High Prevalence of qnr and aac(6’)-Ib-cr Genes in Both Water-Borne Environmental Bacteria and Clinical Isolates of Citrobacter freundii in China

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We investigated the prevalence of qnr and aac(6’)-Ib-cr genes in water-borne environmental bacteria and in clinical isolates of Enterobacteriaceae, as well as the subtypes of qnr. Environmental bacteria were isolated from surface water samples obtained from 10 different locations in Hangzhou City, and clinical isolates of Citrobacter freundii were isolated from several hospitals in four cities in China. qnrA, qnrB, qnrS, and aac(6’)-Ib-cr genes were screened using PCR, and the genotypes were analyzed by DNA sequencing. Ten of the 78 Gram-negative bacilli isolated from water samples were C. freundii and 80% of these isolates carried the qnrB gene. qnrS1 and aac(6’)-Ib-cr genes were detected in two Escherichia coli isolates and qnrS2 was detected in one species, Aeromonas punctata. The qnr and aac(6’)-Ib-cr genes were present in 75 (72.8%) and 12 (11.6%) of 103 clinical isolates of C. freundii, respectively. Of the clinical C. freundii isolates with the qnr gene, 65 isolates (63.1%) carried qnrB, but only three (2.9%) and one (1%) carried qnrA1 and qnrS2, respectively, while five isolates carried both qnrA1 and qnrB, and one isolate carried both qnrS1 and qnrB. The qnrB9 gene was the dominant qnrB subtype, followed by qnrS8 and qnrB6. Southern hybridization studies indicated that the qnr genes are located on different plasmids. Plasmids isolated from both environmental and clinical C. freundii isolates appeared to be homogenous.

Key words: Enterobacteriaceae, environmental bacteria, quinolone resistance gene

Quinolones, which have a broad spectrum of antibacterial activity, have been widely used for chemotherapy and have led to increased resistance of bacteria. Resistance to quinolones is mainly due to chromosomally mediated mechanisms, including mutations in quinolone targets (DNA gyrase and topoisomerase IV) and decreased accumulation of quinolones (porin alternation or overexpression of efflux pump systems) (21). The first plasmid-mediated quinolone resistance (PMQR) determinant was identified in Klebsiella pneumoniae in 1998 (11). Cloning of the gene identified this determinant as a 657-bp fragment encoding a protein with 218 amino-acid residues, which was named Qnr (more recently termed qnr) (29); QnrB and QnrS were discovered subsequently (5, 6). Very recently, two novel qnr genes, qnrC and qnrD, were reported (3, 30). In addition to Qnr, two new types of PMQR determinants have been described. The aac(6’)-Ib-cr aminoglycoside acetyltransferase gene, whose product is capable of acetylating ciprofloxacin and norfloxacin, was discovered in qnrA-positive Escherichia coli in 2006 (18). QepA, a plasmid-mediated fluoroquinolone efflux pump, was identified in two clinical isolates of E. coli, one from Belgium and one from Japan, in 2007 (14, 33).

Qnr and aac(6’)-Ib-cr determinants have now been identified worldwide in many different enterobacterial species (17, 19, 26). These determinants can also be detected in E. coli isolates from poultry and swine (35) and in Enterobacteriaceae from pets, livestock and poultry (10). To better understand the transfer and prevalence of drug-resistant pathogens and determinants in the human-environment system, we collected bacteria from both water samples and clinical patients, and analyzed the qnr and aac (6’)-Ib-cr genes. We found that a high percentage of the environmental samples shared the qnrB gene common with the clinical samples.

Materials and Methods

Bacterial strains

Water samples were collected from 10 distinct aquatic environments (including West Lake, Qiantang River, Jinghang Grand Canal, Xixi Wetland, Jiefang River, Huajiaxi Lake, Jiuxi River, Tiesha River, two fountains in Qingchun Square and the 2nd Affiliated Hospital of Zhejiang University [SAHZU]) in Hangzhou, China during October to November 2008. Five to ten representative sites in each locality were selected for sample collection. We selected those sampling location to represent the main water environments of the city, including artificial fountain, rivers with running water, large volume lakes, and small ponds. Bacteria in water samples (1 L samples) were concentrated by centrifugation and inoculated onto blood-, MacConkey-, and thiourea citrate bile salts sucrose (TCBS)-agar plates. Clinical isolates of Citrobacter spp. were isolated from SAHZU and collected from several other hospitals in four cities (Beijing, Shanghai, Hangzhou, and Wenzhou) in China during January to December 2008, in a drug resistance surveillance program. All the collected isolates were from sources such as sputum, urine and bodily secretions. All of these isolates were identified using the Vitrek System (bioMérieux, Hazelwood, MO, USA).

Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) of ciprofloxacin,
levofoxacin and nalidixic acid against bacteria was determined using the agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) recommendations (4). MIC results were determined after incubation at 35°C for 16–20 hours. Muller-Hinton agar was purchased from Oxoid (Hampshire, UK).

PCR amplification and sequencing

Screening of \( qnrA \), \( qnrB \), \( qnrS \) and \( aac(6')-Ib \) genes was carried out by PCR amplification using specific primers (8, 20). Colonies were boiled to prepare DNA templates for PCR. The reaction was conducted in a Tpersonal thermal cycler (Whatman Biometra, City, NJ, USA). The DNA was trans-blotted to a nylon membrane from ethidium bromide (EB)-free 0.8% agar gel after 1.5 h of electrophoresis, and was then hybridized to the probe using the DIG High Primer DNA Labeling and Detection Starter Kit I according to the manufacturer’s instructions. The amplified products of \( qnrB \) and \( qnrS \) of the environmental isolates were labeled using the DIG High Primer DNA Labeling and Detection Starter Kit I according to the manufacturer’s instructions. The amplified products of \( qnrB \) and \( qnrS \) were sequenced using an ABI3730 Sequencer (Applied Biosystems, Carlsbad, CA, USA), and the obtained sequences were compared with the sequences deposited in GenBank.

Transconjugation and transformation studies

For these studies the donors were the \( qnr \)-carrying strains isolated in this study, while rifampicin-resistant \( E. coli \) J53 was used as the acceptor. The transconjugated strains were screened on a medium including sulfamethoxazole or levofloxacin. The detailed experimental method was as described by Wang et al. (30).

Southern hybridization

The amplified products of \( qnrB \) and \( qnrS \) of the environmental isolates were labeled using the DIG High Primer DNA Labeling and Detection Starter Kit I (Roche, Diagnostics, Mannheim, Germany), according to the manufacturer’s instructions. Plasmid and chromosomal DNA was extracted from the \( qnr \)-carrying waterborne strains using a kit from Axygen (Axygen Scientific, Union City, NJ, USA). The DNA was trans-blotted to a nylon membrane from ethidium bromide (EB)-free 0.8% agar gel after 1.5 h of electrophoresis, and was then hybridized to the probe using the DIG High Primer DNA Labeling and Detection Starter Kit I according to the manufacturer’s instructions.

Results

Bacteria isolated from aquatic environments

Seventy-eight Gram-negative bacilli were isolated from water samples, including 33 Enterobacteriaceae, 21 Aeromonas spp., ten Acinetobacter spp., ten Pseudomonas spp., two Alcaligenes spp., two Plesiomonas spp. and ten Citrobacter freundii (Table 1). Gram-negative cocci or Gram-positive bacteria were not obtained in the current study.

Quinolone susceptibility of environmental and clinical isolates

The MIC\(_{50}\) and MIC\(_{90}\) of ciprofloxacin against 78 water-borne environmental isolates were \( \leq 0.125 \mu \text{g mL}^{-1} \) and 16 \( \mu \text{g mL}^{-1} \), respectively. The MICs of ciprofloxacin, levofloxacin and nalidixic acid, as well as the MIC\(_{50}/\text{MIC}_{90}\) ratios and the MIC range against each genus are shown in Table 1. The MIC\(_{50}\) and MIC\(_{90}\) of ciprofloxacin and levofloxacin against the water-borne environmental \( C. freundii \) were \( \leq 0.125 \mu \text{g mL}^{-1} \) and 0.25 \( \mu \text{g mL}^{-1} \); 0.125 \( \mu \text{g mL}^{-1} \) and 0.25 \( \mu \text{g mL}^{-1} \), respectively, while those of nalidixic acid were 4 \( \mu \text{g mL}^{-1} \) and 8 \( \mu \text{g mL}^{-1} \), respectively.

