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# Prevalence of Quinolone Resistance Genes in *Klebsiella pneumoniae* Strains Isolated from Hospitalized Patients During 2013 - 2014

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## Abstract

**Background:** The increasing emergence of resistance among clinical isolates of *Klebsiella pneumoniae* (*K. pneumoniae*) has limited the therapeutic options used to treat infections caused by these bacteria.
**Objectives:** The aim of this study was the molecular detection of quinolone resistance genes *acrA*, *acrB*, *qepA*, and *aac(6')-Ib-cr* in *K. pneumoniae* strains isolated from hospitalized patients in selected hospitals in Tehran during 2013 - 2014.
**Methods:** One hundred and seventeen strains of *K. pneumoniae* were isolated between August 2013 and March 2014 from hospitalized patients in Taleghani hospital, Mofid children's hospital, and Imam Hossein hospital in Tehran. Antimicrobial susceptibility tests were performed using disk diffusion and broth microdilution methods based on CLSI guidelines. The identification of the genes that encode efflux pumps *acrA*, *acrB*, *qep*A, and *aac(6')-Ib-cr* was done using the PCR technique.
**Results:** Antimicrobial susceptibility tests showed that colistin and tigecycline had the best effect against clinical isolates of *K. pneumoniae*. The PCR assay detected the *acrA* and *acrB* genes in 110 (94%) and 102 (87%) isolates, respectively. Additionally, the *qepA* and *aac(6')-Ib-cr* genes were detected in 5 (4%) and 100 (85%) isolates, respectively.
**Conclusions:** The prevalence of the *acrA*, *acrB*, *qepA*, and *aac(6')-Ib-cr* genes in *K. pneumoniae*, which causes resistance to fluoroquinolones, in this study is cause for concern. Based on our results, accurate identification of resistant Gram-negative bacteria such as *K. pneumoniae* and detection of its susceptibility to common antibiotics could lead to proper treatment and control of resistant nosocomial infections.

*Keywords:* Efflux Pump, Fluoroquinolone, Antibiotic Resistance, *Klebsiella pneumoniae*

## 1. Background

*Klebsiella pneumoniae* (*K. pneumoniae*) is one of the important nosocomial infections, causing respiratory, urinary, and wound infections (1-3). Resistance to quinolones in hospitalized isolates was first studied in 1998 in a *K. pneumoniae* strain isolated in Birmingham, USA (4, 5). Over the past three decades, quinolone resistance has expanded among *K. pneumoniae* strains isolated from hospitalized patients. Thus, the spread of resistant *K. pneumoniae* is becoming a global threat to public health (6, 7).

Fluoroquinolone resistance mainly occurs as a result of mutations in chromosomal gene-containing DNA gyrase, topoisomerase IV, and an overexpression of the AcrAB efflux system. Two plasmid-mediated quinolone resistance mechanisms have also been reported in QepA, a plasmid-mediated fluoroquinolone efflux pump, and the Aac(6')-Ib-cr enzyme, which acetylates aminoglycosides and cipro [U+FB02] oxacin (8-11). Efflux pumps are transport proteins that extrude toxic substrates from the intracellular environment into the extracellular environment. These proteins are found in both Gram-positive and Gram-negative bacteria. The AcrAB and QepA efflux pumps act on the bacterial membrane by extruding antibiotics to the extracellular environment so that the intracellular concentration of antibiotic decreases, causing antibiotic resistance (12, 13).

The AcrAB efflux system consists of the outer membrane channel TolC; the transporter AcrB, which is placed in the inner membrane; and the periplasmic AcrA, which bridges these two integral membrane proteins. The AcrAB efflux pump is able to transport various compounds with little chemical similarity, thus conferring resistance to a broad spectrum of antibiotics. The plasmid-mediated QepA efflux belongs to the main facilitator superfamily-type group and confers diminished susceptibility to hydrophilic fluoroquinolone. The *aac(6')-Ib-cr* gene encrypts an aminoglycoside acetyltransferase AAC(6')-Ib variant marked by Trp102Arg and Asp179Tyr substitutions. These

changes afford the new enzyme the capability to acetylate fluoroquinolones that are harboring an unsubstituted piperazinyl group, such as ciprofloxacin and norfloxacin. As a consequence, this gene confers decreased susceptibility to some fluoroquinolones, tobramycin, kanamycin, and amikacin.

## 2. Objectives

The aim of this study was to detect AcrAB and QepA efflux pumps and the Aac(6')-Ib-cr enzyme among *K. pneumoniae* clinical isolates using the PCR method in three teaching hospitals in different parts of Tehran.

