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Coastal Seawater Bacteria Harbor a Large Reservoir of Plasmid-Mediated Quinolone Resistance Determinants in Jiaozhou Bay, China

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Abstract Diversity and prevalence of plasmid-mediated quinolone resistance determinants were investigated in environmental bacteria isolated from surface seawater of Jiaozhou Bay, China. Five *qnr* gene alleles were identified in 34 isolates by PCR amplification, including *qnrA3* gene in a *Shewanella algae* isolate, *qnrB9* gene in a *Citrobacter freundii* isolate, *qnrD* gene in 22 *Proteus vulgaris* isolates, *qnrS1* gene in 1 *Enterobacter* sp. and 4 *Klebsiella* spp. isolates, and *qnrS2* gene in 1 *Pseudomonas* sp. and 4 *Pseudoalteromonas* sp. isolates. The *qnrC*, *aac(6')-Ib-cr*, and *qepA* genes could not be detected in this study. The 22 *qnrD*-positive *Proteus vulgaris* isolates could be differentiated into four genotypes based on ERIC-PCR assay. The *qnrS1* and *qnrD* genes could be transferred to *Escherichia coli* J53 Azi^R or *E. coli* TOP10 recipient strains using conjugation or transformation methods. Among the 34 *qnr*-positive isolates, 30 had a single point mutation in the QRDRs of GyrA protein (Ala67Ser, Ser83Ile, or Ser83Thr), indicating that cooperation of chromosome- and plasmid-mediated resistance contributed to the spread and evolution of quinolone resistance in this coastal bay. Eighty-five percent of the isolates were also found to be resistant to ampicillin, and bla_{CMY}, bla_{OXY}, bla_{SHV}, and bla_{TEM} genes were detected in five isolates that also harbored the *qnrB9* or *qnrS1* gene. Our current study is the first identification of *qnrS2* gene in *Pseudoalteromonas* and *Pseudomonas* strains, and *qnrD* gene in *Proteus vulgaris* strains. High prevalence of diverse *qnr* genes in Jiaozhou Bay indicates that coastal seawater may serve as an important reservoir, natural source, and dissemination vehicle of quinolone resistance determinants.

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Introduction

Quinolones, a class of synthetic broad-spectrum antimicrobial agents, have been used for treatment of bacterial infections for several decades. They bind specifically to the bacterial type II topoisomerases and form a drug-enzyme-DNA complex to interfere with DNA replication and kill the bacteria [31]. Extensive and excessive use of quinolones in medication stimulates the propagation of antibiotic-resistant clinical pathogens. More than 50% of *Escherichia coli* strains isolated from Chinese hospitals in Shanghai were resistant to ciprofloxacin [80]. Quinolones are also used in veterinary, agriculture, and aquaculture in some developing countries. In Chile, more than 100 tons of quinolones were used annually as veterinary medicines, nearly ten times of those used as human medicines [6]. Most of the applied quinolones may end up into the environment eventually [21, 44, 62, 76], creating a huge selective pressure on environmental bacteria, which may, in turn, form a huge reservoir and dissemination source of quinolone resistance genetic determinants.

The molecular mechanisms of quinolone resistance involve both chromosome- and plasmid-mediated resistance. Chromosomal mutations of gyrases, type IV topoisomerases, outer membrane proteins, membrane efflux pumps, and other regulatory proteins may reduce the affinity of quinolones to their targets or reduce the accumulation of quinolones inside bacterial cells [30]. Plasmid-mediated

quinolone resistance (PMQR) involves target protection by pentapeptide-repeat Qnr (plasmid-mediated quinolone resistance) proteins, enzymatic inactivation by aminoglycoside acetyltransferase AAC(6′)-Ib-cr, and active efflux by QepA or OqxAB pump, with the *qnr* genes recognized as the most common PMQR determinants [63]. PMQR dramatically accelerates the global propagation of quinolone-resistant pathogens. Similarly, PMQR may also constitute an important mechanism for the transmission of quinolone resistance in environment.

The first *qnr* gene, designated *qnrA*, was discovered on a transferable plasmid pMG252 in a multidrug-resistant clinical *Klebsiella pneumoniae* isolate [47]. This gene could be transferred into different *Enterobacteriaceae* and *Pseudomonas aeruginosa* recipient strains by conjugation, reducing quinolone susceptibility in the transconjugants. The *qnrA* gene encodes a pentapeptide-repeat protein that binds to the type II topoisomerases to prevent these enzymes from quinolone inhibition [67]. Plasmid-borne genes *qnrS*, *qnrB*, *qnrD*, and *qnrC* were subsequently identified in *Shigella flexneri*, *K. pneumoniae*, *Salmonella enterica*, and *Proteus mirabilis*, respectively [11, 28, 36, 72]. The differences between these *qnr* genes were more than 30% in their amino acid sequences [33]. Meanwhile, diverse *qnr* gene alleles and their transferable genetic elements were described in different clinical bacterial isolates. Among the five groups of *qnr* genes, *qnrB* with 42 gene alleles appeared more prevalent in the *qnr*-positive bacterial isolates reported [63]. Some *qnr* genes were detected in *E. coli* or *K. pneumoniae* isolates from pediatric patients without receiving quinolone treatment [70]. Broad distribution of quinolone-resistant environmental strains and cross-selection by other antimicrobial agents, such as β-lactam antibiotics, may play an important role in the global dissemination of the *qnr* genes [29].

