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Correlation of quinolone-resistance, *qnr* genes and integron carriage in multidrug-resistant community isolates of *Klebsiella* spp.

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ABSTRACT

Objective(s): Plasmid-mediated quinolone resistance (PMQR) determinants and integrons have a considerable contribution to bacterial drug resistance in Gram-negative pathogens. We studied the prevalence of PMQR genes and integron carriage in multidrug-resistant community isolates of *Klebsiella* spp.

Materials and Methods: Two hundred and fifty *Klebsiella* spp. isolates were collected from outpatient specimens between August 2015 and October 2017 in Yazd central Laboratory, Iran. Antibiotic susceptibility was determined against 17 antibiotics and minimum inhibitory concentration (MIC) of ciprofloxacin was measured by E-test. Polymerase chain reaction (PCR) was employed for detection of *qnrA, qnrB, qnrS, aac(6')-Ib-cr, oqxAB* and *qepA* genes.

Results: Disc diffusion results showed that 17 isolates (6.8%) were multidrug resistant (MDR), two of which were *Klebsiella oxytoca* and 15 were *Klebsiella pneumoniae*. MIC measurements revealed 11 ciprofloxacin-resistant isolates (including the two *K. oxytoca*), three intermediately-resistant and three ciprofloxacin-susceptible isolates. All ciprofloxacin-resistant and intermediately-resistant isolates carried at least one and up to four PMQR genes. The most prevalent PMQR gene was oqxAB (93.75%) followed by aac(6')-ib-cr (50.0%), *qnrB* (25.0%) and *qnrS* (18.75%) but *qnrA* and *qepA* were not detected. Class 1 integron was observed in 14 (82.3%) isolates including nine ciprofloxacin-resistant, two intermediately-resistant, and three susceptible isolates. Class 2 and 3 integrons were not observed.

Conclusion: Presence of MDR, multiple PMQR determinants as well as class 1 integron in community isolates of *Klebsiella* spp. can be an important source of transmission of these opportunistic pathogens.

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Introduction

Klebsiella pneumoniae is an opportunistic pathogen and the cause of a significant number of nosocomial infections including pneumonia, wound, blood, intra-abdominal and urinary tract infections (1). Over the past few years, the most common treatment for these infections has been the use of extended-spectrum β-lactam antibiotics and fluoroquinolones (2). However, multidrug resistant (MDR) isolates are rapidly spreading not only in nosocomial infections but also more recently in the community isolates (2, 3). Previously, fluoroquinolone resistance was thought to be solely due to the mutations occurring within its DNA gyrase target gene (4). However, the discovery of plasmid-mediated quinolone resistance (PMQR) in clinical isolates of *Enterobacteriaceae* has shown to play an important role in quinolone resistance (5). PMQR genes, frequently found in clinical isolates of *Enterobacteriaceae*, include: *qnrA*, *qnrB*, *qnrS*, *qnrC* and *qnrD* which their pentapeptide protein products protect quinolones from inhibition by DNA gyrase and topoisomerase IV, as well as the aminoglycoside acetyltransferase (A*ac(6')-Ib-cr*) which is responsible for enzymatic modification of fluoroquinolones (5). Furthermore, fluoroquinolone-resistance is often observed in extended spectrum β-lactamase (ESBL) producing *K. pneumoniae* (6, 7). Over-expression of efflux pumps such as *QepA*, *AcrAB* and *OqxAB* also contribute to quinolone and fluoroquinolone resistance (8-10). Among the efflux pumps discovered in Gram-negative bacteria, the *oqxAB* was initially detected on a conjugative plasmid in *Escherichia coli* isolated from swine manure (11). Dissemination of *oqxAB*, a PMQR determinant, was later shown in clinical isolates of *K. pneumoniae* from Spain, South Korea and China (10, 12, 13). Furthermore, presence of PMQR genes on mobile genetic elements such as antibiotic resistant integrons, often carried by conjugative plasmids, has been shown to contribute to the dissemination of multidrug resistance in pathogens as well as environmental bacteria (14, 15). Among these mobile genetic elements, class 1 integron is the most commonly detected among Gram-negative MDR clinical isolates followed by class 2 integron (16, 17). However, there are few studies on integron carriage by community acquired isolates of *Klebsiella* spp. The aim of this study was to investigate the presence of multiple antibiotic resistance determinants in *Klebsiella* spp. isolates from outpatient specimens and the possible

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correlation between quinolone-resistance, PMQR and integron carriage in these organisms.

