ORIGINAL ARTICLE

Emergence of plasmid-mediated quinolone-resistant determinants in *Klebsiella pneumoniae* isolates from Tehran and Qazvin provinces, Iran

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Key words

Klebsiella pneumoniae • Quinolones resistance • *qnr*

Summary

Background. *Plasmid-mediated quinolone resistance is an increasing clinical concern, globally. The major objective of the present study was to identify the qnr-encoding genes among the quinolone non-susceptible K. pneumoniae isolates obtained from two provinces in Iran.*
Methods. *A total of 200 K. pneumoniae isolates were obtained from hospitals of Qazvin and Tehran, Iran. The identification of bacterial isolates was carried out by standard laboratory methods and API 20E strips. Susceptibility to quinolone compounds were examined by standard Kirby-Bauer disk diffusion method according to the CLSI guideline. PCR and sequencing were employed to detect qnrA, qnrB and qnrS-encoding genes.*
Results. *Of 200 K. pneumoniae isolates, 124 (62%) were non-susceptible to quinolone compounds among those 66 (53.2%) and 58 (46.8%) isolates showed high and low-level quinolone resistance rates, respectively. 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.*
Conclusions. *This study, for the first time, revealed the high appearance of qnrB1, qnrS1 and qnrB4 genes among the clinical isolates of K. pneumoniae in Iran. Therefore, the application of proper infection control measures and well-established antibiotic administration guideline should be strictly considered within our medical centers.*

Introduction

Klebsiella pneumoniae (K. pneumoniae) is an opportunistic pathogen causing several nosocomial infections such as urinary tract infections, pneumonia, septicemia, and soft tissue infections [1]. This organism is also known as a community-acquired potential pathogen [2]. Health care associated infection caused by this organism has been linked to high mortality and morbidity especially among the patients admitted to intensive care units [3, 4].
Quinolones are a group of synthetic antibacterial agents that are widely used in routine clinical practice [5]. The new quinolones compounds (6-fluoroquinolones) exhibit broad spectrum of antibacterial activity against Gram-negative, mycobacterial pathogens, and anaerobes. Moreover, these agents show a good-to-moderate oral absorption and tissue penetration with favorable pharmacokinetics in humans, creating desirable clinical efficacy in treating many kinds of infections [6, 7]. Quinolones inhibit the function of bacterial DNA gyrase and topoisomerase IV [8]. While the first and second generation fluoroquinolones selectively inhibit the topoisomerase II ligase domain or DNA gyrase activity, the quinolones of third and fourth generations are with more tendency for topoisomerase IV ligase [9]. Excessive and inappropriate administration of antimicrobial agents such as quinolones has increased the emergence of multidrug resistant *K. pneumoniae* isolates which makes the process of antimicrobial therapy to become marginal and problematic [10, 11]. In recent years, several studies have demonstrated that the appearance of quinolone-resistant *K. pneumoniae* is rising at a faster rate, worldwide [12-15]. Infections caused by resistant organisms are often due to extensive cross-resistance with other antimicrobials, including beta-lactams and aminoglycosides [16]. Quinolone resistance in *Enterobacteriaceae* mainly occurs through chromosomal mutations in the genes coding for DNA gyrase and topoisomerase IV, changes in outer membrane and efflux proteins or in their regulatory mechanisms [17]. Findings from recent studies show that plasmid-mediated resistance, associated with the pentapeptide proteins of the *qnr* family, might play a crucial role in quinolone compound resistance [18]. Three major groups of *qnr* determinants, *qnrA*, *qnrB*, and *qnrS*, are increasingly being identified in the clinical isolates of various enterobacterial species, worldwide [19]. It was in 1998 that the first plasmid-mediated quinolone resistance determinant, *qnrA*, was reported in a *Klebsiella pneumoniae* strain from the United States [20]. Since then two *qnr* determinants, *qnrB* and *qnrS* have been discovered in other *Enterobacte-*

riaceae species such as *Citrobacter Koseri*, *Escherichia coli*, *Enterobacter cloacae*, and *Klebsiella pneumoniae* from Asia and Europe [21-24]. To date, there has been no report for the frequency of *qnr* genes among *K. pneumoniae* isolates in Iran. In the current study, for the first time, we described the frequency of *qnr* determinants (*qnrA, qnrB,* and *qnrS*) among the isolates of quinolone non-susceptible *K. pneumoniae* collected from hospitals of Qazvin and Tehran provinces.