Investigations on epidemiology of *qnr* genes were carried out in more than 30 countries, and most surveys focused on clinical *Enterobacteriaceae* isolates. Beyond clinical settings, quinolone-resistant bacteria with elevated abundance were identified from several mariculture ponds in China [17–20]. Furthermore, the *qnrA*, *qnrB*, and *qnrS* genes were detected in bacterial isolates from fish farms in Egypt [32]. The *qnrS* genes were also detected in *Aeromonas* spp. isolates from the Seine River in France and the Lugano Lake in Swiss, and in *E. coli* and *K. pneumoniae* isolates from northern rivers in Turkey [9, 49, 53]. Studies in the last five years indicate that environmental bacteria may form a natural source of antibiotic resistance gene pool (the "resistome") [7, 75], and the *qnr* genes may originate in the chromosomes of some aquatic bacteria, such as *Shewanella* and *Vibrio* [40, 54, 55, 57]. In aquatic environment, bacteria from different origins could mix without geographic limits, promoting frequent exchange of genetic materials, such as antimicrobial resistance genes and transferable genetic elements [15, 16, 71]. Aquatic environment is thus recognized as a reservoir, natural source, and dissemination vehicle of antimicrobial resistance determinants [3]. The exchange of antibiotic resistance determinants between natural environment and clinical setting may promote the creation of new resistance determinants and the distribution of resistance in clinically important pathogens, aggravating the environmental quality and the difficulty and cost of bacterial infection disease control and treatment. Nowadays, antibiotic resistance determinants have been recognized as important environmental contaminants [45]. However, environmental PMQR bacteria were seldom studied.

Jiaozhou Bay is a typical semi-enclosed coastal bay in China, surrounded by several sewage processing plants, small rivers, maricultural zones, and bathing beaches. Due to rapid urbanization and development of marine economics in the surrounding areas, environmental quality of this coastal bay was dramatically deteriorated by various chemical and biological contaminations [13–15]. This study was carried out to investigate the current status of PMQR in this coastal environment. To explore the possible mechanisms of persistence and dissemination of quinolone resistance in Jiaozhou Bay, molecular techniques were employed to determine the diversity and prevalence of PMQR bacteria and their gene determinants.

Materials and Methods

Strains and Culture Conditions

Seven hundred four tetracycline-resistant bacteria and 348 chloramphenicol-resistant bacteria were isolated from the surface seawater of Jiaozhou Bay in September and October of 2004 [15, 16]. These isolates were collected from ten sampling stations associated with different anthropogenic disturbances as described in previous publications [13–16]. From these antibiotic-resistant bacteria collections, quinolone-resistant strains were screened on tryptic soy agar (TSA, Difco formula) plates supplemented with 3% NaCl and 32-μg ml^{-1} nalidixic acid and further cultivated at 25°C in tryptic soy broth (TSB) containing 30-μg ml^{-1} tetracycline or chloramphenicol to avoid possible mutations induced by quinolone. The antimicrobial agents used in this study were purchased from Sigma, USA.

Detection of PMQR Gene Determinants

A simple boiling method was used for rapid bacterial genomic DNA extraction [17]. The *qnrA*, *qnrB*, and *qnrS* genes were screened using a multiplex PCR method as described previously [10]. The *qnrC*, *aac(6')-Ib-cr*, and

qepA genes were also screened [39, 43, 51]. A pair of specific primers was designed for detection of the *qnrD* gene. For the bacterial isolates carrying a *qnrA*, *qnrB*, or *qnrS* gene, further *qnr* gene allele determination was performed by PCR amplification and gene sequencing. The primers used in this study were listed in Table 1.

Primers 27 F and 1492R [74] were used for bacterial 16S rRNA gene amplification and sequencing for phylogenetic analysis of the bacterial isolates bearing PMQR genes.

Multiple Antibiotic Resistance Assay

Nalidixic-acid-resistant isolates that harbored the *qnr* genes were selected for further determination of their susceptibility to other typical antimicrobial agents. These isolates were screened on TSA plates supplemented with 30-μg ml^{-1} tetracycline (TET_{30}), 30-μg ml^{-1} chloramphenicol (CHL_{30}), 30-μg ml^{-1} streptomycin (STR_{30}), 30-μg ml^{-1} kanamycin (KAN_{30}), 30-μg ml^{-1} gentamicin (GEN_{30}), 15-μg ml^{-1} erythromycin (ERY_{15}), 5-μg ml^{-1} ciprofloxacin (CIP_5), and 100-μg ml^{-1} ampicillin (AMP_{100}), respectively, for 48-h incubation at 25°C, based on the method described previously [17].

Molecular Typing

Enterobacterial repetitive intergenic consensus (ERIC)-PCR assay was carried out to analyze the clonal relatedness of the *Proteus vulgaris* isolates that harbored the *qnr* genes. Bacterial genomic DNA was extracted by TIANamp bacteria DNA kit (Tiangen, China) according to the manufacture's protocol. Primers ERIC1 and ERIC2 [69] were used in

Table 1 Primers used for screening the PMQR and *gyrA* genes in this study

Primers	Sequences (5′-3′)	Target genes	Sources
qnrAF	AGAGGATTTCTCACGCCAGG	*qnrA*	[10]
qnrAR	TGCCAGGCACAGATCTTGAC		
qnrBF	GGMATHGAAATTCGCCACTG	*qnrB*	[10]
qnrBR	TTTGCYGYYCGCCAGTCGAA		
qnrSF	GCAAGTTCATTGAACAGGGT	*qnrS*	[10]
qnrSR	TCTAAACCGTCGAGTTCGGCG		
qnrCF	GGGTTGTACATTTATTGAATCG	*qnrC*	[39]
qnrCR	CACCTACCCATTTATTTTCA		
qnrDF	GGGTTGATTTAACTGATAC	*qnrD*	This study
qnrDR	TTCGCACTTTTCTAATATGAC		
aacIbF	TTGCGATGCTCTATGAGTGGCTA	*aac(6')-Ib-cr*	[51]
aacIbR	CTCGAATGCCTGGCGTGTTT		
qepAF	GCAGGTCCAGCAGCGGGTAG	*qepA*	[43]
qepAR	CTTCCTGCCCGAGTATCGTG		
qnrAwF	ATGGATATTATTGATAAAG	Whole *qnrA3* gene	This study
qnrAwR	CTAATCCGGCAGCACTAT		
qnrBwF	ATGGCTCTGGCACTCGTTGGCGAA	Whole *qnrB9* gene	This study
qnrBwR	CTAGCCAATAATCGCGATGCCAAG		
qnrS1wF	TGGAAACCTACAATCATAC	Whole *qnrS1* gene	This study
qnrS1wR	TTAGTCAGGATAAACAAC		
qnrS2wF	ATGGAAACCTACCGTCACAC	Whole *qnrS2* gene	This study
qnrS2wR	CTAGTCAGGAAAAACAACAATAC		
qnrDwF	ATGGAAAAGCACTTTATCAATGAAAAG	Whole *qnrD* gene	This study
qnrDwR	TTATCGGTGAACAATAACACCTAAAC		
gyrEF	CGACCTTGCGAGAGAAAT	*gyrA* of *Enterobacter* and *Klebsiella*	This study
gyrER	TTCGCGTCAACTTCCACTTC		
gyrCF	GATTATGCGATGTCGGTCATTG	*gyrA* of *Citrobacter*	This study
gyrCR	CAGGTTCATGATCTTCGGCTG		
gyrPF	TGGATTATGCGATGTCCG	*gyrA* of *Proteus*	This study
gyrPR	CCGTCTTTATCAGACTCGTC		
gyrPaF	TTAGATTACGCCATGAGTGT	*gyrA* of *Pseudoalteromonas* and *Pseudomonas*	This study
gyrPaR	GTAATGGTCAATTCAGGGTT		
gyrSF	ATGACTGATYTGGCWTCATCTAT	*gyrA* of *Shewanella*	This study
gyrSR	ATRCGRATTTCGGTRTAACGCAT		

