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ORIGINAL RESEARCH

The Phylogenetic Relatedness of bla_{NDM-1} Harboring Extended-Spectrum β-Lactamase Producing Uropathogenic *Escherichia coli* and *Klebsiella pneumoniae* in the North of Iran

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Background: *Escherichia coli* and *Klebsiella pneumoniae* as an important part of *Enterobacterales* family are important causes of both community- and hospital-acquired infections. The present study aimed to investigate the prevalence of antibiotics resistance and molecular characteristics of uropathogenic isolates of *E. coli* and *K. pneumoniae* in Iranian patients.

Methods: This cross-sectional study performed on 223 *Escherichia coli* and 68 *Klebsiella pneumoniae* isolates obtained from hospitalized patients in the north of Iran. The isolates were identified by standard microbiologic tests and confirmed by API 20E strip. Disk diffusion method was applied to determine antibiotic susceptibility pattern. The presence of β-lactamases encoding genes was evaluated by PCR method. Analysis of the mutations and homology among sequences was done by the CLC sequence viewer (Qiagen, Denmark), and phylogenetic trees were constructed by the neighbor-joining method (Bootstrap: 1000 times).

Results: The overall rates of extended-spectrum β-lactamases (ESBLs)-producing *E. coli* and *K. pneumoniae* isolates were 37.7% and 32.4%, respectively. The overall presence of bla_{SHV}, bla_{NDM-1}, and bla_{OXA-1} genes was detected in 16 (5.5%), 12 (4.1%), and 48 (16.4%) of isolates, respectively. The neighbor-joining analysis for *E. coli* KU985246.1 strain showed that the most related bla_{NDM-1} sequences were from China, Singapore, UK, Thailand, and Bangladesh. While *K. pneumoniae* KU985245.1 strains were mostly related to bla_{NDM-1} sequences form Myanmar, and China.

Conclusion: In summary, the remarkable rate of ESBL-producing uropathogenic *Enterobacterales* along with the first prevalence of NDM-1 β-lactamases can be a serious concern in our region.

Keywords: uropathogenic, *Escherichia coli*, *Klebsiella pneumoniae*, antibiotic resistance, ESBL, NDM-1 β-lactamases

Introduction

Escherichia coli and *Klebsiella pneumoniae* are Gram negative, facultative anaerobic rods belonging to the family of *Enterobacterales* which are part of the gastrointestinal tract microbiota.[1] These opportunistic pathogens lead to both community- and hospital-acquired infections with a wide range of complications, such as blood stream, respiratory tract, surgical wounds, gastrointestinal, and urinary tract infections.[1,2]

Beta-lactam (β-lactam) antibiotics are amongst the widely prescribed agents for managing *E. coli* and *K. pneumoniae* related infections.[3,4] However, recently extended-

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spectrum beta-lactamases (ESBLs) producing strains have emerged as a critical health issue.[3] The hydrolyzing activity of ESBLs which results in the inactivation of penicillins, broad-spectrum cephalosporins, and monobactams.[3,4] These enzymes are divided into several main groups, including SHV, TEM, and CTX-M, which are able to inactivate the b-lactams by hydrolyzing the b-lactam ring.[3]

ESBLs cannot efficiently inactivate cephamycins, beta-lactamase inhibitors, and particularly carbapenems. So, carbapenems act as important therapeutic options against multiple-drug resistant (MDR) strains.[5] However, recently the trend of carbapenemase producing *Enterobacterales* has increased all around the world, as well as in Iran.[6–8] According to the Ambler classification of β-lactamases, two main classes of carbapenemases were introduced. One of them is class B Zinc metallo-beta-lactamase (MBL), susceptibleto metallic ion chelator such as EDTA,[9] in which New Delhi metallo-β-lactamase (NDM) is the most important enzyme.[10] Secondly come oxacillinase (OXA) enzymes or class D β-lactamases, subclassified based on single amino acid substitutions or deletions that are responsible for their effective hydrolyzing activity.[10]

The β-lactamase production is mostly associated with achievement of resistance determinant genes carried on large plasmids whose dissemination by horizontal gene transfer among different species has become a serious global health problem.[11]

In regard to the importance of epidemiological data on ESBLs and carbapenems producing strains, we aimed to analyze the molecular characteristics of uropathogenic *E. coli* and *K. pneumoniae* and its antibiotics resistance pattern in northern Iran.

