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Note

First clinical cases of NDM-1-producing *Klebsiella pneumoniae* from two hospitals in Bulgaria

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ABSTRACT

We report the first confirmed cases of NDM-1-producing *Klebsiella pneumoniae* infections in two hospitals in Bulgaria. The isolates were diverse in terms of plasmid and co-resistance gene content. *K. pneumoniae* PR2682, causing sepsis in patient with polytrauma due to traffic accident, harbored bla_{NDM-1}, bla_{CMY-4}, $bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1b}, *qnrB*, and *aac(6′)-Ib*. bla_{NDM-1} was transferable by conjugation and located on an IncA/C plasmid of 176-kb, which also carried bla_{CMY-4}, $bla_{CTX-M-15}$, bla_{TEM-1b}, and *qnrB*. *K. pneumoniae* PR2830, causing urinary tract infection in prostate cancer patient, harbored bla_{NDM-1}, bla_{SHV-1}, bla_{TEM-1}, and *aac(6′)-Ib*. bla_{NDM-1} was carried on an 86-kb IncA/C plasmid transferable by conjugation together with bla_{TEM-1}, and *aac(6′)-Ib*. Multilocus sequence typing indicated that the two isolates belonged to sequence type ST11. The emergence of NDM-1-producing *K. pneumoniae* indicates that bla_{NDM-1}-mediated resistance is already disseminated among *Enterobacteriaceae* in Bulgaria. Our results further confirm the role of the Balkans as a secondary reservoir where NDM-encoding genes originate.

Klebsiella pneumoniae exhibiting antibiotic resistance has been notable pathogen in hospital settings for more than four decades [1]. One of the most recently established resistance determinants is the New Delhi Metallo-β-lactamase (NDM), conferring resistance to all β-lactams except aztreonam. Since its first description in 2009 [2], reports continue to emphasize the expanding spread of NDM producers and the increased reliance on therapy with polymyxins or tigecycline [1,3]. Apart from the Indian subcontinent, the Balkan countries are now considered to be a secondary reservoir where NDM-like-encoding genes originate [3].

In Bulgaria, the limited reports on carbapenem resistance in *Enterobacteriaceae* were attributed to KPC-2 and VIM-1-producing *K. pneumoniae* isolates from the same hospital [4], VIM-1-producing *Proteus mirabilis* isolates from two hospitals [5,6], NDM-1- producing *Escherichia coli* isolates recovered from a hospital outbreak [7], and a clinical case of OXA-48-producing *K. pneumoniae* [8]. The described carbapenemase producers were isolated between 2007 and 2014, VIM-1-positive *P. mirabilis* was the first detected [6]. In January and July 2014, two carbapenem-resistant and epidemiologically unrelated clinical isolates of *K. pneumoniae* recovered in two hospitals in Sofia were sent to the National Reference Laboratory for Control and Monitoring of Antibiotic Resistance for confirmation of carbapenemase production. The isolates proved to be NDM producers were genetically different. In this study, we describe the identification and molecular characterization of the first confirmed cases of NDM-producing *K. pneumoniae* infections in two Bulgarian hospitals.

The first patient was a 52-year-old man, who presented in January 2014 to the Emergency Medical Institute "Pirogov" in Sofia with traumatic shock and multiple fractures due to traffic accident. He was transferred to the intensive care unit for continuous

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intensive therapy until stabilization of his general condition. One month after surgical interventions the patient's status deteriorated with septic signs and multiple organ dysfunction. Cultures made from urine, wound specimens, and blood yielded carbapenem-resistant *K. pneumoniae* isolate PR2682 as determined by Vitek-2 (bioMérieux, Marcy l'Etoile, France). During six months of hospitalization, he was treated with continuous antibiotic courses including meropenem, piperacillin/tazobactam, cefoperazone/sulbactam, gentamicin, amikacin, levofloxacin, linezolid, and tigecycline. The patient was discharged in satisfactory general condition with negative culture results.

The second patient was an 81-year-old man with a 5-year history of prostate cancer, who presented to the urology unit of "Tokuda Hospital Sofia" because of difficult and painful urination. He was diagnosed with urinary tract infection due to urethral stricture and admitted for urethrotomy. Urine culture, taken on the day of admission, yielded carbapenem-resistant *K. pneumoniae* isolate PR2830 as identified by Phoenix (BD Diagnostics, Sparks, MD, USA). Treatment was carried out with colistin and cold knife internal urethrotomy. The patient was discharged on hospital day 10 after restoration of micturition with sterile urine culture.

Susceptibility of isolates to antimicrobials was determined by disc diffusion and microdilution methods following Clinical and Laboratory Standards Institute (CLSI) recommendations [9]. Results were interpreted according to CLSI guidelines [9], except for colistin and tigecycline MICs that were interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (http://www.eucast.org/clinical-breakpoints/). Isolates were screened for carbapenemase production using the Carba NP test [10]. In addition, combination disk tests were performed to differentiate between class A, B, and D carbapenemases in accordance with manufacturer's procedure (Rosco Diagnostica, Taastrup, Denmark). Suggestive evidence of Extended-spectrum β-lactamase (ESBL) production was revealed by the double-disk synergy tests [11].

