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Short communication

First report of New Delhi metallo-β-lactamase-6 (NDM-6) among *Klebsiella pneumoniae* ST147 strains isolated from dialysis patients in Iran

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ABSTRACT

There has been an alarming health-related concern about the growth of New Delhi metallo-β-lactamase. The aims of this study include the phenotypic detection of β-lactamases and molecular characterization of NDM in *Klebsiella pneumoniae* isolates in Tehran, Iran. A total of 120 *K. pneumoniae* isolates were collected from hospitalized haemodialysis patients, Tehran, Iran from March 2014 to February 2017. Antibiotic susceptibility tests were conducted using Kirby-Bauer disc diffusion and Broth Microdilution methods according to Clinical and Laboratory Standards Institute guidelines. Metallo-β-lactamase was detected using the Combined Disc Diffusion Test (CDDT), and production of carbapenemase was screened using the Modified Hodge Test. NDM-producing *K. pneumoniae* strains were screened for the presence of *mcr*-1 gene, β-lactamase genes, and 16S rRNA methylase genes by Polymerase Chain Reaction and sequencing. Molecular typing of the strains was determined using Repetitive Sequence Based-PCR and Multilocus Sequence Typing. The *bla*$_{\text{NDM-6}}$ gene was detected in 3 (2.5%) out of 120 isolates from dialysis patients. Also, the three isolates were positive for *bla*$_{\text{CTX-M-15}}$,*bla*$_{\text{TEM}}$ extended-spectrum β-lactamase genes, *arm*A type plasmid-mediated 16S rRNA methylase and CMY-type plasmid-mediated AmpC β-lactamase.

The isolates were identified as MLST sequence type 147 (ST147). This is the first report of *bla*$_{\text{NDM-6}}$ in *K. pneumoniae* strains, isolated in Iran.

1. Introduction

Hospitalized patients are prevalently subject to infections that result from carbapenem-resistant *Klebsiella pneumoniae*, which is gravely hazardous to their health. Carbapenems as important therapeutic agents are used to treat infections induced by ESBL-producing isolates (Williamson et al., 2012). Different carbapenem-resistance mechanisms have been identified, and the carbapenemase production is an important mechanism in Gram-negative bacteria, particularly those coding for Ambler Class B Metallo-β-Lactamases (MBLs) (Bocanegra-Ibarias et al., 2017). These carbapenemases contain genetic determinants that are widely detected on mobile genetic elements, such as plasmids; in this way, they are easily transferred to other organisms. In 2008, NDM-1 was detected in a *K. pneumoniae* isolate from a Swedish patient that was hospitalized in India. A vast range of β-lactam antibiotics are hydrolyzed by NDM-1 including carbapenems (Bosch et al., 2017). Since 2009, the spread of NDM-positive isolates has been reported worldwide. The *bla*$_{\text{NDM-1}}$ has been detected in *Enterobacteriaceae* and also in *Pseudomonas* and *Acinetobacter* spp. (Rahman et al., 2014). NDM-6 was identified in *Escherichia coli* with substitution of a single amino acid at position 233 (Ala → Val). The NDM-producing bacteria show resistance to almost all available antibiotics except polymyxin E; however, emergence of *mcr*-1 as a resistance element has limited the therapeutic options to this last resort antibiotic (Erfanimanesh and Hashemi, 2016). Different NDM variants were identified and reported around the world. In Iran, the most prevalent variant was NDM-1 (Solgi et al., 2017). So far, the existence of NDM-6-producing *K. pneumoniae* has not been reported in Iran.

The aims of the present study include the phenotypic detection of β-lactamases and molecular characterization of NDM-producing *Klebsiella*

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pneumoniae strains isolated from urine samples of dialysis patients hospitalized in Tehran, Iran during 2014–2017.

2. Materials and methods

2.1. Ethical statement

The present study was acknowledged by the Ethics Committee of Shahid Beheshti University of Medical Sciences with reference number IR.SBMU.SM.REC.1396.38.

