Fluoroquinolone Resistance in Clinical Isolates of *Klebsiella Pneumoniae*

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Abstract

Introduction Fluoroquinolones are widely used broad-spectrum antibiotics. Recently, increased rate of resistance to this antibiotic has been observed in Klebsiella pneumoniae. The aim of the present study was to determine the presence of quinolone resistance determining regions (QRDR) mutation genes and plasmid-mediated quinolone resistance (PMQR) determinants in clinical isolates of ciprofloxacin-resistant K. pneumoniae.

Material and Methods The study included 110 nonduplicate ciprofloxacin-resistant K. pneumoniae clinical isolates. Antibiotic susceptibility testing by disk diffusion method and minimum inhibitory concentration (MIC) by agar dilution methods for ciprofloxacin was performed according to the recommendations of Clinical Laboratory Standards Institute. The presence of QRDR genes and PMQR genes was screened by polymerase chain reaction (PCR) amplification.

Result All 110 isolates were resistance to ciprofloxacin, levofloxacin, and ofloxacin. As much as 88% of the isolates exhibited high-level of MIC to ciprofloxacin. Among the 110 isolates, 94(85%) harbored gyrA and 85 (77%) gyrB. The parC and parE genes were detected in 88 (80%) and 64 (58%) isolates. qnrB was detected in 13 (12%) isolates and qnrS in 5 (4.5%) isolates. Two (1.8%) isolates carried both qnrB and qnrS genes. The acc (6')-Ib-cr gene was found in 98 (89%) isolates and oqxAB was detected in 7 (6.3%) isolates. One (0.9%) isolate carried qnrB, acc(6')-Ib-cr and oqxAB genes.

Conclusion The prevalence of acc (6')-Ib-cr gene is high among PMQR determinants, followed by qnrB, oqxAB and qnrS.

Keywords
- QRDR
- Plasmid
- GyrA

Introduction

Klebsiella pneumoniae is a clinically important pathogen which causes a wide range of infections.[1] It is the most common of the fluoroquinolone-resistant bacteria among *Enterobacteriaceae*.[2] Ciprofloxacin is a fluoroquinolone frequently administered to treat bacterial infections.[3] The emergence of fluoroquinolone resistance is rapidly rising due to its broad-spectrum of activity and consequent high-usage in the treatment of infectious disease.[4]

Resistance to fluoroquinolone is mediated by several mechanisms. The major mechanism is the chromosomal mutation at quinolone resistance determining regions (*QRDR*) encoded by DNA gyrases (*gyrA* and *gyrB* genes) and topoisomerase IV (*parC* and *parE* genes).[5]

The other mechanism of resistance is plasmid-mediated quinolone resistance (PMQR) and this was first reported in 1998 in a clinical isolate of *K. pneumoniae*.[6] The three PMQR mediators are the *qnr* proteins (*qnrA*, *qnrB* and *qnrS*) that protect the target enzymes encoding DNA gyrase and topoisomerase IV. Yet another mechanism attributed to fluoroquinolone resistance is the *acc(6')-Ib-cr* gene, encoding a variant of aminoglycoside transferase which acetylates certain fluoroquinolones also. The *qepA* and *oqxAB* are specific

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efflux pump encoding genes that extrude fluoroquinolone from bacterial cell, thus contributing to resistance.[7]

PMQR determinants confer low-level resistance to fluoroquinolones, and they provide a favorable background for the selection of additional chromosomally encoded fluoroquinolone resistance mechanisms.[8] Recently, PMQR is increasingly being reported worldwide.

The purpose of this study was to determine the presence of QRDR mutation genes and PMQR determination in clinical isolates of ciprofloxacin-resistant *K. pneumoniae*.

Methods

Bacterial Isolates

This study included 110 nonduplicate clinical isolates of ciprofloxacin-resistant *K. pneumoniae* obtained from hospitalized patients admitted to a tertiary healthcare hospital. The source of the clinical isolates were exudates (n = 88), respiratory secretions (n = 5), and blood (n = 17), and these were collected from June 2014 to May 2015. The bacterial identity was performed by automated (VITEK2 GN-card; BioMerieux, Brussels, Belgium) and conventional methods.

