IJMS
Vol 40, No 6, November 2015

Original Article

Prevalence of *qnr* and *aac(6')-Ib-cr* Genes in Clinical Isolates of *Klebsiella Pneumoniae* from Imam Hussein Hospital in Tehran

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Abstract

Background: Plasmid mediated quinolone resistance (PMQR) has been shown to play an important role in resistance not only to quinolones, but also β-lactams and aminoglycosides. In fact, *qnr* genes are frequently carried along with β-lactamase determinants on the same plasmids. We studied the prevalence of *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr* genes among quinolone and cephalosporin resistant clinical isolates of *Klebsiella pneumoniae (K. pneumoniae)*, as well as the association between PMQR genes with resistance to quinolones, cephalosporins and aminoglycosides.

Methods: The study was conducted on 79 *K. pneumoniae* clinical isolates collected from Imam Hussein hospital in Tehran between July 2010 and January 2011, based on their resistance to quinilones and cephalosporins. Antibacterial susceptibility was determined to 15 antibiotics by disc diffusion. Presence of *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr* genes were investigated using specific primers and PCR.

Results: Of the 79 *K. pneumoniae* isolates, 47 (59.5%) carried the PMQR determinants. Among these, 42 (89.4%) carried *aac(6')-Ib-cr* of which, 21 (50%) also harbored *qnrB*. Three isolates carried *qnrB* alone, two (4.2%) harbored *qnrS* and none had *qnrA*. Resistance to aminoglycosides and cephalosporins was significantly higher in the isolates carrying both *qnrB* and *aac(6')-Ib-cr* genes compared to *aac(6')-Ib-cr* alone.

Conclusion: This study showed a high prevalence of *aac(6')-Ib-cr* and *qnrB* genes among the Iranian *K. pneumoniae* clinical isolates as well as co-carriage of the two genes. There was a significant association between *qnrB* gene carriage and resistance to quinolones, cephalosporins, and aminoglycosides.

Please cite this article as: Eftekhar F, Seyedpour SM. Prevalence of *qnr* and *aac(6')-Ib-cr* Genes in Clinical Isolates of *Klebsiella Pneumoniae* from Imam Hussein Hospital in Tehra. Iran J Med Sci. 2015;40(6):515-521.

Keywords ● *Klebsiella pneumoniae* ● Quinolone resistance ● *Qnr* protein ● Iran

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Received: 27 October 2013
Revised: 7 December 2013
Accepted: 26 January 2014

Introduction

Klebsiella pneumoniae is an opportunistic pathogen responsible for up to 10% of nosocomial infections.[1] Extensive use of extended-spectrum cephalosporins, fluoroquinolones and carbapenems for treatment of these infections, has led to a significant increase of resistance in these bacteria. Resistance mechanisms such as production of β-lactamases,

plasmid-mediated quinolone resistance (PMQR) and carbapanemases have caused serious therapeutic problems.[2,3]

Quinolones were introduced into clinical use in 1962 in the form of nalidixic acid, a fully synthetic agent with bactericidal effects on most members of *Enterobacteriaceae*. These broad-spectrum antibacterial agents are commonly used for treatment of infections, both in humans and in veterinary medicine. As a result, enhanced levels of quinolone resistance has occurred in recent years.[4] For example, ciprofloxacin, previously shown to have excellent activity against clinical isolates of *Klebsiella*, has become less effective due to its extensive use.[5,6] Early studies showed that quinolone resistance arises by mutations occurring in topoisomerase subunits, changes in the expression of efflux pumps and porins that provide entry for these agents into the bacterial cell.[7] PMQR-mediated resistance was discovered in the late 1990s in clinical isolates of *Enterobacteriaceae*, including *K. pneumoniae*. It has been shown to play not only an important role in quinolone resistance, but also resistance to other antibiotics, particularly β-lactams and aminoglycosides.[7,8] The first PMQR gene in *K. pneumoniae* was reported in 1998 in USA. Since then, plasmids harboring *qnr* genes have been found in clinical isolates of *Enterobacteriaceae* worldwide.[8-11] PMQR determinants include: *Qnr* proteins (*QnrA*, *QnrB*, *QnrS*, *QnrC*, and *QnrD*), which protect DNA gyrase and topoisomerase IV from inhibition by quinolones, the aminoglycoside acetylteransferase, Aac(6')-Ib-cr, and more recently, the fluoroquinolone specific efflux pump protein, QepA.[12] The *aac(6')-Ib-cr* (cr for ciprofloxacin resistance) is a variant of *aac(6')-Ib* (responsible for resistance to kanamycin, tobramycin and amikacin) with two amino acid substitutions compared to the wild-type allowing it to acetylate and subsequently reduce the activity of norfloxacin and ciprofloxacin.[13-15] Therefore, in addition to quinolone resistance, PMQR determinants can play an important role in resistance to other antibiotics, particularly β-lactams and aminoglycosides. In fact, a number of studies have shown plasmids which carry *qnr* genes along with various β-lactamase determinants.[15,16] We studied the prevalence of *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr* genes among quinolone and cephalosporin resistant clinical isolates of *Klebsiela pneumoniae*, as well as the association between PMQR gene carriage with resistance to quinolones, cephalosporins and aminoglycosides.