2.2. Bacterial isolates

From March 2014 to February 2017, 120 *K. pneumoniae* isolates were recovered from hospitalized haemodialysis patients with positive urine cultures in Tehran Hospitals, Iran. The isolates were detected using biochemical tests such as oxidase test, reaction on TSI culture medium, indole production and motility on SIM medium, reaction in MR-VP medium, growth on Simmon-citrate medium, and urease production on urea agar (Merck Darmstadt, Germany). The isolates were stored at − 80° C in Trypticase soy broth (Merck, Germany) with 20% glycerol.

2.3. Antimicrobial susceptibility testing

The Kirby-Bauer disk diffusion method was employed here to determine antimicrobial susceptibility of *K. pneumoniae* isolates on Mueller Hinton agar (Merck, Germany). Results were interpreted as advised by the Clinical and Laboratory Standards Institute (CLSI), 2016 guidelines (Clinical and Laboratory Standards Institute.Performance standards for antimicrobial susceptibility testing; Twenty-Six informational supplement. Document M100-S26 Wayne). Penicillins [Ampicillin (AMP,10 μg)], Cephems [Ceftazidime (CAZ, 30 μg), Cefotaxime (CTX, 30 μg), Cefpodoxime (CPD, 30 μg), Cefoxitin (FOX), 30 μg], Carbapenems [Imipenem (IPM, 10 μg), Meropenem (MEM, 10 μg), Ertapenem (ETP, 10 μg), Doripenem (DOR, 10 μg)], Aminoglycosides [Gentamicin(GEN,10 μg), Amikacin (AK, 30 μg)], Fluoroquinolones [Ciprofloxacin (CIP, 5 μg), levofloxacin (LVX, 5 μg)], Inhibitors [Trimethoprim-sulfamethoxazole (TS, 1.25/23.75 μg)], Polymyxins [Colistin (CO,10 μg)], and Fosfomycins [Fosfomycin/Trometamol (FOT, 200 μg)], were used. The value of Minimum Inhibitory Concentration (MIC) ranging from 2 to 256 μg/mL for the following antibiotics (Imipenem, Meropenem, Ceftazidime, Ciprofloxacin, and Amikacin) was determined by broth microdilution method following CLSI, 2016 guidelines (Clinical and Laboratory Standards Institute.Performance standards for antimicrobial susceptibility testing; Twenty-Six informational supplement. Document M100-S26 Wayne). European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016) guidelines were used to interpret the MIC results of Colistin. MIC values were measured just for carbapenemase producing strains. The antibiotic powders were purchased from Sigma-Aldrich. *E. coli* ATCC 25922 was used as a quality control strain for disk diffusion and MIC results.

2.4. Screening tests for ESBL and AmpC production

All *K. pneumoniae* isolates were evaluated for production of ESBLs by the phenotypic confirmatory disc diffusion method using ceftazidime (CAZ, 30 μg) and cefotaxime (CTX, 30 μg) with CAZ + clavulanic acid and CTX + clavulanic acid (CA, 10 μg) per disc (Mast Group, Merseyside, UK)(Ghalavand et al., 2018; Taherpour and Hashemi, 2013). Also, AmpC detection was accomplished using cefepime (CPM), cefoxitin (FOX), CAZ and CTX discs (30 μg) alone and in combination with 400 μg of phenylboronic acid (BA) (Sigma-Aldrich) (Mansouri et al., 2014).

2.5. Screening of carbapenemases

By employing the modified Hodge test following (CLSI, 2016) guidelines, the production of Carbapenemase was screened (Clinical and Laboratory Standards Institute.Performance standards for antimicrobial susceptibility testing; Twenty-Six informational supplement. Document M100-S26 Wayne). Combined disk diffusion test were used to detect Metallo-β-Lactamase (MBL) activity as described before (Shariati et al., 2018). *P. aeruginosa* ATCC 27853 and *P. aeruginosa* PA40 (Accession number: KM359725) were used as negative and positive controls for MBL production, respectively.

