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ORIGINAL RESEARCH

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*$_{NDM-1}$-positve *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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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}), caebapenemase (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://bigsdb.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://bigsdb.pasteur.fr/klebsiella/klebsiella.html.

Molecular typing of bla_{NDM}-positive isolates by ERIC-PCR

ERIC-PCR using ERIC2 primer (5′-AAGTAAGTGACTGGGGTGAGC-3′) was used for molecular typing of NDM-positive isolates.[30] The results of ERIC-PCR were

Table 1 Sequence of primers used in this study for the detection of resistance genes in PCR method

Genes	Primer sequence (5′–3′)	Annealing temperature (°C)	Product size (bp)	Reference
bla_{CTX-M}	F-ATGTGCAGYACCAGTAARGTKATGGC R-TGGGTRAARTARGTSACCAGAAYCAGCGG	58	593	14
bla_{TEM}	F-CTTCCTGTTTTTGCTCACC R-AGCAATAAACCAGCCAGC	54	636	15
bla_{SHV}	F-TCAGCGAAAAACACCTTG R-TCCCGCAGATAAATCACC	51	472	15
bla_{KPC}	F-CGTCTAGTTCTGCTGTCTTG R-CTTGTCATCCTTGTTAGGCG	58	798	16
bla_{OXA-48}	F-GCGTGGTTAAGGATGAACAC R-CATCAAGTTCAACCCAACCG	58	438	16
bla_{VIM}	F-ATGTTAAAAGTTATTAGTAGT R-CTACTCGGCGACTGAGCGAT	53	801	16
bla_{IMP}	F-GGAATAGAGTGGCTTAAYTCTC R-GGTTTAAYAAAACAACCACC	58	232	16
bla_{NDM}	F-GGTTTGGCGATCTGGTTTTC R-CGGAATGGCTCATCACGATC	58	621	16
bla_{OXA}	F-GCGTGGTTAAGGATGAACAC R-CATCAAGTTCAACCCAACCG	52	438	17
bla_{AIM}	F-CTGAAGGTGTACGGAAACAC R-GTTCGGCCACCTCGAATTG	59	322	18
bla_{PER}	F-GGGACARTCSKATGAATGTCA R-GGYSGCTTAGATAGTGCTGAT	47	926	19
bla_{GIM}	F-TCGACACACCTTGGTCTGAA R-AACTTCCAACTTTGCCATGC	58.5	477	18
bla_{SPM}	F-AAAATCTGGGTACGCAAACG R-ACATTATCCGCTGGAACAGG	59	271	20
bla_{SIM}	F-TACAAGGGATTCGGCATCG R-TAATGGCCTGTTCCCATGTG	61	570	20
bla_{GES}	F-ATGCGCTTCATTCACGCAC R-CTATTTGTCCGTGCTCAGG	56	844	21
oqxA	F-CTCGGCGCGATGATGCT R-CCACTCTTCACGGGAGACGA	57	392	22
oqxB	F-TTCTCCCCCGGCGGGAAGTAC F-CTCGGCCATTTTGGCGCGTA	56	512	23
rmtA	F-CTAGCGTCCATCCTTTCCTC R-TTTGCTTCCATGCCCTTGCC	56	635	24
rmtB	F-CCCAAACAGACCGTAGAGGC R-CTCAAACTCGGCGGGCAAGC	56	584	25
rmtC	F-CGAAGAAGTAACAGCCAAAG R-ATCCCAACATCTCTCCCACT	61	711	26
armA	F-AGGTTGTTTCCATTTCTGAG R-TCTCTTCCATTCCCTTCTCC	56	590	27
mcr-1	F-CGGTCAGTCCGTTTGTTC R-CTTGGTCGGTCTGTAGGG	53	309	28

analyzed in http://insilico.ehu.eus/dice_upgma/ using the Dice similarity coefficient. Clusters were defined as DNA patterns sharing ≥80% similarity.

Results

In this study, 175 nonduplicated isolates of *K. pneumoniae* were recovered from hospitalized patients in four referral hospitals in Kerman, Iran. The isolates were collected from different specimens including burning wounds 9 (5.1%), 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: [a]Left and right brackets indicate the lowest and highest MICs tested, respectively. [b]No. of isolates.
Abbreviation: MIC, minimum inhibitory concentration.

