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ORIGINAL ARTICLE

Description and plasmid characterization of the *qnrD* determinant in *Proteeae* in Wenzhou, Southern China

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KEYWORDS
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Abstract *Background/Purpose:* Only limited information is available about the detailed characteristics of *qnrD*, a plasmid-mediated quinolone resistance (PMQR) gene. This study aimed to understand the distribution of *qnrD* and the characterization of *qnrD*-carrying plasmids in *Proteeae*.

Methods: The distribution of *qnrD* genes was investigated by polymerase chain reaction (PCR) amplification in 203 consecutive nonduplicate clinical isolates of *Proteeae* collected from inpatients at the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China. The minimum inhibitory concentrations (MICs) of antibiotics were measured by agar dilution method and other PMQR determinants were also determined by PCR. *qnrD* was positioned via Southern hybridization and the transferability of *qnrD*-carrying plasmids was achieved by conjugation experiment. The genetic environment of *qnrD* was investigated by sequencing, and chromosomal polymorphism for *qnrD*-positive strains was analyzed by pulsed-field gel electrophoresis (PFGE).

Results: Forty strains carried *qnrD*, showing decreased fluoroquinolone susceptibility or low-level fluoroquinolone resistance. *qnrD* was encoded on the plasmid of about 2.7 kb or 5.2 kb in length, which cannot be transferred by liquid conjugation or filter mating, but can be successfully transferred by transduction. The transformants showed 62.5–300-fold increases in the MICs of quinolones compared with the recipient. The plasmids carrying *qnrD* showed a high similarity with that of *Providencia* spp. and *Proteus vulgaris*. PFGE analysis demonstrated that these isolates were divergent and not clone related.

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Conclusion: *qnrD* could have originated from *Proteeae* or presented in these bacteria as a reservoir; furthermore, *qnrD* could be transferred and spread within the same or across different bacterial species if the plasmids acquired mobile elements under antimicrobial selective pressures.

Introduction

Fluoroquinolones have a wide spectrum of *in vitro* antibacterial activity, particularly against Gram-negative bacteria. Unfortunately, the widespread use of this antibiotic in clinical medicine has resulted in the resistance to these agents becoming more common among Enterobacteriaceae. Quinolone resistance is mainly due to mutations in the subunits of DNA gyrase and topoisomerase IV.[1–3] Although plasmid-mediated quinolone resistance (PMQR) determinants confer only low-level resistance to quinolones, they can increase the minimum inhibitory concentrations (MICs) of quinolones; moreover, these changes are sufficient to facilitate the selection of mutants with higher levels of resistance.[4] Besides, PMQR genes are located on transferable plasmids and play an important role in quinolone resistance. To date, a number of PMQR determinants have been detected and studied, including the *qnr* genes (A, B, S, C, D, and VC); the *aac(6′)-Ib-cr* gene; the *qepA* gene; and *oqxAB*, a new PMQR determinant.[5,6]

The *qnrD* gene was first described on a 4270-bp plasmid in a human clinical *Salmonella enterica* isolate from China in 2009, but there has been only little information about the detailed characteristics of *qnrD* due to the low prevalence of this PMQR gene in clinical isolates.[5] The *qnrD* gene was found on small plasmids, whose size was about 2.7 kb or 4.2 kb; meanwhile, the *qnrD*-carrying plasmids typically had a simple backbone and were stably maintained. Thomas Guillard had hypothesized that *qnrD* could be more closely related to the *Proteeae* bacteria than the other *qnr* genes and perhaps originated from them.[7] In this study, we investigated 203 *Proteeae* strains isolated from humans to understand the distribution of *qnrD* gene and the characterization of the *qnrD*-carrying plasmids.

Methods

qnrD detection in *Proteeae* clinical isolates

A total of 203 consecutive nonduplicate *Proteeae* clinical isolates (100 *Morganella morganii*, 85 *Proteus mirabilis*, 15 *Proteus vulgaris*, and 3 *Providencia rettgeri* isolates) were collected and screened from inpatients at the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China. All isolates were identified by a VITEK 2 system (bioMérieux, France). The total DNAs of all isolates were obtained using an AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen Scientific, Union City, CA, USA) and were used as polymerase chain reaction (PCR) templates. The presence of *qnrD* was detected for all isolates by PCR, and PCR products were confirmed by sequencing.

Antimicrobial susceptibility testing

The MICs of ciprofloxacin, norfloxacin, levofloxacin, and nalidixic acid were determined by the agar dilution method or broth microdilution method for *qnrD*-positive strains and *qnrD*-negative strains according to the relevant Clinical and Laboratory Standards Institute 2014 guidelines.[8] Quality control for the MIC analyses was performed with *Escherichia coli* ATCC 25922. The MIC_{50} and MIC_{90} were also reported to provide preliminary information about how the *qnrD* gene contributes to the MIC of quinolones.

Investigation of other related resistance determinants

All *qnrD*-positive strains were also screened for other PMQR determinants, including *qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib-cr*, *qnrC*, *qepA*, *oqxA*, and *oqxB* by PCR using primers as described previously.[9] The positive PCR products were screened by electrophoresis on 1.0% agarose gel and were sequenced by Shanghai Majorbio Bio-Pharm Technology Co. (Shanghai, China). Nucleotide sequences were analyzed and compared using Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST).

