High prevalence of plasmid-mediated quinolone resistance determinants in Enterobacter cloacae isolated from hospitals of the Qazvin, Alborz, and Tehran provinces, Iran

Amir Peymani[1], Taghi Naserpour Farivar[1], Reza Najafipour[1] and Samaneh Mansouri[1]

[1]. Celular e Molecular Research Center, Qazvin Universidade de Ciências Médicas, Qazvin, Iran.

Abstract

Introduction: Plasmid-mediated quinolone resistance (PMQR) is a growing clinical concern worldwide. The main aims of this study were to detect qnr-encoding genes and to evaluate the clonal relatedness of qnr-positive Enterobacter cloacae isolates.

Methods: A total of 116 E. cloacae isolates that were not susceptible to quinolone were obtained from seven hospitals in Tehran, five hospitals in Qazvin, and two hospitals in Karaj (Iran). Bacterial identification was performed using standard laboratory methods and API 20E strips. Quinolone resistance was determined using the Kirby-Bauer disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI) guidelines. PCR and sequencing were employed to detect qnrA, qnrB, and qnrS genes, and clonal relatedness was assessed using the enterobacterial repetitive intergenic consensus (ERIC)-PCR method.

Results: In total, 45 (38.8%) and 71 (61.2%) of isolates showed high- and low-level quinolone resistance, respectively, and qnr-encoding genes were detected in 70 (60.3%) of them. qnrB1 [45 (38.8%) isolates] was the most commonly detected gene, followed by qnrS1 [28 (24.1%) isolates] and qnrB4 [18 (15.5%) isolates] either alone or in combination with other genes. The results of the ERIC-PCR revealed that 53 (75.7%) qnr-positive isolates were genetically unrelated.

Conclusions: This study describes, for the first time, the high prevalence of the qnrB1, qnrS1, and qnrB4 genes among E. cloacae isolates in Iran. The detection of qnr genes emphasizes the need for establishing tactful policies associated with infection control measures in hospital settings in Iran.

Keywords: Enterobacter cloacae. Enterobacterial repetitive intergenic consensus-PCR. Plasmid-mediated quinolone resistance.

INTRODUCTION

Enterobacter cloacae is a clinically significant gram-negative bacterium that can cause several clinical diseases such as urinary tract infections, bacteremia and sepsis, lower respiratory tract infections, pneumonia, and soft tissue infections[1]. Health care for patients with infections caused by this organism has been associated with high mortality and morbidity, especially among patients admitted in intensive care units (ICUs)[2,3].

Nowadays, quinolones are being frequently used to treat serious infections caused by enterobacteria in hospital settings[4,5]. Extensive and inappropriate use of quinolones and other such antimicrobial agents has increased multidrug resistance in Enterobacter cloacae (MDREC) isolates, which complicates and limits the process of antimicrobial therapy[6,7].

Resistance to quinolone compounds is often due to chromosomal point mutations in deoxyribonucleic acid (DNA) gyrase and/or topoisomerase IV[8]. However, plasmid-mediated quinolone resistance (PMQR) has also been reported in several parts of the world[9,10,11,12,13,14]. Plasmids carrying qnr genes widely vary in size and typically carry multiple resistance determinants[15,16]. Qnr proteins are members of a pentapeptide repeat protein family, which is capable of protecting DNA gyrase and DNA topoisomerase IV from quinolone compounds[17]. For example, QnrB4 is a characterized pentapeptide repeat protein that interacts with DNA gyrase[18]. Antibiotic treatment against infections caused by qnr-positive isolates is more complicated because of the remarkable ability of these organisms to develop resistance to different antibiotic classes as well as their high potential for transmitting antibiotic resistance between different bacterial species[19,20]. Three major groups of qnr determinants, qnrA, qnrB, and qnrS, are increasingly being identified in clinical isolates of various enterobacterial species worldwide[21]. The first PMQR gene was reported in a Klebsiella pneumoniae isolate from Birmingham in 1994[22]. Later, these genes were also reported in

Corresponding author: Dr. Reza Najafipour.
e-mail: maja.jipour@gmail.com
Received 18 January 2016
Accepted 28 April 2016

other clinical isolates such as Enterobacter spp., Escherichia coli, Salmonella spp., and Citrobacter freundii.

To the best of our knowledge, there has been no report so far on the frequency of qnr genes among E. cloacae isolates in Iran. In this study, for the first time, we described the frequency of qnr determinants (qnrA, qnrB, and qnrS) among E. cloacae isolates that are non-susceptible to quinolone isolates collected from hospitals of the Qazvin, Alborz, and Tehran provinces in Iran. This study emphasizes the need for establishing specific policies for infection control measures in hospital settings in Iran.