Materials and Methods

Study Design and Bacterial Isolates

This cross-sectional study was performed at 5 teaching hospitals in the north of Iran within six months. This study has been designed in accordance with the declaration of Helsinki and also the regional approval obtained by University Ethics Committee (IR.GUMS.REC.1394.641). The presumptive *E. coli* and *K. pneumoniae* isolates were isolated from clean-catch urine specimens using standard microbiologic tests and API 20E strips (API-bioMérieux, France).

Antimicrobial Susceptibility Testing

The antibiotic susceptibility of isolates was tested by standard disk diffusion method on Mueller-Hinton agar medium (Merck, Germany) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[12] The antibiotic disks were ampicillin (10 µg), amoxicillin-clavulanate (20/10 µg), cefepime (30 µg), aztreonam (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), cefoxitin (30 µg), cefixime (5 µg), ceftazidime (30 µg), cefotaxime (30 µg), imipenem (10 µg), tetracycline (30 µg), trimethoprim/sulfamethoxazole (25 µg), and nitrofurantoin (300 µg). *E. coli* ATCC 25922 strain was employed for quality check purpose. Multiple-drug resistant (MDR) isolates were defined non-susceptible (resistant or intermediate) to at least 1 agent in ≥3 antimicrobial categories according to previously mentioned definitions.[13] ESBL testing was performed by double-disk synergy test using cefotaxime (30 µg) and ceftazidime (30 µg), alone and in combination with clavulanate (10 µg).[12] *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive control strains, respectively.

Plasmid DNA Extraction and Molecular Assays

Plasmid DNA extraction from the fresh isolates was done using gene JET plasmid miniprep Kit (Fermentas, Lithuania) according to its instructions. PCR was performed to detect bla_{SHV}, bla_{OXA-1}, and bla_{NDM} genes by a Veriti 96-well thermal cycler instrument (Applied Biosystems at Life Technologies, Foster City, CA) as previously described.[14–16] Primers were provided by Metabion Co, Germany. The list of used primers is shown in Table 1. PCR was done in a final volume of 20 µL containing Master Mix (Bioneer, South Korea), primers at concentrations of 0.3–0.4 µM, 3µL DNA templates and ddH_2O. All controls for PCR were kindly provided by Pasteur Institute, Tehran, Iran. The PCR setup was an initial denaturation step at 94°C for 3 min, followed by 30 cycles of DNA denaturation at 94°C for 30 s, primer

Table 1 Primer Sequences Used in the Present Study

Primer	Oligonucleotide Sequence (5′ to 3′)	Gene	Reference
SHV-F	ATGCGTTATATTCGCCTGTG	bla_{SHV}	13
SHV-R	TGCTTTGTTATTCGGGCCAA		
NDM-F	GCAGCTTGTCGGCCATGCGGGC	bla_{NDM}	14
NDM-R	GGTCGCGAAGCTGAGCACCGCAT		
OXA-F	TTTTCTGTTGTTTGGGTTTT	bla_{OXA-1}	15
OXA-R	TTTCTTGGCTTTTATGCTTG		

annealing for 45 sec Temperatures varied based on the sequences of primers, and primer extension at 72°C for 50 sec, followed by a final extension at 72°C for 5 min. The products were separated by electrophoresis in 1% agarose gels with 1 X TAE (Tris/Acetate/EDTA) buffer, stained with safe stain load dye (CinnaGen Co., Iran), and visualized under ultraviolet illumination.

DNA Sequencing

To confirm PCR products, a purified amplicon of tested genes was sent for sequencing by the ABI capillary system (Macrogen Research, Seoul, Korea). Afterwards, sequences were confirmed by comparison with available sequences on NCBI database (http://www.ncbi.nlm. nih.gov/BLAST/). These sequences were submitted in the GenBank nucleotide database under accession numbers: MG797557 (OXA-1, *E. coli*), KU985247.1 (OXA-1, *K. pneumoniae*), MG797558 (SHV-1, *E. coli*), MG797559 (SHV-1, *K. pneumoniae*), KU985246.1 (NDM-1, *E. coli*), and KU985245.1 (NDM-1, *K. pneumoniae*) available at National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov). The mutations and homology among sequences were analyzed by the CLC sequence viewer (Qiagen, Denmark), and phylogenetic trees were constructed by the neighbor-joining method (Bootstrap: 1000 times).