## 3. Methods

### 3.1. Bacterial Isolates

One hundred and seventeen strains of *K. pneumonia*e were isolated between August 2013 and March 2014 from hospitalized patients in Taleghani hospital, Mofid children's hospital, and Imam Hossein hospital in Tehran. Conventional biochemical tests were performed according to well-recognized methods: ornithine and lysine decarboxylation, a motility test, an indole test, a methyl red test, the Voges-Proskauer test, the Simmons' citrate test, and the triple sugar iron test (Merck, Germany) (14). *Escherichia coli* (*E. coli*) ATCC 25922 was used as positive control strain for bacterial detection.

### 3.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was determined using the microdilution and Kirby-Bauer disk diffusion (Mast group, Merseyside, UK) methods in accordance with the recommendations of the clinical and laboratory standards institute (CLSI 2013) (14). The antimicrobial agents tested with the microdilution method were imipenem, meropenem, ampicillin, cefotaxime, and ceftazidime. The agents tested with the Kirby-Bauer disk diffusion method were ciprofloxacin (CIP, 30 $\mu$g), aztreonam (ATM, 30 $\mu$g), imipenem (IPM,10 $\mu$g), meropenem (MEM, 10 $\mu$g), doripenem (DOR, 10 $\mu$g), ertapenem (ETP, 10 $\mu$g), gentamicin (GEN, 10 $\mu$g) amikacin (AK, 30 $\mu$g), ampicillin (AMP, 10 $\mu$g), ceftazidime (CAZ, 30 $\mu$g), cefotaxime (CTX, 30 $\mu$g), cefpodoxime (CPD, 30 $\mu$g), tetracycline (TET, 10 $\mu$g), tigecycline (TGC, 15 $\mu$g), piperacillin (PIP, 100 $\mu$g), and colistin (CT, 10 $\mu$g). E. coli ATCC25922 was used as a quality control strain in both methods.

**Table 1.** Primer Sequence and Product Size

| Primer | Sequence, 5' - 3' | Product Size, bp |
|---|---|---|
| ***QepA*** | | 403 |
| F | 5'- CTGCAGGTACTGCGTCATG -3' | |
| R | 5'- CGTGTTGCTGGAGTTCTTC -3' | |
| ***AcrA*** | | 157 |
| F | 5'- TCTGATCGACGGTGACATCC -3' | |
| R | 5'- TCGAGCAATGATTTCCTGCG -3' | |
| ***AcrB*** | | 64 |
| F | 5'- CAATACGGAAGAGTTTGGCA -3' | |
| R | 5'- CAGACGAACCTGGGAACC -3' | |
| ***Aac(6')-Ib*** | | 611 |
| F | 5'- TTGCGATGCTCTATGAGTGGCTA -3' | |
| R | 5'- CTCGAATGCCTGGCGTGTTT -3' | |

### 3.3. PCR Detection and DNA Sequencing Analysis

The DNA was extracted by the GeNet Bio company (Korea, Cat. no. K-3000) and was used as a template for the PCR reaction (Eppendorf, Mastercycler gradient). Briefly, the 25 $\mu$L PCR mixture contained 12.5 $\mu$L of master mix (Bioneer company, Korea, Cat. number K-2016), 7.5 $\mu$L of deionized water, 1 $\mu$L of each primer, and 3 $\mu$L of bacterial DNA. Amplification was achieved using the following thermal cycling conditions: five minutes at 94°C for the initial denaturation and for 36 cycles of amplification consisting of 45 seconds at 94°C, 45 seconds at 51 - 57°C, and 45 seconds at 72° C with five minutes at 72°C for the final extension.

The results of the PCR were compared with positive controls. The *qepA* genes were screened using a polymerase chain reaction PCR technique. The presence of *aac(6')-Ib* was detected using the primers Aac(6')-Ib-F (5'-TTGCGATGCTCTATGAGTGGCTA-3') and *Aac(6')-Ib-R* (5'-CTCGAATGCCTGGCGTGTTT-3'). The specific primers for the acrA gene (AcrA-F, 5'-TCTGATCGACGGTGACATCC-3' and AcrA-R, 5'-TCGAGCAATGATTTCCTGCG-3') and the *acrB* gene (AcrB-F 5'-CAATACGGAAGAGTTTGGCA-3' and AcrB-F 5'-CAGACGAACCTGGGAACC-3') were used to evaluate the presence of the AcrAB efflux pump. The obtained amplicons were sequenced. The PCR products were analyzed using electrophoresis in a 1% $vw^{-1}$ agarose gel. One of the PCR products was purified, and direct sequencing was done (Bioneer company, Korea). The designed target-specific primers, PCR amplification products, and the conditions of the PCR assay and for the *acrAB*, *aac(6')-Ib-cr*, and *qepA* genes among the clinical isolates are shown in Table 1 and 2.