combination for PCR with *Ex Taq* polymerase (TaKaRa, Japan). The PCR program consisted of an initial denaturation step at 94°C for 5 min,followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 46°C for 1 min, and primer extension at 72°C for 3 min. After the last cycle, a final extension step at 72°C for 10 min was performed. PCR products of 8 μl each were then electrophoresed directly on 1.5% agarose gel containing 0.5-μg ml^{-1} ethidium bromide at 70 V for 3 h. The gel image was photographed by an AlphaImager HP system (Alpha Innotech, USA), and band detection and normalization were analyzed by software Quantity One (version 4.6.2, Bio-Rad, USA). The presence or absence of each band was presented by a binary code (1 or 0), and a binary data sheet was generated according to the band distribution. Similarity between the ERIC-PCR profiles was determined by using the Jaccard coefficient, and a dendrogram was produced by UPGMA method using the software NTSYSPC (version 2.1, Exeter Software, USA) [56].

Assay of *qnr* Gene Transfer

Transfer of the *qnr* genes from quinolone-resistant environmental isolates to *E. coli* was attempted, with *E. coli* J53 Azi^R (resistant to sodium azide) and *E. coli* TOP10 used as recipient strains for conjugation and transformation experiments, respectively.

Conjugation experiments between the *qnr*-positive *Enterobacteriaceae* isolates and *E. coli* J53 Azi^R were performed by the liquid mating assay as previously described, with minor modifications [70]. The transconjugants were selected on TSA plates supplemented with 100-μg ml^{-1} sodium azide and 6-μg ml^{-1} nalidixic acid. For the other *qnr*-positive isolates, the donor and recipient strains were grown in TSB medium to logarithmic phase (OD_{600}=1), respectively. Donor cells (0.1 ml) and recipient cells (1 ml) were mixed together and added to fresh TSB medium (3.9 ml) and then incubated overnight at 25°C without shaking. The transconjugants were selected on TSA plates containing 300-μg ml^{-1} sodium azide and 6-μg ml^{-1} nalidixic acid, at which the growth of the donor bacterial cells was suppressed, and then replica-plated onto Chromocult® coliform agar (Merck, USA) plates with 6-μg ml^{-1} nalidixic acid.

For transformation experiments, plasmids were extracted individually from the *qnr*-positive environmental isolates by alkaline lysis method [58], checked by gel electrophoresis, and then electroporated into *E. coli* TOP10 cells in a 0.2-cm cuvette at a capacity of 25 μF, resistance of 200 Ω, and current at 2.5 kV. Transformants were selected on Luria-Bertani agar (LB, Difco formula) plates containing 3-μg ml^{-1} nalidixic acid or 0.06-μg ml^{-1} ciprofloxacin.

The *E. coli* transconjugants and transformants were confirmed to carry the same *qnr* gene as that from their donors by PCR experiments.

Screening of Mutations in *gyrA* Gene

For the *qnr*-positive environmental isolates, PCR assay was carried out to amplify the quinolone resistance-determining regions (QRDRs) of the *gyrA* gene. Based on known *gyrA* gene sequences of the closest-match bacterial species in GenBank, primers (Table 1) were designed and used for PCR gene amplification and DNA sequencing to analyze possible mutations in QRDRs.

Screening of *bla* Genes

Various extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase gene variants, such as bla_{SHV}, bla_{TEM}, bla_{OXA}, bla_{MOX}, bla_{CMY}, bla_{LAT}, bla_{BIL}, bla_{DHA}, bla_{ACC}, bla_{FOX}, bla_{ACT}, bla_{MIR}, bla_{KPC}, and $bla_{CTX\text{-}M}$, were screened for the ampicillin-resistant *qnr*-positive isolates using multiplex PCR-based techniques described previously [4, 12, 22, 52]. The amplified gene products were sequenced to determine the *bla* genes.

Bioinformatic Analysis

DNA sequence alignments were processed using Blast [1] and ClustalX [66] programs. Phylogeny of *qnr* gene or partial 16S rRNA gene sequences from the *qnr*-positive isolates was constructed using MEGA5 software [65].

GenBank Accession Numbers

The bacterial 16S rRNA gene sequences were submitted to GenBank with the accession nos. JN384129–JN384161 and HM371197. The representative *qnr* gene sequences of quinolone-resistant isolates were submitted to GenBank with the accession nos. JN384125–JN384126 and JN384196–JN384207. The *gyrA* gene sequences were submitted to GenBank with the accession nos. JN384162–JN384195. The *bla* gene sequences were submitted to GenBank with the accession nos. JN384127–JN384128, JN384208–JN384210, and JN587513.

