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Frequency of Quinolone Resistance and *qnrB* and *qnrC* Genes in Clinical Isolates of *Klebsiella pneumoniae*

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ABSTRACT

***Background*:** *Klebsiella pneumoniae* is among the most common and significant agents of community and hospital-acquired infections. Plasmid mediated quinolone resistance (PMQR) was increasingly identified in *Enterobacteriaceae* family worldwide. Plasmid-mediated quinolone resistance (PMQR) was first reported in 1998 from a *Klebsiella pneumoniae* isolate in the USA. Till date, five Qnr proteins have been identified; QnrA, QnrB, QnrC, QnrD and QnrS. Quinolone are broad spectrum antibiotics the resistant to which has increasingly been reported among many bacterial species including *Klebsiella*. The aim of this study was the antibiotic resistance profile was determined based on resistance and molecular characterization of *qnrB* and *qnrC* genes in *Klebsiella pneumoniae* clinical isolated.

***Methods*:** In this cross sectional study, 94 samples of *K. pneumoniae* were collected. Isolates were screened for quinolone antibiotics resistance using disk diffusion method according to clinical and laboratory standards institute (CLSI) guidelines. Isolates with resistance to at least one of the quinolone antibiotics, examined for presence of the *qnrB* and *qnrC* genes.

***Results*:** Based on the obtained results by the Agar disk diffusion test, 29.78%, 27.65%, 28.72% and 27.65% of the isolates were resistant to nalidixic acid, ciprofloxacin, norfloxacin and ofloxacin, respectively. Of these 46.66% carried *qnrB*, 3.33% carried *qnrC* genes.

***Conclusion*:** The identification of *qnrB* gene among quinolone-resistant *K. pneumoniae* isolates shows that the emergence of PMQR in this region requires serious preventive measures.

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Introduction

Klebsiella pneumoniae is a Gram-negative non motile, enteric rod and a member of the family *Enterobacteriaceae* that produces capsule (1, 2). It is known as an opportunistic pathogen and one of the most important species involved in nosocomial infections (2, 3). *K. pneumoniae* is among the body micro flora (1). The mortality rate related to it is high and causes a variety of diseases such as urinary tract infection, septicemia, pneumoniae and intra-abdominal infections in hospitalized patients in various hospital settings; however infections associated to *K. pneumoniae* in the community is less observed (4, 5).

From clinical aspect, *K. pneumoniae* strains colonize vastly and seen in hospitalized patients with deficiency in immune system such as diabetic individuals, patients with acquired immune deficiency, elderly patients and children. Severe epidemics due to *Klebsiella* usually occur in children and endemic infections are mostly in patients with renal disease. Although pneumonia by *Klebsiella* include a small part of total pneumonia, it is highly fatal and encounters approximately 90% (6). Quinolones are extended spectrum anti-bacterial synthetic agents that extensively used for treatment purposes which inhibit DNA replication and transcription. The anti-bacterial activity of fluoro-quinolones is because of inhibition of two enzymes called DNA gyrase and topoisomerase IV (1). DNA gyrase in gram negative and topoisomerase IV in gram positive bacteria are more susceptible to the quinolones (7, 8). The both of them are tetramer with different subunits. DNA gyrase subunits are GyrA and GyrB and in case of topoisomerase IV these include ParC and ParE (7). In the year 1998, the plasmid mediated quinolone resistance was described for the first time in one *K. pneumoniae* strain in the United States (9). This plasmid (pMG252) which contained *qnr* gene had molecular weight of 56kb (10-12). The *qnr* genes are responsible for plasmid resistance to quinolones and *qnrA* encoding QnrA 218 amino acid protein belonged to Penta-peptide family. This protein binds to subunits of DNA gyrase and topoisomerase IV and prevents attachment of quinolones to these enzymes and thus leads to the resistance with protecting DNA (13, 14). The *qnr* gene causes resistance to quinolones such as nalidixic acid and increase MIC of fluoro-quinolones up to more than 20 times. Agents of qnr have been reported in *Enterobacteriaceae* worldwide and some variants have been determined (14). Four more Qnr proteins with Penta-peptide repeats including QNRB, QNRC, QNRD and QNRS have been identified which those amino acid sequences are similar to QNRA 43%, 64%, 48% and 59%, respectively (9, 10)

