

Endemic dissemination of different sequence types of carbapenem-resistant *Klebsiella pneumoniae* strains harboring bla_{NDM} and *16S rRNA* methylase genes in Kerman hospitals, Iran, from 2015 to 2017

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Introduction: The emergence and spread of *Klebsiella pneumoniae* strains resistant to multiple antimicrobial agents are considered as a serious challenge for nosocomial infections.

Materials and methods: In this study, 175 nonrepetitive clinical isolates of *K. pneumoniae* were collected from hospitalized patients in Kerman, Iran. Extended-spectrum β -lactamases (ESBLs), AmpC, and carbapenemase-producing isolates were recognized by phenotypic methods. The resistance genes including efflux pumps *oqxA/oqxB*, *16S rRNA* methylase, ESBL, AmpC, and carbapenemase were detected by PCR-sequencing method. Molecular typing was performed by enterobacterial repetitive intergenic consensus-PCR and multilocus sequence typing methods among bla_{NDM} -positive isolates.

Results: Thirty-seven (21.14%) isolates along with sequence types (STs): ST43, ST268, ST340, ST392, ST147, and ST16 were harbored bla_{NDM} . ST43 in 2015 and ST268 during 2016–2017 were the most frequent STs among New Delhi metallo-beta-lactamase (NDM)-positive isolates. We found the distribution of some isolates with bla_{NDM} , bla_{CTX-M} , bla_{SHV} , bla_{OXA} , bla_{TEM} , bla_{CMY} , *rmtC*, and *oqxA/oqxB*. Enterobacterial repetitive intergenic consensus-PCR represented seven clusters (A–G) plus four singletons among NDM-positive isolates. This study provides the first report of bla_{NDM-1} -positive *K. pneumoniae* along with ST268 as well as the spread of nosocomial infections with six different STs harboring bla_{NDM-1} and other resistance genes in hospital settings especially neonatal intensive care unit.

Conclusion: The dissemination of various clones of NDM-producing *K. pneumoniae* can contribute to increase the rate of their spread in health care settings. Therefore, molecular typing and detection of resistance genes have an important role in preventing and controlling infection by limiting the dissemination of multidrug-resistant isolates.

Keywords: bla_{NDM} , *16S rRNA* methylase, MLST, ERIC-PCR

Introduction

Infections caused by multidrug-resistant bacteria have declared a substantial threat to public health worldwide.¹ Carbapenems are the most important antibiotics used for the treatment of infections caused by extended-spectrum β -lactamases (ESBLs) and AmpC-producing Gram-negative bacteria.² Several mechanisms including the loss of outer membrane proteins and carbapenemase such as KPC, GES, VIM, IMP, GIM, New Delhi metallo-beta-lactamase (NDM), and OXA-types are involved in resistance to carbapenems in Enterobacteriaceae.³ Carbapenemase-producing bacteria usually cause life-threatening infections and long-time hospitalization in health care settings.¹ For the first time, the NDM has been identified in carbapenem-resistant *Klebsiella*

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<http://dx.doi.org/10.2147/IDR.S186994>

Infection and Drug Resistance 2019:12 45–54



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pneumoniae in Sweden and then has been reported in other Gram-negative bacteria.⁴⁻⁷ In Iran, the first NDM-producing *K. pneumoniae* was identified in March 2011 from Tehran.¹ NDM-producing *K. pneumoniae* are broadly considered as multidrug-resistant bacteria that have been commonly associated with additional resistance mechanisms such as AmpC, ESBLs, and methylation of 16S rRNA by *armA*, *rmtA*, *rmtB*, and *rmtC*.⁸ Several typing methods have been introduced and developed for epidemiological investigation of *K. pneumoniae* including enterobacterial repetitive intergenic consensus amplification (ERIC-PCR) and multilocus sequence typing (MLST).^{9,10} MLST is one of the best molecular typing methods for long-term and global epidemiological investigations, and ERIC-PCR is usually used for local outbreaks over a short period of time.¹⁰ In this study, we investigated the molecular epidemiology from NDM-1-producing clones among carbapenem-resistant *K. pneumoniae* isolates in Kerman hospitals, Iran, and we emphasized on the clonal relatedness of these isolates.