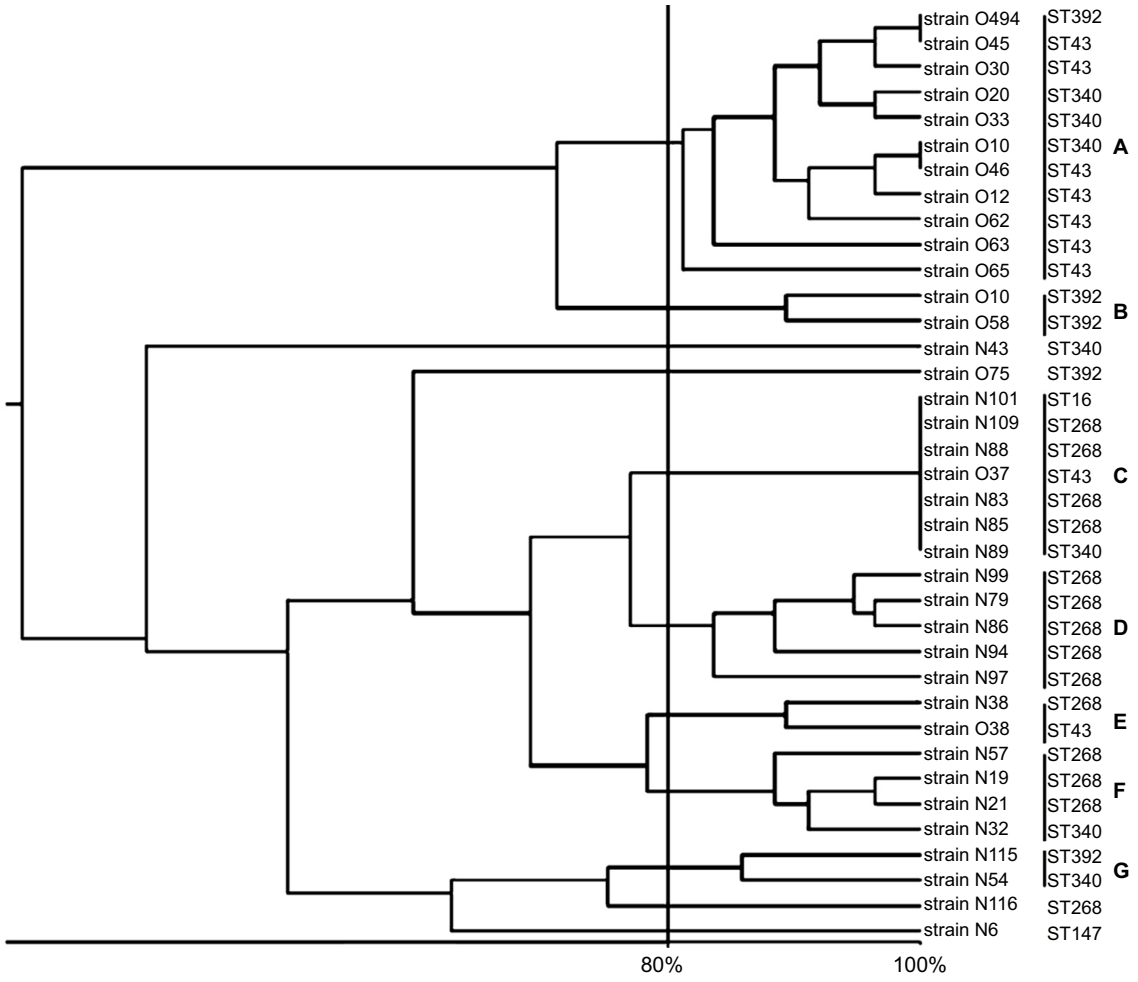

Figure 1 Corresponding dendrogram generated with UPGMA clustering method in clinical bla_{NDM}-positive isolates of *Klebsiella pneumoniae*.
Note: ERIC-PCR represented seven clusters (**A**–**G**) plus four singletons among NDM-positive isolates.
Abbreviations: ERIC-PCR, enterobacterial repetitive intergenic 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 cefoxitin 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	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{TEM}, *oqxA, oqxB*
O12	Shafa	2015	Burning	Wound	32	2,048	1,024	A/43	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{SHV}, bla_{TEM}, *oqxA, oqxB*
O18	Shafa	2015	Burning	Wound	64	1,024	64	B/392	bla_{NDM}, bla_{CTX-M}, *oqxA, oqxB*
O20	Afzalipoor	2015	Internal	Urine	128	2,048	512	A/340	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{SHV}, bla_{TEM}, *oqxA, oqxB*
O30	Afzalipoor	2015	NICU	BAL	16	2,048	1,024	A/43	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{SHV}, bla_{TEM}, *oqxA*
O33	Shafa	2015	Internal	Urine	16	1,024	64	A/340	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{TEM}, *oqxA, oqxB*
O37	Afzalipoor	2015	NICU	Blood	4	32	64	C/43	bla_{NDM}, bla_{CTX-M}, *oqxA, oqxB*
O38	Afzalipoor	2015	NICU	Urine	16	2,048	1,024	A/43	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{TEM}, *oqxA*
O45	Afzalipoor	2015	NICU	Blood	128	2,048	1,024	A/43	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{SHV}, bla_{TEM}, *oqxA, oqxB*
O46	Afzalipoor	2015	NICU	Blood	16	2,048	1,024	A/43	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{TEM}, bla_{SHV}, *oqxA, oqxB*
O58	Shafa	2015	ICU	BAL	8	512	256	B/392	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{TEM}, *oqxA, oqxB*
O62	Bahonar	2015	Surgery	Wound	16	2,048	1,024	A/43	bla_{NDM}, bla_{CTX-M}, *armA*, bla_{SHV}, bla_{TEM}, *oqxA, oqxB*
O63	Afzalipoor	2015	NICU	Blood	16	2,048	1,024	A/43	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{TEM}, *oqxA, oqxB*
O65	Afzalipoor	2015	Surgery	Wound	16	512	1,024	A/43	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{TEM}, bla_{SHV}, *oqxA*
O75	Shafa	2015	ICU	Urine	4	2,048	256	S/392	bla_{NDM}, bla_{CTX-M}, bla_{SHV}, bla_{TEM}, bla_{OXA}, bla_{ACT}
O494	Shafa	2016	Burning	Wound	16	2,048	1,024	A/392	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{SHV}, bla_{TEM}, *oqxA, oqxB*
N6	Shafa	2016	Neurosurgery	Urine	64	2,048	512	S/147	bla_{NDM}, bla_{CTX-M}, bla_{SHV}, *oqxA, oqxB*, bla_{FOX}, bla_{CMY}
N19	Afzalipoor	2016	NICU	Blood	16	2,048	512	F/268	bla_{NDM}, bla_{CTX-M}, bla_{SHV}, bla_{TEM}, *oqxA, rmtC*
N21	Afzalipoor	2016	NICU	Urine	8	2,048	512	F/268	bla_{NDM}, bla_{CTX-M}, bla_{TEM}, bla_{OXA}, *oqxA, rmtC*
N32	(Kashani)	2016	Internal	Urine	16	1,024	512	F/340	bla_{NDM}, bla_{CTX-M}, bla_{SHV}, bla_{OXA}, *oqxA*