Materials and methods

Isolates

During one year August 2014 to August 2015, a total 94 isolates of *K. pneumoniae* were collected from several samples of hospitalized patients with different clinical infections, in two hospital of Boroujerd. All isolates were identified by conventional bacteriological tests. The bacterial isolates were kept frozen at -70 ºC before tested.

This study has been performed on 94 *K. pneumoniae* isolates from various hospital settings. For confirmation of isolates with resistance to quinolones, phenotypic method using antibiotic disks of norofloxacin (10 µg), ciprofloxacin (5 µg), ofloxacin (5 µg) and nalidixic acid (30 µg) (Rosco, Taastrup, Denmark) was conducted by preparation of half McFarland and culture on Muller Hinton agar based on disc diffusion (Kirby Bauer) method. *Escherichia coli* ATCC25922 was used as the quality control for antibiotic disks (15, 16).

Molecular detection of qnr genes

After detecting isolates which were phenotypically positive, they were assessed with the molecular test. For this purpose, we firstly extracted the total DNA from quinolone resistant isolates with the boiling method. The polymerase chain reaction (PCR) method was performed for

amplification of genes with specific primers shown in table 1. The master mix adjusted in 25 µl including 12.5 µl 2X Taq Master mix (Amplicon), 1ul forward primer, 1ul reverse primer with 5 picomol/L, 3 µl template DNA and 7.5 µl double distilled sterile water and PCR was performed with thermocycler (BioRad) device.

Results

Isolates

Of the 94 clinical isolates included in our study, 70 (74.5%) were isolated from urine, 15 (16%) from tracheal aspiration, 5 (5.3%) from blood culture, 2 (2.1%) from wound, 2 (2.1%) from sputum.

Antibiotic susceptibility testing and identification of qnr genes

In the antibiotic susceptibility test, 30 (31.91%) isolates of *K. pneumoniae* were resistant to quinolones (Table 2). Twenty-seven (28.72%), 26 (27.65%) and 28 (29.78%) isolates were resistant to norfloxacin, ciprofloxacin/ or ofloxacin and nalidixic acid, respectively. PCR products were observed after electrophoresis on 2% gel agarose. Among 30 examined isolates, 14 (46.66%) and 1 (3.33%) were positive for *qnrB* and *qnrC* genes, respectively (Figure 1).

Of 27 norfloxacin resistant isolates, 12 and 1 carried *qnrB* and *qnrC* genes, respectively. Among 26 ofloxacin resistant isolates, 12 were positive for *qnrB*, but none carried *qnrC*. Moreover, of 26 ciprofloxacin resistant isolates, 12 and 1 could amplify *qnrB* and *qnrC* genes, respectively. Of 28 isolates with resistance to nalidixic acid, 13 and 1 were positive for *qnrB* and *qnrC* genes, respectively.

Discussion

In this study, the resistance of *K. pneumoniae* isolates to nalidixic acid, norfloxacin, ciprofloxacin and ofloxacin was 29.78%, 28.72%, 27.56% and 27.56%, respectively that the highest was to nalidixic acid.

Plasmid mediated quinolone resistance (PMQR) in the family of *Enterobacteriaceae* is one kind that is transmitted among strains rapidly in horizontal manner. In addition to this, horizontal transmission of these plasmids to normal flora is a concern over this kind of resistance (19-21). It seems that a higher level use of quinolones in animals treatment and fish farms have had important role in development of this kind of resistance (9). If the *qnr* containing plasmids can transmit to pathogenic strains, exposure of these strains to quinolones causes development of resistance. One important concern about advent of quinolone resistance is their close relation with other agents especially beta-lactamases and aminoglycosides. Unfortunately, this biologic relationship among these agents has caused a suitable opportunity for dissemination of multidrug-resistant Enterobacteriaceae, which restricts treatment choices. Therefore, physicians should consider that when prescribing quinolones, the resistance to cephalosporins and aminoglycosides and other resistance forms which are associated with PMQRs occurs as well (21).