Materials and methods

Bacterial isolates

In this study, 175 nonduplicated isolates of *K. pneumoniae* were collected from hospitalized patients in four referral hospitals (Shafa, Afzalipoor, Bahonar, and Kashani) during February 2015 to November 2017 in Kerman, Iran. All the isolates were identified as *K. pneumoniae* by standard microbiological tests.¹¹

Antibiotic susceptibility testing

Antibacterial susceptibility test of isolates to cefepime (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), ceftizoxime (30 µg), cefpodoxime (10 µg), imipenem (10 µg), meropenem (10 µg), ertapenem (10 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), and norfloxacin (10 µg) (Mast Group Ltd., Bootle, UK) was determined by disk diffusion method on Müller–Hinton agar media (Laboratorios CONDA, Madrid, Spain) according to the Clinical and Laboratory Standards Institute (CLSI).¹² Minimum inhibitory concentration (MIC) of isolates to cefotaxime, cefepime, and imipenem was determined by microbroth dilution method according to CLSI. To determine MIC of colistin and tigecycline by microbroth dilution method, we used the European Committee on Antimicrobial Susceptibility Testing recommendations (<http://www.eucast.org/clinical-breakpoints>). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as standard strains in antibacterial susceptibility testing.

Detection of ESBLs, AmpC, and carbapenemase-producing isolates

ESBLs and carbapenemase-producing isolates were determined according to CLSI recommendations by combination disk with clavulanate and Carba NP test, respectively.¹² AmpC disk test was used to detect AmpC β-lactamase-producing isolates.¹³

Genomic DNA extraction

The genomic DNA was extracted using Exgene Clinic SV (GeneAll Biotechnology, Co., Ltd., Seoul, Republic of Korea; Kat: 106-152) according to the manufacturer's guidelines.

Detection of resistance genes by PCR sequencing

Antibiotic resistance genes including ESBLs (*bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{OXA-1}, and *bla*_{PER}), carbapenemase (*bla*_{KPC}, *bla*_{GES}, *bla*_{OXA-48}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{NDM}, *bla*_{SPM}, *bla*_{SIM}, *bla*_{GIM}, and *bla*_{AIM}), efflux pump (*oxqA/B*), 16S rRNA methylase (*rmtA*, *rmtB*, *rmtC*, and *armA*), and *mcr-1* (colistin resistance gene) were detected by PCR. The primers used for amplification of resistance genes are listed in Table 1. The AmpC β-lactamase genes including *bla*_{CMY}, *bla*_{FOX}, *bla*_{ACC}, *bla*_{ACT}, *bla*_{DHA}, *bla*_{EBC}, and *bla*_{CIT} were detected by using multiplex PCR as previously described, and furthermore, PCR products were confirmed by sequencing (Bioneer Corporation, Daejeon, Republic of Korea).²⁹

MLST of NDM-producing isolates

MLST of isolates was performed using seven conserved housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) according to protocols available at the MLST Pasteur website (<http://bigsdbs.pasteur.fr/klebsiella/klebsiella.html>) for NDM-producing isolates. Products of the above genes in MLST were sequenced by Bioneer, Co. Sequences of each housekeeping gene in both directions were analyzed by Sequence Scanner Software v.2.0 (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA) and assembled by Lasergene 6 software (DNASTAR). The sequence types (STs) of each isolate were determined based on the seven studied loci described at <http://bigsdbs.pasteur.fr/klebsiella/klebsiella.html>.

Molecular typing of *bla*_{NDM}-positive isolates by ERIC-PCR

ERIC-PCR using ERIC2 primer (5'-AAGTAAGT-GACTGGGGTGAGC-3') was used for molecular typing of NDM-positive isolates.³⁰ The results of ERIC-PCR were