2.6. Quantitative assay of biofilm production

Biofilm formation was evaluated for NDM-6 producing isolates by the crystal violet staining method as previously described by Stepanović S et al. Briefly, overnight culture of the isolates adjusted to the turbidity of 1.0 McFarland standard. After dilution 1:100 with Luria Broth (LB) (Merck, Germany) the suspension was transferred to a flat-bottomed 96-well microtitre plate (SPL, Korea). Following 24 h incubation at 37 °C and washing with sterile phosphate buffered saline adherent biofilms were fixed with 95% methanol and stained with 2% (*w/v*) crystal violet. Biofilms were destained by treatment with 250 mL 33% glacial acetic acid for 20 min and the OD was read at 492 nm (OD492). Grouping of isolates was carried out according to the following criteria: strong biofilm producer (4 x ODc < OD), moderate biofilm producer (2 x ODc < OD _ 4 xODc), weak biofilm producer (ODc < OD _2 x ODc), and no biofilm producer (OD _ ODc).

2.7. Polymerase Chain Reaction (PCR) amplification and DNA sequencing

DNA was extracted using the DNA extraction kit (GeNet Bio Company, Daejeon, Korea; Cat. No, K-3000) according to the manufacturer's instruction. PCR analysis for bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{GIM}, and bla_{SPM} MBL genes, $bla_{\mathrm{CTX\text{-}M}}$, bla_{SHV}, and bla_{TEM} ESBL genes was accomplished using specific primers and followed by DNA sequencing. (Heffernan et al., 2009; Williamson et al., 2012). In addition, for plasmid-mediated AmpC β-lactamase and aminoglycoside resistance-conferring 16S rRNA methylase genes, PCR and sequencing were carried out as detailed earlier (Heffernan et al., 2009). Also, PCR was conducted to detect *mcr*-1 and bla_{KPC} genes (Erfanimanesh and Hashemi, 2016). Positive and negative controls were used for bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{SIM}, bla_{GIM}, bla_{KPC}, *mcr-1*, plasmid-mediated AmpC β-lactamase and aminoglycoside resistance-conferring 16S rRNA methylase genes. The PCR purification kit (Bioneer Co., Korea) was applied for purification of PCR products. The Macrogen Company (Korea) conducted the DNA sequencing on the purified PCR products. The FinchTV software was utilized to analyse the nucleotide sequences, and the NCBI basic local alignment search tool was employed to compare them with the sequences in GenBank (www.ncbi.nlm.nih.gov/BLAST).

2.8. Plasmid analysis

Plasmid DNA of NDM-6 producing *K. pneumoniae* isolates was extracted by Roche kit (Cat. No. 11754777001) according to the manufacturer's protocol. Electroporation was used to first transform Plasmids encoding bla_{NDM} into *E. coli* TOP10. The NDM-producing transformants on Luria-Bertani agar (LB agar) supplemented with meropenem (0.5 mg/L) or ceftazidime (4 mg/L) were chosen. Of note, PCR and sequencing are indeed necessary to perform to confirm (Williamson et al., 2012).

2.9. Nucleotide sequence accession numbers

GenBank nucleotide accession numbers of MF624840, MF624841

Table 1
Antimicrobial resistance profile of three NDM-6-producing *K. pneumoniae* isolates from three patients in Tehran, Iran.

Strains	Minimum inhibitory concentration (μg/mL) for antibiotics						Phenotypic tests for carbapenemase	
	IMP	MEM	CAZ	CIP	CO	AK	MHT	CDDT
K4	64	32	≥256	128	0.5	128	+	+
K11	128	64	≥256	128	0.5	256	+	+
K22	64	64	≥256	128	0.5	128	+	+

IMP, imipenem; MEM, meropenem; CAZ, ceftazidime; CIP, ciprofloxacin; CO, colistin; AK, amikacin; MHT, Modified Hodge Test; CDDT, Combined Disk Diffusion Test.

and MH205172 has been assigned to the nucleotide sequence.

