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# Detection of NDM-1 producing *Klebsiella pneumoniae* ST15 and ST147 in Iran during 2019–2020

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## ABSTRACT

Carbapenems are employed to treat infections caused by Gram-negative bacteria including *Klebsiella pneumoniae*. This research is aimed to perform phenotypic detection of  $\beta$ -lactamases and molecular characterization of NDM-1 positive *K. pneumoniae* isolates. Another objective is to investigate NDM-1 producing *K. pneumoniae* among children in Iran. From 2019 to 2020, altogether 60 *K. pneumoniae* isolates were acquired from various patients in certain Iranian hospitals. Antimicrobial susceptibility testing was performed by disk diffusion and broth microdilution methods. In addition, mCIM and eCIM were used to confirm the production of carbapenemases and metallo-beta-lactamases (MBLs), respectively. Detection of resistance genes namely,  $bla_{NDM-1}$ ,  $bla_{IMP}$ ,  $bla_{VIM}$ ,  $bla_{KPC}$ ,  $bla_{OXA-48-like}$ ,  $bla_{CTX-M}$ ,  $bla_{SHV}$ ,  $bla_{TEM}$ , and  $mcr-1$  was performed by PCR and confirmed by DNA sequencing. Multilocus sequence typing (MLST) was employed to determine the molecular typing of the strains. According to the findings, the highest rate of carbapenem resistance was detected against doripenem 83.3% (50). Moreover, 31.7% (19) were resistant to colistin. Further to the above, altogether 80% (48) were carbapenemase-producing isolates and among them 46.7% (28) of the isolates were MBL and 33.3% (20) isolates were serine  $\beta$ -lactamase producer. According to the PCR results, 14 isolates produced  $bla_{NDM-1}$ . Remarkably, four  $bla_{NDM-1}$  positive isolates were detected in children. In addition, these isolates were clonally related as determined by MLST (ST147, ST15). Altogether ten  $bla_{NDM-1}$  positive isolates were ST147 and four  $bla_{NDM-1}$  positive isolates were ST15. Based on the results, the emergence of NDM-producing *K. pneumoniae* among children is worrying and hence, it is necessary to develop a comprehensive program to control antibiotic resistance in the country.

## KEYWORDS

*K. pneumoniae*, carbapenem-resistance, carbapenemase, colistin,  $bla_{NDM-1}$  gene, metallo- $\beta$ -lactamase genes

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## INTRODUCTION

Nowadays, one of the major concerns of healthcare systems in the 21st century is the growing number of infections caused by antibiotic-resistant bacteria [1]. According to