In comparison to this study, a study by Alijani in 2013 showed 16.7% resistance to ciprofloxacin being lower that this study, and this can be because of difference in geographical differences (6). On the other hand, high level use of antibiotics, hospitalization in hospital settings such as ICU and internal medicine for long time and use of infected medical devices such as intravenous and urethral catheters, respiratory trachea and dialysis devices are other causes of resistance. In case of resistance to nalidixic acid, this study did not confirm with a study by Soltan Dallal in Tehran in 2012. This difference may be because of working differences and errors among different locations such as disks, depth and volume of media in plates and components of media. Moreover, use of antibiotics in different areas and climate are other effective factors (22). Amin and coworkers in Pakistan, among 40 *K.*

Table 1. Primer sequences and PCR conditions.

Gene	Sequence (5′-3′)	Temperatures	Amplicon Size (bp)	Reference
qnrB	F: GGMATHGAAATTCGCCACTG R: TTTGCYGYYCGCCAGTCGAA	30 cycle, 95 ˚C 1 min, 55 ˚C 1 min, 72 ˚C 1 min	264	(17)
qnrC	F: GGGTTGTACATTTATTGAATC R: TCCACTTTACGAGGTTCT	40 cycle, 95 ˚C 30s, 52 ˚C 1 min, 72 ˚C 1 min	447	(18)

[c]M = A or C; H = A or C or T; Y = C or T[c]

Table 2. Results of antibiotic susceptibility test.

Antibiotics	Susceptible	Intermediate	Resistant
Norfloxacin	67 (71.27%)		27(28.72)
Ofloxacin	67 (71.27%)	1 (1.06%)	26 (27.65%)
Ciprofloxacin	64 (68.08%)	4 (4.25%)	26 (27.65%)
Nalidixic acid	59 (62.76%)	7 (7.45%)	28(29.78%)

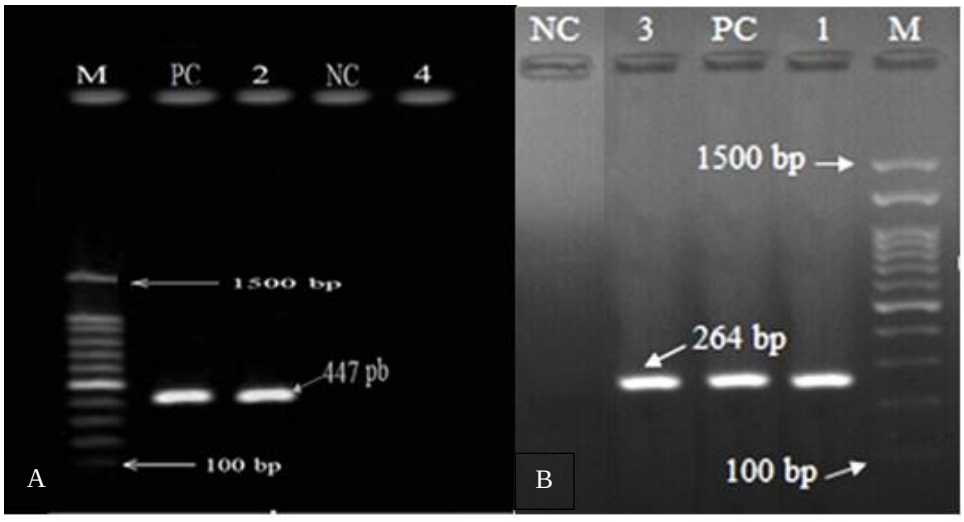

Fig 1. Electrophoresis of PCR products of *qnr* gene in clinical isolates of *K. pneumoniae*, A: Bands with molecular weight of 447bp showing *qnrC*. B: bands with 264bp showing *qnrC* gene.
NC: Negative control. PC: positive control. M: Molecular size marker.