Statistical Analysis

The distribution of antibiotic resistance and genes between groups was calculated by Chi-square and Fisher's exact tests using SPSS^{TM} software, version 21.0 (IBM Corp., USA). A P value of ≤0.05 was considered as statistically significant.

Results

Generally, 291 uropathogenic isolates, consisting of 223 *E. coli* and 68 *K. pneumoniae* isolates were included in our study. *E. coli* isolates were obtained from 169 (75.8%) female and 54 (24.2%) male subjects with a mean age of 35.8 ± 26.3 years. Also, *K. pneumoniae* isolates were obtained from 38 (55.9%) female and 30 (44.1%) male subjects with a mean age of 28.3 ± 26.7 years. In overall, the age range of included patients was from 1 month to 89 years.

The results revealed that *E. coli* isolates were mostly non-susceptible to ampicillin (84.8%) followed by nalidixic acid (78.5%) and tetracycline (65.5%) while the highest susceptibility belonged to imipenem (97.8%). Regarding antibiotic susceptibility among *K. pneumoniae* isolates, the lowest susceptibility rate was held by nalidixic acid (29.4%) followed by nitrofurantoin (47.1%), and the highest susceptibility belonged to imipenem (94.1%). Further analysis revealed that the rate of ESBL-producing *E. coli* and *K. pneumoniae* isolates were 37.7% and 32.4%, respectively. ESBLs producers showed significantly higher antibiotic resistance compared to non-ESBLs (Tables 2 and 3). Moreover, the most effective in vitro agent against ESBL-producing in both uropathogenic isolates was imipenem. The distribution of ESBLs isolates according to patients age was 28 (26.4%) among patients less than 18 years, 55 (51.9%) among 19–64 years, and 23 (21.7%) among older than 65 years old.

The overall presence of bla_{SHV}, bla_{NDM-1}, and bla_{OXA-1} genes was detected in 16 (5.5%), 12 (4.1%), and 48 (16.4%) of isolates, respectively. Full results of investigated genes separated by pathogen, MDR, ESBLs, and carbapenem resistance are presented in Table 4. The investigated genes were significantly more prevalent in ESBLs-producing isolates in both uropathogens whereas only the presence of bla_{NDM-1} gene in *K. pneumoniae* was significantly associated with carbapenem-resistant isolates.

The neighbor-joining analysis for *E. coli* KU985246.1 strain indicated that the most related bla_{NDM-1} sequences were from China, Singapore, UK, Thailand, and Bangladesh. However, *K. pneumoniae* KU985245.1 strains were mostly related to bla_{NDM-1} sequences from Myanmar and China (Figure 1).

The pervasiveness of MDR isolates was estimated at 82.5% in *E. coli* and 60.3% in *K. pneumoniae* while the rate of MDR isolates was significantly higher in ESBLs-producing isolates, as 45.7% of *E. coli*, and 53.7% of *K. pneumoniae* isolates were MDR ($P < 0.001$).

The co-occurrence of β-lactamase genes in *E. coli* isolates was one bla_{SHV-1}/bla_{NDM-1}, and one bla_{OXA-1}/bla_{NDM-1} isolates. This pattern in *K. pneumoniae* isolates was one $bla_{SHV-1}/bla_{NDM-1}/bla_{OXA-1}$, 3 bla_{OXA-1}/bla_{NDM-1}, and 7 bla_{SHV-1}/bla_{OXA-1} isolates.

Discussion

The emergence of MDR *Enterobacterales*, particularly ESBLs-producing strains, is responsible for a large rate of nosocomial outbreaks, related to increased morbidity and mortality.[17] Drug-resistant strains of *E. coli* and *K. pneumoniae* have acquired resistance to most—and in some cases to all antibiotics that are currently available in the clinic. This problem demands looking for novel antibiotics targeting drug-resistant Gram-negative pathogens.