N38	Afzalipoor	2016	NICU	Urine	8	2,048	1,024	E/268	bla_{NDM}, bla_{CTX-M}, bla_{TEM}, bla_{SHV}, *oqxA, oqxB, rmtC*
N43	(Kashani)	2016	Internal	Urine	4	32	64	S/340	bla_{NDM}, bla_{TEM}
N54	(Kashani)	2016	Internal	Urine	4	32	64	G/340	bla_{NDM}, bla_{TEM}, *oqxA, oqxB*
N57	Afzalipoor	2016	NICU	Urine	8	2,048	1,024	F/268	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{SHV}, bla_{TEM}, *oqxA, oqxB, rmtC*
N79	Afzalipoor	2017	Internal	Urine	8	2,048	512	D/268	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{SHV}, bla_{TEM}, *oqxB, rmtC*
N83	Afzalipoor	2017	NICU	Urine	8	2,048	1,024	C/268	bla_{NDM}, bla_{CTX-M}, bla_{SHV}, bla_{TEM}, *oqxA*
N85	Afzalipoor	2017	NICU	Blood	8	2,048	512	C/268	bla_{NDM}, bla_{CTX-M}, bla_{SHV}, bla_{TEM}, *oqxA, oqxB*
N86	Afzalipoor	2017	NICU	CSF	8	2,048	512	D/268	bla_{NDM}, bla_{CTX-M}, bla_{SHV}, bla_{TEM}, *oqxA, rmtC*
N88	Afzalipoor	2017	NICU	Urine	8	2,048	512	C/268	bla_{NDM}, bla_{CTX-M}, bla_{SHV}, bla_{TEM}, *oqxA, oqxB, rmtC*
N89	Afzalipoor	2017	Emergency	Urine	8	64	32	C/340	bla_{NDM}, bla_{CTX-M}, bla_{SHV}
N94	Afzalipoor	2017	Emergency	CSF	8	512	512	D/268	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{SHV}, $bla_{TEM,}$
N97	Afzalipoor	2017	Internal	Urine	8	512	512	D/268	bla_{NDM}, bla_{CTX-M}, bla_{SHV}
N99	Shafa	2017	Internal	Urine	8	128	32	D/268	bla_{NDM}, bla_{CTX-M}, bla_{SHV}, *oqxA, oqxB, rmtC*
N101	Afzalipoor	2017	ICU	Blood	16	128	32	C/16	bla_{NDM}, bla_{CTX-M}, bla_{SHV}, *oqxA, oqxB*
N109	Afzalipoor	2017	Internal	Urine	8	2,048	1,024	C/268	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{TEM}, *oqxA, rmtC*
N115	Shafa	2017	ICU	Blood	4	256	512	G/392	bla_{NDM}, bla_{CTX-M}, bla_{SHV}, bla_{OXA}, bla_{TEM}, *oqxA, oqxB*, bla_{CMY}
N116	Afzalipoor	2017	NICU	Urine	8	512	128	S/268	bla_{NDM}, bla_{CTX-M}, bla_{OXA}, bla_{SHV}, *oqxA, oqxB*, bla_{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. pneumonia*, 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 $bla_{\text{CMY-4}}$ gene and $bla_{\text{OXA-48}}$ described in Tunisia.[42] In our study, ST147 was associated with bla_{NDM}, $bla_{\text{CTX-M}}$, 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}, $bla_{\text{CTX-M}}$, 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 al[34] and Hammerum et al[44] 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 $bla_{\text{NDM-1}}$ is frequently associated with promiscuous plasmids related to a broad host range of clinical variants harboring $bla_{\text{NDM-1}}$, 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 $bla_{\text{NDM-1}}$, bla_{KPC}, and $bla_{\text{OXA-48}}$ 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 MSLT 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.

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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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