I	>32	R
Meropenem	4	I	2	S	1	S	8	I
Ertapenem	16	R	8	R	8	R	16	R
Doripenem	2	I	2	S	2	I	4	I
Gentamicin	2	S	1	S	2	S	2	S
Colistine	1	S	0.5	S	1	S	1	S
Tigecycline	1	S	1	S	1	S	1	S
Cefepime	16	R	32	R	32	R	32	R
Ceftazidime	64	R	128	R	128	R	32	R
Cefotaxime	32	R	16	R	32	R	128	R
Aztreonam	>256	R	>256	R	>256	R	>256	R
Piperacillin/tazobactam	>256	R	>256	R	>256	R	>256	R
Amoxicillin/clavulanate	>256	R	>256	R	>256	R	>256	R

[a] – University Hospital of Bialystok (USK); [b] – Children's University Hospital of Bialystok (DSK); [c] – Specimen source (USK 1/1, urine; USK 2/1, nasal swab; USK 4/1, bronchial secretion; DSK 1/1, anus swab), [d] – R, resistant; S, susceptible; I, intermediate; MIC, minimal inhibitory concentration; mg/L.

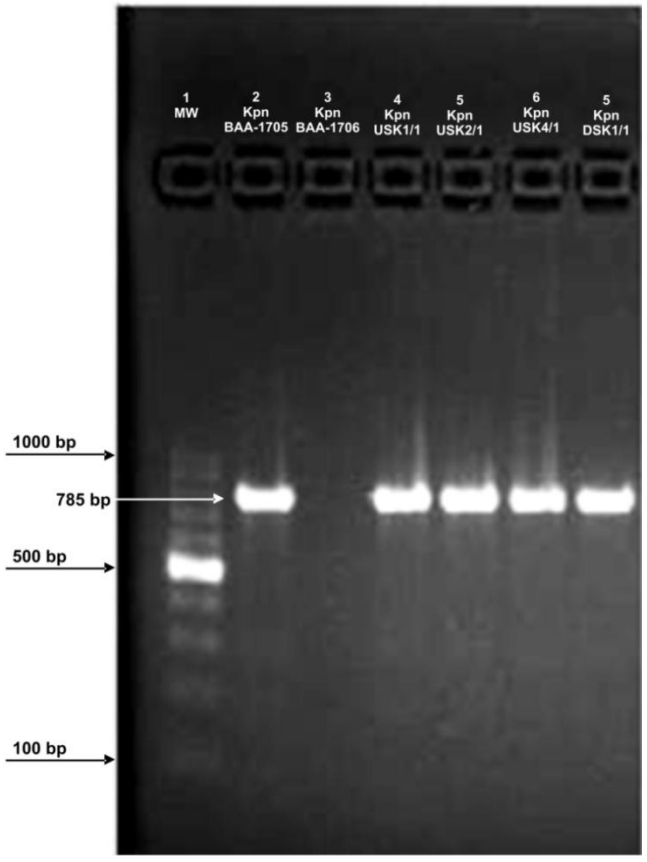

Figure 1. PCR detection of *bla*$_{KPC}$ genes (785bp) in *K. pneumoniae* isolates. Lane 1; MW, DNA molecular size marker (M100-1000 bp, DNA-Gdansk II, Gdansk, Poland); lane 2, Kpn BAA-1705 (isolate, positive control); 3, Kpn BAA -1706 (isolate, negative control); 4 to 6, USK1/1, USK2/1, USK 4/1, isolates from University Hospital; 7, DSK1/1, isolate from Children's University Hospital.

(USK1/1, USK2/1, and USK4/1) were intermediate to imipenem (MICs 4 mg/L), only one isolate (DSK1/1) from University Children's Hospital was resistant to imipenem (MIC >32 mg/L). In addition they were intermediate or susceptible to meropenem (MICs 1-8 mg/L) and doripenem (MICs 2-4 mg/L). For the other antimicrobial agents (gentamicin, colistine, and tigecycline), all of the studied isolates showed susceptibility. Results of antibiotic susceptibility testing for the isolates are summarized in Table 1.

Molecular analysis of KPC-positive isolates

All four isolates were checked for the presence of carbapenemase-encoding genes using PCR methods (Figure 1) described above. All of them were positive for the *bla*$_{KPC-2}$ gene. Rep-PCR distinguishes two different clusters of isolates: A and B (Figure 2). Cluster A contains three similar isolates, USK1/1, USK 2/1, and USK4/1 (similarity >98%), from University Hospital; cluster B contains only one isolate (DSK1/1) from Children's University Hospital (similarity <95% to other isolates) which was dissimilar.

