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Identification of Carbapenem-Resistant *Klebsiella pneumoniae* with Emphasis on New Delhi Metallo-Beta-Lactamase-1 (bla_{NDM-1}) in Bandar Abbas, South of Iran

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Background and Objective: The spread of carbapenem-resistant *Klebsiella pneumoniae* especially bla_{NDM-1}-carrying isolates is a great concern worldwide. In this study we describe the molecular basis of carbapenem-resistant *K. pneumoniae* in three teaching hospitals at Bandar Abbas, south of Iran.
Materials and Methods: A total of 170 nonduplicate clinical isolates of *K. pneumoniae* were investigated. Antimicrobial susceptibility test was performed by disc diffusion method. PCR was carried out for detection of carbapenemase (bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{OXA-48}, and $bla_{OXA-181}$) and extended-spectrum β-lactamase (bla_{CTX-M}, bla_{SHV}, bla_{TEM}, bla_{VEB}, bla_{GES}, and bla_{PER}). Clonal relatedness of bla_{NDM-1}-positive isolates was evaluated by multilocus sequence typing (MLST).
Results: Tigecycline was the most effective antimicrobial agent with 96.5% susceptibility. In addition, 6.5% of the isolates were carbapenem resistant. Bla_{NDM-1} was identified in four isolates (isolate A–D) and all of them were multidrug-resistant. MLST revealed that bla_{NDM-1}-positive isolates were clonally related and belonged to two distinct clonal complexes, including sequence type (ST) 13 and ST 392. In addition to bla_{NDM-1}, isolate A coharbored bla_{SHV-11}, $bla_{CTX-M-15}$, and bla_{TEM-1}, isolate B harbored bla_{SHV-11} and $bla_{CTX-M-15}$, and isolates C and D contained both bla_{SHV-1} and $bla_{CTX-M-15}$.
Conclusion: Our results indicate that NDM-1-producing *K. pneumoniae* ST 13 and ST 392 are disseminated in our region. Moreover, one of our major concerns is that these isolates may be more prevalent in the near future. Tracking and urgent intervention is necessary for control and prevention of these resistant isolates.

Keywords: *Klebsiella pneumoniae*, carbapenem-resistant, New Delhi Metallo-beta-lactamase-1

Background

Klebsiella pneumoniae is a well-recognized human pathogen causing healthcare-associated infections.[1,2] Carbapenem-resistant *K. pneumoniae* has become a new multidrug-resistant nosocomial pathogen and is a serious public health threat.[3,4] Metallo-β-lactamase (MBLs) enzymes have a broad range of substrate spectrum and can hydrolyze penicillins, cephalosporins, and carbapenems.[5] The most frequent type of MBLs in *Klebsiella* spp. includes IMP, VIM, and newly discovered NDM group.[6,7] New Delhi metallo-β-lactamase-1 (NDM-1) was initially identified in *K. pneumoniae* in 2008 and since then *K. pneumoniae* harboring NDMs has been reported from different countries.[4,8–12] NDM-1-producing bacteria are a growing concern for the treatment of infections because these organisms are usually resistant to a broad range of antimicrobial agents.[1,13]

NDM-1 is capable of inactivating approximately all β-lactams, except aztreonam. Unfortunately, eradication of infections caused by NDM-1 producers is difficult because treatment options are very limited.[14] In this study, 170 *K. pneumoniae* isolates were studied to identify antimicrobial susceptibility pattern, to investigate phenotypic test for detection of carbapenemase and to determine carbapenem

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resistance genes particularly bla_{NDM-1} and extended-spectrum β-lactamases (ESBLs). Finally, clonal relatedness of bla_{NDM-1}-positive isolates was examined by multilocus sequence typing (MLST).

Materials and Methods

Bacterial isolates

A total of 170 nonduplicate clinical isolates of *K. pneumoniae* was included in this study. Bacterial isolates were obtained from various clinical specimens of patients admitted to three teaching hospitals (Shahid Mohammadi, Dr. Shariati, and Pediatrics) at Bandar Abbas located on the South of Iran, between June 2015 and November 2016. The isolates were identified as *K. pneumoniae* by colony morphological features, gram stain, and biochemical tests.[15]

Antimicrobial susceptibility test

Disc diffusion assay on Mueller-Hinton agar was used for antimicrobial susceptibility test based on the Clinical and Laboratory Standards Institute (CLSI) guidelines as updated in 2016.[16] Commercially available discs (MAST Group Ltd., Merseyside, United Kingdom) tested against 170 *K. pneumoniae*, were penicillins (piperacillin 100 μg, piperacillin–tazobactam 100/10 μg, ampicillin–sulbactam 10/10 μg), cephalosporins (cefazolin 30 μg, cefoxitin 30 μg, ceftazidime 30 μg, cefotaxime 30 μg, ceftriaxone 30 μg, cefuroxime 30 μg, and cefepime 30 μg), carbapenems (imipenem 10 μg, meropenem 10 μg, ertapenem 10 μg, and doripenem 10 μg), aztreonam 30 μg, aminoglycosides (gentamicin 10 μg, amikacin 30 μg, and tobramycin 10 μg), fluoroquinolones (gemifloxacin 5 μg and ciprofloxacin 5 μg), nalidixic acid 30 μg, tetracycline 30 μg, tigecycline 15 μg, fosfomycin 200 μg, and trimethoprim 5 μg.