The overall MIC\(_{50}\) and MIC\(_{90}\) of ciprofloxacin against 103 clinical isolates of \( C. freundii \) were 2 \( \mu \text{g mL}^{-1} \) and 32 \( \mu \text{g mL}^{-1} \), respectively. There was little difference in the MIC\(_{50}\) and MIC\(_{90}\) for samples collected from hospitals in different cities. The MIC\(_{50}\) and MIC\(_{90}\) against clinical samples from the four cities were 4 \( \mu \text{g mL}^{-1} \) and 16 \( \mu \text{g mL}^{-1} \), \( \leq 0.125 \mu \text{g mL}^{-1} \) and 32 \( \mu \text{g mL}^{-1} \), 0.5 \( \mu \text{g mL}^{-1} \) and 16 \( \mu \text{g mL}^{-1} \), and 1 \( \mu \text{g mL}^{-1} \) and 32 \( \mu \text{g mL}^{-1} \), for samples from Hangzhou, Wenzhou, Shanghai, and Beijing respectively. The MIC\(_{50}\) and MIC\(_{90}\) of ciprofloxacin against all isolates of Citrobacter koseri and Citrobacter amalonaticus was \( \leq 0.125 \mu \text{g mL}^{-1} \).

Prevalence of the \( qnr \) and \( aac(6')-Ib \)-cr genes in environmental and clinical isolates

Ten of the water-borne environmental isolates were \( C. freundii \) and 80% of these carried the \( qnrB \) gene (Table 2). The \( qnrS \) gene was detected in one \( E. coli \) and one Aeromonas sp. The \( aac(6')-Ib \) gene was detected in other \( E. coli \) strain and another Aeromonas

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**Table 1.** Species distribution of bacteria isolated from aquatic environments and their MICs of ciprofloxacin, levofloxacin and nalidixic acid (\( \mu \text{g mL}^{-1} \))

| Strain                | No. | Ciprofloxacin MIC, or MIC\(_{50}/\text{MIC}_{90}\) | MIC Range | Levofloxacin MIC, or MIC\(_{50}/\text{MIC}_{90}\) | MIC Range | Nalidixic acid MIC, or MIC\(_{50}/\text{MIC}_{90}\) | MIC Range | Resource* |
|-----------------------|-----|---------------------------------------------------|------------|-----------------------------------------------|------------|-------------------------------------------------|------------|-----------|
| *C. freundii*         | 10  | ≤0.125/0.25≤0.125/64                              | 0.125/25   | ≤0.125/1                                      | 1/8        | 2/16                                            |            | WL, QTR, JHG, XXW, JFR, HJCL, JHGC |
| *E. coli*             | 9   | 0.25/64                                           | ≤0.125/64  | 0.03/2                                        | 4>256      | 1->256                                          |            | WL, JHG, JFR, HJCL, JX |
| *K. pneumonia*        | 6   | ≤0.125                                            | ≤0.125     | 0.03/0.06                                     | 2/4        | 0.5−4                                           |            | QTR, XXW, JFR, HJCL, JX |
| *E. cloacae*          | 2   | ≤0.125                                            | ≤0.125     | 0.03                                          | 2          | 2                                               | XXW, JFR   |
| *E. aerogenes*        | 1   | ≤0.125                                            | _b         | 0.06                                          | 2          | 2                                               | HJCL       |
| *E. intermedia*       | 1   | 0.25                                               | ≤0.125     | 0.05                                          | 1          | 1                                               | HJCL       |
| *Proteus penneri*     | 1   | ≤0.125                                            | ≤0.125     | 0.05                                          | 1          | 1                                               | HJCL       |
| *Pantoea sp.*         | 1   | ≤0.125                                            | ≤0.125     | 0.06                                          | 128        | JFR                                             |            | |
| *Kluyvera spp.*       | 2   | ≤0.125                                            | ≤0.125     | 0.015/0.03                                    | ≤0.015−0.03| 1/4                                             | 1−4        | QTR, JFR |
| *Aeromonas spp.*      | 21  | 0.25/16                                           | 0.125−32   | 0.25/16                                       | 128>256    | 64->256                                          |            | WL, JHG, XXW, JFR, HJCL, JX, TSR, FQCS, FH |
| *Acinetobacter spp.*  | 10  | 0.5/16                                            | ≤0.125−32  | 0.06/0.5                                      | 2/64       | 0.5−128                                         |            | QTR, XXW, JFR, TSR, FQCS |
| *Pseudomonas spp.*    | 10  | ≤0.125/1                                          | ≤0.125−32  | 0.06/0.5                                      | 1          | 0.5−1                                           |            | WL, QTR, JHG, JFR, HJCL, JX, TSR |
| *Alcaligenes spp.*    | 2   | ≤0.125                                            | ≤0.125−0.25| 0.015                                          | ≤0.015     | 1                                               | 1          | WL |
| *Plesiomonas shigelloides* | 2  | ≤0.125                                            | ≤0.125−0.25| 0.015                                          | ≤0.015     | 1                                               | 1          | HJCL |