Antimicrobial Susceptibility Testing

The Kirby–Bauer Disk diffusion method and minimal inhibitory concentration (MIC) was performed in accordance with the Clinical Laboratory Standards Institute guidelines (CLSI 2017).[9] ATCC *Escherichia coli* 25922 was used as a control for both disc diffusion method and MIC. The antibiotics tested by disc diffusion method were as follows: levofloxacin (5 µg), ciprofloxacin (5 µg), and ofloxacin (5 µg) (Hi-media, Mumbai). MIC was determined by agar dilution assay for ciprofloxacin (Sigma-Aldrich, India).

Preparation of Media and Antibiotic Solution

MIC was determined using concentration derived from serial two-fold dilution indexed to the base 2 (e.g., 1, 2, 4, 8µg/mL). Two mL of various serial two-fold dilutions of the antimicrobial agent was added to 18 mL molten MHA agar. The inoculum was prepared by mixing colonies in peptone water obtained from an overnight culture of Gram negative clinical isolates grown on MacConkey Agar plate (MAC) (Himedia Laboratories, India). The agar plate with the concentration of the drug at which there were was no growth was taken as the minimum inhibitory concentration.

Polymerase Chain Reaction (PCR)

The DNA of the study isolates was extracted by the boiling method.[10] The QRDR mutation genes (*gyrA*, *gyrB*, *parC* and *parE*) were detected by using specific primers,[11] and the PCR conditions were as follows: 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for *gyrA* and *parE*, 58°C for *gyrB,* and 52°C for *parC* for 30 seconds, with extension at 72°C for 50 seconds, and a final extension at 72°C for 10 minutes. The amplification of *qnr* genes (*qnrA*, *qnrB* and *qnrS*) was performed by multiplex PCR using the cyclic profile: initial denaturation at 94°C for 7 minutes; denaturation at 94°C for 50 seconds, annealing at 53°C for 40 seconds, and elongation at 72°C for 60 seconds, repeated for 35 cycles, and a final extension at 72°C for 5 minutes.[12] PCR conditions for *acc (6')-Ib-cr* were: initial denaturation at 94°C for 7 minutes, denaturation at 94°C for 50 seconds, annealing at 55°C for 40 seconds, and elongation at 72°C for 60 seconds, repeated for 35 cycles, and a final extension at 72°C for 5 minutes. The PCR cyclic parameters for *oqxAB* were as follows: initial denaturation at 95°C for 15 minutes; 30 cycles of 94°C for 30 seconds, 63°C for 90 seconds, and 72°C for 90 seconds; followed by a final extension at 72°C for 10 minutes. The PCR condition used for *qepA* were as follows: initial denaturation at 96°C for 1 minute, followed by 30 cycles of amplification at 96°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and the final extension step was at 72°C for 5 minutes.[13,14] The primers used are given in ►**Table 1**. The PCR product was examined by electrophoresis in 1.5% agarose gel containing ethidium bromide and visualized by gel documentation system.

Nucleotide Sequence

The PCR positive amplicons were sequenced by SciGenome Labs PVT. Ltd, India, and analyzed with BLAST tools (www.ncbi.nim.nih.gov).

The assigned Genbank accession numbers for the submitted sequences are as follows: MH709267 (*gyrA*); MH709268 (*gyrB*); MK318818 (*parC*); MK318819 (*parE*); MH709266 (*qnrA*); KY130487 (*qnrB*); KY130488 (*qnrS*); MH709269 (*acc (6')-Ib-cr*); MH709851 (*oqxAB*).

Conjugation Assay

Conjugation assay was performed to study the transfer of plasmid from *qnr* positive isolates which were used as donors.