METHODS
Bacterial isolates and antimicrobial susceptibility
In this cross-sectional descriptive study, 116 quinolone non-susceptible E. cloacae isolates (one isolate per patient) were obtained from the clinical samples of patients admitted to the university hospitals of Tehran (seven hospitals), Qazvin (five hospitals), and Karaj (two hospitals) in Iran. The samples were collected from June 2013 to October 2014. The bacteria were isolated from various clinical specimens including tracheal aspirates, urine, sputum, blood, bronchoalveolar lavage (BAL), wounds, and cerebrospinal fluid. Bacterial identification was initially performed using standard laboratory methods, and the results were then confirmed using the API 20 E (bioMérieux, France) microorganism identification system. The isolates were stored at -70°C in trypticase soy broth containing 20% glycerol and subcultured twice prior to testing. Written informed consent was obtained from all the patients enrolled in this study. Kirby-Bauer disk diffusion testing was performed according to the Clinical Laboratory Standards Institute (CLSI) guideline to detect quinolone resistance using nalidixic acid (10µg) and ciprofloxacin-susceptible isolates were classified as showing low-level quinolone resistance. On the other hand, nalidixic acid-resistant or -intermediate isolates and ciprofloxacin-susceptible isolates were classified as showing low-level quinolone resistance. Escherichia coli American Type Culture Collection (ATCC) 25922 and Pseudomonas aeruginosa ATCC 27853 were used as the quality control strains for antimicrobial susceptibility testing.

PCR and sequencing of qnr-encoding genes
All the isolates were subjected to PCR for the detection of the qnrA, qnrB, and qnrS PMQR genes using specific primers (Table 1). Plasmid DNA was extracted using the plasmid mini extraction kit (Bioneer Company, Korea).

PCR amplifications were performed in a thermocycler (Applied Biosystems, USA) as follows: 95°C for 5 min and 35 cycles of 1 min at 95°C, 1 min at specific annealing temperature for each primer and 1 min at 72°C. A final extension step of 10 min at 72°C was performed. The reaction mixtures 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 10X PCR buffer in final concentration of 1X.

The PCR products were electrophoresed on 1% agarose gel at 100 volts and were then stained with ethidium bromide solution and visualized in a gel documentation system (UVtec, UK). The purified PCR products were sequenced using the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Molecular typing by enterobacterial repetitive intergenic consensus-PCR
All the qnr-positive E. cloacae isolates were tested for epidemiological relationships using enterobacterial repetitive intergenic consensus (ERIC)-PCR as previously described by Smith et al. The PCR cycling conditions were as follows: denaturation at 94°C for 1 s, annealing at 52°C for 10 s, and extension at 72°C for 35 s for 30 cycles, followed by a final extension at 72°C for 4 min. The resulting products were analyzed on 1.5% agarose gels. Fingerprints were compared visually, and patterns differing by two or more bands were classified as different.

| TABLE 1 - Primers used for the detection of qnr genes in Enterobacter cloacae isolates. |
|-----------------------------------------------|-----------------------------------------------|-----------------------------|
| Target genes                                  | Primer sequence (5'-3')                        | Annealing temperature (°C)  |
| qnrA-6                                        | Forward: ACGCCAGGATTTGAGTGAC                  | 53                          |
|                                               | Reverse: CCAGGCACAGATCTTGTAC                  |                             |
| qnrB1-3, 5, 6, 8                              | Forward: GGCACCTGAATTATCCGGC                  | 49                          |
|                                               | Reverse: TCCGAATTGGTCAGATCG                   |                             |
| qnrB4                                         | Forward: AGTTGTGATCTCTCCATGGC                 |                             |
|                                               | Reverse: CGGATATCATAATCGCCCCAG                |                             |
| qnrS1-2                                       | Forward: CCTACATCATCATATCCGCC                 |                             |
|                                               | Reverse: GCTTCGAGAATCAGTCTTGT                |                             |

qnr: quinolone resistance.
Statistical analysis

Statistical analysis was performed for descriptive statistics including frequencies and cross tabulation of microbiological, clinical, and demographic characteristics using the computer software program Statistical Package for the Social Sciences (SPSS) version 16.

RESULTS

During the study period, 116 quinolone non-susceptible E. cloacae isolates were recovered from different clinical specimens; 42 (36.2%) were isolated from urine specimens, 28 (24.1%) from wound specimens, 18 (15.5%) from tracheal specimens, 17 (14.7%) from blood specimens, 5 (4.3%) from sputum specimens, 4 (3.4%) from bronchoalveolar lavage specimens, and 2 (1.7%) from cerebrospinal fluid specimens. The isolates were obtained from 47 (40.5%) patients admitted to ICUs, 31 (26.7%) from those admitted to the internal medicine ward, 14 (12.1%) from the infectious diseases ward, 9 (7.8%) from the surgery ward, 9 (7.8%) from the orthopedic ward, and 6 (5.2%) from the neurology ward. The mean age of the patients was 51.7 ± 17.4 years (range: 17-83 years); 69 (59.5%) patients were male and 47 (40.5%) were female.