O'Neill's estimates of antimicrobial resistance, approximately 700,000 people die worldwide each year owing to infections caused by drug-resistant organisms and by 2050, deaths from antibiotic resistance will surpass cancer-induced deaths [2]. Enterobacteriaceae are opportunistic pathogens. The outbreak of Carbapenemase-Producing Enterobacteriaceae (CPE) remains a global threat to public health [3]. In 2017, World Health Organization (WHO) issued a list of pathogens that prioritized Carbapenem-resistant Enterobacteriaceae (CRE), Carbapenem-resistant *Pseudomonas aeruginosa*, and Carbapenem-resistant *Acinetobacter baumannii* (i.e., critical) [4]. *Klebsiella pneumoniae* as a Gram-negative bacteria originates from the Enterobacteriaceae family. *K. pneumoniae* are opportunist pathogens with a definite link to nosocomial infections and therefore, they are a member of the so-called ESKAPE group [5]. Carbapenems (particularly, imipenem and meropenem) are broad-spectrum beta-lactam agents that are applied to treat severe infections caused by multidrug-resistant Enterobacteriaceae [6] and especially Extended-Spectrum Beta-Lactamase (ESBL) producing Enterobacteriaceae [7]. Among the various agents exhibiting resistance to carbapenems, production of enzymes that degrade carbapenems deserves closer attention [8]. Metallo- $\beta$ -Lactamases (MBLs) belonging to the Ambler class B (Verona imipenemase [VIM], imipenemase [IMP], and New Delhi Metallo- $\beta$ -lactamase [NDM]) have been reported in several countries as a reservoir of various nosocomial outbreaks [9]. The first NDM was detected in an Extensively Drug-Resistant (XDR) *K. pneumoniae* clinical isolate recovered from a urinary specimen of a Swedish patient previously hospitalized in India in 2008 [7]. Multiple antibiotic treatments have not had a significant effect on *bla*<sub>NDM-1</sub>-carrying bacteria, which is unsatisfactory. Thus, bacterial strains that carry *bla*<sub>NDM-1</sub> are the so called superbugs. Superbugs can seriously challenge antibiotic therapy [10]. NDM-producing bacteria are resistant to nearly all accessible antibiotics except polymyxin E, but *mcr-1* emerging as an element of resistance limits the number of available therapeutic options to this last resort antibiotic [11]. The *bla*<sub>NDM-1</sub>-resistance gene encodes the NDM-1 enzyme and is traced in mobile genetic structures, mainly plasmids, which can facilitate *bla*<sub>NDM-1</sub> dissemination [12]. *bla*<sub>NDM-1</sub> plasmids are isolated from various bacterial species, demonstrating the significant role of plasmids in spreading *bla*<sub>NDM-1</sub> [10]. So far, more than 40 countries have reported NDM-1 enzyme-producing pathogens [13]. In this regard, the Asian continent, mainly China and India, remains the biggest reservoir of NDM production with ~58.15% abundance of the NDM-1 variant [12]. Since its first recognition in 2008, NDM-1 has now 29 (NDM 1–29) variants in different bacterial species, most of which are nosocomial pathogens [14]. Due to such capacities as pilgrimage, tourism, and trade, Iran and neighboring countries including Iraq, Turkey, Afghanistan, Pakistan, and the Persian Gulf countries remain the hub of many commutes and trips. This is the reason why transmission and spread of resistant strains (e.g., CPE) among these countries pose high risks [15, 16]. In light of the above concerns, the major objective of this study is to

investigate the prevalence of NDM-1-producing *K. pneumoniae* in Iran among adults and children.

## MATERIALS AND METHODS

### Ethical approval

This research was ethically endorsed by the Ethics Committee of Shahid Beheshti University of Medical Sciences with reference number IR.SBMU.MSP.REC.1398.783. Note that the anonymity of clinical isolates and personal data was ensured before analysis and no human or animal trials were conducted.

### Bacterial strains

This study was carried out at the Microbiology Laboratory of Shahid Beheshti University of Medical Sciences (Tehran, Iran). In total, 60 non-repetitive clinical *K. pneumoniae* isolates were taken from various clinical specimens existing in 9 hospitals in 4 provinces in Iran from 2019 to 2020. Further testing was employed to identify *K. pneumoniae* clinical isolates using biochemical tests such as oxidase test, the reaction on TSI culture medium, indole production and motility on SIM medium, the reaction in MR-VP medium, growth on Simmon-citrate medium, and urease production on urea agar (Merck Darmstadt, Germany) [17]. The isolates were maintained in Tryptic Soy Broth (TSB) (Merck, Germany) with 20% glycerol at  $-70^{\circ}\text{C}$ .

### Antimicrobial Susceptibility Testing (AST)

The Kirby-Bauer disk diffusion technique was employed to perform AST on Mueller-Hinton agar (Merck, Germany) in line with the guidelines proposed by Clinical and Laboratory Standards Institute [CLSI] (2019) [18].