Isolates that were resistant to meropenem were defined as carbapenem resistant. The minimum inhibitory concentration (MIC) of imipenem and meropenem against carbapenem-resistant isolates was determined by using E-test (Liofilchem, Italy) range 0.002–32 μg/ml on Mueller-Hinton agar according to the manufacturer's recommendation. Colistin MIC was measured for NDM-1-positive isolates by E-test (Liofilchem, Italy) range 0.016–256 μg/ml. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control. Results were interpreted according to CLSI criteria. Colistin breakpoint was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST), breakpoint tables for interpretation of MICs and zone diameters, Version 7.0, 2017.[17]

Screening of carbapenemase production by Modified Hodge Test (MHT) and Triton Hodge Test (THT)

MHT was carried out for all isolates as recommended by CLSI.[18] Briefly, a 0.5 McFarland standard suspension of *E. coli* ATCC 25922 (the indicator organism) was prepared in 5 ml saline using the direct colony suspension method. Then, a 1:10 dilution of this suspension was inoculated on the surface of Mueller-Hinton agar plate (Merck, Germany) as the routine disc diffusion. After drying, a 10 μg of ertapenem (MAST Group Ltd., Merseyside, United Kingdom) was placed in the center of the agar plate. Test isolates were streaked in a straight line starting from the edge of ertapenem disc to the edge of the plate in four different directions. The plate was incubated overnight at 37°C in ambient air. The enhanced growth of *E. coli* ATCC 25922 at the intersection of test organism growth streak and the zone of inhibition is considered a positive result for carbapenemase production.[18] A newly described protocol for MHT named as ''Triton Hodge test'' was performed according to Pasteran *et al.*[19]

Phenotypic detection of KPC

Boronic acid disc test was conducted to detect the KPC-possessing *K. pneumoniae* isolates. A 0.5 McFarland bacterial suspension was inoculated on freshly prepared Mueller-Hinton agar plate (Merck, Germany) using a cotton swab. Two 10 μg meropenem discs containing 400 μg of boronic acid and one without boronic acid were placed on the inoculated plate.[20] After incubation at 37°C for 18 hr, they were examined and the growth-inhibitory zone produced around each of the discs were compared. The test was considered positive when the inhibition zone diameter around meropenem plus phenylboronic acid was ≥5 mm greater than the inhibition zone diameter around the meropenem disc alone.[20]

Screening of metallo-β-lactamase production by dipicolinic acid (DPA)

To identify MBLs producer, all bacterial isolates were evaluated by a combined disc method using DPA as MBLs inhibitor instead of ethylenediaminetetraacetic acid.[21] Meropenem disc (10 μg) containing DPA and a 10 μg meropenem disc without DPA were used. After overnight incubation periods at 35°C, the inhibition zone diameter around meropenem + DPA and meropenem disc alone were measured. The difference of ≥5 mm between the inhibition zone diameter of Meropenem + DPA as compared with the meropenem only disc was interpreted as a positive result for metallo-β-lactamase production.[22] We also used EDTA disc synergy test for detection of metallo-β-lactamase as previously described.[23]

Detection of carbapenemase and extended-spectrum β-lactamase genes

Extraction of bacterial DNA was performed by the boiling method.[24] All isolates were screened using PCR for the presence of carbapenemase genes bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{OXA-48}, and $bla_{OXA-181}$ according to the previously described condition and primers listed in Table 1. All PCR reactions were carrying out in a final volume of 25 μl containing 1× PCR buffer, 1 U Taq polymerase, 1.5 mM $MgCl_2$, 200 μM of dNTP (SinaClon, BioScience Co., Iran), 10 pmol of each primer (TAG; Copenhagen A/S, Denmark), and 1 μl of the extracted DNA. Amplification parameters were performed in a DNA thermal cycler (Bio-Rad My Cycler Thermal Cycler). Annealing temperature for each gene is shown in Table 1. The PCR products were screened by electrophoresis on 1.5% (w/v) agarose gel (SinaClon, BioScience Co., Iran) in 1× TBE buffer, stained by 5× GelRed (Biotium) and visualized on an ultraviolet transilluminator. In addition, for each gene, a positive control was purchased from the Pasteur Institute of Iran. Furthermore, PCR was carried out on all isolates for detection of ESBL genes by specific primers listed in Table 1.[9] Direct sequencing of PCR products was performed using an ABI 3730 XL DNA Analyzer (Bioneer, South Korea).

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Table 1. Primers Sequences and Annealing Temperature Used in This Study