Table 2 The Results of Antibiotic Susceptibility Pattern of *E. coli* Isolates

Antibiotic	Total No. 223			ESBLs-Positive No. 84			P value[a]
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant	
Ampicillin	34 (15.2)	8 (3.6)	181 (81.2)	1 (1.2)	1 (1.2)	82 (97.6)	<0.001
Amoxicillin-clavulanate	98 (43.9)	58 (26)	67 (30)	19 (22.6)	35 (41.7)	30 (35.7)	<0.001
Cefoxitin	176 (78.9)	21 (9.4)	26 (11.7)	61 (72.6)	13 (15.5)	10 (11.9)	0.073
Cefixime	99 (44.4)	16 (7.2)	108 (48.4)	1 (1.2)	0	83 (98.8)	<0.001
Ceftazidime	115 (51.6)	20 (9)	88 (39.5)	6 (7.1)	10 (11.9)	68 (81)	<0.001
Cefotaxime	111 (49.8)	9 (4.0)	103 (46.2)	3 (3.6)	1 (1.2)	80 (95.2)	<0.001
Cefepime	148 (66.4)	19 (8.5)	56 (25.1)	21 (25)	15 (17.9)	48 (57.1)	<0.001
Ciprofloxacin	114 (51.1)	13 (5.8)	96 (43)	21 (25.0)	3 (3.6)	60 (71.4)	<0.001
Ofloxacin	126 (56.5)	3 (1.3)	94 (42.2)	25 (29.8)	0	59 (70.2)	<0.001
Nalidixic acid	48 (21.5)	16 (7.2)	159 (71.3)	5 (6)	3 (3.6)	76 (90.5)	<0.001
Aztreonam	123 (55.2)	16 (7.2)	84 (37.7)	4 (4.8)	13 (15.5)	67 (79.8)	<0.001
Tetracycline	77 (34.5)	3 (1.3)	143 (64.1)	22 (26.2)	1 (1.2)	61 (72.6)	0.042
Gentamicin	191 (85.7)	7 (3.1)	25 (11.2)	63 (75)	4 (4.8)	17 (20.2)	<0.001
Trimethoprim sulfamethoxazole	85 (38.1)	0	138 (61.9)	17 (20.2)	0	67 (79.8)	<0.001
Nitrofurantoin	199 (89.2)	6 (2.7)	18 (8.1)	66 (78.6)	3 (3.6)	15 (17.9)	<0.001
Imipenem	218 (97.8)	5 (2.2)	0	82 (97.6)	2 (2.4)	0	0.91

Note: [a]Compared with antibiotic resistance pattern of non-ESBL-producing isolates.

Table 3 The Results of Antibiotic Susceptibility Pattern of *K. pneumoniae* Isolates

Antibiotic	Total No. 68			ESBLs-Positive No. 22			P value[a]
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant	
Amoxicillin-clavulanate	35 (51.5)	7 (10.3)	26 (38.2)	1 (4.5)	6 (27.3)	15 (68.2)	<0.001
Cefoxitin	55 (80.9)	2 (2.9)	11 (16.2)	16 (72.7)	2 (9.1)	4 (18.2)	0.24
Cefixime	39 (57.4)	0	29 (42.6)	0	0	22 (100)	<0.001
Ceftazidime	38 (55.9)	2 (2.9)	28 (41.2)	0	1 (4.5)	21 (95.5)	<0.001
Cefotaxime	39 (57.4)	1 (1.5)	28 (41.2)	0	0	22 (100)	<0.001
Cefepime	44 (64.7)	2 (2.9)	22 (32.4)	3 (13.6)	1 (4.5)	18 (81.8)	<0.001
Ciprofloxacin	47 (69.1)	8 (11.8)	13 (19.1)	9 (40.9)	3 (13.6)	10 (45.5)	<0.001
Ofloxacin	53 (77.9)	2 (2.9)	13 (19.1)	12 (54.5)	1 (4.5)	9 (40.9)	0.001
Nalidixic acid	20 (29.4)	26 (38.2)	22 (32.4)	4 (18.2)	6 (27.3)	12 (54.5)	0.16
Aztreonam	40 (58.8)	2 (2.9)	26 (38.2)	1 (4.5)	1 (4.5)	20 (90.9)	<0.001
Tetracycline	42 (61.8)	0	26 (38.2)	9 (40.9)	0	13 (59.1)	0.014
Gentamicin	48 (70.6)	1 (1.5)	19 (27.9)	9 (40.9)	1 (4.5)	12 (54.5)	<0.001
Trimethoprim/sulfamethoxazole	42 (61.8)	1 (1.5)	25 (36.8)	7 (31.8)	0	15 (68.2)	<0.001
Nitrofurantoin	32 (47.1)	12 (17.6)	24 (35.3)	5 (22.7)	5 (22.7)	12 (54.5)	0.005
Imipenem	64 (94.1)	1 (1.5)	3 (4.4)	19 (86.4)	1 (4.5)	2 (9.1)	0.06

Note: [a]Compared with antibiotic resistance pattern of non-ESBL-producing isolates.