Eleven different antibiotic disks (Mast, Company) were used: carbapenems [imipenem (IMI, 10  $\mu\text{g}$ ), meropenem (MEM, 10  $\mu\text{g}$ ), doripenem (DOR, 10  $\mu\text{g}$ ), ertapenem (ETP, 10  $\mu\text{g}$ )], cephalosporins [ceftazidime (CAZ, 30  $\mu\text{g}$ ), cefotaxime (CTX, 30  $\mu\text{g}$ ), ceftaroline (CPT, 30  $\mu\text{g}$ )], aminoglycosides [gentamicin (GEN, 10  $\mu\text{g}$ ), amikacin (AK, 30  $\mu\text{g}$ )], fluoroquinolones [ciprofloxacin (CIP, 5  $\mu\text{g}$ ), and levofloxacin (LEV, 5  $\mu\text{g}$ )]. Minimum Inhibitory Concentration (MIC) was obtained by the broth microdilution method for imipenem (IMI), meropenem (MEM), ceftazidime (CAZ), cefotaxime (CTX), and colistin(CO) (Sigma-Aldrich); moreover, we interpreted the results in accordance with the guidelines presented by the Clinical and Laboratory Standards Institute [CLSI] (2019) [18]. *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were assumed to be quality control strains for antimicrobial susceptibility testing. The guidelines offered by European Committee on conducting Antimicrobial Susceptibility Testing (EUCAST, 2020) were applied to interpret colistin MIC values [19].

### Screening tests for ESBL

Phenotypic confirmatory disc diffusion test was employed to assess the production likelihood of ESBL. To this end,



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) were used [11]. To identify ESBL-producing isolates, *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were assumed as control strains.

### Screening tests for Carbapenemase and Metallo-β-Lactamase (MBL)

In our study mCIM was used to detect carbapenemase in Enterobacterales, while eCIM and mCIM were employed in conjunction to distinguish metallo-β-lactamases from serine carbapenemase in Enterobacterales. In terms of mCIM, to test each isolate, a sterile 10 µL loopful of bacteria for *K. pneumoniae* from a blood agar plate maintained overnight was emulsified and added to a 2 mL tryptic soy broth (TSB)-containing tube (Merck, Germany). Then, we vortexed bacterial suspension within 10–15 seconds. Next, a 10 µg meropenem disk was added to each tube using sterile forceps. Then, the tube was incubated for 4 h ± 15 min in ambient air at 35 °C ± 2 °C. Before or directly upon finalization of the TSB meropenem disk suspension incubation, we provided a 0.5 McFarland suspension of *E. coli* ATCC® 25922 using the colony suspension method in nutrient broth or saline and a Mueller Hinton Agar (MHA) (Hi-Media, Mumbai, India) was inoculated, which is a plate with *E. coli* ATCC® 25922 as for the routine disk diffusion procedure. The meropenem disk was isolated from TSB-meropenem disk suspension through a 10 µl inoculating loop; it was inserted on the inoculated MHA plate incubated in an inverted position for 18–24 h at 35–2°C in ambient air. In the eCIM test, a second 2 mL TSB tube was labeled for each isolate and 20 µL of 0.5 M EDTA was added to it to determine the final EDTA concentration. The steps above were taken for the mCIM procedure [18].

### Polymerase Chain Reaction (PCR) amplification and DNA sequencing

In compliance with the manufacturer's instruction, the DNA extraction kit (GeNet Bio Company, Daejeon, Korea; Cat. No, K-3000) was employed to extract total DNA. We conducted PCR analysis and sequencing for *bla*<sub>NDM-1</sub>, *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>KPC</sub>, and *bla*<sub>OXA-48</sub>-like genes for carbapenemases as well as for *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>TEM</sub> for ESBL genes. In addition, PCR was applied to identify *mcr-1* genes, as detailed earlier [11]. Positive and negative controls were applied to *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>NDM-1</sub>, *bla*<sub>KPC</sub>, and *mcr-1*. The PCR purification kit (Bioneer Co., Korea) helped to purify PCR products. In this regard, Macrogen Co. (Korea) applied DNA sequencing to purified PCR products. Chromas software (version 1.45) was used to analyze the nucleotide sequences and BLAST in NCBI ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)).