The results of the disk diffusion test showed that 93 (80.2%) isolates were fully resistant to nalidixic acid and 23 (19.8%) showed intermediate resistance. Forty-three (37.1%) isolates were fully resistant to ciprofloxacin and 2 (1.7%) showed intermediate resistance. Forty-three (37.1%) isolates were fully resistant to nalidixic acid and 23 (19.8%) showed intermediate resistance. Forty-three (37.1%) isolates were fully resistant to ciprofloxacin and 2 (1.7%) showed intermediate resistance. Forty-three (37.1%) isolates were fully resistant to ciprofloxacin and 2 (1.7%) showed intermediate resistance. Forty-three (37.1%) isolates were fully resistant to ciprofloxacin and 2 (1.7%) showed intermediate resistance.

PCR and sequencing revealed that the qnr-encoding genes were present in 70 (60.3%) of the quinolone non-susceptible E. cloacae isolates; among them, qnrB1 [45 (38.8%) isolates] was the most commonly detected gene, followed by qnrS1 [28 (24.1%) isolates] and qnrB4 [18 (15.5%) isolates] either alone or in combination with other genes. The qnrB1 gene was found to coexist with qnrS1 in 6 (8.6%) isolates; 5 (7.1%) isolates also carried both qnrB1 and qnrB4, and 3 (4.3%) isolates carried the qnrS1, qnrB1, and qnrB4 genes. In this study, the presence of the qnrA gene was not detected. In total, 38 (54.3%) isolates showing high-level quinolone resistance and 32 (45.7%) showing low-level quinolone resistance carried the qnr genes. The most common gene found among the isolates showing high- and low-level quinolone resistance was qnrB1 (Table 2). qnr-positive isolates were mostly recovered from wound specimens (19%), followed by urine samples (15.5%). The patients infected by these organisms were mostly admitted to the ICU (26.7%) and the internal medicine (12.1%) wards (Table 3). The ERIC-PCR results revealed that 53 (75.7%) qnr-encoding isolates showed different genotypes and had distinct ERIC-PCR patterns, indicating clear heterogeneity in their genetic profiles (Table 4).

DISCUSSION

Enterobacter cloacae has been increasingly recognized as a clinically significant nosocomial pathogen(29). Quinolones are among the most commonly prescribed antimicrobials for the treatment of serious infections caused by E. cloacae and other members of the Enterobacteriaceae family. However, the development of resistance to these antibiotics in the causative bacteria has complicated treatment and may lead to treatment failures(30). In recent years, PMQR has been reported in several studies, especially in enterobacteria(8). However, there is limited data on the prevalence of qnr genes among enterobacteria isolates in Iran(4). To the best of our knowledge, this is the first report on the detection of qnrB1, qnrB4, and qnrS1 determinants among quinolone non-susceptible E. cloacae isolates in Iran.

The present study demonstrated a high (60.3%) prevalence of PMQR determinants among quinolone non-susceptible E. cloacae isolates collected from several educational hospitals in Iran. The prevalence rate of these genes found in our study is higher than that reported by Dahmen et al(32) from Tunisia (16%) (32), Wu et al(10) from Taiwan (16.3%) (10), Kim et al(33) from Korea (17%) (33), and Robicsek et al(34) from the United States (31%) (34). However, the

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<th>qnr-encoding genes</th>
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<th>Total</th>
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<td>qnrB1</td>
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<td>qnrB4</td>
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<td>qnrB1 + qnrS1</td>
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<td>2.9</td>
<td>4</td>
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<td>qnrB1 + qnrB4</td>
<td>4</td>
<td>5.7</td>
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<td>qnrS1 + qnrB4</td>
<td>3</td>
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Total 32 45.7 38 54.3 70 100.0

qnr: quinolone resistance.
prevalence rate reported in our study is lower than that reported by Bouchakour et al. in their study in Morocco, in which 62.5% of the E. cloacae isolates were found to carry qnr determinants\(^{35}\). This may indicate that the rate of PMQR is increasing among enterobacteria. In a previous study, we reported that 53 (44.2%) of the E. cloacae isolates studied were extended-spectrum β-lactamase (ESBL) producers\(^{36}\). Taken together, these data suggest that inappropriate and extensive use of broad-spectrum antibiotics has resulted in the emergence of resistant isolates in our hospital settings. Moreover, the high resistance rate found among the isolates in this study emphasizes the need for a local and national antimicrobial resistance surveillance system for monitoring the administration of antimicrobials and emergence of antibiotic resistance in bacterial isolates present in our hospital settings. The data on antibiotic resistance received from the hospital infection and antibiotic control committees can help encourage physicians to discontinue excessive use of antibiotics, which will eventually lead to better preventive measures for controlling hospital infections.