2.10. Repetitive-Sequence-Based PCR (Rep-PCR) typing method

According to Versalovic et al., the single primers BoxA1R (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) were utilized (Adamek et al., 2011).

2.11. Molecular typing

Multilocus sequence typing (MLST) was performed on NDM-6-producing *K. pneumoniae* isolates using seven housekeeping genes (*gap*A, *inf*B, *mdh, pgi*, *pho*E, *rpo*B, and *ton*B). Seven gene fragments of the *K. pneumoniae* strains were amplified by PCR and sequencing. The allele sequences and sequence types (STs) were determined by the Institute Pasteur *Klebsiella* MLST database (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

3. Results

The resistance rate of *K. pneumoniae* isolates to the tested antibiotics was 87 (72.5%) to cefotaxime, 65(54.2%) to ceftazidime, 12(10%) to ertapenem, 12(10%) to doripenem, 12(10%) to meropenem, 12(10%) to imipenem, 62(51.6%) to gentamicin, 15(12.5%) to amikacin, 90(75%) to ciprofloxacin, 112(93.4%) to cefpodoxime, 120(100%) to ampicillin, 80(66.6%) to cefoxitin, 8(6.6%) to fosfomycin, 95(79.2%) to cotrimoxazole, 40(33.4%) to levofloxacin and 8(6.6%) to colistin. Of the 120 strains, 86 (71.6%) were identified as ESBL producers, 30 (25%) were determined as AmpC β-lactamase producers, and 24 (20%) had both ESBL and AmpC β-lactamase enzymes. The existence of *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{CTX-M}}$ was detected in 35 (40.69%), 46 (53.4%) and 75 (87.20%) ESBL-producing isolates, respectively. In this study, *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{SIM}}$, *bla*$_{\text{GIM}}$,*bla*$_{\text{SPM}}$,*bla*$_{\text{KPC,}}$ and *mcr-1* genes were not detected in isolates. Out of 120 isolates, 12 (10%) isolates were found to be carbapenems-resistant.

Of 12, five were MBL-producing strains and three of them were identified as *K. pneumoniae*, carrying *bla*$_{\text{NDM-6}}$ gene, reported for the first time in Iran. NDM-6-producing isolates recovered from the urine samples (k4 of a 37 year old male), (k11 of a 42 year old female), and (k22 of a 59 year old female). These three isolates (k4, k11, and k22) were greatly resistant to the common antibiotics including carbapenems (imipenem, meropenem, ertapenem, and doripenem), extended-spectrum cephalosporins (cefoxitin, ceftazidime, cefpodoxime, and cefotaxime), aminoglycoside (gentamicin and amikacin), fluoroquinolone (ciprofloxacin), cotrimoxazole; three isolates were affected by fluoroquinolone (levofloxacin), tetracycline (minocycline and tigecycline), fosfomycin, and colistin. Table 1 shows the data for MIC. The three strains of *K. pneumoniae* (k4, k11, and k22) were found positive for carbapenemase in MHT (Table 2). The three isolates were assigned as MBL producers in CDDT (Table 1). The *bla*$_{\text{NDM-6}}$ gene conferring carbapenemase resistance was identified in three isolates. In addition, the three isolates with *bla*$_{\text{NDM-6}}$ gene carried the *bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CMY-2}}$ and *armA* genes (Table 2). In addition, these isolates presented strong biofilm and were clonally related as determined by rep-PCR (Fig. 1) and MLST (ST147). Transformants containing *bla*$_{\text{NDM-6}}$ carrying plasmids were successfully obtained.