Results

qnr Genes in Multidrug-Resistant Environmental Isolates

In the current study, 71 tetracycline-resistant and 53 chloramphenicol-resistant bacterial isolates obtained from Jiaozhou Bay were screened for quinolone resistance, and

82 isolates (66%) were found to be resistant to 32-μg ml^{-1} nalidixic acid. These nalidixic-acid-resistant isolates were screened for the *qnr*, *aac(6')-Ib-cr*, and *qepA* genes. The *qnr* genes were detected in 34 isolates, including the *qnrA* gene in 1 isolate, the *qnrB* gene in 1 isolate, the *qnrS* gene in 10 isolates, and the *qnrD* gene in 22 isolates. Other PMQR genes, such as *qnrC*, *aac(6')-Ib-cr*, and *qepA*, could not be detected in this study. Therefore, 41% of the nalidixic-acid-resistant environmental isolates carried the *qnr* genes, 27% carried the predominant *qnrD* gene, 12% carried the *qnrS* gene, and 1% carried the *qnrA* or *qnrB* gene.

So far, 7 *qnrA*, 42 *qnrB*, and 5 *qnrS* gene alleles with a few amino acid substitutions have been identified (http://www.lahey.org/qnrStudies/). The *qnrA*-, *qnrB*-, and *qnrS*-positive isolates in this study were selected for further determining their *qnr* gene subtypes. The complete *qnr* gene sequences of these isolates were obtained by PCR amplification and DNA sequencing. As a result of the amino acid sequence alignments with known Qnr proteins (Fig. 1 and Table 2), *qnrA3* gene was identified in isolate QC39, *qnrB9* gene was identified in isolate Q15, *qnrS1* genes were identified in isolates Q6, Q11, Q13, Q14, and Q19, and *qnrS2* genes were identified in isolates Q24, Q29, QC43, QC44, and QC45.

These 34 *qnr*-positive isolates were also analyzed for their susceptibility to other antibiotics (Table 3). All isolates were resistant to at least five different classes of antimicrobial agents, and 23 isolates (68%) were resistant to 9 different antimicrobial agents tested, including the *qnrS1*-positive isolate Q11 and all of the *qnrD*-positive isolates. Thirty-one isolates (91%) were resistant to the fluoroquinolone class antibiotic ciprofloxacin. Twenty-nine isolates (85%) were resistant to ampicillin, excluding the five *qnrS2*-positive isolates.

As a result of the 16S rRNA gene sequence alignments, all of the 34 *qnr*-positive isolates shared more than 99% sequence identities of the 16S rRNA genes with their closest-match sequences retrieved from the GenBank database. The phylogenetic tree constructed verified their phylogenetic affiliations (Fig. 2). All isolates belonged to the γ-*Proteobacteria* subdivision. Bacteria affiliated to *Enterobacteriaceae* contributed to 82% of the 34 isolates, including species closely related to *Citrobacter freundii*, *Enterobacter* sp., *K. pneumoniae*, and *Proteus vulgaris*. The remaining six isolates are indigenous estuarine or marine bacteria, affiliated with *Pseudoalteromonas* sp., *Pseudomonas* sp., and *Shewanella algae*. The *Shewanella algae* isolate carried the *qnrA3* gene, the *C. freundii* isolate carried the *qnrB9* gene, and the *Proteus vulgaris* isolates carried the *qnrD* genes. The *qnrS*-positive isolates were quite diverse. The *Enterobacter* sp. and *Klebsiella* spp. isolates harbored the *qnrS1* genes, and the *Pseudoalteromonas* sp. and *Pseudomonas* sp. isolates harbored the *qnrS2* genes.

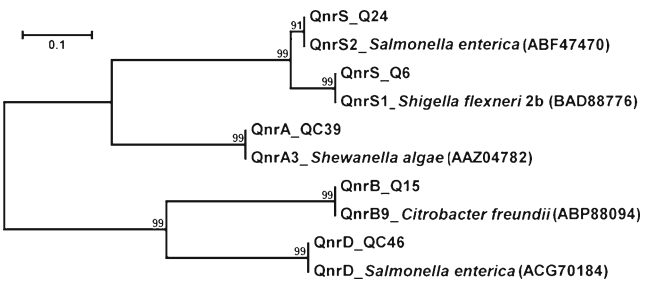

Figure 1 Phylogenetic tree of the representative Qnr protein sequences from the *qnr*-positive isolates with their closest-match GenBank Qnr protein sequences. The reconstruction was computed by the distance method (Neighbor-Joining Poisson correction distance model) with interior branch length supports from 1,000 replicates using MEGA5 software. The reference Qnr protein sequences are according to Jacoby et al. [33], with their GenBank accession numbers labeled *in parentheses*

Molecular Types of *Proteus vulgaris* Isolates

Molecular typing was carried out to investigate the genetic diversity of the 22 *qnrD*-positive *Proteus vulgaris* isolates. A series of PCR amplification conditions were tested to optimize the ERIC-PCR result. Eventually, 46°C was chosen as the optimal annealing temperature. Several different PCR product profiles were observed by electrophoresis (Fig. 3). Cluster analysis was made based on the banding types of ERIC-PCR fingerprinting. These 22 *Proteus vulgaris* isolates were classified into four distinct groups. The isolates QC46, QC48, and QC51 were unique individually, and the remaining 19 isolates were highly related (Fig. 4).

Transfer of *qnr* Genes

Due to multidrug resistance of the 34 *qnr*-positive isolates, nalidixic acid and ciprofloxacin were used as selective pressure to study the transfer capacity of *qnr* genes between nalidixic-acid-resistant isolates and *E. coli* recipients. The *qnrS1* gene could be transferred from isolates Q6, Q11, Q13, Q14, and Q19 to *E. coli* J53 Azi^R by conjugation and to *E. coli* TOP10 by electroporation. The *qnrD* gene in the 22 *Proteus vulgaris* isolates could be transferred to *E. coli* TOP10 by electroporation, but conjugation experiments failed. The same *qnr* gene as that from their donors could be verified in the *E. coli* transconjugants or transformants by PCR amplification.