pneumonia isolates detected resistance to ciprofloxacin (55%), ofloxacin (47.5%), nalidixic acid (42.5%) and norfloxacin 35% (23). With the comparison of results from this study and in Pakistan, it is observed that the latter showed higher prevalence, mainly because of misuse consumption of antibiotics without supervision. Moreover, several other factors include population studied (impatient or outpatient), age, hospitalization period, invasive works for diagnosis or treatment, antibiotic used and corporation are reasons of differences (24).

In a study by Kim in Boston in 2009, plasmid mediated quinolone resistance genes were detected in 13 (5%) of 261 *E. coli* isolates and 13 (10%) of 135 *K. pneumonia*, which 22 and 4 isolates were positive for *qnrB* and *qnrS* genes, respectively, while none of isolates could amplify *qnrA* and *qnrC* genes (25). In this study, one isolate was *qnrC* positive and 14 were positive for *qnrB* gene. The prevalence of *qnrB* was higher in the mentioned study, that the difference may be because of diversity in specimens, time of study, treatment strategy in every area, and lower prevalence may be because of low spread of genes. In Minarini's study, none of clinical isolates were *qnrA* positive, and *qnrB* was the predominant among *qnr* genes, which was not consistent with the present study (26). In the investigation of Azadpour et al (2014), among 107 clinical isolates of *K. pneumoniae* isolates in Khorramabad, 34 were resistant to ciprofloxacin, 7 isolates were intermediate and 66 were susceptible. Eighteen of 107 isolates (16.8%) were qnr positive, among which, 16 (88.9%) and 1 (5.55%) were *qnrB* and *qnrS* positive, respectively and one isolate was positive for both of genes. These genes were detected in 8 (23.5%) ciprofloxacin resistant, 1 (14.3) intermediate and 9 (13.6%) from susceptible isolates. no significant relationship was detected between ciprofloxacin resistance and qnr genes (27).

In this study, from 94 clinical isolates of *K. pneumonia*, 26 isolates were resistant, 4 were intermediate and 64 were susceptible to ciprofloxacin. 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. The comparison of the results from this study and Azadpour showed a higher rate of these genes in the present study that may be because of differences in geographical areas, higher antibiotic consumption or abuse prescription, and also transmission of resistance determinants via plasmids, bacteriophages, transposons and integrons.

It can be noted that *Enterobacteriaceae* mechanisms against fluoroquinolones are not similar to those for *Staphylococcus aureus* like *mecA* gene as a powerful defense, but there are the cumulating of multiple mechanisms each with a lower resistance to resist quinolones. Moreover, the relation between *qnr* and other resistance genes and beta-lactamases and plasmid born aac (6) Ib cr and qepA causes the advent of multiple resistant agents. This makes difficulties in the treatment and restricts the choices of antibiotics against these strains (21).

Conclusion

Investigation and study of antibiotic resistance mechanisms in bacteria especially those nosocomial pathogens are very important. Because correct understanding of these mechanisms we can choose suitable drugs via amendments and alteration of instructions in the treatment process and with management of extended spectrum antibiotics consumption we can prevent prevalence of resistance and spread of nosocomial infections. From these studies we can conclude that dependent on the bacterial species and criteria of bacteria choosing, the *qnr* gene prevalence and subtypes are different. Moreover, these genes are different in different geographical regions. The prevalence of *qnrB* is greater than other plasmid born *qnr* genes according to studies from most areas of the world. Moreover, because of placing on plasmids with wide spectrum of host, they are more able spread. The results of this study can be used as a base for other studies to

justify antibiotic resistance mechanisms in resistant isolates and be able to prevent the dissemination of them.

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Conflict of interest

None declared conflicts of interest.

Financial disclosure

None declared.

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