Genes	Primer sequence (5'–3')	Annealing temperature (°C)	Product size (bp)	Reference
<i>bla</i> _{CTX-M}	F-ATGTGCAGYACCAGTAARGTKATGGC R-TGGGTRAARTARGTSACCAGAAACAGCGG	58	593	14
<i>bla</i> _{TEM}	F-CTTCCTGTTTTGCTCACC R-AGCAATAAACCCAGCCAGC	54	636	15
<i>bla</i> _{SHV}	F-TCAGCGAAAAACACCTTG R-TCCCGCAGATAAATCACC	51	472	15
<i>bla</i> _{KPC}	F-CGTCTAGTTCTGCTGTCTTG R-CTTGTCATCCTTGTTAGGCG	58	798	16
<i>bla</i> _{OXA-48}	F-GCGTGGTTAAGGATGAACAC R-CATCAAGTTCAACCCAACCG	58	438	16
<i>bla</i> _{VIM}	F-ATGTTAAAAGTTATTAGTAGT R-CTACTCGGCGACTGAGCGAT	53	801	16
<i>bla</i> _{IMP}	F-GGAATAGAGTGGCTTAAYTCTC R-GGTTTAAYAAAAACAACCACC	58	232	16
<i>bla</i> _{NDM}	F-GGTTTGCGCATCTGGTTTTTC R-CGGAATGGCTCATCACGATC	58	621	16
<i>bla</i> _{OXA}	F-GCGTGGTTAAGGATGAACAC R-CATCAAGTTCAACCCAACCG	52	438	17
<i>bla</i> _{AIM}	F-CTGAAGGTGTACGGAACAC R-GTTCGGCCACCTCGAATTG	59	322	18
<i>bla</i> _{PER}	F-GGGACARTCSKATGAATGTCA R-GGYSGCTTAGATAGTGCTGAT	47	926	19
<i>bla</i> _{GIM}	F-TCGACACACCTTGGTCTGAA R-AACTTCCAACCTTGCCATGC	58.5	477	18
<i>bla</i> _{SPM}	F-AAAACTGGGTACGCAAACG R-ACATTATCCGCTGGAACAGG	59	271	20
<i>bla</i> _{SIM}	F-TACAAGGGATTGCGCATCG R-TAATGGCCTGTTCCCATGTG	61	570	20
<i>bla</i> _{GES}	F-ATGCGCTTCATTCACGCAC R-CTATTTGTCCGTGCTCAGG	56	844	21
<i>oqx</i> A	F-CTCGGCGCGATGATGCT R-CCACTCTTCACGGGAGACGA	57	392	22
<i>oqx</i> B	F-TTCTCCCCCGGCGGGAAGTAC F-CTCGGCCATTTTGCGCGGTA	56	512	23
<i>rmt</i> A	F-CTAGCGTCCATCCTTTCCTC R-TTTGCTTCCATGCCCTTGCC	56	635	24
<i>rmt</i> B	F-CCCAAACAGACCGTAGAGGC R-CTCAAACCTCGGCGGGCAAGC	56	584	25
<i>rmt</i> C	F-CGAAGAAGTAACAGCCAAAG R-ATCCCAACATCTCTCCCACT	61	711	26
<i>arm</i> A	F-AGGTTGTTTCCATTTCGAG R-TCTCTTCCATTCCTTCTCC	56	590	27
<i>mcr-I</i>	F-CGGTCAGTCCGTTTGTTT R-CTTGGTCGGTCTGTAGGG	53	309	28

urine 126 (72%), blood 21 (12%), bronchoalveolar lavage 16 (9.1%), and cerebrospinal fluid 3 (1.7%).

Antimicrobial susceptibility testing

The rate of resistance to antibiotics was the following: cefpodoxime 83 (47.4%), cefotaxime 80 (45.7), ceftizoxime 78 (44.6%), ceftazidime 68 (38.9%), cefoxitin 64 (36.6%), cefepime 71 (40.5%), imipenem 45 (25.7%), meropenem 33 (18.9%), ertapenem 30 (17.1%), amikacin 68 (38.9%), genta-

micin 59 (33.7%), norfloxacin 33 (18.9%), and ciprofloxacin 31 (17.7%). The ranges of MIC to imipenem, cefepime, and cefotaxime were 4–128 µg/mL, 16–2,048 µg/mL, and 8–2,048 µg/mL, respectively. MIC to colistin was increased in seven (4%) isolates with range 2–16 µg/mL and among other isolates were ≤0.5 µg/mL. All isolates were sensitive to tigecycline with MIC ≤0.5 µg/mL. The MIC results of the clinical isolates are shown in Table 2.

Phenotypic confirmatory tests

Among the 175 *K. pneumoniae* isolates, 72 (41.1%) strains produced ESBLs, 12 (6.8%) isolates produced AmpC, and 8 (4.5%) isolates produced both ESBLs and AmpC β-lactamase. Out of 175 *K. pneumoniae* isolates, 37 (21.1%) isolates were considered as positive carbapenemases with Carba NP test.

PCR amplification of antibiotic resistance genes

Based on the PCR assays, the prevalence of ESBL genes was as follows: *bla*_{CTX-M} 46.28% (n=81), *bla*_{SHV} 41.1% (n=72), *bla*_{TEM} 38.9% (n=68), and *bla*_{OXA-1} 21.7% (n=38). The only carbapenemase gene found in isolates was *bla*_{NDM-1} 21.14% (n=37). The major AmpC β-lactamase genes found were *bla*_{CMY} 2.85% (n=5), followed by *bla*_{FOX} 1.1% (n=2) and *bla*_{ACC}, *bla*_{ACT} 0.6% (n=1). The efflux pump genes including *oqxA/oqxB* were detected in 36.6% (n=64) and 19.4% (n=34) of isolates. Aminoglycoside-resistant genes (*16S rRNA methylase*) including *rmtC* and *armA* were observed in 5.7% (n=10) and 1.1% (n=2) of isolates, respectively. The rest of the antibiotic resistance genes (*bla*_{EBC}, *bla*_{CIT}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{GIM}, *bla*_{AIM}, *bla*_{SPM}, *bla*_{SIM}, *bla*_{GES}, *bla*_{KPC}, *bla*_{OXA-48}, *bla*_{PER}, *bla*_{DHA}, *rmtA*, *rmtB*, and *mcr-1*) were negative.

