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

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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.

Key words
Klebsiella pneumoniae • Quinolones resistance • qnr

Summary

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: 95°C for 5min and 35 cycles of 1min at 95°C, 1min at specific annealing temperature for each primer and 1min at 72°C. A final extension step of 10 min at 72°C 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 (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,

<table>
<thead>
<tr>
<th>PCR targets</th>
<th>Primer sequence (5'→3')</th>
<th>Annealing temperatures (ºC)</th>
<th>References</th>
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</table>
| qnrA1–6     | F: ACCCAGGATTGTGATGAC  
R: CCACCCACACATCTTGC  | 49                           | 27         |
| qnrB1-3, 5, 6, 8 | F: GCCACTGATTATATCACC  
R: TCCGAATTGTCAGATCC  | 49                           | 27         |
| qnrB4       | F: AGTTGTGATCCTCTCATGAC  
R: CGGATATCTAAATCGCCAG  | 53                           | 27         |
| qnrS1-2     | F: CCTACAACTACATATCACC  
R: GCTTCGAGAATCAGTCTTG  | 53                           | 27         |
Qnr determinants in K. pneumoniae

In the present study, 58% and 34.5% of isolates were mostly 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 resistance. As plasmid-mediated resistance determinants produce only low-level resistance to quinolones, it can be hypothesized that high level resistant pattern is possibly

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 enterobacterial isolates is 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.

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Embryonic stem cells (ESC) represent a promising source of differentiation. ESCs are pluripotent cells that can be differentiated into any cell type in the body. The process of differentiation involves the activation of lineage-specific genes that are repressed in ESCs. The Wnt/β-catenin signaling pathway plays a key role in the regulation of ESC differentiation. Wnt signaling promotes the expression of transcription factors that ultimately lead to the activation of lineage-specific genes. The Wnt/β-catenin pathway is activated by binding of Wnt proteins to Frizzled receptors on the cell surface. This leads to the activation of disheveled (Dsh) and axin (AX) proteins, which in turn inhibit the activity of glycogen synthase kinase 3β (GSK3β). The inhibition of GSK3β allows the activation of β-catenin, which translocates into the nucleus and binds to T-cell factor (TCF) orly (lymphoma) factors to activate target genes. The role of ESCs in regenerative medicine is under investigation, with research focusing on their potential use in the treatment of diseases such as Parkinson’s disease, spinal cord injury, and diabetes.

This unique stem cell property is a result of the Wnt/β-catenin signaling pathway, which is essential for maintaining the self-renewal and pluripotency of ESCs. The pathway is a complex network that includes multiple proteins and pathways. The role of Wnt signaling in ESC differentiation is well understood, and many research studies aim to elucidate the mechanisms by which this pathway regulates cell fate decisions. The Wnt pathway can be activated by various factors, including extracellular matrix proteins, growth factors, and cytokines. This activation leads to the phosphorylation and inactivation of GSK3β, which allows for the stabilization and nuclear translocation of β-catenin.

The importance of ESC differentiation cannot be overstated, as it is a critical step in the development of new therapies for various diseases. The potential of ESCs to differentiate into any cell type in the body holds promise for the treatment of a wide range of conditions, from neurodegenerative diseases to congenital defects. However, the ethical and safety concerns surrounding the use of ESCs have limited their clinical applications. Recent advancements in induced pluripotent stem cell (iPSC) technology have provided an alternative approach to ESCs, allowing researchers to generate pluripotent cells without the ethical concerns associated with ESCs.

The Wnt/β-catenin signaling pathway is a key regulator of ESC differentiation. This pathway is crucial for maintaining the self-renewal and pluripotency of ESCs. By understanding the mechanisms by which this pathway regulates cell fate decisions, researchers can develop more effective strategies for ESC differentiation. The potential of ESCs to differentiate into any cell type in the body holds promise for the treatment of various diseases. However, ethical and safety concerns have limited their clinical applications. Recent advancements in iPSC technology have provided an alternative approach to ESCs, allowing researchers to generate pluripotent cells without the ethical concerns associated with ESCs.

In summary, the Wnt/β-catenin signaling pathway plays a critical role in the differentiation of ESCs. By understanding the mechanisms by which this pathway regulates cell fate decisions, researchers can develop more effective strategies for ESC differentiation. The potential of ESCs to differentiate into any cell type in the body holds promise for the treatment of various diseases. However, ethical and safety concerns have limited their clinical applications. Recent advancements in iPSC technology have provided an alternative approach to ESCs, allowing researchers to generate pluripotent cells without the ethical concerns associated with ESCs.
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 described 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 ceftriaxime-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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References


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