Moreover, we found that 54.3% of the qnr-positive isolates in this study showed high-level quinolone resistance. Since PMQR determinants confer only low-level resistance to quinolones, we hypothesize that the high-level resistance pattern is due to another mechanism such as chromosomal mutation; however, we could not test this hypothesis in the present study. Further, most of the qnr-positive E. cloacae isolates were frequently collected from patients admitted to ICUs, which is consistent with the reports of some previous studies\(^{37,38}\). This result suggests that prolonged stay in ICUs, exposure to broad-spectrum antibiotics, chronic underlying conditions, and the use of invasive techniques and devices might predispose patients to infections caused by the aforementioned quinolone-resistant isolates.

In the present study, 38.8%, 24.1%, and 15.5% of the quinolone non-susceptible E. cloacae isolates carried the qnrB1, qnrS1, and qnrB4 genes, respectively, alone or in combination with other genes. In a previous study conducted...
in Iran, Pakzad et al.\(^{14}\) reported that 9 (37.5%) and 4 (20.8%) of the ESBL-producing \(E.\ coli\) isolates studied were positive for the \(qnrA\) and \(qnrB\) genes, respectively\(^{14}\). In another study conducted in Iran, Saboohi et al.\(^{31}\) showed the presence of the \(qnrA\) (25.8%), \(qnrB1\) (1.2%), and \(qnrS\) (1.2%) genes among ESBL-producing \(Salmonella\) isolates\(^{31}\). In Taiwan, Wu et al.\(^{10}\) reported that 10.1%, 6.5%, and 0.6% of the clinical \(E.\ cloacae\) isolates studied contained the \(qnrB2\), \(qnrS1\), and \(qnrA1\) genes, respectively\(^{10}\).

In the United States, Robicsek et al.\(^{34}\) showed that 20% and 11% of the \(Enterobacter\) isolates studied harbored the \(qnrA\) and \(qnrB\) genes, respectively\(^{34}\). In Algeria, Iabadene et al.\(^{15}\) reported the presence of \(qnrB1\), \(qnrB4\), and \(qnrS1\) in clinical isolates of \(E.\ cloacae\)\(^{15}\). Dahmen et al. from Tunisia reported that \(qnrB1\) was the most prevalent gene among \(E.\ cloacae\) isolates, followed by \(qnrB2\) and \(qnrS1\), whereas \(qnrA\) was more prevalent among \(K.\ pneumoniae\) isolates\(^{32}\). Thus, to the best of our knowledge, this is the first report on the detection of the \(qnrS1\), \(qnrB4\), and \(qnrB1\) genes among \(E.\ cloacae\) isolates collected from three distinct provinces of Iran.

In addition, the ERIC-PCR analysis conducted in this study showed that more than 75% of the \(qnr\)-positive isolates were epidemiologically unrelated, suggesting that the dissemination of \(qnr\)-positive isolates was not due to a clonal outbreak. This is in agreement with the fact that the isolates in the present study were collected from seven hospitals in Tehran, five hospitals in Qazvin, and two hospitals in Karaj – three different locations in Iran.

In conclusion, the results of this study revealed a high prevalence of PMQR due to the presence of \(qnr\) genes among clinical isolates of \(E.\ cloacae\) in Iran. The emergence and spread of these resistance determinants in clinical settings raises serious concerns about infection control management and antibiotic therapy. The fact that PMQR determinants confer only low-level resistance to quinolones and our finding that 54.3% of the \(qnr\)-positive isolates in our study showed high-level quinolone resistance suggest that another mechanism such as chromosomal mutation may be responsible for the high-level resistance observed. This hypothesis needs to be tested in future studies. Limitation of our study was laboratory resources. Nevertheless, our findings highlight the need for adopting appropriate infection control policies formulated by infection control committees and rational antibiotic administration by physicians to reduce the further spread of these resistant bacteria in our hospitals.

Acknowledgments

The authors would like to appreciate the acting head and the staff of the Cellular and Molecular Research Center, Qazvin University of Medical Sciences, for providing assistance for completing this research project.

Conflict of interest

The authors declare that there is no conflict of interest.

Financial Support

This study was funded by the Cellular and Molecular Research Center and Research Deputy of Qazvin University of Medical Sciences (Grant number 90/437).

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