**Table 2.** Temperature and Time of the PCR Assay

| PCR Steps | Temperature, °C | | | | Time | | | |
|---|---|---|---|---|---|---|---|---|
| | *Aac(6')-Ib* | *QepA* | *AcrA* | *AcrB* | *Aac(6')-Ib* | *QepA* | *AcrA* | *AcrB* |
| **Initial denaturation** | 94 | 94 | 94 | 94 | 5 min | 5 min | 5 min | 5 min |
| **Denaturation** | 94 | 94 | 94 | 94 | 45 s | 45 s | 45 s | 45 s |
| **Annealing** | 55 | 51 | 57 | 52 | 45 s | 45 s | 45 s | 45 s |
| **Extension** | 72 | 72 | 72 | 72 | 45 s | 45 s | 45 s | 45 s |
| **Final extension** | 72 | 72 | 72 | 72 | 5 min | 5 min | 5 min | 5 min |
| **PCR steps** | 36 | 36 | 36 | 36 | 36 | - | - | - |

### 3.4. Statistical Analysis

This study was a descriptive study. MINITAB16 software was used for the analysis of the study's results. The P value and confidence intervals were $P < 0.05$ and 95%, respectively.

## 4. Results

In total, 117 strains were recovered. Seventy strains were isolated from Taleghani HOSPITAL (51.2%), 45 were isolated from Mofid children's hospital (38.6%), and 12 were isolated from Imam Hossein hospital (10.2%). Sixty-four strains were isolated from females (55%) and 53 were isolated from males (45%). The ages of the patients ranged from 1 to 90 years old. The patients included 67 (57.26%) males and 50 (42.74%) females. The studied strains were isolated from the following wards: pediatrics, 49 (42%); outpatient, 16 (13.5%); intensive care units (ICUs), 13 (11.1%); surgery, 9 (7.5%); neonatal ICUs, 12 (10.1%); bone marrow transplant unit, 4 (3.6%); hematology, 4 (3.6%); endocrine, 3 (2.5%); gastrology, 3 (2.5%); and other wards, 4 (3.6%). The distribution of the antibiotic resistance genes in the *K. pneumoniae* isolates is shown in Table 3. The MIC results for the studied strains are shown in Table 4. The PCR assay, using specific primers, demonstrated that among the 117 isolates, 110 (94%) and 102 (87%) isolates were positive for the *acrA* and *acrB* gene, respectively, showing that different acr types were circulating with a high prevalence. Additionally, for the *qepA* and *aac(6')-Ib-cr* genes, 5 (4%) and 100 (85%) isolates were detected, respectively. Both the *acrAB* and *aac(6')-Ib-cr* genes were significantly more prevalent among the *K. Pneumonia* isolates.

## 5. Discussion

A multiresistant strain of *K. pneumoniae* was described in Birmingham two decades ago. It included a broad host range plasmid that contributed to a decline in vitro activity of quinolones. In the presence of this plasmid, resistance to quinolones due to efflux pump systems, DNA gyrase alterations, and porin loss increased eightfold. The gene of quinolone efflux pump resistance, named *qepA*, is located on a plasmid containing a transposable element on both sides (15).

In our survey, the *qepA* gene was reported in 5 (4%) isolates. These results are similar to a study conducted by Kim et al. in Korea (16). Although the prevalence of the *qepA* gene in the current study was low, this plasmid encoding gene can be moved between individuals, hospitals, and environments, increasing the resistance rate.

The *aac(6')-Ib-cr* gene confers decreased susceptibility to some fluoroquinolones and to aminoglycosides. In our study, 100 (85%) *K. pneumoniae* strains isolated from hospitalized patients carried the *aac(6')-Ib-cr* gene. This finding indicates an alarming trend in the increasing frequency of *K. pneumoniae* that is resistant to fluoroquinolones and aminoglycosides, which are two important anti-Gram negative agents that are commonly used in our practice.

Ma et al. performed a study in China reported that found that the most antibiotic resistance belonged to ciprofloxacin and levofloxacin and that among a total of 101 isolates, 35 (34.7%) genes were *aac(6')-Ib-cr*, *qepA*, and *qnr* (17).