### Molecular typing

Multilocus Sequence Typing (MLST) was done for NDM-1-producing *K. pneumoniae* isolates utilizing such

Table 1. Demographic information and clinical characteristics of patients infected with *K. pneumoniae* (n = 60)

Demographics	Number (%)
Sex	
Male	34 (56.7)
Female	26 (43.3)
Children	14 (23.3)
City	
Tehran	49 (81.7)
Ahwaz	4 (6.7)
Tabriz	3 (5.0)
Bandar Abbas	4 (6.7)
Specimens	
Urine	15 (25.0)
Blood	14 (23.3)
Sputum	24 (40.0)
Wound	2 (3.3)
Trachea	1 (1.7)
Bronchoalveolar lavage	1 (1.7)
Drainage fluid	1 (1.7)
Throat culture	2 (3.3)

housekeeping genes as *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*. These genes were replicated through PCR and sequencing. The sequences were given in the MLST database (<https://bigsd.b.pasteur.fr/klebsiella/klebsiella.html>) to access allele sequences and sequence types (STs) [20].

### Statistical analysis

Phenotypic and molecular tests were analyzed using SPSS software, 26.0 (SPSS Inc., Chicago, IL, USA).

## RESULTS

Out of 247 samples, 60 *K. pneumoniae* isolates were obtained from patients hospitalized in Iranian hospitals. Table 1 presents patients' clinical characteristics and demographic information. According to the CLSI 2019 interpretive protocol, the obtained antibiotic susceptibility testing and MIC results are given in Table 2. According to results, most *K. pneumoniae* clinical strains had substantial resistance to many classes of both β-lactam and non-β-lactam antibiotics. The results showed that among carbapenem antibiotics, the highest rate of resistance was assessed to doripenem 83.3% (50), followed by meropenem and ertapenem 78.3% (47) and imipenem 75% (45). Levofloxacin and amikacin were the most effective antibiotics with resistance rates of 68.3% (41) and 73.3% (44), respectively. Moreover, according to MIC results, a much lower level of resistance to colistin was observed at 31.7 % (19) in this study (Table 2).

Of the 60 strains, 76.6% (46) were ESBL producers. Of the total CRKP tested, 80% (48) reportedly produced carbapenemase through the mCIM test and according to the eCIM test, 46.7% (28) and 33.3% (20) of the isolates produced MBL and serine β-lactamase, respectively. In line with



Table 2. Antibiotic susceptibility of the *K. pneumoniae* clinical isolates ( $n = 60$ )

Antibiotics	Disc diffusion $n$ (%)			MIC $n$ (%)			
	S	I	R	S	I	R	R
Meropenem	11(18.3)	2(3.3)	47(78.3)	9(15.0)	2(3.3)	49(81.7)	
Imipenem	12(20.0)	3(5.0)	45(75.0)	10(16.7)	3(5.0)	47(78.3)	
Doripenem	3(5.0)	7(11.7)	50(83.3)	–	–	–	
Ertapenem	12(20.0)	1(1.7)	47(78.3)	–	–	–	
Ceftazidime	9(15.0)	4(6.7)	47(78.3)	12(20.0)	2(3.3)	46(76.7)	
Cefotaxime	–	1(1.7)	59(98.3)	–	–	60(100.0)	
Ceftaroline	8(13.3)	2(3.3)	50(83.3)	–	–	–	
Gentamicin	13(21.7)	1(1.7)	46(76.7)	–	–	–	
Amikacin	11(18.3)	5(8.3)	44(73.3)	–	–	–	
Ciprofloxacin	4(6.7)	5(8.3)	51(85.0)	–	–	–	
Levofloxacin	10(16.7)	9(15.0)	41(68.3)	–	–	–	
Colistin	–	–	–	41(68.3)	–	19(31.7)	

Table 3. An overview of molecular features related to NDM-1-producing *K. pneumoniae* isolates in Iran