Screening for carbapenemase-encoding genes (bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{SIM}, bla_{GIM}, bla_{SPM}, $bla_{OXA-48-like}$, bla_{GES}, bla_{KPC}), associated ESBL genes (bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{PER}, bla_{VEB}), plasmid-mediated *ampC* genes, and plasmid-mediated quinolone resistance determinants was done by multiplex and simplex polymerase chain reactions (PCRs) as previously described [8,12,13]. Positive PCR products were confirmed on a high-resolution QIAxcel capillary gel electrophoresis system (Qiagen, Hilden, Germany). Amplicons were purified using the Agencourt AMPure XP beads (Beckman Coulter, Fullerton, CA, USA) and sequenced with the GeXP Genetic Analysis System (Beckman Coulter, USA). Sequence analyses and comparison with known sequences were performed with the BLAST programs at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

Transferability of carbapenem resistance was analyzed by conjugation using rifampicin-resistant *E. coli* ML4909 (F^- *galK2 galT22 hsdR metB1 relA supE44* Rif^r) as the recipient [11]. Transconjugants were selected on MacConkey agar plates containing imipenem (0.5 mg/L) or cefoxitin (10 mg/L) and 200 mg/L rifampin (Sigma Chemical Co., St Louis, MO). Plasmids of NDM-producing isolates and their respective transconjugants were analyzed by S1 nuclease digestion of genomic DNA and separation by pulsed-field gel electrophoresis (PFGE). Plasmid incompatibility groups were identified by a PCR-based replicon typing (PBRT) method designed to detect 21 replicons [8]. Relatedness among the isolates was assessed by the BOX-PCR fingerprinting technique as previously described [14]. PCR products were resolved using the QIAxcel capillary gel electrophoresis system (Qiagen, Hilden, Germany) and compared visually. Multilocus sequence typing (MLST) of NDM-producing *K. pneumoniae* isolates was performed according to Protocol 2 described in *K. pneumoniae* MLST database (http://bigsdb.web.pasteur.fr/klebsiella/primers_used.html).

The results of antimicrobial susceptibility testing for PR2682 and PR2830 showed that both isolates were resistant to penicillins, penicillin/inhibitor combinations, cephalosporins, cephamycins, carbapenems, amikacin, tobramycin, kanamycin, netilmicin, quinolones, nitrofurantoin, and tetracycline, and both were susceptible to gentamicin, tigecycline and colistin. Difference in susceptibilities was observed for aztreonam, chloramphenicol, and trimethoprim/sulfamethoxazole, where PR2682 exhibited resistance while PR2830 remained susceptible (Table 1). The Carba NP test was

Table 1
Antimicrobial susceptibility of NDM-1-producing *Klebsiella pneumoniae* clinical strains, their respective transconjugants (Tc), and *Escherichia coli* recipient strain ML4909.

Antimicrobial MIC (μg/ml)[a] for:	*K. pneumoniae*		*E. coli* ML4909			
	PR2682	PR2830	TcPR2682-1	TcPR2682-2	TcPR2830	Parent
Piperacillin/tazobactam	>64	>64	>64	≤8	>64	≤8
Ticarcillin/clavulanic acid	>128	>128	>128	>128	>128	≤16
Cefotaxime	>32	>32	>32	>32	>32	≤1
Ceftazidime	>16	>16	>16	>16	>16	≤1
Cefepime	>16	>16	>16	8	>16	≤2
Aztreonam	>16	≤2	>16	>16	≤2	≤2
Imipenem	>8	8	4	≤1	4	≤1
Meropenem	>8	>8	4	≤1	8	≤1
Ertapenem	>4	>4	4	≤0.25	>4	≤0.25
Doripenem	>2	>2	>2	≤0.12	>2	≤0.12
Gentamicin	≤1	4	≤1	≤1	4	≤1
Tobramycin	>8	>8	≤1	≤1	>8	≤1
Amikacin	16	>32	≤4	≤4	>32	≤4
Ciprofloxacin	>2	>2	≤0.25	≤0.25	≤0.25	≤0.25
Levofloxacin	>8	>8	≤1	≤1	≤1	≤1
Trimethoprim/sulfamethoxazole	>4	≤0.5	>4	>4	≤0.5	≤0.5
Colistin	0.5	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Polymyxin B	1	≤0.25	0.5	0.5	≤0.25	≤0.25
Doxycycline	16	16	4	4	≤2	≤2
Minocycline	8	8	≤2	≤2	≤2	≤2
Tigecycline	1	0.5	≤0.25	≤0.25	≤0.25	≤0.25

MIC, minimum inhibitory concentration.
[a] MIC values were determined using Sensititre® GNX2F MIC plates (Trek Diagnostics Systems, East Grinstead, UK).