Table 4 The Full Results of Investigated Genes Separated by Pathogen, MDR, ESBLs, and Carbapenem Resistance

Gene	*E. coli*				*K. pneumoniae*			
	Total No. 223	ESBLs No. 84	IMI-R No. 5	MDR No. 184	Total No. 68	ESBLs No. 22	IMI-R No. 4	MDR No. 41
bla_{SHV}	6	6[a]	0	6	10	9[a]	0	9[a]
bla_{NDM}	4	4[a]	0	4	8	7[a]	3[a]	8[a]
bla_{OXA-1}	33	31[a]	0	32[a]	15	15[a]	0	15[a]

Note: [a]P value compared to susceptible isolates was statistically significant ($P < 0.05$).
Abbreviations: MDR, multiple-drug resistant; IMI-R, imipenem-resistant.

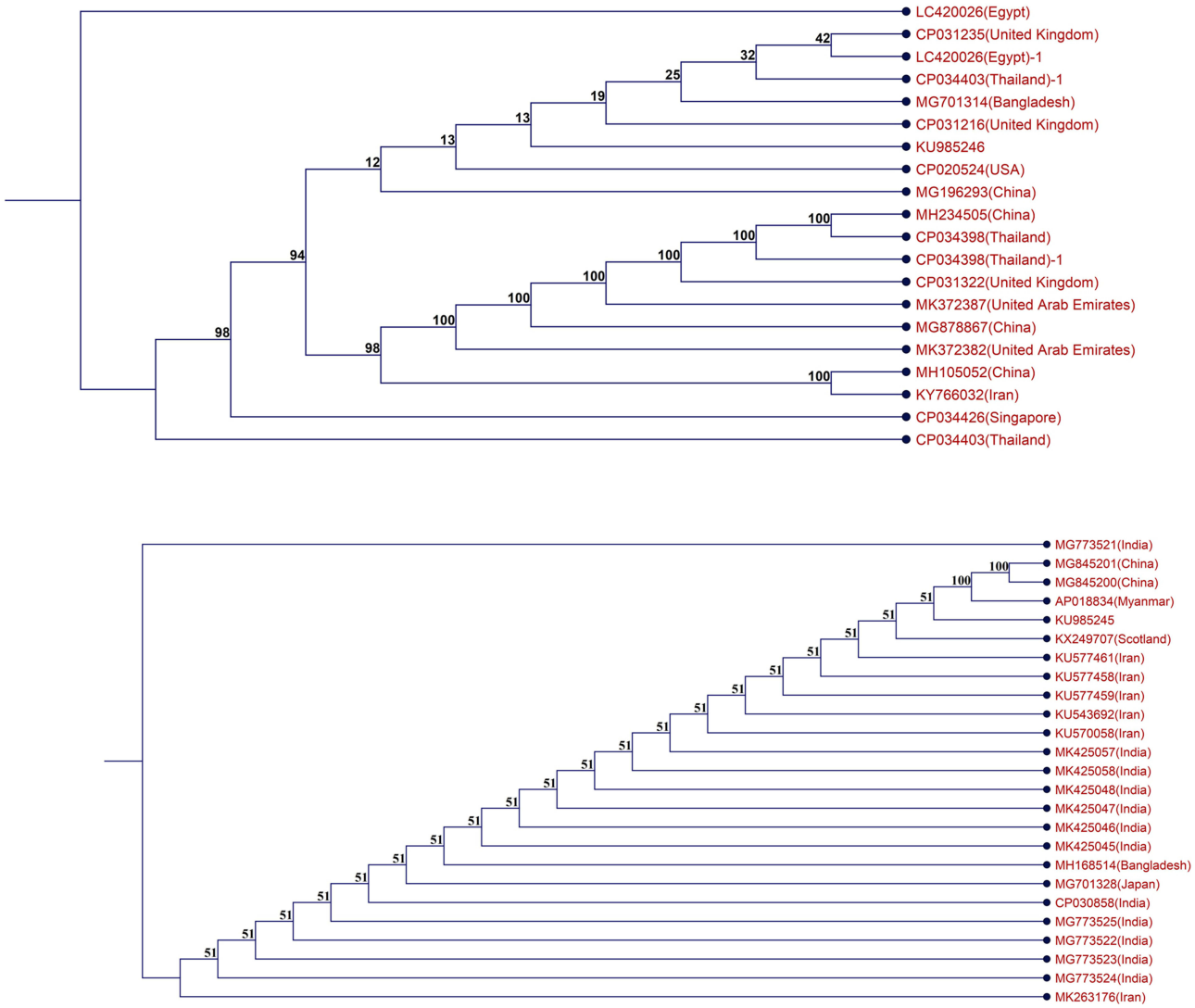

Figure 1 The neighbor-joining dendrogram (Boot strap: 1000) of two bla_{NDM-1} harboring isolates.

