



The Emergence of Qnr-resistance Among *Klebsiella pneumoniae* spp in Zahedan

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Abstract

Although quinolone resistance results mostly from chromosomal mutations in *Klebsiella pneumoniae*, it may also be mediated by plasmid – encoded qnr determinants. Plasmid-mediated quinolone resistance (PMQR) was increasingly identified in Enterobacteriaceae family worldwide. The aim of this study was to investigate the prevalence of qnr genes in clinical isolates of *Klebsiella pneumoniae* spp in Zahedan, south-East of Iran. In this sectional-descriptive study which was performed in 2013, clinical isolates of *K. pneumoniae* (n=184) were collected from patients referred to 3 hospitals of Zahedan. The presence of the qnr gene was screened by PCR using specific primers for qnrA, qnrB, qnrS and qnrC in extracted plasmid DNA. Of 184 *K. pneumoniae* clinical isolates, 45 isolates were positive for the qnr gene. The prevalence of qnrA, qnrB and qnrS clusters among these isolates were 8 (17.7%), 22 (48.8%), 4(8.88%) respectively and qnrC was not identified in any isolate. Another 6(13.33%) possessed both qnrA and qnrB genes and 5(11.11%) possessed both qnrB and qnrS. qnr are widely distributed worldwide. Community-acquired and nosocomial pathogens and the emergence of qnr-mediated quinolone resistance among clinical *K.pneumoniae* isolates are described for the first time in Iran.

Key words: *Klebsiella pneumoniae*, Drug resistance, PMQR, Fluoroquinolone, PCR, Zahedan

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1. Introduction

Klebsiella spp are opportunistic pathogens that cause hospital and community acquired infections such as pneumonia, urinary tract infection, septicemia, soft tissue infection, liver abscess and meningitis [1] Multidrug resistance in *Enterobacteriaceae* including resistance to quinolones is rising worldwide [2]. The development of quinolone resistance

by Gram-negative pathogens constitutes a remarkable bacterial success story [3]. Quinolones are a group of synthetic antibacterial agents that are widely used in routine clinical practice. The new quinolones compounds (6-fluoroquinolones) exhibit broad spectrum of antibacterial activity against Gram-negative, mycobacterial pathogens, and anaerobes [4,19]. The emergence of FQ resistance in *Escherichia coli* and *Klebsiella pneumoniae* is of great concern, because these pathogens account for >20% of all hospital-acquired infections [4]. Quinolones or fluoroquinolones are among the most important antimicrobial drugs, used extensively for treatment of bacterial infections both in human and veterinary medicine [5]. Although plasmid-mediated quinolone resistance (PMQR) was thought not to exist before its discovery in 1998, the past decade has seen an explosion of research characterizing this phenomenon. The best-described form of PMQR is determined by the *qnr* group of genes [6]. Qnr-type plasmid-mediated quinolone resistance determinants belong to the pentapeptide-repeat family of proteins and they confer low-level resistance to quinolones probably by protecting DNA gyrase directly from quinolone inhibition [7]. Five *qnr* proteins have been described: *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS*. Qnr genes shows high diversity with 7*qnrA*, 25*qnrB*, 1*qnrC*, 1*qnrD* and 4*qnrS* identified to date [8]. The objective of this study was to screen for the presence of the *qnr* gene in clinical ciprofloxacin or norfloxacin resistant isolates

of *Klebsiella pneumoniae* from hospitalized patients in Zahedan, south-east of Iran.

2. Materials and Methods

2.1. Bacterial Isolates

In this cross-sectional study, a total number of 184 specimens of urine, respiratory tract secretions, blood, ulcer and tracheal lavage, fluid on urinary catheters, joint effusion, and phlegm collected during 2013 and 2014 from patients referring to Khatamal-Anbia, Bou-Ali, and Imam Ali hospitals in Zahedan, south-east of Iran. Urine was the most common among specimens (132=71.7%) while there were two joint effusion fluid specimens and one phlegm specimen accounted for the lowest numbers among our samples.

One hundred thirty six samples provided by Khatam-al-Anbia hospital, 30 samples from Imam Ali and 18 samples from Bou-Ali hospitals respectively. Isolates were identified by colony growth on Mac Conkey agar, and motility- indole- lysine media incubated at 37°C and compared to the standard *E.Coli* strain, ATCC 25992.

2.2. Susceptibility Testing

A disk diffusion susceptibility test was performed on Mueller-Hinton agar by quinolone antibiotics (nalidixic acid, ciprofloxacin, norfloxacin, ofloxacin, levofloxacin, and getifloxacin disks were used according to the Clinical and Laboratory Standards Institute (CLSI) 2010. Disks were ordered from MAST co. England. Minimal inhibitory concentrations (MICs) of the

antibiotics were determined according to CLSI. *E. coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as quality control reference strains. MICs of ciprofloxacin were determined by agar dilution assay.