None plasmid could be extracted from the *qnrA*-positive isolate QC39 or the *qnrS2*-positive isolates Q24, Q29, QC43, QC44, and QC45 using alkaline lysis method, and these *qnr* genes failed to be transferred into *E. coli* recipient strains. Although five plasmids were isolated from the *qnrB9*-positive isolate Q15 (data not shown), no *E. coli*

Table 2 Representative *qnr* and *bla* gene sequences determined in the current study and their closest-match GenBank sequences

Resistance gene	Strain	Closest-match GenBank sequence	Amino acid sequence identity	GenBank accession number
qnr	QC39	*Shewanella algae*, *qnrA3* gene	100%	AAZ04782
	Q15	*Citrobacter freundii*, *qnrB9* gene[a]	100%	ABP88094
	Q6	*Shigella flexneri* 2b, *qnrS1* gene	100%	BAD88776
	Q24	*Salmonella enterica* subsp. enterica serovar Anatum, *qnrS2* gene	100%	ABF47470
	QC46	*Salmonella enterica* subsp. enterica serovar Bovismorbificans Gss-HN-2007057, *qnrD* gene	100%	ACG70184
bla	Q11	*Klebsiella oxytoca*, bla_{OXY} gene	100%	CAA82916
	Q11	*Escherichia coli* UMNF18, bla_{TEM} gene	100%	AEJ60089
	Q13	*Klebsiella pneumoniae*, bla_{SHV} gene	100%	ADG08161
	Q15	*Salmonella enterica* subsp. enterica serovar Typhimurium, bla_{CMY} gene	100%	ACS54274

[a] Gene nomenclature according to Jacoby et al. [33]

transconjugant or transformant could be obtained in this study.

Mutations in GyrA Protein

Mutations in the QRDRs of type II topoisomerases are frequently associated with the *qnr* genes in quinolone-resistant or less susceptible bacteria [5]. For quinolone resistance, the most common point mutations of Gram-negative bacteria occur in the *gyrA* gene. In the current study, the *gyrA* genes of the 34 *qnr*-positive isolates were PCR amplified and sequenced. Compared with the amino acid sequences in the QRDRs of GyrA in the closest-match bacterial species, the isolates Q11, Q24, Q29, QC43, QC44, and QC45 and the 22 *Proteus vulgaris* isolates had a Ser83-Ile substitution, the isolate Q15 had a Ser83Thr substitution, and the isolate QC39 had an Ala67Ser substitution. No point mutation in the QRDRs of GyrA was found in isolates Q6, Q13, Q14, and Q19 (Table 3). Therefore, 88% of the *qnr*-positive isolates had a single point mutation in the QRDRs of GyrA protein.

bla Gene Screening

Considering the possibility of cross-selection of environmental antimicrobial-resistant strains by quinolones and β-lactam antibiotics, 29 ampicillin-resistant isolates that harbored the *qnr* genes were screened for the ESBL and AmpC β-lactamase genes by PCR amplification. Four different types of *bla* genes were detected in five environmental isolates, including the bla_{CMY} gene in isolate Q15, the bla_{OXY} and bla_{TEM} genes in isolate Q11, and the bla_{SHV} gene in isolates Q13, Q14, and Q19. The bla_{OXY}, bla_{SHV}, and bla_{TEM} genes combined with the *qnrS1* gene were present in four *Klebsiella* isolates, and the bla_{CMY} gene combined with the *qnrB9* gene was present in the *C. freundii* isolate. The other *bla* gene variants tested in this study could not be detected.

Discussion

The PMQR determinants have been identified in a number of clinically important pathogens and environmental bacteria in different geographic regions of the world [9, 32, 41, 49, 53]. Studies of PMQR determinants as a new type of biological contaminants in aquatic environment, especially in coastal areas that receive high anthropogenic activities, are very limited and need more efforts. Previous studies on the incidence and persistence of tetracycline-, chloramphenicol-, ampicillin-, and streptomycin-resistant bacteria and their resistance determinants in Jiaozhou Bay found that the antimicrobial resistance status in the coastal seawater was very severe and complicated [15, 16, 71, 79]. Our current study adds more evidences to the exacerbated antimicrobial resistance status by providing new data about the diversity and prevalence of PMQR determinants in multidrug-resistant bacterial isolates from this coastal bay.

Diverse *qnr* genes, including *qnrA*, *qnrB*, *qnrS*, and *qnrD*, were identified in seawater bacterial isolates of Jiaozhou Bay, and surprisingly, the predominant PMQR gene was *qnrD* that was carried by 27% quinolone-resistant isolates. The prevalence of different PMQR genes in Jiaozhou Bay was different from the statistical data of the global prevalence of PMQR genes, as the *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib-cr* genes were found to be more common in clinical *Enterobacteriaceae* isolates [63]. The *qnrD* gene is relatively a new member of the PMQR determinants, and it has been identified in a few isolates from human and animals [11, 48, 68, 78]. Our study is the first report about the occurrence of *qnrD* in marine environment, indicating the importance of this newly discovered quinolone resistance

Table 3 Bacterial taxonomic affiliation, antibiotic resistance spectra, and resistance genes detected of the *qnr*-positive isolates