Materials and Methods

Bacterial isolates and identification

Two hundred and fifty non-duplicate community acquired *Klebsiella* isolates were collected from outpatient specimens in Yazd Central Laboratory between August 2015 and October 2017 (mean age between 35 and 81 years old). Conventional biochemical tests were used for identification of the isolates which were then maintained in Tryptic Soy Broth (TSB; Merck, Germany) containing 4% glycerol at −70 ˚C.

Antimicrobial susceptibility testing

Susceptibility testing against various antibiotics was performed by disc diffusion according to the CLSI guidelines (2017) using commercially available discs (Mast, UK) including: amoxicillin (AMX, 10 µg), cefalotin (CF, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), gentamicin (GM, 10 µg), nalidixic acid (NA, 30 µg), kanamycin (KM, 30 µg), amikacin (AN, 30 µg), trimethoprim/sulfamethoxazole (SXT, 23.75/1.25 µg), ciprofloxacin (CIP, 30 µg), ofloxacin (OFX, 5 µg), norfloxacin (NOR, 10 µg), nitrofurantoin (NF, 300 µg), meropenem (MEM, 10 µg), ertapenem (ETP, 10 µg), tetracycline (TE, 30 µg) chloramphenicol (CM, 30 µg) (18). *K. pneumoniae* ATCC 10031 was used as the susceptible control.

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) were measured for ciprofloxacin-resistant and intermediately-resistant isolates by E-test (Liofilchem, Italy) according to the CLSI 2017 guidelines (18).

DNA Extraction and PCR amplification

DNA extraction was carried out by boiling. Briefly, a loopful of bacteria grown overnight on MacConkey agar (Merck, Germany) were resuspended in 500 ml sterile double distilled water, boiled at 100 ºC for 10 min, and were centrifuged at 10,000 g for 10 min. The supernatant used as DNA template for detection of *qnr* genes (*qnr*A, *qnr*B and *qnr*S) as well as the presence of integrase genes (*Int1, Int2* and *Int3*) by PCR using specific protocols and primers shown in Table 1 (3, 10, 19, 20). Each PCR reaction mixture (25 µl) contained 1.5 µl DNA template, 1.5 mM $MgCl_2$, 0.25 mM of dNTP mix (Cinnagen, Iran), 1 unit of DFS-Taq DNA polymerase (Bioron, Germany), and 20 pmol of each primer (Faza Biothec, Iran). PCR amplifications were carried out in a thermo cycler (Applied Biosystems, USA). PCR products were separated on 1.5% agarose gels and visualized using gel a documentation system (ATP, Iran). PCR products were sequenced (Pishgam company, Tehran, Iran) to determine the subclasses of *qnr* genes as well as confirmation of integrase amplification products.

Results

Of the 250 *Klebsiella* outpatient isolates, nine were *K. oxytoca* (3.6%) and the rest were *K. pneumoniae*. The majority of the isolates were urinary (98%) including all *K. oxytoca* and 96.4% of *K. pneumoniae* isolates. Three isolates (1.2%) were from wounds and two (0.8%) were from sputum specimens. Disc diffusion results showed that 17 isolates (6.8%) were resistant to at least three antibiotic classes and were considered multidrug resistant (MDR) (Table 2). Fifteen MDR isolates were obtained from urine of female subjects over 60 years of age. One isolate was recovered from the sputum of a

Table 1. Primers and thermocycler conditions used for amplification of plasmid-mediated quinolone resistance (PMQR) and integron genes in ciprofloxacin-resistant community isolates of *Klebsiella* spp.