Darobactin is an example of the current efforts that showed a reasonable activity against important drug-resistant pathogens, *E. coli* and *K. pneumoniae*, including polymyxin-resistant, ESBLs and carbapenem-resistant isolates with a minimum inhibitory concentration (MIC) of 2 μg mL^{-1}.[18] The periodic surveillance for knowing the distribution and mechanisms of antibiotic resistance has an important role in preventing and overcoming the risk of complications attributed to ESBL-producing strains.[3]

In our study, 36.4% of all isolates were ESBL producers with a high rate of antibiotic resistance. Despite the great discrepancy in the rate of ESBLs-producing *Enterobacterales*, our finding is consistent with the median values (range 13.4% to 69.2%) reported in different uropathogens and regions of Iran.[19–22] The variations in the occurrence of ESBL producers can be in account of different infection control measures, infection types and studied population.

Regarding the emergence of ESBL-producing strains, the main concern is the spread of resistance determinants due to the horizontal gene transfer.[3] bla_{TEM}, bla_{SHV}, and bla_{CTX-M} are the most frequent ESBLs encoding genes.[23] In the present research, the proportion of bla_{SHV} genotype was low and found in 5.5% (2.7% in *E. coli* vs 14.7% in *K. pneumoniae*) of isolates. The prevalence of ESBLs genes among *Enterobacterales* was varied. Previously, the rates of bla_{SHV} containing *Enterobacterales* with a focus on uropathogenic isolates were reported 12.2% to 54.8% by Iranian authors.[24–29] Meanwhile, several authors have shown the global spared of SHV type β-lactamases in uro-

pathogenic *Enterobacterales* from China,[30] India,[31] Saudi Arabia,[32] Turkey,[33] Ukraine,[34] and Morocco.[35]

In our results, 4.1% of uropathogenic *Enterobacterales* contained $bla_{\text{NDM-1,}}$ which to the best of our knowledge, is the first prevalence of this gene in Guilan province (North of Iran). Previously, the most reported origin of NDM-1 producing bacteria belonged to Asian countries, including China, India, Turkey, Pakistan and our neighboring countries in Persian Gulf region.[10] Unfortunately, reports point out the increasing emergence of $bla_{\text{NDM-1}}$ harboring *Enterobacterales* in different regions of Iran. Concerning the emergence of $bla_{\text{NDM-1}}$ harboring *Enterobacterales*, there are some reports from the Capital (Tehran),[6,36,37] central part (Isfahan),[7] and southwest (Shiraz) of Iran.[10]

In the present study, the most prevailing β-lactamases encoding genes, generally 16.4% of isolates, and 44.4% of ESBLs-producing isolates were $bla_{\text{OXA-1}}$ positive. Previously, Alizadeh et al, in a study conducted in the southwest of Iran (Kerman), showed that 17.2% of uropathogenic isolates were OXA-1 producers.[20] Reviewing global reports such as Brazil (60%),[38] Portugal (55%),[39] Saudi Arabia (54.8%, and 48.3%),[32,40] and Bangladesh (47.5%),[41] indicate that the occurrence of OXA-1 β-lactamases can be varied based on type and source of isolates and the prevalence of ESBLs in each region.

The present work has some limitations: firstly, the β-lactamase expression was not assessed by real time-PCR, particularly concerning those isolates genetically positive for investigated genes yet phenotypically β-lactam susceptible. Secondly, other β-lactamases encoding genes reported in other Iranian studies should be further investigated. Finally, the availability of clinical features of the patient populations including treatments and outcomes of treatments could provide important information.

In summary, the prevalence of ESBL-producing uropathogenic *Enterobacterales* containing NDM-1 β-lactamases could be a major concern in our region. Carbapenems are still effective on our MDR isolates, but the co-occurrence of β-lactamases genes suggests restricted infection control policies and the rational prescription and use of antibiotics.

Disclosure

The authors report no conflicts of interest in this work.

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