Some sequences of the antibiotic resistance genes including *bla*_{NDM}, *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{OXA-1}, *bla*_{SHV}, *armA*, and *rmtC* were submitted to the GenBank under accession numbers MG515599, MG515594, MG515597, MG515600, MG515593, MG515596, and MG515592, respectively.

Molecular typing of NDM-producing isolates

In this study, we described the first NDM-producing *K. pneumoniae* isolates belonging to the ST268 (n=14), which was the major ST. The other STs were as follows: ST43 (n=9), ST340 (n=7), ST392 (n=5), ST147 (n=1), and ST16 (n=1).

According to the eBURST results, ST268 is triple-locus variants of ST16 reporting NDM-producing *K. pneumoniae* previously. In this study, in comparison with other STs, most isolates of *K. pneumoniae* ST268 carrying *rmtC* gene were associated with neonatal intensive care unit (NICU), whereas one of *K. pneumoniae* ST43 isolate coproducing *armA* and *bla*_{NDM} genes was associated with surgical unit (Table 3).

Table 3 showed distribution and genetic characterization of 37 NDM-producing *K. pneumoniae* strains. ERIC-PCR findings showed that the 37 NDM-producing strains were divided into 7 clusters A to G (11 strains in clusters A, 2 strains in clusters B, E, G, 7 strains in cluster C, 5 strains in cluster D, 4 strains in cluster F, and 3 strains were selected to represent sporadic strains) (Figure 1). ST43 was divided into three clusters (A, C, E), ST268 divided into four clusters (C, D, E, F), ST340 divided into four clusters (A, C, F, G), ST392 divided into three clusters (A, B, G), ST16 was subdivided into one cluster, and ST147 showed as one singleton (Figure 1 and Table 3).

Discussion

During the past decades, carbapenem resistance among *K. pneumoniae* is typically caused by the emergence of transmissible carbapenemases, such as *bla*_{KPC} and *bla*_{NDM}.⁵ NDM specially comprises of the most rapidly growing group of metallo-beta-lactamases.⁵ They have been increasingly detected in different countries, suggesting a worldwide dissemination.¹ Here, we reported the distribution of nosocomial infections caused by NDM-producing *K. pneumoniae*, especially in NICU from four referral hospitals in Kerman, Iran.

According to the previous studies, our findings showed that the most effective antibiotics against isolates were colistin and tigecycline.^{31,32} However, most isolates exhib-

Table 2 The MIC of clinical isolates of *Klebsiella pneumoniae* resistance to imipenem, cefotaxime, cefepime, and colistin

Antibiotic agents		MIC level (µg/mL) ^a														
		0.5	1	2	4	8	16	32	64	128	256	512	1,024	2,048	MIC ₅₀	MIC ₉₀
Imipenem (n)	No. of isolates	–	–	–	[10 ^b	17	13	1	2]	2	–	–	–	–	8	32
Cefotaxime (n)		–	–	–	–	[3	1	1	5	5	4	6	7]	48	2,048	2,048
Cefepime (n)		–	–	–	–	–	[4	5	5	2	3	23	27]	2	512	1,024
Colistin (n)		–	–	[3	2]	–	2	–	–	–	–	–	–	–	4	16

Notes: ^aLeft and right brackets indicate the lowest and highest MICs tested, respectively. ^bNo. of isolates.

Abbreviation: MIC, minimum inhibitory concentration.