Strain	Sampling station[a]	Sampling month	Species	Antibiotic resistance profile	*qnr* gene	*bla* gene	Mutation in GyrA
QC39	C4	September	*Shewanella algae*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, ERY_{15}, KAN_{30}, AMP_{100}	*qnrA3*		Ala67Ser
Q15	C4	September	*Citrobacter freundii*	TET_{30}, NAL_{30}, $STR_{30,}$ ERY_{15}, KAN_{30}, AMP_{100}	*qnrB9*	bla_{CMY}	Ser83Thr
Q6	A5	September	*Enterobacter* sp.	TET_{30}, CHL_{30}, NAL_{30}, $STR_{30,}$, AMP_{100}	*qnrS1*		
Q11	C4	September	*Klebsiella* sp.	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrS1*	bla_{OXY}, bla_{TEM}	Ser83Ile
Q13	C4	September	*Klebsiella pneumoniae*	TET_{30}, CHL_{30}, NAL_{30}, ERY_{15}, AMP_{100}	*qnrS1*	bla_{SHV}	
Q14	C4	September	*Klebsiella pneumoniae*	TET_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, AMP_{100}	*qnrS1*	bla_{SHV}	
Q19	Y1	September	*Klebsiella pneumoniae*	TET_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrS1*	bla_{SHV}	
Q24	D1	October	*Pseudomonas* sp.	TET_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}	*qnrS2*		Ser83Ile
Q29	D1	October	*Pseudoalteromonas* sp.	TET_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}	*qnrS2*		Ser83Ile
QC43	D1	September	*Pseudoalteromonas* sp.	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ KAN_{30}, GEN_{30}	*qnrS2*		Ser83Ile
QC44	D1	September	*Pseudoalteromonas* sp.	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ KAN_{30}, GEN_{30}	*qnrS2*		Ser83Ile
QC45	D1	September	*Pseudoalteromonas* sp.	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ KAN_{30}, GEN_{30}	*qnrS2*		Ser83Ile
Q3	A5	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
Q5	A5	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
Q9	C3	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
Q10	C3	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
Q28	A5	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC14	D1	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC15	D1	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC17	C4	October	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC29	Y1	October	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC32	B2	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC33	C1	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC34	C1	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC35	C1	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC36	C1	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC46	D5	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC47	D5	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC48	D6	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC49	D6	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC50	D1	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, $STR_{30,}$ ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile

Table 3 (continued)

Strain	Sampling station[a]	Sampling month	Species	Antibiotic resistance profile	*qnr* gene	*bla* gene	Mutation in GyrA
QC51	D1	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, STR_{30}, ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC52	D1	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, STR_{30}, ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile
QC53	D1	September	*Proteus vulgaris*	TET_{30}, CHL_{30}, NAL_{30}, CIP_5, STR_{30}, ERY_{15}, KAN_{30}, GEN_{30}, AMP_{100}	*qnrD*		Ser83Ile

[a] The locations of the sampling stations were described in detail in Dang et al. [15]

mechanism, especially in coastal settings. Given that some bacteria with *qnr* genes could not be selected via antimicrobial susceptibility tests, especially with nalidixic acid [26], higher prevalence and diversity of PMQR genes should be expected from Jiaozhou Bay.

In our current study, the *qnrA3* gene was found to be carried by a *Shewanella algae* isolate with no plasmid detected, and this gene could not be transferred into *E. coli* recipients, consistent to the speculation of other studies that this gene may be located on the chromosome of *Shewanella algae* stains [55]. The ubiquitous distribution of *qnrA*-bearing *Shewanella algae* in aquatic environments suggests their important role in *qnr* gene origination and evolution in a global scale. The *qnrB9* gene in Jiaozhou Bay was found to be carried by *C. freundii* isolate Q15 that carried five plasmids, but this gene could not be transferred into *E. coli* cells using conjugation or transformation technique. It is likely that the *qnrB9* gene may be situated on the bacterial chromosome or a plasmid that could not replicate in *E. coli* recipients. Currently, a great number of *qnrB* gene allelic variants, located on both plasmids and chromosomes, have been identified in *C. freundii* strains [2, 8, 35, 37, 61, 63, 64]. In China, most *C. freundii* isolates collected from other aquatic environments or clinical settings were found to carry the *qnrB* gene, and *qnrB9* was a dominant quinolone resistance determinant for this bacterial species [77]. Therefore, *C. freundii* bacteria may provide a large gene pool with diverse *qnrB* gene alleles for the evolution and transmission of the *qnr* genes.

In Jiaozhou Bay, two *qnrS* gene alleles, *qnrS1* and *qnrS2*, were identified in ten isolates affiliated to *Enterobacter*, *Klebsiella*, *Pseudoalteromonas*, and *Pseudomonas*. The *qnrS1* gene in *Enterobacter* and *Klebsiella* isolates could