Target	*Primer*	*Forward sequence*	*Reverse sequence*	*Product size (bp)*	*Annealing, °C*	*Reference*
Carbapenemase genes	KPC	ATTTTCAGAGCCTTACTGCCC	TATCGTTGATGTCACTGTATCG	901	55	3
	IMP	GGAATAGAGTGGCTTAAYTCTC	GGTTTAAYAAAACAACCACC	232	52	26
	VIM	GATGGTGTTTGGTCGCATA	CGAATGCGCAGCACCAG	390	52	
	SPM	AAAATCTGGGTACGCAAACG	ACATTATCCGCTGGAACAGG	271	52	
	NDM	CCGTATGAGTGATTGCGGCG	GCCCAATATTATGCACCCGG	779	60	27
	OXA-48 & OXA-181	TATATTGCATTAAGCAAGGG	CACACAAATACGCGCTAACC	847	60	28
ESBL genes	SHV	AGCCGCTTGAGCAAATTAAAC	ATCCCGCAGATAAATCACCAC	713	60	29
	TEM	CATTTCCGTGTCGCCCTTATTC	CGTTCATCCATAGTTGCCTGAC	800	60	
	CTX-M-1	TTAGGAARTGTGCCGCTGYA	CGATATCGTTGGTGGTRCCAT	688	60	
	CTX-M-2	CGTTAACGGCACGATGAC	CGATATCGTTGGTGGTRCCAT	404	60	
	CTX-M-9	TCAAGCCTGCCGATCTGGT	TGATTCTCGCCGCTGAAG	561	60	
	CTX-Mg8/25	AACRCRCAGACGCTCTAC	TCGAGCCGGAASGTGTYAT	326	60	
	GES	AGTCGGCTAGACCGGAAAG	TTTGTCCGTGCTCAGGAT	399	57	
	PER	GCTCCGATAATGAAAGCGT	TTCGGCTTGACTCGGCTGA	520	60	
	VEB	CATTTCCCGATGCAAAGCGT	CGAAGTTTCTTTGGACTCTG	648	60	
MLST	rpoB	VIC3:GGCGAAATGGCWGAGAACCA	VIC2:GAGTCTTCGAAGTTGTAACC	501	50	25
	gapA	gapA173:TGAAATATGACTCCACTC ACGG	gapA181:CTTCAGAAGCGGCTTTGATGGCTT	450	60	
	Mdh	mdh130:CCCAACTCGCTTCAGGTTCAG	mdh867:CCGTTTTTCCCCAGCAGCAG	477	50	
	Pgi	pgi1F:GAGAAAAACCTGCCTGTACTGCTGGC pgi2F(seq):CTGCTGGCGCTGATCGGCAT	pgi1R:CGCGCCACGCTTTATAGCGGTTAAT pgi2R(seq):TTATAGCGGTTAATCAGGCCGT	432	50	
	phoE	phoE604.1:ACCTAC CGCAACACCGACTTCTTCGG	phoE604.2:TGATCAGAACTGGTAGGTGAT	420	50	
	infB	infB1F:CTCGCTGCTGGACTATATTCG infB2F(seq):ACTAAGGTTGCCTCCGGCGAAGC	infB1R:CGCTTTCAGCTCAAGAACTTC	318	50	
	tonB	tonB1F:CTTTATACCTCGGTACATCAGGTT	tonB2R:ATTCGCCGG CTGRGCRGAGAG	414	50	

ESBLs, extended-spectrum β-lactamases; MLST, multilocus sequence typing.

Nucleotide sequences were compared with sequences in GenBank database and were analyzed using BLAST (www.ncbi.nlm.nih.gov/BLAST).

Nucleotide sequence accession number

The nucleotide sequences obtained in this study were deposited in the EMBL nucleotide sequence database and are available under the accession numbers: LT615329, LT615330, LT615331, and LT615332 for bla_{NDM-1}; LT628514, and LT628513 for bla_{SHV-1}; LT628511, LT628512, and LT854835 for bla_{SHV-11}; LT853894 for bla_{SHV-12}; LT628518, LT628519, LT628520, and LT628521 for $bla_{CTX-M-15}$; LT853895, LT854834, and LT853900 for $bla_{CTX-M-28}$; and LT628525, LT853896, LT853897, LT853898, and LT853899 for bla_{TEM-1}.

Multilocus sequence typing

MLST was performed on *K. pneumoniae* isolates harboring bla_{NDM-1}. Seven housekeeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, *and tonB*) were investigated by PCR and sequencing according to the guidelines of the Pasteur Institute *K. pneumoniae* MLST Database (http://bigsdb.pasteur.fr/klebsiella) and Dinacort *et al.*'s procedure.[25] The primer sequences are shown in Table 1.

Statistical analysis

Statistical analysis was performed by SPSS software (Version.17 IBM, Chicago, IL). PCR was considered as the gold standard and sensitivity and specificity of each test was compared with PCR.

Results

Bacterial isolates

During the study period from June 2015 to November 2016, a total of 170 nonduplicate *K. pneumoniae* isolates were recovered from urine (68.2%), tracheal aspirates (8.8%), wound (8.2%), blood (4.1%), sputum (4.1%), secretions (2.4%), eye infection (2.4%), bronchoalveolar lavage (1.2%), and ascites (0.6%).

Antimicrobial susceptibility testing

Of the 25 antimicrobials tested, the most effective was tigecycline (96.5% of isolates were susceptible) followed by imipenem (91.8% susceptible) and fosfomycin (88.8% susceptible). The results of the antimicrobial susceptibility test are shown in Table 2. Isolates that were nonsusceptible to meropenem disc were considered as carbapenem resistant. The antimicrobial susceptibility pattern of bla_{NDM-1}-positive isolates is shown in Table 3. In addition, carbapenem-resistant isolates were tested for MIC by E-test for imipenem and meropenem. Colistin MIC was measured only for bla_{NDM-1}-positive isolates. The MIC of imipenem was varied among carbapenem-resistant isolates from 0.19 to 32 μg/ml. The MIC_{50} and MIC_{90} for imipenem was 32 μg/ml.