Materials and Methods

Bacterial Isolates

Seventy-nine isolates of *K. pneumoniae,* which were resistant to quinolones and/or cephalosporins, were chosen from a collection of 104 clinical isolates obtained from Imam Hussein hospital in Tehran between July 2010 and January 2011. Majority of the specimens were from urine (44.3%), followed by sputum (25.3%), catheters (11.4%), wound and blood (7.6% each), and other miscellaneous sources (3.8%). All isolates were identified by conventional biochemical tests and were maintained at -20°C in brain heart infusion broth (Oxoid, UK) containing 10% (v/v) dimethyl sulfoxide (Merck, Germany) until use.

Antimicrobial Susceptibility

Susceptibility to 15 antibiotics was determined by disc diffusion using the CLSI recommendations.[17] The antibiotics (Himedia, India) were: amoxicillin/clavulanic acid (AMC, 20+10 µg), cefepime (CPM, 30 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), aztreonam (ATM, 30 µg), imipenem (IPM, 10 µg), ciprofloxacin (CP, 30 µg), levofloxacin (LOM, 5 µg), norfloxacin (NOR, 10 µg), ofloxacin (OFX, 5 µg), nalidixic acid (NA, 30 µg), amikacin (AK, 30 µg), gentamicin (GM, 10 µg), kanamycin (KM, 30 µg) and nitrofurantoin (NF, 300 µg). *K. pneumoniae* ATCC 10031 (obtained from Persian type culture collection, Iran) was used as control for antimicrobial susceptibility.

ESBL Production

The isolates were initially screened for ESBL production by the double disc synergy test (DDST) using cefotaxime (30 µg), ceftazidime (30 µg) and cefepime (30 µg), placed 20 mm (center to center) from an amoxicillin/clavulanic acid disc (20+10 µg). ESBL production was detected when synergy was observed between the inhibition zones of cephalosporins and amoxicillin/clavulanic acid and was further confirmed by the phenotypic confirmatory test (PCT) using ceftazidime and cefotaxime alone or in combination with clavulanic acid.[18,19]

DNA Extraction and PCR Amplifications

Extraction of plasmid DNA was performed using an improved phenol/chloroform method where the cells were directly lysed by phenol.[20] Presence of *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr* genes were detected by PCR using the primers shown in Table 1.[21,22]

PCR reaction mixtures (25 µl) contained 1.5 µl DNA template, 1.5 mM $MgCl_2$, 0.25 mM of

dNTP mix, 1 unit of DFS-Taq DNA polymerase (Bioron, Germany) and 20 pmol of each primer. PCR amplifications were performed in a thermal cycler (Bioer TC25/H, Bioer Technology, China) using the following program. Initial denaturation at 94ºC for 5 min followed by 30 cycles of 1 min at 94ºC, 1 min at annealing temperature (57ºC for *qnrA*, *qnrB* and *qnrS*, 54ºC for *aac(6')-Ib-cr* genes), 1 min at 72ºC and a final extension period of 10 min at 72ºC. The amplified PCR products were resolved by electrophoresis on 1.5% agarose gel and visualized after staining with ethidium bromide. Antibiotic resistance rates in PMQR harboring isolates were analyzed by the two-way ANOVA test.

Results

Antibiotic resistance rates (including intermediate resistance) of the isolates were: amoxicillin/clavulanic acid (100%), ceftazidime (81%), cefotaxime (74.7%) cefepime (72.1%), aztreonam (73.4%), nitrofurantoin (59.5%), kanamycin (78.5%), gentamicin (53.2%), amikacin (20.2%), norfloxacin (74.7%), ciprofloxacin (58.2%), nalidixic acid (45.6%), ofloxacin (43%) and levofloxacin (38%). All isolates were susceptible to imipenem.

Overall, PMQR determinants were found in 47 isolates (59.5%). Figure 1 shows the amplification product of the *qnr* genes. Among these, 24 isolates (30.4%) carried *qnrB* of which, the majority (n=21, 87.5%) also co-harbored the *aac(6')-Ib-cr* gene. Two isolates (2.5%) harbored *qnrS* and none carried the *qnrA* gene. Figure 2 presents the PCR product of the *aac(6')-Ib-cr* gene. Forty two isolates (53.2%) carried *aac(6')-Ib-cr* of which, half also had the *qnrB* as mentioned above. There was no association between the specimen source and gene carriage (data not shown).