* WL, West Lake; QTR, Qiantang River; JHG, Jinghang Grand Canal; XXW, Xixi Wetland; JFR, Jiefang River; HJCL, Huaiachi Lake; JX, Jiuxi River; TSR, Tiexia River; FQCS, fountain in Qingchun Square; FH, fountain in 2nd Affiliated Hospital of Zhejiang University.

* only one isolate.
sp. The two Aeromonas spp. were identified as Aeromonas punctata based on the results of sequencing of the gyrB gene (32). The gyrS1 gene in E. coli and the gyrS2 gene in A. punctata were identified by sequencing. One of the three isolates that carried the aac(6’)-Ib-cr gene carried the -cr variant. E. coli and A. punctata were all isolated from Huajia Lake. Three of the eight C. freundii isolates that carried the gyrB gene were isolated from Huajia Lake, two were from West Lake and one each was isolated from the Qiantang River, Jinhang Grand Canal and the Xixi Wetland.

Of the 103 clinical isolates of C. freundii, 75 (72.8%) and 12 (11.6%) carried the gyr and aac(6’)-Ib-cr genes, respectively. GyrA-, gyrB- and gyrS-type alleles were detected in eight (7.8%), 71 (68.9%), and two (1.9%) isolates. Some of these isolates carried two types of gyr alleles (Table 2). The rate of gyr carriage among C. freundii isolates from Beijing, Shanghai, and Wenzhou was a little higher than 50%, while that from Hangzhou was 74.5%. The sequences of the gyrA genes of the isolates all matched that of gyrA1 (12), and the sequences of the gyrS genes of the isolates from Hangzhou and Shanghai matched those of gyrS1 and gyrS2 (12), respectively. We used three different restriction endonucleases (ApaI, HindIII and XhoI) to digest the two plasmids. The restriction fingerprinting of the plasmids was different, indicating that although the two plasmids are of a similar size, they are different. The common subtypes of gyrB detected in 79 water-borne environmental and clinical isolates of C. freundii were gyrB9 (27 isolates), gyrB8 (16 isolates), gyrB16 (11 isolates), and gyrB4 (6 isolates), and other subtypes, including gyrB10, gyrB11, gyrB12, gyrB13, gyrB16, gyrB17, and gyrB18, were also detected (7). We compared the gyrB sequences of the clinical and environmental strains, but no mutations in the gyrB gene were found in genes that originated from either the environmental or clinical strains.

Interestingly, 11 of 12 isolates that carried aac(6’)-Ib-cr also carried one or two types of gyr. Four isolates carried gyrA1, gyrB8, and aac(6’)-Ib-cr, three isolates carried gyrA1 and aac(6’)-Ib-cr, two isolates carried gyrB6 and aac(6’)-Ib-cr, two isolates carried gyrB9 and aac(6’)-Ib-cr, and one isolate carried only aac(6’)-Ib-cr. Of the clinical isolates of other Citrobacter spp., two of the seven C. braakii isolates carried both gyrA1 and aac(6’)-Ib-cr; one C. braakii isolate carried gyrB11; one C. braakii isolate carried aac(6’)-Ib-cr; one of three C. amalonaticus isolates carried gyrB9. No gyr or aac(6’)-Ib-cr gene was detected in C. koseri.