Isolate	From adults/children	ESBL genes	Colistin	<i>mcr-1</i>	City
K2	Adults	CTX-M, SHV	S	–	Ahvaz
K3	Adults	CTX-M, SHV	R	–	Ahvaz
K5	Adults	CTX-M, SHV	R	–	Ahvaz
K11	Adults	CTX-M, SHV	S	–	Tabriz
K12	Adults	TEM, CTX-M, SHV	R	–	Tabriz
K13	Adults	TEM, CTX-M, SHV	S	–	Tabriz
K33	Adults	TEM, CTX-M, SHV	R	–	Tehran
K43	Adults	TEM, CTX-M	S	–	Tehran
K45	Adults	TEM, CTX-M	S	–	Tehran
K46	Adults	TEM, CTX-M, SHV	S	–	Tehran
K57	Children	TEM, CTX-M	S	–	Tehran
K58	Children	TEM, CTX-M	R	–	Tehran
K59	Children	TEM, CTX-M	R	–	Tehran
K60	Children	TEM, CTX-M	S	–	Tehran

the PCR results, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>CTX-M</sub> were detected in 45% (27), 58.3% (35), and 63.3% (38) of the ESBL-producing isolates, respectively. Of note, this study did not find *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>KPC</sub>, and *mcr-1* genes in the isolates. Among the isolates resistant to carbapenem antibiotics, 28 (46.7%) were MBL-producing ones and 14 (23.3%) of them were recognized as *K. pneumoniae*, carrying *bla*<sub>NDM-1</sub> gene. Fourteen NDM-1-positive isolates were identified in three cities of Tehran, Ahvaz, and Tabriz. Among the NDM-1-positive isolates in Tehran, four isolates from hospitalized children were identified. As shown in Table 3, it is noteworthy that out of 14 NDM-1-positive isolates, six isolates were resistant to colistin and out of four NDM-1-positive isolates for children, two isolates were resistant to colistin. In addition, these isolates were clonally related as determined by MLST (ST147, ST15). Altogether ten positive *bla*<sub>NDM-1</sub> isolates were ST147 and four positive *bla*<sub>NDM-1</sub> isolates were ST15.

## DISCUSSION

This is the first study on the *bla*<sub>NDM-1</sub>-resistant gene prevalence rate in different cities of Iran and it is revealed that

the gene has spread to different regions of the country, from which a number of positive cases have been observed among children. Carbapenems are the proposed drug for treating those severe infections that have been induced by Gram-negative bacteria, including *K. pneumoniae*, which have become a more challenging treatment due to the growing resistance to carbapenems [21, 22]. Among the MBL genes, NDM-1 confers resistance to beta-lactam antibiotics. The *bla*<sub>NDM-1</sub>-resistant gene has spread widely through the transmission of plasmids and other mobile genetic elements [23]. Previous studies have reported the primary reservoir of *bla*<sub>NDM-1</sub> producers in the Indian subcontinent, the Balkans regions, and the Middle East. It was first recognized in 2008 in a Swedish patient hospitalized in India [7, 16]. Thanks to routine international trips among Iraq, Turkey, Pakistan, and Iran, strains of *K. pneumoniae* carrying *bla*<sub>NDM-1</sub> gene have emerged in Iran [15, 16]. Shahcheraghi et al. found the first case of *bla*<sub>NDM-1</sub> gene in Iran in 2012 when studying Enterobacteriaceae family isolates taken from five hospitals in Tehran [24]. The prevalence rate of CRKP strains in different hospitalized patients was 78.7%. In the study of Jalalvand et al. in Tehran in 2019–2020, a prevalence rate of 35% was reported for CRKP [25]. However, in the study of

Solgi et al. in 2015–2016 done in Isfahan, the prevalence rate of CRKP was 41.7% [15]. In comparison with these studies, we reported high resistance to carbapenems. The highest antibiotic resistance against carbapenems and cephalosporins was detected, while the least resistance was seen for levofloxacin and amikacin. Despite high resistance to  $\beta$ -lactams (78.7%), a low rate of resistance to colistin (31.7%) was observed, although this drug is not a common option for children due to its side effects. The most striking observation based on the data was that six isolates from children turned out to be resistant to colistin.