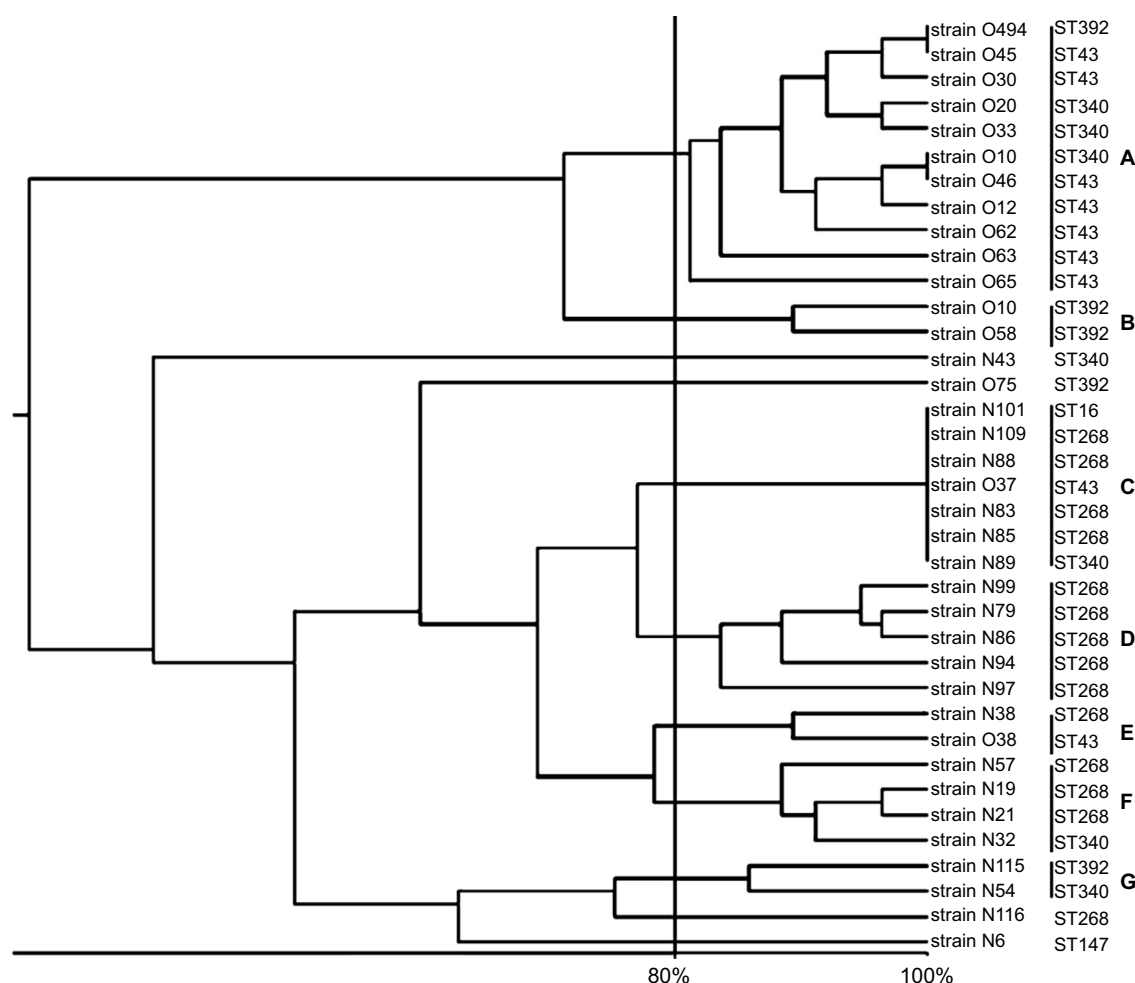


Figure 1 Corresponding dendrogram generated with UPGMA clustering method in clinical *bla_{NDM}*-positive isolates of *Klebsiella pneumoniae*.

Note: ERIC-PCR represented seven clusters (A–G) plus four singletons among NDM-positive isolates.

Abbreviations: ERIC-PCR, enterobacterial repetitive intergenic consensus amplification; NDM, New Delhi metallo-beta-lactamase.

ited a high resistance level to other antimicrobial agents including extended spectrum cephalosporins, carbapenems, quinolones, and aminoglycosides. Similar to our findings, in the study in Egypt, all carbapenem-resistant *K. pneumoniae* isolates were sensitive to colistin and tigecycline.³²

In this study, eleven (17.46%) isolates indicated positive results for AmpC disk test. In our study, non-AmpC-producing isolates might be associated with other resistance mechanisms. Shi et al reported that ceftioxin resistance could be related to the change of cellular permeability to antibiotics, resulting from the loss or deficiency of outer membrane proteins.^{33,34}

In our hospital settings, we found the emergence and establishment of NDM-producing *K. pneumoniae* along with *rmtC* and *armA*. Sporadic dissemination of NDM-1 in Iran was first described in 2013, which was resistant to the majority of antibiotics except for colistin.¹ In the current findings, we detected four clinical isolates being resistant to colistin, although one of them only harbored *bla_{NDM-1}* gene. This study also focused on epidemiological investigation of MLST and

ERIC-PCR in the NDM-positive *K. pneumoniae*. To the best of our knowledge, the obvious report of the ST prevalence has not been yet accounted for NDM-producing *K. pneumoniae* in Iran. However, we reported the prevalence of different STs of NDM-producing *K. pneumoniae* among hospitalized patients during 3 years from March 2015 to November 2017 in Kerman.