Distribution of the MIC of ciprofloxacin against isolates carrying gyr- or aac(6’)-Ib-cr-genes

A wide range of MICs of ciprofloxacin against isolates that carried the gyr- or aac(6’)-Ib-cr-genes was observed, which varied from ≤0.125 μg mL−1 to 128 μg mL−1 (Fig. 1). The MIC of ciprofloxacin against most gyrA1- or aac(6’)-Ib-cr-carrying isolates was ≥0.25 μg mL−1, except for one clinical isolate of C. freundii that carried gyrA1,

### Table 2. Prevalence of gyr and aac(6’)-Ib-cr genes in water-borne environmental and clinical bacteria

| Resource       | Strain       | No. | qnrA | qnrB | qnrS | gyrB | gyrS+ gyrB | gyrS+ gyrB | aac(6’)-Ib | aac(6’)-Ib | aac(6’)-Ib-cr | aac(6’)-Ib-cr |
|----------------|--------------|-----|------|------|------|------|-----------|-----------|-------------|-------------|---------------|---------------|
| Aquatic environment | C. freundii | 10  | 0    | 8    | (80.0) | 0  | 0         | 0         | 0           | 0           | 0             | 0             |
|                | E. coli      | 9   | 0    | 0    | (11.1) | 0  | 0         | 0         | 0           | 1           | (11.1)        | (11.1)        |
|                | Aeromonas spp. | 21  | 0    | 0    | (4.8)  | 0  | 0         | 0         | 0           | 2           | (9.5)         | 0             |
|                | Others       | 38  | 0    | 0    | 0      | 0  | 0         | 0         | 0           | 0           | 0             | 0             |
| Hospital       | C. freundii | Hangzhou | 51  | 3    | (5.9) | 38 | (74.5)    | 0         | 4           | (7.8)       | 1 (2.0)       | 25 (49.0)     |
|                | Wenzhou      | 14  | 0    | 0    | (7.0)  | 0  | 0         | 0         | 2           | 1 (4.3)     | 1 (4.3)       | 1 (4.3)       |
|                | Shanghai     | 7   | 0    | 0    | (5.7)  | 0  | 0         | 1 (14.3)  | 1 (14.3)    | 1 (14.3)    | 1 (14.3)      |               |
|                | Beijing      | 31  | 0    | 0    | (51.6)| 0  | 0         | 0         | 5           | (16.1)     | 0             |               |
|                | Total        | 103 | 3    | (2.9)| 65   | (63.1)| 1 (1.0)  | 5 (4.8)   | 1 (1.0)     | 33 (32.0)   | 12 (11.6)     |               |
|                | C. braakii   | 7   | 2    | (28.6)| 1 (14.3)| 0  | 0         | 4 (57.1)  | 3 (42.9)    |               |               |               |
|                | C. koseri    | 9   | 0    | 0    | 0      | 0  | 0         | 0         | 0           | 0           | 0             | 0             |
|                | C. amalonaticus | 3  | 0    | 1   | (33.3)| 0  | 0         | 0         | 0           | 0           | 0             | 0             |

Fig. 1. Distribution of MIC of ciprofloxacin among isolates with gyr or aac(6’)-Ib-cr gene. (A) Clinical isolates. (B) Water-borne environmental isolates.

qnrB8, and aac(6’)-Ib-cr (MIC ≤0.125 μg mL−1). Similarly, the MIC of ciprofloxacin against four gyrS-carrying isolates (4/200, 2.0%) was ≥0.5 μg mL−1; however, for gyrB-carrying isolates, the MIC of ciprofloxacin against 16 of 71 clinical isolates (21.9%), and five of eight water-borne environmental isolates of C. freundii (62.5%) was ≤0.125 μg mL−1.