Backed by the AST results, our research revealed that the patients hospitalized in different Iranian hospitals were subject to a high prevalence of carbapenemase-producing *K. pneumoniae*. In this study, according to CLSI 2019, mCIM, and eCIM tests were used to identify carbapenemases and MBL, respectively. Performing and interpreting these tests is quite quick (24 h) and easy, even with limited resources. Previous studies have shown that mCIM and eCIM tests have a sensitivity and specificity rate of 100% and based on CLSI 2019, the eCIM test is a method that can be applied in conjunction with the mCIM to spot MBL-producing Enterobacteriaceae [26]. According to the mCIM test, carbapenemase producer was found in 48 cases (80%). Outcome of the eCIM test revealed that MBL and serine beta-lactamase were found in 28 (46.7%) and 20 (33.3%) cases, respectively. Among 28 (46.7%) MBL-producing isolates, 14 (23.3%) were NDM-1 positive, four of which were isolated from children. Being in agreement with previous reports, the present findings showed that the most common mechanism of CRKP isolates was the production of NDM-1 carbapenemases [15, 25]. According to the prevalence of carbapenem resistance genes, the highest frequency was related to *bla*<sub>NDM-1</sub> gene at 23.3% (14), and *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>KPC</sub>, and *mcr-1* genes were not identified in the isolates. Furthermore, Fazeli and Solgi et al. reported NDM [16, 27]. In agreement with the study of Jalalvand et al., the frequency of genes was OXA-48 in 28.88% of cases, KPC in 2.2% of cases, and NDM in 6.66% of cases. Further to the above, co-produced OXA-48 and NDM in 24.44% of cases and parallel expression of co-produced NDM, OXA-48, and KPC in 24.44% of cases were observed [25]. On the contrary, co-production of NDM, OXA-48, and KPC were not observed in our study. Solgi reported the co-production of OXA-48 and NDM in 35.4% of the cases in Isfahan with no trace of KPC involved [15]. A study in China reported the prevalence rate of 9% for NDM-1 in *K. pneumoniae* in 2020 [3]. Nevertheless, in the Arabian Peninsula, NDM-1 and OXA-48 were detected in 46.5 and 32.5% of cases, respectively, while the co-production of these determinants was observed in 3.5% of total cases [28]. It should be noted that small sample size was the main limitation of this study. Comprehensive epidemiological data on the prevalence of carbapenem-resistance genes (*bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, and *bla*<sub>NDM-1</sub>) in pediatric patients in Iran are limited and so far, no extensive studies have been conducted in this regard in Iran. In accordance with findings of Wang (2020), the frequent switch of predominant carbapenemase genotype occurs

among adults and children for a variety of reasons [29]. Hence, it is of significance to ensure long-term resistance surveillance of CRKP among pediatric patients and adults. Spread of the *bla*<sub>NDM-1</sub> gene in different cities points to the endemicity of the carbapenem-resistant gene in Iran.

Finally, obtained findings are the materials that form new reports concerning the *bla*<sub>NDM-1</sub>-producing *K. pneumoniae* ST147 strains isolated from pediatric patients in Iran. Isolates positive for *bla*<sub>NDM-1</sub> were observed in adults. Among 28 (46.7%) MBL-producing isolates, 14 (23.3%) were NDM-1 positive, four of which were isolated from children. It is now quite clear that distribution of NDM-1 isolates over many provinces of the country among adults and children is the proof of the necessity of implementing a monitoring system and strong programs to control antibiotic resistance in the country so as to prevent the development of a latent endemic situation.

*Conflict of interest:* There is no conflicts of interest.

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