Methods

Bacterial isolates and Antimicrobial susceptibility

In this descriptive study, a total of 200 clinical isolates of *K. pneumoniae* were collected from hospitalized patients in several teaching hospitals in Tehran and Qazvin during 2012-2013. The isolates were obtained from different clinical specimens including urine, wound, trachea, secretions, blood, and ascites. All isolates were identified by standard laboratory methods and confirmed with the API 20 E (bioMérieux, France) strips. All isolates were kept at -70°C in trypticase soy broth containing 20% glycerol and subcultured twice before testing. The mean age of patients (77 (38.5%) male and 123 (61.5%) female) was 51.7±17.4 (range17-83) years. Written informed consent was obtained from all subjects enrolled in this study. Kirby-Bauer disk diffusion technique was performed according to the CLSI guideline to identify quinolone resistance using nalidixic acid (30µg), ciprofloxacin (5 µg), gatifloxacin (5 µg), norfloxacin (10 µg), and levofloxacin (5 µg) disks [25]. In this study the isolates were classified either as high-level quinolone resistant if the resistance to both nalidixic acid and ciprofloxacin disks was observed or low-level quinolone resistant in the cases of resistance to nalidixic acid, presence of intermediate isolates or ciprofloxacin-susceptible organisms [26]. Antibiotic disks were purchased from the Mast (Mast Diagnostics Group Ltd, Merseyside, UK). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains in antimicrobial susceptibility testing.

Detection of *qnr* determinants

Detection of *qnrA*, *qnrB,* and *qnrS* plasmid-mediated quinolone resistance genes was performed using PCR and specific primers (Tab. I). Plasmid DNA was extracted by plasmid mini extraction kit (Bioneer Company, South Korea). PCR amplifications were applied in a thermocycler (Applied Biosystems, USA) as follows: 95ºC for 5min and 35 cycles of 1min at 95ºC, 1min at specific annealing temperature for each primer and 1min at 72ºC. A final extension step of 10 min at 72ºC was performed. Amplification reactions were prepared in a total volume of 25µl (24µl of PCR master mix plus 1µl of template DNA) including 5ng of genomic DNA, 2.0U of Taq DNA polymerase, 10mM dNTP mix at a final concentration of 0.2mM, 50mM MgCl2 at a final concentration of 1.5mM, 1µM of each primer, and 1X PCR buffer (final concentration). PCR products were electrophoresed on 1% agarose gel at 100 volts and later stained with ethidium bromide solution and finally visualized in a gel documentation system (UVtec, UK). The purified PCR products were sequenced by the Macrogen Company (Seoul, South Korea) and the sequence alignment and analysis were performed online using the BLAST program of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Data were summarized using mean ± SD (standard deviation), proportional frequency and confidence interval for microbiological, clinical, and demographic characteristics. All analyses were carried out using a Statistical Software Package, SPSS for windows version 16.0 (Chicago, IL, USA).

Results

In this study, the bacterial isolates were recovered from different clinical specimens including urine (110-55.0%), trachea (59-29.5%), wound (18-9.0%), blood (8-4%), and ascites (5-2.5%). These isolates were obtained from the patients admitted to intensive care units (96-48.0%), internal medicine (54-27.0%), infectious diseases (35-17.5%), surgery (13-6.5%), and orthopaedic (2-1.0%) wards. The results of antimicrobial susceptibility testing showed the resistance rates against the antimicrobial agents used in our study varied between 20% and 58%. Overall, nalidixic acid (58%) and ciprofloxacin (34.5%) revealed the highest rates of resistance among the antimicrobials tested whereas levofloxacin and norfloxacin also demonstrated high susceptibility rates of 80% and 77%, respectively (Tab. II). In total,

Tab. I. The primers used for detection of qnr genes in K. pneumoniae isolated from Qazvin and Tehran hospitals.

PCR targets	Primer sequence (5′-3′)	Annealing temperatures (ºC)	References
qnrA1–6	F: ACGCCAGGATTTGAGTGAC R: CCAGGCACAGATCTTGAC	49	27
qnrB1-3, 5, 6, 8	F: GGCACTGAATTTATCGGC R: TCCGAATTGGTCAGATCG	49	27
qnrB4	F: AGTTGTGATCTCTCCATGGC R: CGGATATCTAAATCGCCCAG	53	27
qnrS1–2	F: CCTACAATCATACATATCGGC R: GCTTCGAGAATCAGTTCTTGC	53	27

Tab. II. Antibiotic susceptibility of *K. pneumoniae* against quinolone compounds.