Southern hybridization

Southern hybridization using a gyrS probe identified a specific band (Table 3 and Fig. 2) corresponding to the plasmid DNA of environmental E. coli or Aeromonas spp. isolates, whereas no band that corresponded to chromosomal DNA was observed, suggesting that the gyrS gene is located on the plasmid with an approximate size of 53 kb. Using Southern hybridization we also confirmed that a gyrB probe hybridized to plasmid DNA but not to chromosomal DNA of three of the gyrB-carrying environmental C. freundii isolates. No hybridized band was identified from either the plasmid or the chromosomal DNA of five other gyrB-carrying isolates. Since no hybridization was observed for chromosomal DNA, this indicates that gyrB genes are not found on the chromosomes. It is possible that lower copy numbers of the gyrB gene are located on the plasmids of these strains.
Table 3. Summary of Southern hybridization of environmental isolates carrying qnrB or qnrS genes

| Strain          | qnrB-positive | qnrS-positive |
|-----------------|---------------|---------------|
|                 | No. of positive isolates |               |
| qnrB-positive   |               |               |
| C. freundii     | 8             | 0             |
| qnrS-positive   |               |               |
| E. coli         | 1             | 0             |
| Aeromonas sp.   | 1             | 0             |

Discussion

*C. freundii* is one of the normal flora in human and animal intestines; however, it is also an opportunistic pathogen, which can cause diarrhea, septicemia, meningitis, and brain abscess. It was ranked as No. 13 of the most frequently isolated pathogenic Gram-negative bacteria in 2009 in Zhejiang Province. In this study, the presence of *qnr* and *aac(6’)-Ib-cr* genes in water-borne environmental bacterial isolates was screened by PCR and it was found that the prevalence of *qnrB* in *C. freundii* was as high as 80.0%. The prevalence of such genes in clinical isolates of *C. freundii* was therefore investigated. As expected, the rate of *qnr* and *qnrB* carriage in these isolates was high (72.8% and 63.1%, respectively). Moreover, 11.6% of the clinical isolates of *C. freundii* carried the *aac(6’)-ib-cr* gene, which was not detected in environmental *C. freundii*.

The prevalence of *qnr* and *aac(6’)-ib-cr* genes appears to vary considerably in different studies depending on the criteria used to select the bacterial strains. The overall prevalence of *qnr* in *Enterobacteriaceae* has been reported to range from 0.2% to 50%, and *aac(6’)-ib-cr* may be more prevalent than *qnr* (17, 19). The distribution of *qnr* genes in enterobacterial isolates has been investigated in the UK and Spain (9, 12). The prevalence of *qnr* genes, especially *qnrB*, has also been reported for clinically isolated *K. pneumoniae* and other *Enterobacteriaceae* species in Asian countries (24, 28). In China, *qnr* and *aac(6’)-ib-cr* genes were detected in 8.0% and 9.9% of extended-spectrum β-lactamase (ESBL)-producing *E. coli* and *K. pneumoniae* isolates, respectively, that were collected from six provinces or districts (8). The prevalence of *qnrA*, *qnrB*, *qnrS*, and *aac(6’)-ib-cr* genes in *Enterobacter cloacae* isolates from Anhui Province in China was below 10% for all of the genes (31); however, only a few studies have investigated the prevalence of *qnr* and *aac(6’)-ib-cr* genes in *C. freundii*, possibly due to the relatively low rate of *Citrobacter* sp. isolation in a clinical setting compared with that of *E. coli*, *K. pneumoniae*, and *E. cloacae*. A Korean study showed that 53 (38.4%) of 138 AmpC-producing *C. freundii* isolates harbored Qnr determinants (13). Another Korean study detected QnrB determinants in 67.9% of *C. freundii*, 62.5% of *K. pneumoniae*, 15.8% of *E. cloacae*, and 9.4% of *E. coli* isolates that were resistant to nalidixic acid and to at least one extended-spectrum β-lactam (27). In another study, *Enterobacteriaceae* isolates from nine teaching hospitals in China were investigated, and the MIC of ciprofloxacin against these isolates was >0.25 μg mL⁻¹. Of the isolates for which the MIC of cefotaxime was >2.0 μg mL⁻¹, *qnr* was present in 63.3% of *C. freundii*, 65.5% of *K. pneumoniae*, 65.7% of *E. cloacae*, and 6.5% of *E. coli* isolates. The prevalence of the *aac(6’)-ib-cr* gene in these four bacterial species was 26.7%, 21.8%, 8.6%, and 16.9%, respectively (34). In our study, the 103 clinical isolates of *C. freundii* investigated were collected without any selection criteria; however, the prevalence of *qnr* and *qnrB* was as high as 72.8% and 68.9%, respectively, which is similar to that of its prevalence in water-borne environmental *C. freundii* (80.0%) (Table 2). Clinical isolates of *C. braakii* displayed a high prevalence of *qnr* and *aac(6’)-ib-cr* (42.9% for both) (Table 2), which may be because *C. braakii* is a member of the *C. freundii* complex or may be due to the high MIC of ciprofloxacin against these isolates. It was noted that *qnrA1* was always combined with *aac(6’)-ib-cr*, nine of ten isolates with *qnrA1* also carried *aac(6’)-ib-cr*. Of the 15 isolates with *aac(6’)-ib-cr*, 13 isolates carried *qnr*, nine of which were *qnrA* (Table 2). Six variants of *qnrA*, 19 variants of *qnrB*, and three variants of *qnrS* have been identified worldwide (7). All of the *qnrA* detected in this study were identified as *qnrA1*. Of the four *qnrS*-carrying isolates, two isolates carried *qnrS1* and two isolates carried *qnrS2*. The most common subtypes of the *qnrB* subtype detected in this study were *qnrB9*, *qnrB8*, and *qnrB6*, which is quite different from the results of previous studies (13, 27, 34).