2.3. Plasmid Extraction and Detection of Qnr Genes

Bacterial plasmid DNA was conducted according to AccuPrep (Bioneer, South Korea) plasmid nano-plus plasmid mini extraction kit (Cat. No.: K-3112) protocols. The kit contained 6 buffers (named 1-5 and D), RNase A powder, and DNA extraction columns. The quantity of DNA was measured by spectrophotometry after extraction. For quality control, 3 µL of DNA was electrophoresed on 2% agarose gel.

the presence of *qnrA*, *qnrB*, *qnrS* and *qnrC* genes was performed by PCR.

Their sequences and references are listed in Table 1. Using the following protocol: Initial

denaturation at 94°C for 30s, denaturation at 94°C for 30s, annealing at primer-specific temperature for 30s, elongation at 72°C for 7min. qnr positive control strain *K.pneumoniae* UAB1 (GenBank AY070235) was used as positive controls and tubes without DNA template served as negative controls in each run. PCR product were separated by electrophoresis in a 2% agarose gel for 15 minute at 150v and 200mA, stained with ethidium bromide and detected by UV transillumination. Amplified genes were identified on the basis of fragment size compared to the positive controls and kb marker.

2.4. Statistical Analysis

The data was analyzed statistically using chi-square test and exact test fisher. A P value of ≤ 0.05 was considered statistically significant.

Table 1. Sequences of the primers used in this study.

Target	Primer	Sequence	Annealing temperature (°C)	Product size (bp) Nucleotide position	Reference
qnr A	qnrAF	5'-TCAGCAAGAGGATTTCTCA-3	48°C	516 bp	18
	qnrAR	5'-GGCAGCACTATTA CTCCCA-3			
qnr B	FQ1	5'-ATG ACG CCA TTA CTG TAT AA-3'	53°C	562 bp	2
	FQ2	5'-GAT CGC AAT GTG TGA AGT TT-3'			
qnr C	qnrS-Fu	ATTACGGGTTGTAATTTGTCCTTATG	59.9°C	144 bp	17
	qnrS-Ru	ATCAGAAAATGATCCCCTACT			
qnr S	qnrS-1A	CAATCATACATATCGGCACC	60°C	9748-9767 10 389-10 369	16
	qnrS-1B	TCAGGATAAACAACAATACCC			

Table 2. Antibiotic resistance patterns of isolates to antibiotics used.

Antibiotics	S N(%)	I N(%)	R N(%)
Nalidixic acid	98(53.2%)	28(15.2%)	58(31.5%)
Ciprofloxacin	103(55.9%)	47(25.5%)	34(18.4%)
Norfloxacin	114(61.9%)	38(20.6%)	32(17.3%)
Ofloxacin	159(86.4%)	17(9.2%)	8(4.3%)
Levofloxacin	161(87.5%)	17(9.2%)	6(3.2%)
Gatifloxacin	165(89.6%)	15(8.1%)	4(2.1%)

3. Result and Discussion

One hundred three patients (55.9%) were female and 81 patients (44.2%) were male and the average age for males was 27.5 ± 18.2 years and for females was 24.8 ± 16 years. About disk diffusion in agar method findings, this method was done using 184 strain of *K. pneumoniae* isolated from clinical samples, according to the proposed methods of CLSI for the evaluation of nalidixic acid, ciprofloxacin, norfloxacin, ofloxacin, levofloxacin and gatifloxacin antibiotics. The result of disk agar diffusion has been shown in table 2.

3.1. Prevalence of Qnr Genes

During the study period, 38 patients with FQ-resistant *K.pneumoniae* isolates were identified from the database of the clinical laboratory. Forty-five (24.4%) of these isolates were qnr-positive, of which 17.77% housed the qnrA gene only, 48.8% qnrB only, 8.88% qnrS only.. Another six isolates (13.33%) were positive for qnrA and qnrB and five isolates (11.11%) were positive for qnrB and qnrS and none of the clinical

isolates harbored all three or four determinants. The prevalence of any qnr gene was 45 (24.4%) of all isolates. A typical gel of PCR results for qnrA (left) and qnrB (right) are shown in Figure1&2.