Imipenem MIC for bla_{NDM-1}-positive isolates was as follows: isolate A had an MIC = 32 μg/ml;, isolate B MIC = 8 μg/ml; isolate C MIC = 32 μg/ml, and isolate D MIC = 2 μg/ml. The MIC of meropenem was varied among carbapenem-resistant isolates from 4 to 32 μg/ml, and MIC $_{50}$ and MIC $_{90}$ of this antimicrobial agent was 32 μg/ml. Meropenem MIC for bla_{NDM-1}-positive isolates was as follows: It was MIC = 16 μg/ml for isolate A, isolate B MIC = 32 μg/ml, isolate C MIC = 32 μg/ml, and isolate D MIC = 32 μg/ml. Furthermore, colistin MIC for bla_{NDM-1}-positive isolates was as follows: isolate A, MIC = 0.5 μg/ml; isolate B, MIC = 0.75 μg/ml; and isolates C and D, MIC ≤0.016 μg/ml. Tigecycline and colistin had excellent activity *in vitro* against all NDM-1-producing isolates with a susceptibility of 100%.

Table 2. Result of Antimicrobial Susceptibility Test by Disc Diffusion on 170 *Klebsiella pneumoniae* Isolates

Disc	*Resistant (%)*	*Intermediate (%)*	*Susceptible (%)*
Ampicillin–sulbactam	40.6	5.3	54.1
Piperacillin	49.4	22.4	28.2
Piperacillin–tazobactam	20	21.8	58.2
Cefazolin	45.9	—	54.1
Cefuroxime	42.4	5.9	51.8
Cefoxitin	17.6	6.5	75.9
Ceftriaxone	42.9	2.4	54.7
Ceftazidime	42.4	4.7	52.9
Cefotaxime	45.3	12.4	42.4
Cefepime	41.2	—	58.8
Imipenem	5.9	2.4	91.8
Meropenem	6.5	6.5	87.1
Doripenem	7.6	2.4	90
Ertapenem	8.2	7.6	84.1
Aztreonam	39.4	0.6	60
Amikacin	13.5	2.4	84.1
Tobramycin	32.9	5.3	61.8
Gentamicin	24.7	2.4	72.9
Tetracycline	24.1	1.2	74.7
Tigecycline	1.2	2.4	96.5
Nalidixic acid	34.7	15.3	50
Ciprofloxacin	25.3	10.6	64.1
Gemifloxacin	15.9	21.8	62.4
Trimethoprim	44.7	2.9	52.4
Fosfomycin	4.7	6.5	88.8

Hodge test and THT

All NDM-1-producing isolates showed positive results in Hodge test (sensitivity and specificity 100%). Both tests were negative among imipenem nonsusceptible isolates. Interestingly, seven imipenem-resistant isolates (isolates 1–7) had a negative Hodge test, but they showed positive results in THT. No carbapenemase genes (bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{Oxa-48}, and $bla_{Oxa-181}$) were found in these isolates, but they carried ESBL genes. Isolate 1 contains bla_{SHV-12} and $bla_{CTX-M-28}$. Isolates 2, 3, and 4 contain bla_{TEM-1}. Isolate 5 contains bla_{TEM-1} and $bla_{CTX-M-28}$. Isolate 6 contains $bla_{CTX-M-28}$, and isolate 7 contains bla_{SHV-11}. False positive in a THT may be related to ESBL/AmpC production. THT had a sensitivity of 100% and specificity of 95.78% for detection of NDM-1-positive isolates.

Phenotypic detection of KPC

Boronic acid disc yielded negative results for all isolates and no synergy was observed using meropenem containing

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TABLE 3. ANTIMICROBIAL SUSCEPTIBILITY PATTERN, SOURCE AND WARD OF ISOLATION FOR NDM-1-PRODUCING *K. PNEUMONIAE*

Disc	*Isolate A*	*Isolate B*	*Isolate C*	*Isolate D*
Ampicillin–sulbactam	R	R	R	R
Piperacillin	R	R	R	R
Piperacillin–tazobactam	R	R	R	R
Cefazolin	R	R	R	R
Cefuroxime	R	R	R	R
Cefoxitin	R	R	R	R
Ceftriaxone	R	R	R	R
Ceftazidime	R	R	R	R
Cefotaxime	R	R	R	R
Cefepime	R	R	R	R
Imipenem	R	R	R	I
Meropenem	R	R	R	R
Doripenem	R	R	R	R
Ertapenem	R	R	R	R
Aztreonam	R	R	R	R
Amikacin	S	S	R	R
Tobramycin	R	R	R	R
Gentamicin	R	R	R	R
Tetracycline	R	S	R	R
Tigecycline	S	S	S	S
Nalidixic acid	R	R	R	R
Ciprofloxacin	R	R	R	R
Gemifloxacin	R	R	R	R
Trimethoprim	R	R	R	R
Fosfomycin	S	S	R	S
Type of specimen	Secretions	Wound	Urine	Tracheal aspirate
Ward	Neurology ICU	Internal Medicine	Neurology	General ICU
Hospital	Shahid mohammadi			

S, sensitive; I, intermediate; R, resistant.

400 μg of boronic acid. This result suggests that our isolates were negative for KPC production. The sensitivity and specificity of the test was 100% for identification of KPC-producing isolates.