Gene	Primer Sequence	PCR Product Size (bp)	Thermocycler conditions				Ref
			Denaturation (°C)	Annealing (°C)	Extension (°C)	Number of cycles	
qnrA-F *qnrA*-R	5′-TTCTCACGCCAGGATTTGAG-3′ 3′-TGCCAGGCACAGATCTTGAC-5′	571	94 1 min	57 1 min	72 1 min	30	3
qnrB-F *qnrB*-R	5′-TGGCGAAAAAATTGAACAGAA-3′ 3′-GAGCAACGATCGCCTGGTAG-5′	594	94 1 min	57 1 min	72 1 min	30	3
qnrS-F *qnrS*-R	5′-GACGTGCTAACTTGCGTGAT-3′ 3′-AACACCTCGACTTAAGTCTGA-5′	388	94 1 min	57 1 min	72 1 min	30	3
aac(6′)-Ib-cr-F *aac(6′)-Ib-cr*-R	5′-TTGCGATGCTCTATGAGTGGCTA-3′ 3′-CTCGAATGCCTGGCGTGTTT-5′	482	94 1 min	54 1 min	72 1 min	30	3
oqxA-F *oqxA*-R	5′-CTCGGCGCGCGATGATGCT-3′ 3′-CCACTCTTCACGGGAGACGA-5′	392	94 45 sec	57 45 sec	68 1 min	34	10
oqxB-F *oqxB*-R	5′-TTCTCCCCCGGCGGGAAGTAC-3′ 3′-CTCGGCCATTTTGGCGCGTA-3′	512	94 45 sec	64 45 sec	72 1 min	32	10
qepA-F *qepA*-R	5′-CTGCAGGTACTGCGTCATG-3′ 3′-CGTGTTGCTGGAGTTCTTC-5′	709	94 45 sec	56 45 sec	72 45 sec	30	19
Int1-F *Int1*-R	5′-CCTCCCGCACGATGATC-3′ 3′-TCCACGCATCGTCAGGC-5′	280	94 1 min	60 1 min	72 1 min	35	20
Int2-F *Int2*-R	5′-TTATTGCTGGGATTAGGC-3′ 3′-ACGGCTACCCTCTGTTATC-5′	233	94 1 min	60 1 min	72 1 min	35	20
Int3-F *Int3*-R	5′-AGTGGGTGGCGAATGAGTG-3′ 3′-TGTTCTTGTATCGGCAGGTG-5′	600	94 1 min	60 1 min	72 1 min	35	20

Table 2. Antibiotic resistance profiles, plasmid-mediated quinolone resistance (PMQR) and class 1 integron carriage in 17 community *Klebsiella* isolates

Isolate No	CIP MIC (µg/ml)	PMQR genes	Class 1 Integron	Antibiotic resistance profile (disc diffusion)
Kp 31	0.25	*qnrB, oqxAB*	+	AMX, CF, TE, CAZ, SXT, CIP[I]
Kp 143	1.0	*oqxAB*	+	AMX, NA, TE, SXT, CIP[I]
Kp 172	1.0	*qnrS, oqxB*	+	AMX, CF, CIP, OFL, NA, TE, SXT
Kp 11	1.5	*qnrB, aac(6')-ib-cr*	+	AMX, CF, CTX, CAZ, MEP, GM, AK, NA, TE, SXT, CIP[I], OFL[I]
Kp 9	3.0	*oqxAB, aac(6')-ib-cr*	-	AMX, CF, CTX, CM, NA, TE, SXT CIP[I], OFL[I]
Kp 63	3.0	*qnrB, qnrS , aac(6')-ib-cr, oqxA*	+	AMX, CF, NF, TE, SXT, CIP[I], OFL[I]
Kp 102	6.0	*oqxAB*	+	AMX, CF, NF, TE, SXT, CIP[I], OFL[I]
Ko 141	12	*oqxAB*	+	AMX, CF, NA, CIP, OFL, NF, TE
Kp 182	12	-	-	AMX, CIP, OFL, NOR, NA, NF, GM, SXT
Kp 42	>32	*qnrB, oqxAB, aac(6')-ib-cr*	+	AMX, CF, CAZ, CTX, CIP, OFL, NA, GM, AK, KM, NF, TE, SXT
Ko 55	>32	*oqxAB, aac(6')-ib-cr*	+	AMX, CF, CAZ, CTX, CIP, OFL, NA, KM, NF, TE, SXT
Kp 142	>32	*qnrS , aac(6')-ib-cr, oqxA*	+	AMX, CF, CAZ, CIP, OFL, NA, KM, NF, CM, TE, SXT
Kp 192	>32	*oqxA*	+	AMX, CF, CTX, CIP, OFL, NA, NF, SXT
Kp 229	>32	*aac(6')-ib-cr*	+	AMX, CF, CIP, OFL, NA, GM, NF, SXT
Kp 231	>32	*oqxAB*	-	AMX, CIP, OFL, NA, NF, TE, SXT
Kp 234	>32	*oqxAB, aac(6')-ib-cr*	+	AMX, CF, CAZ, CTX, CIP, OFL, NA, GM, KM, NF, TE, SXT
Kp 236	>32	*oqxAB*	+	AMX, CIP, OFL, NOR, NA, GM, NF, TE, SXT