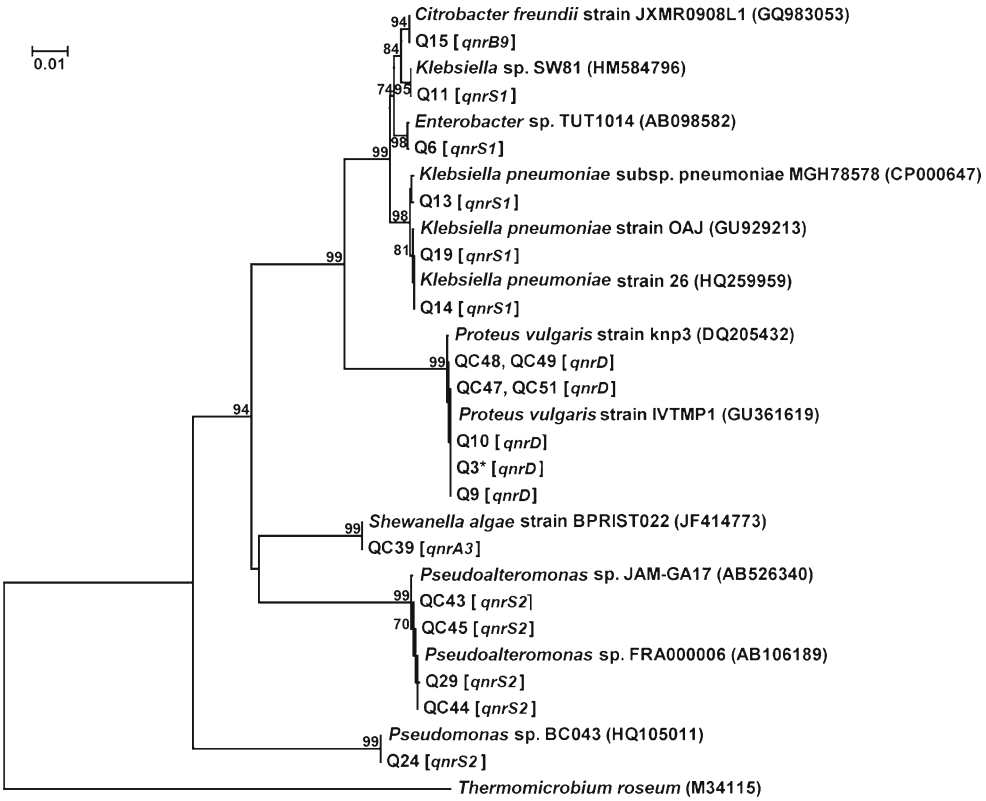

Figure 2 Phylogeny of partial 16S rRNA gene sequences from the *qnr*-positive isolates. The reconstruction was computed by the distance method (Neighbor-Joining Poisson correction distance model) with interior branch length supports from 1,000 replicates using MEGA5 software. The GenBank accession numbers of the reference sequences were labeled *in parentheses*. The *qnr* genes detected were labeled *in brackets* for the corresponding isolates. Asterisk, the 16S rRNA gene sequences of isolates Q5, Q28, QC14, QC15, QC17, QC29, QC32, QC33, QC34, QC35, QC36, QC46, QC50, QC52, and QC53 were identical to that of isolate Q3

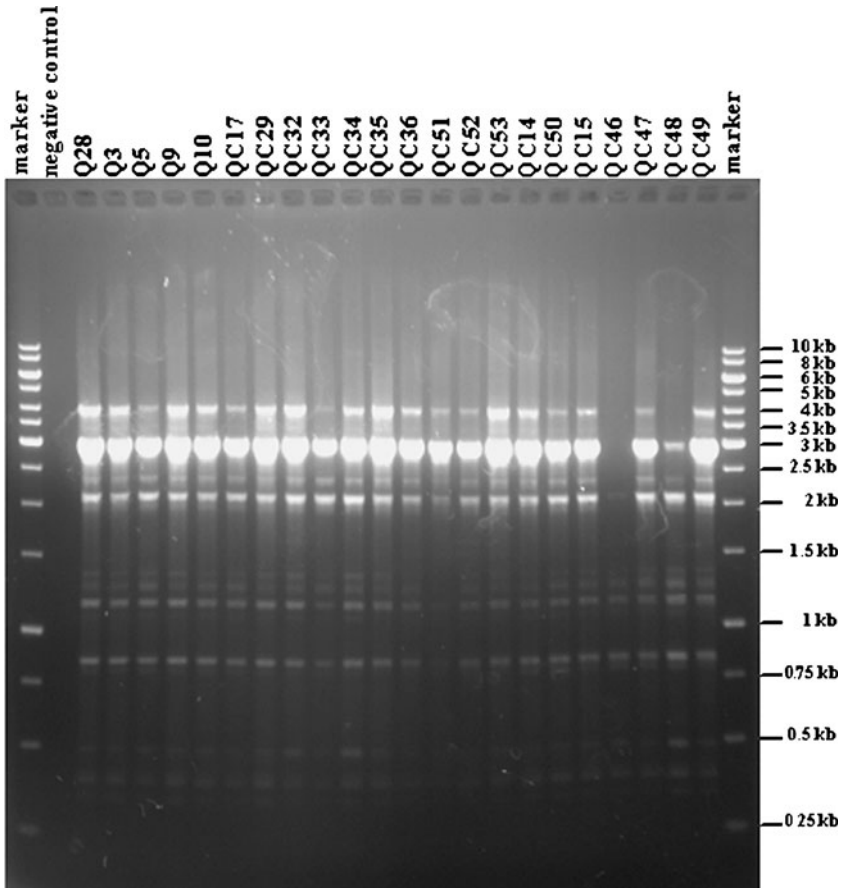

Figure 3 Enterobacterial repetitive intergenic consensus (ERIC)-PCR assay of the 22 *Proteus vulgaris* isolates with ERIC primers ERIC1 and ERIC2. No template DNA was added to the negative control. The DNA molecular weight marker was 1-kb DNA Ladder (Fermentas, USA)

be transferred into *E. coli* recipients, suggesting a potential role of plasmids in the spread of *qnrS1* gene in Jiaozhou Bay. The *qnrS2* genes were identified in four *Pseudoalteromonas* and one *Pseudomonas* isolates, which were found to be without plasmids using alkaline lysis method. As these genes could not be transferred into *E. coli* recipients, the *qnrS2* genes may be situated on the bacterial chromosomes or large plasmids that were difficult to transfer or detect. Our study is the first report of *qnrS2* in *Pseudoalteromonas* and *Pseudomonas* strains, which may serve as an origin of the

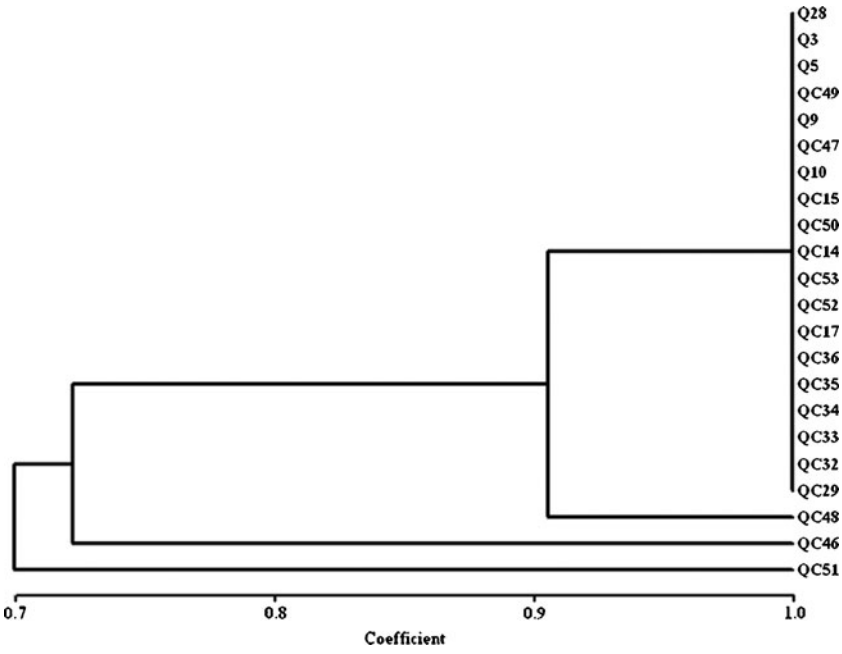

Figure 4 Dendrogram of the cluster analysis based on ERIC-PCR fingerprinting of the 22 *Proteus vulgaris* isolates. The numbers on the *horizontal axis* indicate the percentage similarities as determined by the Jaccard coefficient and UPGMA method using the software NTSYSPC2.1

qnrS gene. Our study also indicates the importance of the aquatic *Pseudoalteromonas* and *Pseudomonas* strains in the evolution and dissemination of quinolone resistance in coastal environments and related clinical settings.