Screening of metallo-β-lactamase production by DPA and EDTA

Of the 170 *K. pneumoniae,* 10 isolates showed positive results in both DPA and EDTA combined tests and were considered MBLs producer. However, only four isolates were NDM-1 positive and the six remaining isolates were negative for MBLs genes (bla_{IMP}, bla_{VIM}, bla_{NDM}, and bla_{SPM}). It should be noted that probably these isolates may carry MBLs genes other than bla_{IMP}, bla_{VIM}, bla_{NDM}, and bla_{SPM}. The combined disc tests of DPA and EDTA yielded positive results for all NDM-1-positive isolates suggesting MBLs production. Furthermore, both of the tests had 100% sensitivity and 95.78% specificity in detection of NDM-1-producing isolates.

Molecular detection of carbapenemase and ESBL genes

PCR was performed on all isolates to determine carbapenemase genes, including bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, $bla_{Oxa\text{-}48}$, and $bla_{Oxa\text{-}181}$. Among studied genes, $bla_{NDM\text{-}1}$ was detected in four isolates (isolates A–D). It was negative in remaining isolates. No PCR product was found for bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{SPM}, $bla_{Oxa\text{-}48}$, and $bla_{Oxa\text{-}181}$ in all of the isolates. PCR amplification and sequencing of ESBL genes (bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}Mg8/25}$, bla_{VEB}, bla_{GES}, and bla_{PER}) revealed that all of the $bla_{NDM\text{-}1}$-positive isolates possessed ESBL genes. Isolate A coharbor $bla_{SHV\text{-}11}$, $bla_{CTX\text{-}M\text{-}15}$, and $bla_{TEM\text{-}1}$; isolate B harbored $bla_{SHV\text{-}11}$ and $bla_{CTX\text{-}M\text{-}15}$; and isolates C and D contained both $bla_{SHV\text{-}1}$ and $bla_{CTX\text{-}M\text{-}15}$. Moreover, among the 166 NDM-1-negative isolates, bla_{SHV} was identified in 138 (83.1%) of the isolates and was the dominant gene in ESBLs. Fifty-eight (34.9%) and 45 (27.1%) of isolates were positive for $bla_{CTX\text{-}M}$ and bla_{TEM} type β-lactamase, respectively.

MLST result

To understand the clonal relationship of the isolates, MLST was carried out and analysis of the results revealed that four NDM-1-positive isolates belonged to two different sequence types (STs), namely ST 392 and ST 13. Isolates A and B belonged to ST 392 and isolates C and D belonged to ST 13.

Discussion

Carbapenem-resistant *K. pneumoniae* is the focus of significant attention in the healthcare settings worldwide.[30,31] Currently, NDM-producing *K. pneumoniae* have been reported from different parts of the world.[13,32]

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Currently 16 variants of NDM (NDM-1 to NDM-16) have been reported on the Lahey Clinic β-lactamase website (www.lahey.org/Studies/); in our study we found NDM-1 in only four isolates (isolates A–D). It has been established that NDM-1-producing bacteria are usually resistant to broad ranges of antimicrobial agents, except aztreonam.[13] Although, aztreonam cannot be hydrolyzed by the NDM-1 enzyme, due to coproduction of ESBLs (frequently by CTX-M types) and plasmid-mediated AmpC cephalosporinase, over 80% of bacteria with NDM-1 are resistant to aztreonam.[13]

In our study, NDM-1-positive isolates were resistant to the most antimicrobial agents and all of them coharbored $bla_{\text{CTX-M-15}}$ and $bla_{\text{SHV-types}}$ ESBLs ($bla_{\text{SHV-1}}$ and $bla_{\text{SHV-11}}$). Aztreonam resistance in these isolates is probably due to the simultaneous production of ESBLs particularly CTX-M-15 that is capable to hydrolyze aztreonam. It is interesting to note, in accordance with our result, coexistence of NDM-1 with CTX-M-15, TEM-1, and SHV-11 has previously been described in *K. pneumoniae*.[33,34] In our study, MLST analysis indicated that four NDM-1-positive isolates belonged to ST 392 and ST 13 and are clonally related. *K. pneumoniae* ST 392 harboring NDM-1, SHV-11, and CTX-M-15 have been reported from Columbia.[35] Furthermore, we found that tigecycline, colistin, and fosfomycin were the most effective agents *in vitro* against NDM-1-producing isolates. Unfortunately, these antimicrobial agents had some limitation for extensive clinical usage[36] and it seems that treatment options for these resistant isolates are very limited.

In our study, all of NDM-1-positive isolates were recovered only from Shahid Mohammadi Hospital's wards (Table 2). It seems that these organisms are disseminated in different wards of this hospital and are not related with a particular ward. It is noteworthy that one of our major concerns is that, these resistant isolates become endemic and will be disseminated to other hospitals. While the epidemiological link between infection with NDM-1-producing bacteria and the Indian subcontinent has been established,[32] we were unable to find whether our patients had a history of hospitalization in the Indian subcontinent and the Balkan, or had traveled to these regions. Therefore, we believe that probably these patients have been infected locally.