Fluoroquinolone have been extensively used since their introduction in the 1980s. The high level of use and misuse is certainly accountable for the rapid emergence of quinolone resistance [10, 11]. The present study demonstrated the high prevalence of plasmid – mediated quinolone resistance among *k.pneumoniae* isolates in Zahedan university hospital. A considerably high frequency of qnr detection (24.4%) was observed in this study with *k.pneumoniae* isolates accounting for the majority of this. This is similar to the prevalence reported in the USA but is much higher than China, Canada and Australia [9- 11]. Frequency rates of 32.5% in Jamaica, 48% in Thailand, 86% in Vietnam have been reported [12-14]. In the present study, the criterion used for selecting these isolates was resistance or reduced susceptibility to ciprofloxacin and levofloxacin based on NCCLS criteria. Three

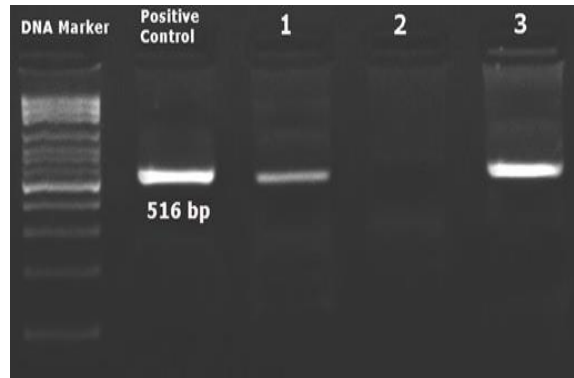


Figure 1. Agarose gel electrophoresis (2%) used for the separation of multiplex PCR products. As shown, 516 bp molecular weight marker *K pneumoniae* *QnrA*+ve control; Lane 1 and 3 clinical isolate.

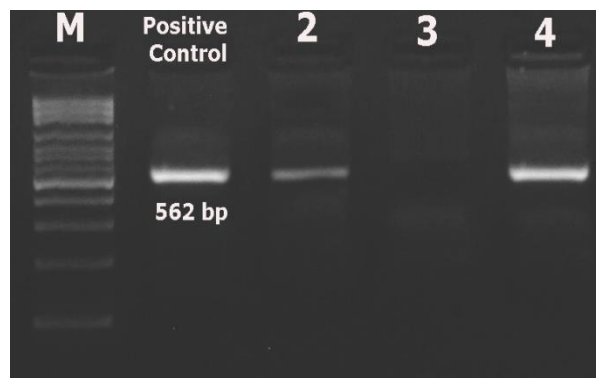


Figure 2. Agarose gel electrophoresis (2%) used for the separation of multiplex PCR products. As shown, 562 bp molecular weight marker *K pneumoniae* *QnrB*+ve control; Lane 2 and 4 clinical isolate.

qnr groups were detected and described in this report. qnrA was detected in a majority of the isolates in this study compared to qnrB, qnrS and qnrC. In this study, the prevalence of *qnrA*, *qnrB*, *qnrS* were 17.7%, 48.8% and 8.88%, respectively, and 13% contained both *qnrA* and *qnrB* genes, and 11% contained both *qnrB* and *qnrS* genes, and *qnrC* was absent in any of the quinolone generators. The dominance of qnrA gene is similar to studies from the UK, Spain, Jamaica and Morocco [12,15] but contrast with other studies from Europe and China [9]. In a study by Peymani et al reported from Iran(Tehran and Qazvin), Out of 124 quinolone non-susceptible isolates,

qnr-encoding genes were present in 49 (39.5%) isolates with qnrB1 (30.6%) as the most dominant gene followed by qnrB4 (9.7%), and qnrS1 (1.6%) either alone or in combination [20]. In another study from Iran, Seyedpour et al showed that 30.4% of community isolates of *K. pneumoniae* harbored *qnr* and/or *aac (6')-Ib-cr* genes [21]. In a study by Moghadasi et al in Boroujerd during one year 2014 to 2015, among resistant isolates, 13 (13.83%) were positive for *qnr* genes. Twelve (92.30%) were *qnrB* positive and one isolate was *qnrC* positive. Furthermore, one isolate amplified both the genes [22]. Wang et al in a study carried out

in China reported that 62 (15.1%), 25 (6.1%), and 10 (2.4%) of ESBL-producing *K. pneumoniae* isolates were positive for *qnrS*, *qnrB*, and *qnrA* genes, respectively [23]. In our study *qnrC* was not detected by PCR. We suggested that real-time PCR can result in the more precise detection of low number of *qnrC* gene. The emergence of plasmid – mediated quinolone resistance thus may contribute by several means to the rapid increase in bacterial resistance to quinolones and may explain why an increase has been noted among *k.pneumoniae* in Zahedan. We strongly recommended *qnr* gene sequencing in order to further identify the details regarding this gene and its characteristics in further studies.

4. Conclusion

This study is the first report of detection of *qnr*-mediated fluoroquinolone resistance in clinical *k.pneumoniae* isolates in Zahedan, South-East of Iran. Because fluoroquinolone are second line antibiotics, it is of concern that such a high prevalence was noted in this isolates. The possibility of *k.pneumoniae* transferring these resistant plasmids to other Enterobacteriaceae and non-fermenting gram negative bacilli is a serious consideration in the care of hospitalized patients.

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