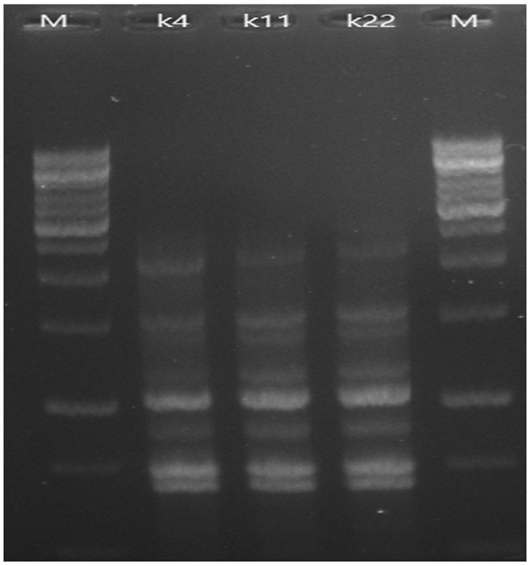

Fig. 1. BOX-PCR of NDM-6-producing *K. pneumoniae* isolates, M; Marker, k4, k11, and k22 positive isolates.

4. Discussion

There has been an increasing number of reports on NDM β-lactamase-producing *K. pneumoniae* from around the world. Currently, 17 NDM (NDM-1 to–17) variants have been found (https://www.lahey.org/Studies/other.asp) in > 40 countries (Principe et al., 2017; Rojas et al., 2017) and several reports have shown the dissemination of such isolates in Europe, Asia, Australia, North and South America, New Zealand, and the Middle East (Savov et al., 2018). To our knowledge, it was reported here for the first time that NDM-6 in *K. pneumoniae* isolated from Iran. To date, NDM-6-producing *E. coli* and *K. pneumoniae* have been reported in New Zealand (ST101) (Williamson et al., 2012) and India (Rahman et al., 2014). The three NDM-6-producing isolates were isolated during a short period of time which makes it a notable concern. In this study, the NDM-6-producing *K. pneumoniae* isolates were sensitive to colistin, levofloxacin, and fosfomycin. However, it is notable that resistance to other antibiotics may cause significant

Table 2
Molecular features related to NDM-6-producing *K. pneumoniae* isolates in Tehran, Iran.

Isolate	Phylogenetic group	MLST	ESBL genes	Plasmid mediated AmpC genes	16S rRNA methylase genes	*mcr*-1	Biofilm	Genebank accession No.
K4	A	ST147	TEM,CTX-M-15	CMY	*armA*	–	++++	MH205172
K11	A	ST147	TEM,CTX-M-15	CMY	*armA*	–	++++	MF624840
K22	A	ST147	TEM,CTX-M-15	CMY	*armA*	–	++++	MF624841

MLST, Multilocus Sequence Typing; ESBL, Extended-Spectrum β-Lactamase.

therapeutic challenges. In this study, all three isolates were highly resistant to aminoglycosides and cephalosporins, an issue that may corresponds with the presence of the *arm*A 16S rRNA methylase and (*bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-15}}$) genes, respectively (Williamson et al., 2012). In this study, the co-existence of genes, encoding the four groups of antibiotic resistance enzymes (carbapenemase, ESBL, AmpC, and 16S rRNA methylase), was detected in NDM-producing isolates, similar to other recent findings (Rahman et al., 2014; Williamson et al., 2012). All three isolates were identified as MLST sequence type 147 (ST147). BOX-PCR results revealed that three NDM-6-producing *K. pneumoniae* strains isolated were clonally related. In this study, Transformants containing *bla*$_{\text{NDM-6}}$ carrying plasmids were successfully obtained. Williamson and et al., showed that transformants containing *bla*NDM-carrying plasmids were successfully obtained from the three *K. pneumoniae* isolate (Williamson et al., 2012). In conclusion, this is the first report on the *bla*$_{\text{NDM-6}}$-producing *K. pneumoniae* ST147 strains isolated from dialysis patients in Iran. Isolates positive for *bla*$_{\text{NDM-6}}$ also harboured genes encoding four other distinct categories of resistance enzymes. The coexistence of NDM-6 with other antibiotic resistance genes may further limit the treatment options and makes infection control procedures more challenging.

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Competing interests

There is no conflict of interest.

Ethical approval

This study was acknowledged by the Ethics Committee of Shahid Beheshti University of Medical Sciences with reference number IR.SBMU.SM.REC.1396.38.

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