NDM-1-producing *K. pneumoniae* has been reported from neighboring countries, such as Iraq, Kuwait, Turkey, Oman, and Pakistan.[33,37–40] Furthermore, NDM-1-producing *K. pneumoniae* has recently been sporadically reported from some parts of Iran.[41–43] According to these findings, we speculate that NDM-1-positive isolates have already disseminated and are now present in our region. Among the aforementioned studies from Iran and neighboring countries, only one study used MLST for typing of NDM-1-positive isolates. In that study, Poirel *et al*. reported ST 14 and ST 340 among NDM-1-positive *K. pneumoniae* in the Sultanate of Oman.[31] Since, there is no information about ST of NDM-1-positive isolates in other studies, we were unable to define the epidemiological link between our isolates with NDM-1-positive *K. pneumoniae* that reported from Iran and our neighboring countries.

In the microbiological laboratory, phenotypic tests are needed for accurate detection and reporting of carbapenemase producer's bacteria. The MHT has been proposed for detection of carbapenemase, but it has been proved that this test had poor sensitivity in detecting NDM-1 producers. Recently, a sensitive Hodge test named as THT was recommended for detection of the NDM-1 producers.[19] We used both of them (Hodge and Triton test) for identification of NDM-1 producers. Our results indicated that MHT had excellent sensitivity and specificity (100%) in the identification of NDM-1 producers. Compared with MHT, Triton test had low specificity (95.78%) in detection of NDM-1 producers, but the sensitivity of this test was similar to MHT (100% sensitivity).

Interestingly, among 170 studied isolates, seven imipenem-resistant isolates had a negative Hodge test, but they were positive in Triton test. It has been previously reported that ESBL/AmpC production can give false positive results in THT.[19,44] Since these seven isolates harbored $bla_{\text{CTX-M-28}}$, $bla_{\text{SHV-11}}$, $bla_{\text{SHV-12}}$, and $bla_{\text{TEM-1}}$ genes, probably false positive in THT may be related to these genes. Also, AmpC production can lead to false positive results in THT, but this gene was not investigated in our isolates and this is one limitation of our study. We used the DPA and EDTA disc synergy for detection of MBLs production. Although, DPA and EDTA detected all of the NDM-1-positive isolates, six imipenem-resistant isolates showed false positive in both tests. It should be noted that no MBLs genes were detected in these isolates. Due to false positive results, it seems that these tests cannot discriminate between metallo-β-lactamase genes. Thus, results suggested that DPA and EDTA could be helpful for the identification of MBLs producers, but these tests cannot discriminate the type of MBLs genes. Since PCR followed by sequencing is the gold standard test for the detection of NDM-1-positive organisms,[13] it is suggested that this method be used routinely in microbiology laboratories for the correct and timely detection of NDM-1-producing *K. pneumoniae* strains.

Conclusion

In conclusion, we identified four NDM-1-harboring *K. pneumoniae* isolates in Bandar Abbas. These isolates were resistant to almost all treatment options. Furthermore, tracing, accurate identification, and reporting of NDM-1- producing *K. pneumoniae* are very important to prevent the dissemination of such resistant isolates in the near future. Active recognition and appropriate screening of patients who had been colonized or infected with NDM-1-positive *K. pneumoniae* isolates are very vital in controlling such resistant isolates.

This study has several limitations that should be considered. Additional samples should be investigated in the near future. Determination of MIC of all antibiotics could help us to find pan-drug-resistant isolates. Since other resistance mechanisms such as AmpC hyperproduction and porin loss were not investigated in our study, we were not able to define sensitivity and specificity of each phenotypic test because these mechanisms can affect the results. It is suggested that these mechanisms be investigated in future studies.

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Disclosure Statement

No competing financial interests exist.