Table 1 Primers used in this study

Gene	Primers	Product size
gyrA	GGATAGCGGTTAGATGAGC CGTTCACCAGCAGGTTAGG	521
gyrB	CAGCAGATGAACGAACTGCT AACCAAGTGCGGTGATAAGC	376
parC	AATGAGCGATATGGCAGAGC TTGGCAGACGGGCAGGTAG	487
parE	GCTGAACCAGAACGTTCAG GCAATGTGCAGACCATCAGA	426
qnrA	5-TCAGCAAGAGGATTTCTCA-3 5-GGCAGCACTATTA CTCCCA-3	516
qnrB	5-GATCGTGAAAGCCAGAAAGG3 5-ACGATG CCTGGTAGTTGTCC-3	469
qnrS	5-ACGACATTCGTCAACTGCAA-3 5-TAAATTGGCACCCTGTAGGC-3	417
acc (6')-Ib-cr	5-TTGGAAGCGGGGACGGAM-3 5-ACACGGCTGGACCATA -3	260
oqxAB	5- CCGCACCGATAAATTAGTCC-3 5-GGCGAGGTTTTGATAGTGGA-3	313
qepA	5 -GCA GGT CCA GCA GCG GGT AG-3 5 -CTT CCT GCC CGA GTA TCG TG-3	199

Escherichia coli J53 AziR strain was used as recipient. The mating was performed in logarithmic phase by adding the donor and recipient cells (0.5 mL each) in 3 mL of Luria–Bertani broth and incubated overnight at 37 °C. Transconjugants were selected on Macconkey agar plates containing sodium azide (100 µg/mL) and ciprofloxacin (0.5 µg/mL).[15] The transconjugants were analyzed by PCR to determine the transferability of PMQR determinants.

Results

Antimicrobial Susceptibility Testing

MIC to ciprofloxacin ranged from 4 µg/mL to ≥256 µg/mL. MIC_{50} and MIC_{90} were 32 µg/mL and 128 µg/mL, respectively. All 110 isolates were resistant to ciprofloxacin, levofloxacin, and ofloxacin.

Distribution of QRDR and PMQR Genes

Among the 110 study isolates, 94 (85%) harbored *gyrA* and 85 (77%) *gyrB*. The *parC* and *parE* genes were detected in 88 (80%) and 64 (58%) isolates. Combination of the above four genes was found in 56 (51%) isolates. (►**Table 2**). Of the eighteen (16%) isolates which harbored the *qnr* genes, *qnrB* was detected in 13 (12%) isolates and *qnrS* in 5 (4.5%) isolates. Two (1.8%) isolates carried both *qnrB* and *qnrS* genes. The *acc (6')-Ib-cr* gene was found in 98 (89%) isolates and *oqxAB* was detected in7 (6.3%) isolates. One (0.9%) isolate carried *qnrB*, *acc (6')-Ib-cr* and *oqxAB* genes (►**Table 3**). Notably, *qnrA* and *qepA* were not detected in any of the study isolates.

PMQR Gene Transfer

Of the 18 *qnr* determinants, 11 (61%) were successfully transconjugated. Among them, eight (44%) harbored the *qnrB* and three (17%) harbored *qnrS* gene.

Among the *qnrB* transconjugants, four (22%) also carried *acc (6')-Ib-cr*, and one coharbored the *acc (6')-Ib-cr* and *oqxAB*. *qnrB* alone was present in three transconjugants. Of the three *qnrS* transconjugants, one (5.5%) coharbored *acc (6')-Ib-cr*.