AMX: Amoxicillin; CF: Cefalotin; CAZ: Ceftazidime; CTX: Cefotaxon; GM: Gentamicin; AN: Amikacin; KM: Kanamycin; NA: Nalidixic acid; CP: Ciprofloxacin; OFX: Ofloxacin; NOR: Norfloxacin; NF: Nitrofurantoin; MEM: Meropenem; CM: Chloramphenicol; Te: Tetracycline; SXT: Trimethoprim-Sulfamethaxazole; Ko: *Klebsiella oxytoca*; Kp: *Klebsiella pneumoniae*. All isolates were obtained from urine specimens of female subjects except for Kp 9 (sputum of a female subject and Kp 172 (urine of a male subject)

female subject and one from the urine of a male subject, both also over 60 years old. Among the MDR isolates, two were identified as *K. oxytoca* and 15 were *K. pneumoniae.* MIC measurements revealed 11 ciprofloxacin-resistant (including the two *K. oxytoca* isolates), with MICs, 6 to >32 µg/ml, three intermediately-resistant (MIC, 1.5 to 3.0 µg/ml) and three ciprofloxacin-susceptible (MIC, 0.25 to 1.0 µg/ml) isolates.

Figure 1 shows representatives of the PMQR and *int1* gene amplification products. Of the 11 ciprofloxacin-resistant isolates, four carried the *oqxAB* genes and one had *oqxA* alone, two had *oqxAB/aac(6')-ib-cr* genes, one carried *aac(6')-ib-cr*, one had *qnrB/oqxAB/aac(6')-ib-cr*, one harbored *qnrS/oqxA/aac(6')-ib-cr* and finally one did not carry any of the tested genes (Table 2). Among the three intermediately ciprofloxacin-resistant isolates,

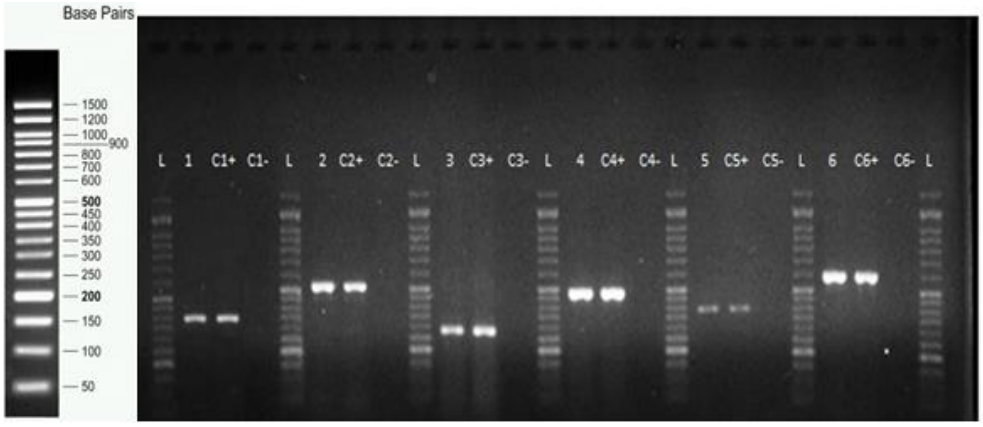

Figure 1. Representatives of polymerase chain reaction (PCR) amplification products of plasmid-mediated quinolone resistance (PMQR) and *int1* genes in outpatient isolates of *Klebsiella* spp. Lane 1, *oqxA* (392 bp); lane 2, *oqxB* (512 bp); lane 3, *int1* (280 bp); lane 4, *aac(6')-Ib-cr* (482 bp); lane 5, *qnrS* (388 bp); lane 6, *qnrB* (594 bp). L; 50 bp DNA ladder; C-, negative control; C+, positive control

Table 3. Identification of qnr genes detected in outpatents isolates of *Klebsiella* spp

Isolate No	*Qnr* gene type	GenBank No	GenBank accession number
Kp 63	*qnr*S1	Y63S	MH369800
Kp 118*	*qnr*S1	Y118S	MH369801
Kp 142	*qnr*S1	Y142S	MH369802
Kp 172	*qnr*S1	Y172S	MH369803
Kp 230*	*qnr*S1	Y230S	MH369804
Kp 11	*qnr*B1	Y11B	MH333285
Kp 31	*qnr*B4	Y31B	MH369805
Kp 42	*qnr*B1	Y42B	MH369806
Kp 63	*qnr*B1	Y63B	MH369807