References

1. Hu, L., Q. Zhong, J. Tu, Y. Xu, Z. Qin, C. Parsons, B. Zhang, X. Hu, L. Wang, F. Yu, and J. Pan. 2013. Emergence of blaNDM-1 among *Klebsiella pneumoniae* ST15 and novel ST1031 clinical isolates in China. Diagn. Microbiol. Infect. Dis. 75:373–376.
2. Siu, L.K., K.M. Yeh, J.C. Lin, C.P. Fung, and F.Y. Chang. 2012. *Klebsiella pneumoniae* liver abscess: a new invasive syndrome. Lancet Infect. Dis. 12:881–887.
3. Corbellini, S., F. Caccuri, M. Gelmi, M.A. De Francesco, S. Fiorentini, A. Caruso, and C. Giagulli. 2014. Emergence of carbapenem-resistant *Klebsiella Pneumoniae* strains producing KPC-3 in Brescia Hospital, Italy. New Microbiol. 37:177–183.
4. Lee, C.R., J.H. Lee, K.S. Park, Y.B. Kim, B.C. Jeong, and S.H. Lee. 2016. Global dissemination of carbapenemase-producing *Klebsiella pneumoniae*: epidemiology, genetic context, treatment options, and detection methods. Front. Microbiol. 7:895.
5. Dahiya, S., P. Singla, U. Chaudhary, and B. Singh. 2015. Carbapenemases: a review. Int. J. Adv. Health Sci. 2:11–17.
6. Chauhan, K., A. Pandey, A.K. Asthana, and M. Madan. 2015. Evaluation of phenotypic tests for detection of *Klebsiella pneumoniae* carbapenemase and metallo-beta-lactamase in clinical isolates of Escherichia coli and Klebsiella species. Indian J. Pathol. Microbiol. 58:31–35.
7. Jeon, J.H., J.H. Lee, J.J. Lee, K.S. Park, A.M. Karim, C.R. Lee, B.C. Jeong, and S.H. Lee. 2015. Structural basis for carbapenem-hydrolyzing mechanisms of carbapenemases conferring antibiotic resistance. Int. J. Mol. Sci. 16:9654–9692.
8. Arpin, C., P. Noury, D. Boraud, L. Coulange, A. Manetti, C. André, F. M'Zali, and C. Quentin. 2012. NDM-1-producing *Klebsiella pneumoniae* resistant to colistin in a French community patient without history of foreign travel. Antimicrob. Agents Chemother. 56:3432–3434.
9. Pérez, J.A.E., N.M.O. Escobar, B. Castro-Cardozo, I.A.V. Márquez, M.I.G. Aguilar, L.M. de la Barrera, E.R.B. Barreto, R.A. Marquez-Ortiz, M.V.M. Guayazán, and N.V. Gómez. 2013. Outbreak of NDM-1-producing *Klebsiella pneumoniae* in a neonatal unit in Colombia. Antimicrob. Agents Chemother. 57:1957–1960.
10. Pillai, D.R., A. McGeer, and D.E. Low. 2011. New Delhi metallo-beta-lactamase-1 in Enterobacteriaceae: emerging resistance. CMAJ 183:59–64.
11. Poirel, L., G. Revathi, S. Bernabeu, and P. Nordmann. 2011. Detection of NDM-1-producing *Klebsiella pneumoniae* in Kenya. Antimicrob. Agents Chemother. 55:934–936.
12. Poirel, L., M. Yilmaz, A. Istanbullu, F. Arslan, A. Mert, S. Bernabeu, and P. Nordmann. 2014. Spread of NDM-1-producing Enterobacteriaceae in a neonatal intensive care unit, Istanbul, Turkey. Antimicrob. Agents Chemother. 58:2929–2933.
13. Nordmann, P., L. Poirel, T.R. Walsh, and D.M. Livermore. 2011. The emerging NDM carbapenemases. Trends Microbiol. 19:588–595.
14. Dortet, L., L. Poirel, and P. Nordmann. 2014. Worldwide dissemination of the NDM-type carbapenemases in Gram-negative bacteria. BioMed Res. Int. 2014:249856.
15. Winn Washington, C., S.D. Allen, W.M. Janda, E.W. Koneman, G.W. Procop, P.C. Schreckenberger, and G.L. Woods. 2006. Koneman's Color Atlas and Textbook of Diagnostic Microbiolgy. Lippincott, Williams & Wilkins, Philadelphia.
16. Clinical and Laboratory Standards Institute. 2016. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-sixth informational supplement, CLSI document M100-S26. Clinical and Laboratory Standards Institute, Wayne, PA.
17. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.0 hweocb.
18. Clinical and Laboratory Standards Institute. 2014. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-fourth informational supplement, CLSI document M100-S24. Clinical and Laboratory Standards Institute, Wayne, PA.
19. Fernando Pasteran A.L.J.G., B. Ezequiel Albornoz, A. Guillermo Bahr, B. Alejandro, J. Vila, B. Alejandra Corsoa. 2016. Triton Hodge Test: improved protocol for modified Hodge Test for enhanced detection of NDM and other carbapenemase producers. J. Clin. Microbiol. 54:640–649.
20. Tsakris A., I. Kristo, A. Poulou, K. Themeli-Digalaki, A. Ikonomidis, D. Petropoulou, S. Pournaras, and D. Sofianou. 2009. Evaluation of boronic acid disk tests for differentiating KPC-possessing *Klebsiella pneumoniae* isolates in the clinical laboratory. J. Clin. Microbiol. 47:362–367.
21. Shina, K.S. 2008. Dipicolinic acid-based disk methods for detection of metallo-β-lactamase–producing *Pseudomonas* spp. and *Acinetobacter* spp. Diagn Microbiol. Infect. Dis. 62:102–105.
22. Giske, C.G. 2011. A sensitive and specific phenotypic assay for detection of metallo-blactamases and KPC in *Klebsiella pneumoniae* with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin. Clin. Microbiol. Infect. 17:552–556.
23. Petropoulou, D., K. Tzanetou, V.P. Syriopoulou, G.L. Daikos, G. Ganteris, and E. Malamou-Lada. 2006. Evaluation of imipenem/imipenem+ EDTA disk method for detection of metallo-β-lactamase-producing *Klebsiella pneumoniae* isolated from blood cultures. Microb. Drug Resist. 12:39–43.
24. Chen, L., J.R. Mediavilla, A. Endimiani, M.E. Rosenthal, Y. Zhao, R.A. Bonomo, and B.N. Kreiswirth. 2011. Multiplex real-time PCR assay for detection and classification of *Klebsiella pneumoniae* carbapenemase gene (bla KPC) variants. J. Clin. Microbiol. 49:579–585.
25. Diancourt, L., V. Passet, J. Verhoef, P.A. Grimont, and S. Brisse. 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J. Clin. Microbiol. 43: 4178–4182.
26. Poirel, L., T.R. Walsh, V. Cuvillier, and P. Nordmann. 2011. Multiplex PCR for detection of acquired carbapenemase genes. Diagn. Microbiol. Infect. Dis. 70:119–123.
27. Lascols, C., M. Hackel, S.H. Marshall, A.M. Hujer, S. Bouchillon, R. Badal, D. Hoban, and R.A. Bonomo. 2011. Increasing prevalence and dissemination of NDM-1 metallo-β-lactamase in India: data from the SMART study (2009). J. Antimicrob. Chemother. 66:1992–1997.
28. Potron, A., P. Nordmann, E. Lafeuille, Z. Al Maskari, F. Al Rashdi, and L. Poirel. 2011. Characterization of OXA-181, a carbapenem-hydrolyzing class D beta-lactamase from *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. 55: 4896–4899.
29. Dallenne, C., A. Da Costa, D. Decré, C. Favier, and G. Arlet. 2010. Development of a set of multiplex PCR assays for the