DISCUSSION

Since carbapenems are often used as last-resort antibiotics, the high rate of carbapenem resistance is one of the most worrying antibiotic resistance problems in Gram-negative rods (Deshpande et al., 2006; Pfeifer et al., 2010) making treatment of these infections extremely difficult and in some cases impossible. Thus, it is very important to understand rapidly the epidemiology and

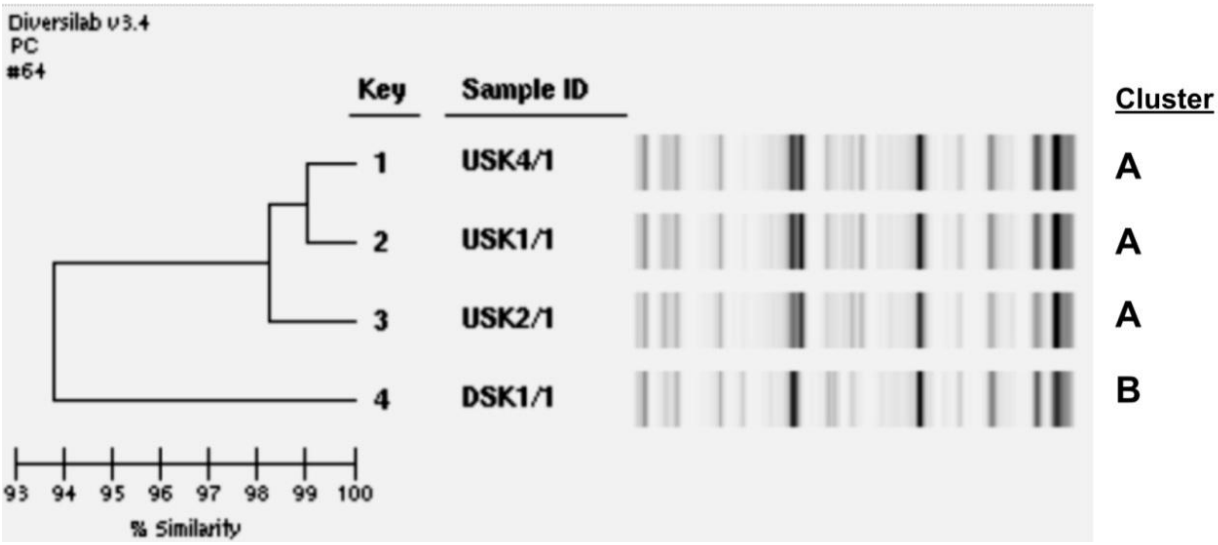

Figure 2. Repetitive sequence-based polymerase chain reaction (rep-PCR) similarity analysis and gel images for four *K. pneumoniae* KPC-positive isolates from two University Hospitals. Isolates USK4/1, USK1/1, and USK2/1 from University Hospital, isolate DSK1/1 from Children's University Hospital.

track in real time the spread of this microorganism (Gupta et al., 2011).

In this study, the DiversiLab system was used to analyze the genetic similarity of four *K. pneumoniae* KPC-2 isolates collected in two University Hospitals over a three-month period. We detected two clusters (A, three isolates; and B, one isolate) among examined KPC-2-positive bacteria. Cluster A was observed to be the dominant isolate group and had spread between different departments (neurology and intensive therapy unit) in one University Hospital. Cluster A isolates were obtained from different specimens. These results indicated differences in the distribution isolates from University Hospital and Children's University Hospital in the same city. Another aim of this study was to determine antimicrobial susceptibility. All isolates were resistant to the majority of tested antibiotics. Only three antibiotics (tigecycline, colistine, and gentamicin) were effective in all tested KPC-positive isolates.

In conclusion, KPC-positive *K. pneumoniae* is an increasing problem in many hospitals in Poland (Baraniak et al., 2009; Zacharczuk et al., 2011), and in other countries near Poland (Hrabák et al., 2011; Richter et al., 2011; Wendt et al., 2010). Many strains are often resistant to all antibiotics except polymyxins and tigecycline. Extensive use of antibiotics is the major driving force leading to the emergence and spread of KPC isolates (Bogdanovich et al., 2011). Despite the low number of isolates, this study is the first report of the presence of KPC-2-type carbapenemases at University Hospitals in northeastern region of Poland. Further investigations are necessary to gain a better understanding of the epidemiology and genetic background of these enzymes.

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