The *qnrA* and *qnrS* genes have been shown to originate from water-borne environmental bacteria, *Shewanella algae* and *Vibrio splendidus*, respectively (1, 16). A *qnrS2* gene was recently identified in a water-borne bacterial species,
Aeromonas, isolated from the River Seine in Paris (2) and from a Swiss lake (15). The qnrS2 gene has also been detected in a clinical Aeromonas veronii isolate (22). In the present study, the same qnrS2 gene was detected in the same bacterial species (Table 2). This is the first report of an isolate of the Aeromonas sp. harboring qnrS2 outside Europe (Table 2). As shown in Table 2, eight out of ten water-borne C. freundii had the qnrB gene (2, 6, 17, 26). These results support the hypothesis that qnr genes are originated from water-borne bacteria; however, for the majority of qnrB-carrying environmental C. freundii, the MIC for quinolone is low (Fig. 1B), while for the clinical qnrB-carrying environmental C. freundii, the MIC is widely distributed, indicating that qnrB itself does not contribute to the high level of quinolone resistance. On the other hand, environmental C. freundii carrying qnrS and aac(6’)-Ib-cr have a higher MIC for quinolone. Such a phenomenon was also observed for the clinical isolates, indicating that those genes contribute to the higher level of quinolone resistance.

Most qnr genes have been reported to be located on plasmid DNA (25), while a few are located on chromosomal DNA (23); however, in our effort to determine the localization of these genes, we were unable to produce transconjugants and transformants that carry qnr genes. The plasmid carrying the qnrB gene may be a non-conjugable plasmid. Our Southern hybridization and endonuclease digestion experiments results clearly indicated that qnrS1-encoding plasmid and qnrS2-encoding plasmid are heterogeneous, and that in at least three out of the eight environmental C. freundii strains, qnrB is located on plasmid(s) (data not shown); however, no bands corresponding to qnrB were detected in either plasmid or chromosomal DNA by Southern hybridization of the other qnrB-positive environmental C. freundii. We suspect that the gene might be located on the plasmids in these strains with very low abundance.

In summary, there is a high prevalence of plasmid-coded qnr and aac(6’)-Ib-cr genes. The plasmids isolated from both environmental and clinical C. freundii isolates appeared to be homogenous. Further investigations are required to confirm the hypothesis that C. freundii play a role as a reservoir of the qnrB gene and that the aquatic environment is an important vehicle for the spread of PMQR.

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