Four *K. pneumoniae* isolates were detected as ESBL producers, half of which harbored *qnrS* and the other two carried *aac(6')-Ib-cr*.

Figure 3 compares the antibiotic resistance profiles of the *K. pneumoniae* isolates carrying *qnrB* alone, with strains harboring *qnrB*+*aac(6')-Ib-cr* genes. As observed, resistance to nalidixic acid, ciprofloxacin, levofloxacin, norfloxacin, cefotaxime, ceftazidime, cefepime, gentamycin, amikacin and kanamycin were almost twice as high in the isolates carrying both genes compared to the isolates, which harbored *qnrB* alone.

Discussion

Presence of PMQR determinants in quinolone resistant *E. coli* and *K. pneumoniae* is increasingly reported worldwide. Most of these studies report a higher prevalence of PMQR genes in *K. pneumoniae* compared to *E. coli*.[3,16,23-27] However, there are variations found in PMQR carriage rates from different parts of

Table 1: Primers used for detection of *qnr* and *aac (6')-Ib-cr* genes

Gene	Primer	Primer sequence	Product size (bps)	Reference
qnrA	Forward Reverse	TTCTCACGCCAGGATTTGAG TGCCAGGCACAGATCTTGAC	571	(21)
qnrB	Forward Reverse	TGGCGAAAAAATTGAACAGAA GAGCAACGATCGCCTGGTAG	594	(21)
qnrS	Forward Reverse	GACGTGCTAACTTGCGTGAT AACACCTCGACTTAAGTCTGA	388	(21)
aac (6')-Ib-cr	Forward Reverse	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTTT	482	(22)

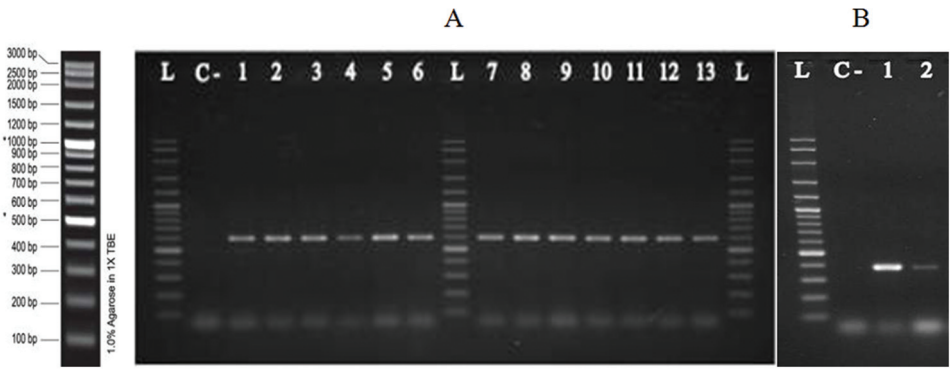

Figure 1: Shows gel electrophoresis of *qnrB* (A; lanes 1-6, and 7-13) and *qnrS* (B: lanes 1 and 2) genes in some isolates of *K. pneumoniae* by PCR. L: 1Kb Ladder, C-: negative control.

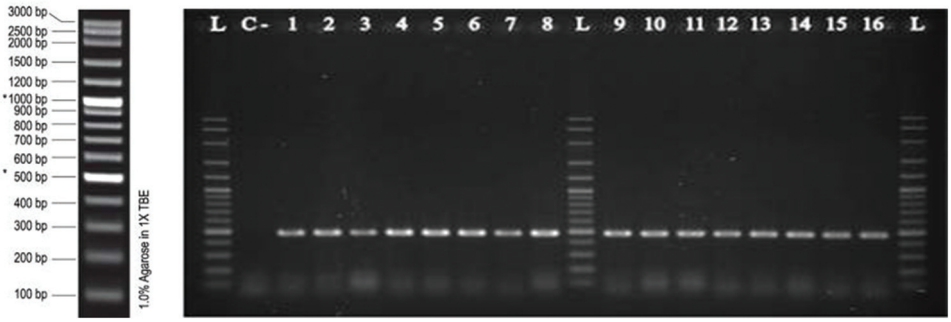

Figure 2: Shows gel electrophoresis of *aac(6')-Ib-cr* gene in some isolates of *K. pneumoniae* by PCR (lanes 1-8 and 9-16). L: 1Kb Ladder, C-: negative control.