* Ciprofloxacin susceptible isolates. The nucleotide sequences of *qnrB* and *qnrS* genes were analyzed by Chromas Pro version 1.7.5 Technelysium
Kp: *Klebsiella pneumoniae*

one carried *qnrB/aac(6')-ib-cr*, one had *oqxAB/aac(6')-ib-cr* and one carried *qnrB/qnrS/oqxA/aac(6')-ib-cr* genes. Of the three ciprofloxacin-susceptible isolates which were resistant to β-lactam antibiotics, one carried the *oqxAB,* and two harbored *qnr* genes (*qnrS/oqxB* and *qnrB/oqxAB)*. Interestingly, the *aac(6')-ib-cr* gene was not observed in the ciprofloxacin-susceptible isolates. None of the isolates carried *qnrA* or *qepA*. Class 1 integron was present in nine of the 11 ciprofloxacin-resistant isolates, one did not have any of the PMQR genes and one carried only the *oqxAB* gene complex (Table 2). Finally, the three ciprofloxacin-susceptible and two of the intermediately-resistant isolates also carried class 1 integron (Table 2). Class 2 and 3 integrons were not observed. Sequencing results showed that among the four *qnrB* positive isolates, three were *qnrB1* and one was *qnrB4*. The five *qnrS* positive strains all were identified as *qnrS1* (Table 3).

Discussion

In this study, 17/250 (6.8%) of the outpatient's isolates were not only resistant to ciprofloxacin but were also MDR. The most prevalent PMQR gene among these isolates was *oqxAB* (93.75%) followed by *aac(6')-ib-cr* (50.0%), *qnrB* (25.0%) and *qnrS* (18.75%). None of the isolates carried *qnrA* or *qepA*. We also found that 82.3% of the ciprofloxacin-resistant isolates harbored class 1 integron but class 2 and 3 integrons were not observed. Among the few Iranian studies, Hashemi *et al*. reported that 4.1% of community isolates of *Enterobacteriaceae* in Hamadan, Iran, were resistant to ciprofloxacin (21). Seyedpour *et al*. reported that 13.5% of the community isolates collected from outpatients, harbored PMQR genes among which, *aac(6')-Ib-cr*, *qnrB* and *qnrS* were predominant, respectively (3).

There are a limited number of studies on community isolates of *K. pneumoniae* worldwide. In a study performed in 2011 in Morocco, 2/36 (5.6%) of ESBL producing *K. pneumoniae* isolates from outpatients carried the *aac (6')-Ib-cr* gene and *qnrS1* (22). In a larger scale study two years later, the same group showed that among 34 ESBL producing *K. pneumoniae* community isolates, *aac (6')-Ib-cr*, was the most prevalent PMQR gene followed by *qnrB1*, *qnrS1, qnrB2* and *qnrA6* (23). Except for *oqxAB* and *qnrA*, our results were similar to the latter report. A study from Vietnam, showed that among 45 *K. pneumoniae* community isolates from healthy individuals, the prevalence of *qnrS* was 33.3% followed by *aac (6')-Ib-cr* (2.2%) (24). In the present study, the majority of the quinolone resistant outpatient isolates carried an *oqxAB* gene. Rodriguez-Martinez *et al*. showed that a high level expression of this efflux pump decreased quinolone susceptibility in ESBL producing *K. pneumoniae* (10). The majority of studies report the presence of *oqxA* in clinical isolates of *K. pneumoniae.* However, human and veterinary strains can easily spread in communities. Hence, as a PMQR determinant, *oqxA* can have a major contribution in dissemination of fluoroquinolone resistance along with other antibiotic-resistance genes among the community isolates especially if they are located on integron/plasmid. In fact, Dakic *et al*. showed that 17.8% of community-acquired urinary tract isolates of *Enterobacteriaceae* in Greece carried integrons (mostly class 1) and observed a significant association between integron carriage and reduced susceptibility to a range of antibiotics including fluoroquinolones (25). In a recent study from Brazil, PMQR genes (55.5%) as well as frequent presence of the class 1 integron were detected in the community isolates of *K. pneumoniae* and *E. coli* (26). Despite the belief that integron harboring MDR clinical isolates are the source of community acquired infections, Leverstein-van Hall *et al*. showed the spread of antibiotic resistance genes from integron positive community strains of *Enterobacteriaceae* into the hospital strains (27). Interestingly, Kaplan *et al*. showed high percentages of ciprofloxacin-resistant *Enterobacteriaceae* in raw sewage and activated sludge from a waste water plant, the majority of which harbored at least one PMQR determinant (59.6% and 75%, respectively). They also found that PMQR gene presence did not correlate with class 1 integron carriage (28). In this research, class 1 integron was observed in the majority of the MDR isolates regardless of ciprofloxacin susceptibility or PMQR gene carriage. The predominance of class 1 integron has been shown in Gram-positive and Gram-negative clinical isolates as well as a number of different environmental bacteria (15).

Conclusion

Presence of multidrug resistance, multiple PMQR determinants as well as class 1 integron in community

isolates of *Klebsiella* spp. is alarming and presents an important source for cross transmission of these opportunistic pathogens among community members and hospitalized patients.

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Conflicts of Interest

All contributing authors declare no conflicts of interest.

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