Antimicrobial agents	Resistance n (%) [CI]	Intermediate n (%) [CI]	Susceptible n (%) [CI]
Nalidixic acid	83(41.5) [34.7-48.3]	33(16.5) [11.4-21.6]	84(42) [35.2-48.8]
Ciprofloxacin	53(27) [20.8-33.2]	15(7.5) [3.8-11.2]	131(65.5) [58.9-72.1]
Gatifloxacin	39(19.5) [14-25]	27(13.5) [8.8-18.2]	134(67) [60.5-73.5]
Norfloxacin	37(18.5) [13.1-23.9]	9(4.5) [1.6-7.4]	154(77) [71.2-82.8]
Levofloxacin	36(18) [12.7-23.3]	4(2) [0.1-3.9]	160(80) [74.5-85.5]

CI = 95% Confidence interval

66 (53.2%) and 58 (46.8%) of isolates showed high and low-level quinolone resistance, respectively.

PCR and sequencing showed the presence of *qnr*-encoding genes in 49 (39.5%) of quinolone non-susceptible *K. pneumoniae* isolates among those *qnrB1* (38-30.6%) was the most common gene followed by *qnrB4* (12-9.7%) and *qnrS1* (2-1.6%) genes either alone or in combination. The study isolates were negative for *qnrA* gene. As shown in Table III, *qnrB1* was found to coexist with *qnrB4* in 3 (2.4%) isolates. Overall, 25 (37.9%) high level quinolone resistant isolates carried *qnr* genes in which 19 (28.8%), 4 (6.1%), and 2 (3%) isolates carried *qnrB1*, *qnrB4,* and *qnrS1* genes, respectively. In addition, 24 (41.4%) low level quinolone resistant isolates were positive for *qnr* genes among those 19 (32.8%) isolates carried *qnrB1* gene followed by *qnrB4* in 8 (13.8%) isolates. Among the high and low-level quinolone resistance isolates, *qnrB1 was the* most frequent gene compared to other genes. Table IV shows that *qnr*-positive isolates were mostly recovered from urine (42.9%) followed by trachea secretion (36.7%) samples. The patients affected by these organisms were mostly admitted to ICU (59.2%) and internal medicine (20.4%) wards.

Tab. III. Distribution of *qnrB1, qnrB4,* and *qnrS1* genes among *qnr*-positive *K. pneumoniae* isolates.

qnr-encoding genes	N of isolates n (%) [CI]
qnrB1	35 (28.2%) [20.3-36.1]
qnrB4	9 (7.3%) [2.7-11.9]
qnrS1	2 (1.6%) [0-3.8]
qnrB1+qnrB4	3 (2.4%) [0-5.1]
qnr negative	75(60.5%) [51.9-69.1]
Total	124 (100%)

CI: 95% Confidence interval

Tab. IV. Frequency of qnr-positive K. pneumoniae isolates based on hospital wards and source of Specimens (n = 49).

Wards	N° of isolates n (%) [CI]	Specimens	N° of isolates n (%) [CI]
ICU	29 (59.2%) [45.4-73]	Urine	21 (42.9%) [29-56.8]
Internal medicine	10 (20.4%) [9.1-31.7]	Trachea	18 (36.7%) [23.2-50.2]
Infectious diseases	8 (16.3%) [6-26.6]	Wound	6 (12.2%) [3-21.4]
Surgery	2 (4.1%) [0-9.7]	Blood	1 (2%) [0-5.9]
Orthopedic	-	Ascites	3 (6.1%) [0-12.8]

ICU: Intensive Care Unit

CI: 95% Confidence interval

Discussion

K. pneumoniae is being increasingly recognized as a clinically significant nosocomial pathogen [1]. Quinolones are among the most commonly administered antimicrobials routinely used for the treatment of serious infections caused by *K. pneumoniae* and other members of the genus Enterobacteriaceae [6]. However, the development of resistance to these antibiotics makes the treatment decision difficult, leading to treatment failures [5]. In recent years, plasmid mediated quinolone resistance among enterobacterial isolates has been reported in several studies, worldwide. However, the number of reports on prevalence of *qnr* genes among Iranian enterobacteria isolates is only limited to few studies [28, 29].