According to this study, NDM-producing STs including ST16, ST147, and ST340 were found in India and Korea.^{35,36} On the contrary, ST147 was recently observed in NDM-positive *K. pneumoniae* in Iraq.³⁷ Our data showed a dissemination of a novel ST namely 268, which has not been reported in NDM-1-producing *K. pneumoniae*, during February 2016 to November 2017. The ST268 has been established as a major threat to NICUs from two referral hospitals after detecting the following STs including ST43, ST340, ST392, and ST14.

In general, the epidemiological trend of NDM-producing isolates in our hospital might be divided into three stages. From March 2015 to December 2015, the following STs ST43, ST340, ST392, and ST147 were found. From

Table 3 Distribution and genetic characterization of 37 NDM-producing *Klebsiella pneumoniae* strains isolated from hospitalized patients

Strain	Center of isolation	Time of collection	Hospital unit	Specimen	MIC (μg/mL)			ERIC/ST	Profile of resistance genes
					IMI	CTX	FEP		
O10	Afzalipoor	2015	Internal	Urine	8	2,048	512	A/340	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B
O12	Shafa	2015	Burning	Wound	32	2,048	1,024	A/43	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B
O18	Shafa	2015	Burning	Wound	64	1,024	64	B/392	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>oqx</i> A, <i>oqx</i> B
O20	Afzalipoor	2015	Internal	Urine	128	2,048	512	A/340	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B
O30	Afzalipoor	2015	NICU	BAL	16	2,048	1,024	A/43	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A
O33	Shafa	2015	Internal	Urine	16	1,024	64	A/340	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B
O37	Afzalipoor	2015	NICU	Blood	4	32	64	C/43	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>oqx</i> A, <i>oqx</i> B
O38	Afzalipoor	2015	NICU	Urine	16	2,048	1,024	A/43	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{TEM} , <i>oqx</i> A
O45	Afzalipoor	2015	NICU	Blood	128	2,048	1,024	A/43	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B
O46	Afzalipoor	2015	NICU	Blood	16	2,048	1,024	A/43	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>oqx</i> A, <i>oqx</i> B
O58	Shafa	2015	ICU	BAL	8	512	256	B/392	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B
O62	Bahonar	2015	Surgery	Wound	16	2,048	1,024	A/43	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>arm</i> A, <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B
O63	Afzalipoor	2015	NICU	Blood	16	2,048	1,024	A/43	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B
O65	Afzalipoor	2015	Surgery	Wound	16	512	1,024	A/43	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>oqx</i> A
O75	Shafa	2015	ICU	Urine	4	2,048	256	S/392	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>bla</i> _{OXA} , <i>bla</i> _{ACT}
O494	Shafa	2016	Burning	Wound	16	2,048	1,024	A/392	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B
N6	Shafa	2016	Neurosurgery	Urine	64	2,048	512	S/147	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>oqx</i> A, <i>oqx</i> B, <i>bla</i> _{FOX} , <i>bla</i> _{CMY}
N19	Afzalipoor	2016	NICU	Blood	16	2,048	512	F/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>rmt</i> C
N21	Afzalipoor	2016	NICU	Urine	8	2,048	512	F/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{TEM} , <i>bla</i> _{OXA} , <i>oqx</i> A, <i>rmt</i> C
N32	(Kashani)	2016	Internal	Urine	16	1,024	512	F/340	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>bla</i> _{OXA} , <i>oqx</i> A
N38	Afzalipoor	2016	NICU	Urine	8	2,048	1,024	E/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>oqx</i> A, <i>oqx</i> B, <i>rmt</i> C
N43	(Kashani)	2016	Internal	Urine	4	32	64	S/340	<i>bla</i> _{NDM} , <i>bla</i> _{TEM}
N54	(Kashani)	2016	Internal	Urine	4	32	64	G/340	<i>bla</i> _{NDM} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B
N57	Afzalipoor	2016	NICU	Urine	8	2,048	1,024	F/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B, <i>rmt</i> C
N79	Afzalipoor	2017	Internal	Urine	8	2,048	512	D/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> B, <i>rmt</i> C
N83	Afzalipoor	2017	NICU	Urine	8	2,048	1,024	C/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A
N85	Afzalipoor	2017	NICU	Blood	8	2,048	512	C/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B
N86	Afzalipoor	2017	NICU	CSF	8	2,048	512	D/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>rmt</i> C
N88	Afzalipoor	2017	NICU	Urine	8	2,048	512	C/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B, <i>rmt</i> C
N89	Afzalipoor	2017	Emergency	Urine	8	64	32	C/340	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV}
N94	Afzalipoor	2017	Emergency	CSF	8	512	512	D/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{SHV} , <i>bla</i> _{TEM}
N97	Afzalipoor	2017	Internal	Urine	8	512	512	D/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV}
N99	Shafa	2017	Internal	Urine	8	128	32	D/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>oqx</i> A, <i>oqx</i> B, <i>rmt</i> C
N101	Afzalipoor	2017	ICU	Blood	16	128	32	C/16	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>oqx</i> A, <i>oqx</i> B
N109	Afzalipoor	2017	Internal	Urine	8	2,048	1,024	C/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>rmt</i> C
N115	Shafa	2017	ICU	Blood	4	256	512	G/392	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>bla</i> _{OXA} , <i>bla</i> _{TEM} , <i>oqx</i> A, <i>oqx</i> B, <i>bla</i> _{CMY}
N116	Afzalipoor	2017	NICU	Urine	8	512	128	S/268	<i>bla</i> _{NDM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{SHV} , <i>oqx</i> A, <i>oqx</i> B, <i>bla</i> _{CMY}