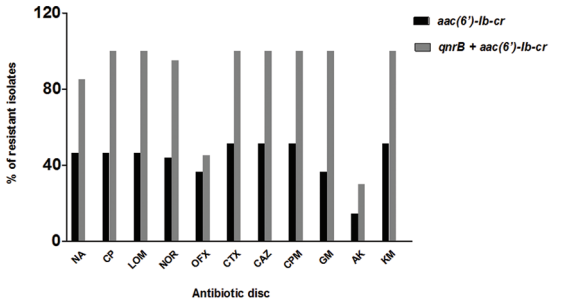

Figure 3: Shows comparison of antibiotic resistance profiles in *K. pneumoniae* isolates carrying only *qnrB* (black bars) with the isolates which harbored both *qnrB+aac(6')-Ib-cr* (grey bars). NA: Nalidixic acid, CP: Ciprofloxacin, LOM: Levofloxacin, NOR: Norfloxacin, OFX: Ofloxacin, CTX: Cefotaxime, CAZ: Ceftazidime, CPM: Cefepime, GM: Gentamycin, AK: Amikacin, KM: Kanamycin.

the world, mostly because the selection of the test bacteria is based on resistance to different antibiotics (e.g., nalidixic acid, ciprofloxacin). Despite that, most studies have shown a higher rate of *aac(6')-Ib-cr* carriage compared to *qnr* genes.[3,16,21-26] We found a high rate of *qnrB* and *aac(6')-Ib-cr* co-carriage among our isolates. These results are supported by the results of previous studies.[3,23,25,27] Co-carriage of *qnr* and *aac(6')-Ib-cr* genes have been reported.[3,23,25,27] Presence of *qnrB* and *aac(6')-Ib-cr* has also been shown in *K. pneumoniae,* but not at the high frequency which was found in our study.[27] Interestingly, the isolates which carried both *qnrB* and *aac(6')-Ib-cr* were significantly more resistant to aminoglycosides, cephalosporins and quinolones (P<0.0001) in comparison to the isolates that harbored the *aac(6')-Ib-cr* alone, suggesting the important role of *qnrB*. Additional resistance mechanisms such as mutations in chromosomal *gyrA* and *gyrC* genes or presence of efflux pumps such as QepA, can also be responsible for the observed high resistance levels.[12,28]

The association of PMQR determinants with ESBL production has been shown by a number of investigators. However, the rates of PMQR carriage vary considerably in ESBL producing *Enterobacteriaceae* (mostly *E. coli* and *K. pneumoniae*). For example, PMQR genes were observed in 48% of the isolates in Thailand, 30.5% in Canada, 10% in Australia, 4.9% in Spain and 1.6% in France.[9,16,27,29,30] Our results showed 5.1% PMQR carriage in ESBL producing *K. pneumoniae* similar to the results of the Spanish study. Co-carriage of PMQR determinants along with ESBL-encoding genes on conjugative plasmids have also been reported by a number of investigators.[16,21,23,29,30]

We did not find any reports on the presence of PMQR genes in *K. pneumoniae* isolates from Iran. Other Iranian studies have shown the presence of *qnr* genes in *E. coli* and *Salmonella.*[31,32] To our knowledge, the only other report on *qnr* gene carriage in Iranian clinical isolates of *K. pneumoniae* was a poster presentation which showed that *qnrB* was the predominant gene (13.4%) followed by *qnrA* and *qnrS* (1.2% each). Presence of *aac(6')-Ib-cr* was not determined in that study, but co-presence of *qnrB* and *qnrS* was observed in one isolate (1.2%).[33] Our results are important in presenting the prevalence of PMQR determinants (specifically *aac(6')-Ib-cr* and *qnrB*) in clinical isolates of *K. pneumoniae* and their association with multiple drug resistance. However, due to the lack of research in this area, there is no basis for comparison of these results with other local or national studies. Hence, further research should be carried out in different parts of Iran to obtain a realistic rate of PMQR gene carriage on the national level. Considering the fact that these genes are often carried on mobile genetic elements and could easily be spread among the members of *Enterobacteriaceae*, such information is needed for choosing a proper antibiotic therapy and may prevent the dissemination of these resistance determinants among important Gram-negative pathogens.

Conclusion

The results of this study showed a high prevalence of *aac(6')-Ib-cr* and *qnrB*, as well as a high rate of co-carriage of the two genes among the *K. pneumoniae* isolates in Tehran. There was a significant association between *aac(6')-Ib-cr* and *qnrB* co-carriage with resistance to quinolones, cephalosporins and aminoglycosides.

Acknowledgment

The authors wish to thank the Shahid Beheshti University Research Council for providing the financial support of this research.

Conflicts of Interest: None declared.

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