In the present study, 58% and 34.5% of isolates were fully or intermediate resistant to nalidixic acid and ciprofloxacin, respectively. These findings were higher than the two previously conducted studies in Iran. Raei et al demonstrated that 36.2% and 34.1% of urinary *K. pneumoniae* isolates were resistant to ciprofloxacin and nalidixic acid, respectively [30]. In another study from Iran, Zamani et al found that 28.57% and 23.8% of *Klebsiella* spp. were resistant to nalidixic acid and ciprofloxacin, respectively [31]. Hence, the emergence of resistant isolates against broad spectrum antibacterial agents in our hospital settings seems to be linked with improper and widespread administration of these antibiotics.

The present study demonstrates a high prevalence (39.5%) for plasmid-mediated quinolone resistance determinants among quinolone non-susceptible *K. pneumoniae* isolates in Iran. The prevalence rate found in our study is higher than those reported by Kim et al from Korea (10%) [32], Wang et al from China (11.9%) [33], Dahmen et al from Tunisia (16%) [34], Yan et al from China (16.2%) [35], and Wang et al from the United States (11.1%) [36] but still lower than that found by Bouchakour et al in Morocco in which 50% of ESBL-producing *K. pneumoniae* isolates were shown to carry *qnr* determinants [37]. This might be indicative of a rising trend in the rate of plasmid mediated quinolone resistance among the genus of Enterobacteriaceae.

In the current study, 25% of *qnr*-positive isolates were shown to have high level quinolone resistance. As plasmid mediated quinolone resistance determinants produce only low-level resistance to quinolones, it can be hypothesized that high level resistant pattern is possibly

caused by another mechanisms such as chromosomal mutation which was not evaluated in the present study. Considering the findings of the present study, it is obvious that most *qnr*-positive *K. pneumoniae* isolates were mostly obtained from the patients admitted to ICUs. Long term ICU stay, broad spectrum antibiotics intake, chronic underlying conditions, and the application of invasive techniques and devices probably make the patients more susceptible to infections caused by these resistant organisms.

In the present study, 30.6%, 9.7%, and 1.6% of quinolone non-susceptible *K. pneumoniae* isolates carried *qnrB1*, *qnrB4,* and *qnrS1* genes alone or in combination, respectively. We believe that this is the first report of *qnrS1*, *qnrB4,* and *qnrB1* genes among the clinical isolates of *K. pneumoniae* collected from two distinct provinces of Iran. In a study by Pakzad et al reported from Iran, 9 (37.5%) and 4 (20.8%) of ESBL-producing *E. coli* isolates were positive for *qnrA* and *qnrB* genes, respectively [29]. The presence of *qnrA* (25.8%), *qnrB1* (1.17%), and *qnrS* (1.17%) genes among ESBL-producing *Salmonella* spp. was also reported in a study by Saboohi et al from Iran [28]. 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 [38]. In Taiwan, Wu et al described the presence of *qnrB4* (3.6%), *qnrS1* (2.8, and *qnrB2* (2.3%) genes in the clinical isolates of *K. pneumoniae* [39]. Robicsek et al in the United States reported that 14% and 6% of ceftazidime-resistant *K. pneumoniae* isolates harbored *qnrA* and *qnrB* genes, respectively [40]. Dahmen et al from Tunisia showed *qnrA* was more prevalent among *K. pneumoniae* isolates whereas *qnrB1* was the most prevalent genes among *E. cloacae* isolates followed by *qnrB2* and *qnrS1* [34]. Similarly, Yan et al in their report from China demonstrated that 8.1%, 4.1%, and 4.1% of ESBL-producing *K. pneumoniae* isolates were positive for *qnrA*, *qnrB*, and *qnrS* genes, respectively [35]. Finally, 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 [33].

Conclusions

Findings of the present study reveal a high prevalence for plasmid-mediated quinolones resistance due to qnr genes among the clinical isolates of *K. pneumoniae* in Iran. The appearance and spread of such resilient organisms within the medical centers around the country not only brings about issues of great concern for human health but also raises questions on how to achieve a successful antibiotic therapy through planning a comprehensive infection control guideline to avoid further spread of these resistant organisms within our medical settings. Our data also highlights the necessity for establishing an appropriate infection control strategy and sensible antibiotic therapy.

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