Recent investigations have expressed various other mechanisms for antimicrobial resistance among *K. pneumoniae* strains. One of these important mechanisms is the efflux systems comprising the AcrAB efflux pumps. Many studies indicated a relationship between the AcrAB efflux system and resistance to quinolones in K. *pneumoniae* (18-21). Now, the AcrAB efflux system is one of the major mechanisms in multidrug resistant.

*K. pneumoniae* strains, and it consists of the transporter AcrB, which is placed in the inner membrane, and the periplasmic AcrA, which bridges these two integral membrane proteins. Our study demonstrated that 110 (94%) and 102 (87%) strains had *acrA* and *acrB* genes, respectively,

**Table 3.** Antibiotic Susceptibility Testing Results

| Antibiotic | Resistant, No (%) | Sensitive, No (%) | Intermediate, No (%) |
|---|---|---|---|
| **Aztreonam** | 75 (64) | 37 (31) | 5 (5) |
| **Meropenem** | 28 (24) | 77 (66) | 12 (10) |
| **Gentamicin** | 51 (43) | 65 (55) | 1 (2) |
| **Amikacin** | 40 (34) | 76 (65) | 1 (2) |
| **Imipenem** | 28 (24) | 77 (66) | 12 (10) |
| **Cefotaxime** | 77 (66) | 39 (33) | 1 (2) |
| **Tetracycline** | 70 (60) | 43 (36) | 4 (4) |
| **Ampicillin** | 73 (62) | 20 (17) | 24 (21) |
| **Piperacillin** | 73 (62) | 40 (34) | 4 (4) |
| **Cefpodoxime** | 84 (72) | 30 (26) | 3 (2) |
| **Tigecycline** | 17 (15) | 35 (30) | 65 (55) |
| **Doripenem** | 28 (24) | 78 (66) | 12 (10) |
| **Ertapenem** | 28 (24) | 77 (66) | 12 (10) |
| **Ceftazidime** | 73 (62) | 39 (33) | 5 (5) |
| **Colistin** | 5 (4) | 112 (96) | 0 (0.0) |

**Table 4.** Minimum Inhibitory Concentration (MIC) Testing Results

| Antibiotics | MIC, $\mu$g/mL | | |
|---|---|---|---|
| | Range | MIC 50 | MIC 90 |
| **Meropenem** | 0.25 - 256 | 1 | 32 |
| **Imipenem** | 0.25 - 256 | 1 | 16 |
| **Ceftazidime** | 1 -> 256 | 64 | > 256 |
| **Cefotaxime** | 0.5 -> 256 | 16 | > 256 |
| **Ampicillin** | 2 -> 256 | 256 | > 256 |

showing that *acrA* and *acrB* were circulating at a high prevalence. Pakzad et al. performed a study in Iran in 2013 that demonstrated that 40 (76.92%) strains of *Klebsiella pneumoniae* (*K. pneumoniae*) that were isolated from burn patients were resistant to ciprofloxacin, and the PCR results in their study showed that all ciprofloxacin-resistant strains also had the *acrA* gene (22).

Another study conducted by Hasdemir et al. in 2004 demonstrated that the AcrAB efflux pump system participated in resistance to fluoroquinolones in multidrug resistant *K. pneumoniae* strains isolated from Turkey (18). Geographical location plays a major role in the distribution of these genes because countries that are geographically closer to our country have a relatively similar distribution of *acrA*, *acrB*, *qepA* and *aac(6')-Ib-cr* genes. Recent surveys exhibited that the emergence of fluoroquinolone-resistant clinical *K. pneumoniae* strains have been extended among hospitalized patients in Iran (23-25).

Additional investigations using PCR or a probe-based assay can help simplify the actual dissemination and prevalence of quinolone resistance. The high prevalence of quinolone resistance-encoding genes implies the need for accurate identification of resistant *K. pneumoniae* strains and for the choice of proper treatment for the prevention of resistant nosocomial infections. Continuous evaluations of the decrease or increase of antibiotic resistance among *K. pneumoniae* strains isolated from hospitalized patients could provide physicians with new therapeutic choices so that treatment with ineffectual drugs can be stopped and replaced by effectual antibiotics.

## Footnote

**Authors' Contribution:** Hossein Goudarzi proposed and supervised the research. Mohsen Heidary performed the experimental work and wrote the manuscript. Ali Hashemi developed the study idea and carried out the statistical analysis. Gita Eslami and Mehdi Goudarzi advised scientifically and technically, and Shokouh Amraei and Alireza Salimi Chirani gathered the strains.

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