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detection of genes encoding important β-lactamases in Enterobacteriaceae. J. Antimicrob. Chemother. 65:490–495.

30. Girmenia, C., A. Serrao, and M. Canichella. 2016. Epidemiology of carbapenem resistant *Klebsiella pneumoniae* infections in mediterranean countries. Mediterr J. Hematol. Infect. Dis. 8:e2016032.
31. Park, S.O., J. Liu, E.Y. Furuya, and E.L. Larson (eds). 2016. Carbapenem-resistant *Klebsiella pneumoniae* infection in three New York City hospitals trended downwards from 2006 to 2014. Open Forum Infect. Dis. 3:ofw222.
32. Johnson, A.P., and N. Woodford. 2013. Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. J. Med. Microbiol. 62:499–513.
33. Poirel, L., Z. Al Maskari, F. Al Rashdi, S. Bernabeu, and P. Nordmann. 2010. NDM-1-producing *Klebsiella pneumoniae* isolated in the Sultanate of Oman. J. Antimicrob. Chemother. 56: 598–599.
34. Poirel, L., J. Schrenzel, A. Cherkaoui, S. Bernabeu, G. Renzi, and P. Nordmann. 2011. Molecular analysis of NDM-1-producing enterobacterial isolates from Geneva, Switzerland. J. Antimicrob. Chemother. 66:1730–1733.
35. Rojas, L.J., M.S. Wright, E. De La Cadena, G. Motoa, K.M. Hujer, M.V. Villegas, M.D. Adams, and R.A. Bonomo. 2016. Initial assessment of the molecular epidemiology of blaNDM-1 in Colombia. Antimicrob. Agents Chemother. 60:4346–4350.
36. Morrill, H.J., J.M. Pogue, K.S. Kaye, and K.L. LaPlante (eds). 2015. Treatment options for carbapenem-resistant Enterobacteriaceae infections. Open Forum Infect. Dis. 2:ofv050.
37. Jamal, W., V. Rotimi, M.J. Albert, F. Khodakhast, E. Udo, and L. Poirel. 2012. Emergence of nosocomial New Delhi metallo-β-lactamase-1 (NDM-1)-producing *Klebsiella pneumoniae* in patients admitted to a tertiary care hospital in Kuwait. Int. J. Antimicrob. Agents 39:183–184.
38. Karabay, O., M. Altindis, M. Koroglu, O. Karatuna, Ö.A. Aydemir, and A.F. Erdem. 2016. The carbapenem-resistant Enterobacteriaceae threat is growing: NDM-1 epidemic at a training hospital in Turkey. Ann. Clin. Microbiol. Antimicrob. 15:6.
39. Perry, J.D., S.H. Naqvi, I.A. Mirza, S.A. Alizai, A. Hussain, S. Ghirardi, S. Orenga, K. Wilkinson, N. Woodford, and J. Zhang. 2011. Prevalence of faecal carriage of Enterobacteriaceae with NDM-1 carbapenemase at military hospitals in Pakistan, and evaluation of two chromogenic media. J. Antimicrob. Chemother. 66:2288–2294.
40. Poirel, L., N. Fortineau, and Nordmann P. 2011. International transfer of NDM-1-producing *Klebsiella pneumoniae* from Iraq to France. Antimicrob. Agents Chemother. 55: 1821–1822.
41. Fazeli, H., M. Norouzi-Barough, A. Ahadi, D. Shokri, and H. Solgi. 2015. Detection of New Delhi Metallo-Beta-Lactamase-1 (NDM-1) in carbapenem-resistant *Klebsiella pneumoniae* isolated from a university hospital in Iran. Hippokratia 19:205.
42. Sadeghi, M.R., R. Ghotaslou, M.T. Akhi, M. Asgharzadeh, and A. Hasani. 2016. Molecular characterization of extended-spectrum β-lactamase, plasmid-mediated AmpC cephalosporinase and carbapenemase genes among Enterobacteriaceae isolates in five medical centres of East and West Azerbaijan, Iran. J. Med. Microbiol. 65:1322–1331.
43. Shahcheraghi, F., S. Nobari, F. Rahmati Ghezelgeh, S. Nasiri, P. Owlia, V.S. Nikbin, and A.A. Imani Fooladi. 2013. First report of New Delhi metallo-beta-lactamase-1-producing *Klebsiella pneumoniae* in Iran. Microb. Drug Resist. 19:30–36.
44. Carvalhaes, C.G., R.C. Picão, A.G. Nicoletti, D.E. Xavier, and A.C. Gales. 2010. Cloverleaf test (modified Hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae*: be aware of false positive results. J. Antimicrob. Chemother. 65:249–251.

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