Abbreviations: BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; CTX, cefotaxime; ERIC/ST, enterobacterial repetitive intergenic consensus/sequence type; FEP, cefepime; ICU, intensive care unit; IMI, imipenem; MIC, minimum inhibitory concentration; NDM, New Delhi metallo-beta-lactamase; NICU, neonatal intensive care unit; S, singleton.

the beginning of February 2016 up till November 2017, the ST268 has been mainly investigated. Based on these findings, we supposed the ST268 was a successful clone to have recently been established in our hospital settings in 2017. This ST has been found in hypermucoviscous *K. pneumoniae*, which was associated with invasive liver abscess syndrome in eastern Asia.³⁸ On the contrary, ST268 was recognized in capsule serotype K20 *K. pneumoniae* isolates, relating to primary meningitis in Taiwan.³⁹ Furthermore, these isolates were significantly associated with the virulence factors such as *rmpA*, *rmpA2*, and aerobactin.³⁹ Importantly, nine of ten *rmtC*-positive isolates in our current study belonged to *K. pneumoniae*, representing ST268, which often exhibited the most predominant ST of carbapenem-resistant *K. pneumoniae* isolates in NICU. Coproduction of *16S rRNA methylase* resistance genes (*rmtC*, *armA*) among carbapenem-resistant *K. pneumoniae* with ST14 and ST340 was reported by Poirel et al.⁴⁰

In our findings, we observed another major ST, namely ST43, identified during 2015. This ST was able to carry virulence factors causing bacteremia.⁴¹ In this study, most isolates belonging to ST43 have been detected from blood samples (Table 3). Recently, ST147 has been associated with *bla*_{CMY-4} gene and *bla*_{OXA-48} described in Tunisia.⁴² In our study, ST147 was associated with *bla*_{NDM}, *bla*_{CTX-M}, and *bla*_{SHV}, although this ST has been recognized as a serious threat to public health worldwide.³⁶ ST16 was the other type represented by one isolate from Afzalipoor Hospital. In this study, ST16 has been associated with *bla*_{NDM}, *bla*_{CTX-M}, and *bla*_{SHV}. This ST has been recently reported in Italy, coproducing NDM-1 and OXA-232, recovering from blood and urine samples of a hospitalized patient with urosepsis.⁴³ However, Lester et al³⁴ and Hammerum et al⁴⁴ in New Zealand and Denmark showed that *K. pneumoniae* ST16 was recognized at several occasions, disseminating ESBLs and NDM-5 carbapenemase genes.