Our study is the first identification of *qnrD* gene in *Proteus vulgaris* isolates that were collected from different months and sampling stations in Jiaozhou Bay (Table 2). The ERIC-PCR analysis revealed that these *Proteus vulgaris* isolates could be divided into four distinct groups. The major group consisted of 19 isolates, the close relatedness of which suggested that the prevalence of *qnrD* gene in Jiaozhou Bay might originate from a common *Proteus vulgaris* ancestor strain that was well adapted to the survival, persistence, and dissemination in this coastal environment.

Acquirement of PMQR genes may only result in reduced susceptibility or low-level resistance to quinolones. However, this process may facilitate the recovery of mutants with high level of quinolone resistance [63]. In our current study, 88% of the *qnr*-positive isolates had a single point mutation at codon 67 or codon 83 in the QRDRs of GyrA protein. As these isolates were previously selected on TSA plates supplemented with tetracycline or chloramphenicol, their *gyrA* genes retained the original statuses and avoided additional mutations induced by the quinolone selection pressure. The combination of chromosome- and plasmid-mediated quinolone resistance may play an important role in the persistence and dissemination of quinolone resistance in Jiaozhou Bay.

It has been found that the *qnr* genes exist in a great number of ESBL- and AmpC-producing *Enterobacteriaceae* [23, 60]. Identification of *qnr* genes in clinical isolates of *E. coli* and *K. pneumoniae* from Chinese pediatric patients without quinolone treatment indicated that the widely used β-lactam antibiotics might contribute to the cross-selection of *qnr* genes [27, 70]. Coexistence of quinolone- and β-lactam-resistant genes on the same genetic element provides one of the mechanisms for this phenomenon [42]. In our current study, 85% of the *qnr*-positive isolates were found to be resistant to ampicillin, and the bla_{CMY}, bla_{OXY}, bla_{TEM}, and bla_{SHV} genes were identified in the *qnrB*- and *qnrS1*-positive isolates. The common ESBL and AmpC β-lactamase genes were not identified in the *qnrA*- and *qnrD*-positive isolates, so some other determinants may be responsible for their resistance to ampicillin. The dominant bla_{SHV} genes were detected in three *K. pneumoniae* isolates, and the bla_{OXY} and bla_{TEM} genes were detected in *Klebsiella* sp. isolate Q11, simultaneously. Coexistence of these ESBL genes and *qnrS* genes was previously reported in Asian and European countries [8]. The plasmid-mediated cephalosporinase gene bla_{CMY} was identified in association with *qnrB9* gene in *C. freundii* isolate Q15 in our current study, in contrary to $bla_{CMY\text{-}1}$ in association with *qnrB2* in an *E. coli* isolate from Korea [50]. Novel combinations may be expected to find in future studies due to the diverse arrays of both genes.

The quinolone-resistant isolates and their *qnr* genes in Jiaozhou Bay might originate from two distinct sources, the terrestrial bacteria related to anthropogenic activities and the indigenous estuarine or marine bacteria. The *qnrB9*, *qnrS1*, and *qnrD* genes were identified in *Enterobacteriaceae* isolates affiliated to *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Proteus*, most of which might potentially relate to human or animal pathogens. Civic wastewater discharges via rivers and sewage processing plants on the seashore may be the major source of quinolone-resistant *Enterobacteriaceae* in Jiaozhou Bay. The *qnrA3* and *qnrS2* genes were detected in *Shewanella*, *Pseudoalteromonas*, and *Pseudomonas* isolates, respectively, most of which are common estuarine or marine bacterial species. Several *qnr*-like genes have been found in the chromosomes of aquatic bacteria and in metagenomes from marine organisms [54, 55, 59]. Expression of some *qnr* genes in environmental bacteria may be induced by cold shock and regulated by the SOS system [38, 73], indicating that the *qnr* gene may contribute to low temperature adaptation of bacteria in aquatic environment. Coastal seawater bacteria may thus provide a natural origin and reservoir of diverse *qnr* genes that may be acquired by or exchanged with pathogenic bacteria, facilitating the creation and transmission of new quinolone resistance determinants. The increasing occurrence of PMQR gene with time indicates that the gene transmission and exchange rate is getting faster recently [34]. Study of antibiotic resistance plasmids isolated from seawater bacteria of Jiaozhou Bay indicates that there is no border for the transmission of antibiotic resistance on a global scale [79]. Our study indicates that coastal environments serve as a dynamic mixture of gene pools from both natural and anthropogenic domains. Antibiotic resistance in these environments should draw a great attention and research effort for winning the battle against the intimidating antibiotic resistance threat [24]. Regional and global antibiotic resistance surveillance programs are present or proposed [25]. However, the focus of these programs is on clinical pathogens. Antibiotic resistance in the environment is seldom concerned. As the clinical setting and natural environment are tightly interlinked in coastal environments [77], the neglect of antibiotic resistance in the environment may be a big mistake in the strategy and implementation of antibiotic resistance surveillance and control [46].

In summary, our pioneering identification of *qnrD* gene in *Proteus vulgaris* and *qnrS2* gene in *Pseudoalteromonas* and *Pseudomonas* enriched the list of microbial diversity of the *qnr* gene hosts. Surveillance survey on prevalence of *qnr* genes in Jiaozhou Bay could not only acquire a basic understanding of the abundance and diversity of these resistance determinants in marine environment, but also help to design better strategy and implementation for coastal environment and human health management.

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