Table 2 Distribution of quinolone resistance chromosomal mutation genes

QRDR genes	No. of positive (*n* = 110)
gyrA	94 (85%)
gyrB	85 (77%)
parC	88 (80%)
parE	64 (58%)
gyrA + gyrB	2 (1.8%)
gyrA + parE	6 (5.4%)
gyrB + parE	4 (3.6%)
gyrA + gyrB + parC	28 (25%)
gyrA + parC + parE	9 (8.1%)
gyrB + parC + parE	2 (1.8%)
gyrA + gyrB + parC + parE	5 (4.5%)

Table 3 Distribution of plasmid-mediated quinolone resistance genes

PMQR genes	No. of positives (*n* = 110)
qnrB	13 (11.8%)
qnrS	5 (4.5%)
acc (6')-Ib- cr	98 (89%)
oqxAB	7 (6.3%)
qnrB + qnrS	2 (1.8%)
qnrB + qnrS+ acc (6')*-Ib– cr + oqxAB*	1 (0.9%)

Discussion

Fluoroquinolones are the most important antibacterial agents used for the treatment of bacterial infections.[16] Recently, bacterial resistance to fluoroquinolones has increased in clinical isolates. The most common resistance mechanism of fluoroquinolones are the chromosomal mutations in QRDR and PMQR.[17] In the present study, 110 ciprofloxacin-resistant clinical isolates of *K. pneumoniae* were screened to determine the prevalence of QRDR mutation genes and PMQR determinants.

In this study, a majority (88%) of isolates exhibited high-level of MIC to ciprofloxacin. The *gyrA* gene (85%) was encountered most frequently followed by *parC* (80%), *gyrB* (77%), and *parE* (58%). Similar high-prevalence rate for mutations in *gyrA* has been reported by Alisha et al from Iran and Muthu et al from India.[18,19] Although the *gyrA* and *parC* are most commonly reported, mutation-resistant genes in the QRDR regions, in the current study, *gyrB* and *parE* genes were also frequently encountered.

In this study, *qnrB* gene (12%) was more prevalent than *qnrS* (4.5%). *qnrA* was not found in any isolate. Our results are consistent with the findings of previous studies.[20-24] A study from Korea reported high-prevalence of *qnrS* (26.6%) as compared with *qnrB* (6.5%) and *qnrA* was not detected.[25] Mahesh et al and Tripathi et al from India observed *qnrA* and *qnrB* in clinical isolates, whereas *qnrS* was not detected.[26,27] In few studies, all the three *qnr* genes (*qnrA*, *qnrB* and *qnrS*) have been found in clinical isolates.[8,28,29] The types of *qnr* genes may vary in different geographical locations.[30] Conjugation experiment demonstrated that 61% of *qnr* determinants are transferable, where one transconjugant carrying multiple PMQR genes was documented. This transferability rate is high compared with previous studies.[31-33]

In the present study, the prevalence of *oqxAB* gene (6.3%) was very low as compared with previous reports.[23,29,31] Thus, indicating that it may not be a major mediator of fluoroquinolone resistance. *qepA* gene was not detected in the present study and similar findings has been documented from Thailand and Iran.[34,35] In contrast, *qepA* gene was detectable in a study conducted by Majida et al, and in the same study, *oqxAB* was notably absent.[30] However, from India, only a few studies have reported *oqxAB* and *qepA* genes in *Enterobacteriaceae*.

In this study, *acc (6')-Ib-cr* was predominantly present along with PMQR genes. Similar to this result, a high-prevalence was noted in Iran, Korea and Israel.[22,35,36] In agreement to the previous reports, all the *qnr* determinants were positive for *acc (6')-Ib-cr gene*.[37-40] A high-frequency of the combined occurrence of *acc (6')-Ib-cr* and QRDR mutations and PMQR determinants in multidrug resistant *K. pneumoniae* has been reported from Brazil.[8] Limited data have been reported on the prevalence of fluoroquinolone resistance in India. These results suggest that the emergence of the PMQR would contribute to a rapid increase and spread in bacterial resistance to fluoroquinolones, which requires continuous surveillance and monitoring of antibiotic use. The limitation of this study is the lack of analysis of efflux pump activity.

Conclusion

The current study demonstrated a high prevalence of *aac (6')-Ib-cr* gene among PMQR determinants. The transferability rate of these determinants is high. This is a cause for concern, since horizontal transfer of PMQR genes can increase the spread of fluoroquinolone resistance among clinical isolates.

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Conflict of Interest
None.

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