Our findings showed that ST340 has different ERIC-PCR patterns, that is a single-locus variant of ST11, which was found in Sweden and the UK.^{45,46} Additionally, NDM-producing isolates from ST340 detected in March 2015 were compared with isolates collected with the same ST in November 2017 to check for ERIC-PCR profile variations within NDM-positive *K. pneumoniae*. As shown in Figure 1, ERIC-PCR pattern from ST340 displayed identical ERIC-PCR profiles among NDM-producing isolates with the other STs (43, 268, 392, and 16) in different clusters. Similar to this study, Richter et al in Italy showed that no ERIC-PCR profile variation was found between

carbapenemase-producing *K. pneumoniae* strains from STs 258 and 37.⁴⁷

Lascols et al in India showed a diverse range of clones harboring carbapenemase-producing *K. pneumoniae* strains representing STs 147 and 340.^{36,40,48,49} The prevalence of *bla*_{NDM-1} is frequently associated with promiscuous plasmids related to a broad host range of clinical variants harboring *bla*_{NDM-1}, hence our findings hypothesized that nosocomial acquisition of *bla*_{NDM} by both outward sources including patients who have traveled to Iran specially neighboring countries and also have acquired a broad spectrum of different resistance genes.⁴⁸ We detected *K. pneumoniae* ST392, which has been previously associated with the dissemination of *bla*_{NDM-1}, *bla*_{KPC}, and *bla*_{OXA-48} genes.^{50,51} However, in this study, ST392 was detected among *bla*_{NDM}- and *bla*_{OXA}-positive isolates, recovering from one hospital with different ERIC-PCR patterns. In this study, some STs were observed to have different ERIC-PCR pattern types; therefore, our molecular typing results revealed that ERIC-PCR and MLST provided measures of genetic diversity, while they were not similar methods. These findings showed that ERIC-PCR displayed pattern discriminations for same STs; however, ERIC-PCR has provided a potential molecular typing method to distinguish greater ranges of genetic changes among NDM-positive *K. pneumoniae* in our hospital settings.⁵² In this study, up to November 2017, 37 patients with at least one NDM-positive *K. pneumoniae* isolates were identified. Most isolates were recovered at Afzalipoor Hospital from NICU (Table 3). Interestingly, the most NDM-positive *K. pneumoniae* isolates belonged to the two STs; 268 and 340 displayed various ERIC-PCR patterns within different clusters. On the contrary, in different hospitals, *K. pneumoniae* isolates with similar STs revealed diverse ERIC-PCR patterns during 2015–2017. It was suggested that these isolates might be affected by coacquisition of some antibiotic resistance plasmids; therefore, it might affect the results of the ERIC-PCR patterns. Moreover, the molecular typing techniques such as ERIC-PCR and pulsed-field gel electrophoresis were used to identify alterations in short-term, while no obvious difference was observed in STs during 3 years, since MLST is considered to evaluate the alternations in the most conserved genes, showing long-term variations.⁵²

Conclusion

In this study, the molecular characterization and epidemiological investigations revealed the dissemination of different clones of NDM-producing *K. pneumoniae* in our hospital settings. Due to the highly resistant nature of bacterial strains carrying the *bla*_{NDM}, there are very limited antibiotics to

combat these bacteria. In this study, MLST differentiated the 37 representative NDM-positive *K. pneumoniae* strains into 7 STs. The STs included ST268 (n=14), ST43 (n=9), ST340 (n=7), ST392 (n=5), and single isolates representing STs 147 and 16. To the best of our knowledge, the clinical isolates of *K. pneumoniae* representing ST268 have not been reported among NDM-producing *K. pneumoniae*. Distribution of *bla*_{NDM} is obviously related to the promiscuity of many plasmids resulting in a wide range of Gram-negative bacteria containing diverse *bla*_{NDM} harboring plasmids. However, our study suggests that dominant clones (STs 43 and 268) have had a potential role to monitor and continue a long-term survival of *bla*_{NDM-1} dissemination. In addition, our data highlight that the potential for local and neighboring countries such as Pakistan, India, and Iraq has been reported as the endemic dissemination of *bla*_{NDM}-producing *K. pneumoniae* clones and plasmid-mediated resistance. Therefore, dissemination of different clones with *bla*_{NDM} among carbapenem-resistant *K. pneumoniae* might have resulted in more frequent opportunities for the emergence of *bla*_{NDM}-positive among other Gram-negative bacteria and it highlighted the need to ongoing epidemiological surveillance and comprehensive infection control guidelines. We also suggested the intrinsic genetic factors, causing a spread and establishment of ST268, as a new NDM-producing *K. pneumoniae* clone identified to increase our knowledge about it.

Ethical statement

The *K. pneumoniae* strains were originally taken as part of routine hospital procedure, and then specifically recovered for this work. This study was approved by ethical numbers: IR:KMU.REC.1395.436 and IR:KMU.REC.1395.806 in ethical committee of Kerman University of Medical Sciences.

Acknowledgments

We thank Student Research Committee Kerman University of Medical Sciences for supporting in financial aid for MLST. This research was supported by the research council and student research committee of Kerman University of Medical Sciences, Kerman, Iran, grant numbers: 95000056 and 95000522.

Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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