Plasmid analysis and Southern hybridization

Plasmids of *qnrD*-positive strains were extracted by the QIAGEN Plasmids Midi Kit (Qiagen, GmbH, Germany). Plasmid DNAs were separated by electrophoresis on a 0.8% agarose gel at a constant voltage of 100 V for 1 hour and then transferred onto a nylon membrane (Roche, Mannheim, Germany). Southern hybridization was performed according to standard protocols.[7] The *qnrD*-specific probe was labeled using a DIG High Prime DNA labeling kit (Roche, Sant Cugat del Vallès, Spain) following the manufacturer's instructions. Plasmids extracted from *E. coli* V517 (sizes, 54 kb, 5.6 kb, 5.1 kb, 3.9 kb, 3.0 kb, 2.7 kb, and 2.1 kb) were used as the standards for plasmid size.

Conjugal transfer experiments

Conjugation experiments were performed using rifampin-resistant *E. coli* C600 and sodium azide-resistant *E. coli* J53 as the recipient strains. In brief, overnight cultures of the donor strain (500 μL) and recipient strain (500 μL) were

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mixed with 10-mL fresh Luria-Bertani broth and incubated for 24 hours at 35°C. Then, the mixture was inoculated on Mueller-Hinton agar plates containing rifampin (600 μg/mL) or sodium azide (100 μg/mL) plus ampicillin (100 μg/mL) or nalidixic acid (50 μg/mL) or ciprofloxacin (0.03 μg/mL and 0.06 μg/mL), or gentamicin (4 μg/mL) for 24 hours at 35°C.

Filter mating was initiated with Luria-Bertani broth suspensions of donor and recipient cells prepared from their 18-hour growth on blood agar, cultured for 4 hours with shaking. Equal portions (1 mL) of donor and recipient cells were mixed and filtered through a 25-mm nitrocellulose filter (0.45-μm pore diameter), which was then incubated on blood agar media for 18 hours at 37°C. The resultant growth was suspended in 2 mL of the broth medium, diluted, and plated on the aforementioned selective media for 24 hours at 35°C.

As previously described, plasmid transfer by transduction was performed by transformation experiments using JM109 as receptor.[8] Transformants were selected on Mueller-Hinton agar plates containing ciprofloxacin (0.06 μg/mL).

PCR analysis was performed to screen the resistant genes, and MICs of quinolones were determined by the agar dilution method for transconjugants and transformants.

Sequence of plasmids

To investigate the genetic environment of *qnrD*, such as mobile elements, the whole sequence of the plasmid harboring the *qnrD* gene was obtained by PCR walking. pM60, pMB5, pMB18, and pMSL46 (extracted from M60, B5, B18, and SL46, respectively) were randomly selected and sequenced. We predicted the open reading frame (ORF) using the ORF Finder website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and matched the whole sequence with sequences in GenBank database. The structure and G+C contents of the plasmid were also investigated.

Detection of mutations in the topoisomerase II and IV genes

The quinolone resistance-determinant regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* in all *qnrD*-positive strains were amplified as described previously.[10] The positive PCR products were sequenced by Shanghai Majorbio Bio-Pharm Technology Co. and sequence data were analyzed using BLAST.

Pulsed-field gel electrophoresis analysis

Chromosomal polymorphism was analyzed by pulsed-field gel electrophoresis (PFGE) using the *Xba*I or *sfi*I (special for *Proteus*) restriction enzyme (Takara Bio, Inc.) for *qnrD*-positive strains. The DNA fragments were separated using a CHEF-Mapper XA PFGE system (Bio-Rad, Hercules, CA, USA) for 22 hours at 6 V/cm and 14°C, with a pulse angle of 120° and pulse duration from 5 seconds to 25 seconds. The restriction patterns were analyzed using the Quality One software (Bio-Rad) and interpreted according to the criteria proposed by Tenover et al.[11]

Results

Detection of resistance determinants and antimicrobial susceptibility testing

The *qnrD* gene screening of the 203 clinical isolates identified 40 (19.7%) strains carrying *qnrD*: 17 *M. morganii* (M36—M110), 20 *P. mirabilis* (B2—B84), two *P. vulgaris* (B85 and B95), and one *P. rettgeri* (SL46). A total of 14 strains were found to co-produce other resistance genes: 20% (5 *M. morganii* and 3 *P. mirabilis*) of the isolates were positive for *aac(6′)-Ib-cr*, 5.0% (1 *P. mirabilis* and 1 *P. vulgaris*) isolates were positive for *qnrB*, 7.5% (3 *M. morganii*) were positive for *qepA*, and 12.5% (2 *M. morganii*, 2 *P. mirabilis*, and 1 *P. vulgaris*) were positive for *oqxAB*. The genotypic characteristics and susceptibilities of *qnrD*-positive isolates are presented in Table 1. The MIC_{50} and MIC_{90} for the *qnrD*-positive strains were at least two or more dilutions higher than that for the *qnrD*-negative strains. The detailed data are presented in Table 2.

Plasmid analysis and Southern hybridization

The plasmid profiles indicated that all the *qnrD*-positive isolates had more than two plasmids and Southern blot analysis of the *qnrD*-positive isolates hybridized with the *qnrD*-specific probe confirmed that *qnrD* was encoded on the 2.7- or 5.2-kb plasmid (Figure 1).

Conjugal transfer experiments

The *qnrD*-positive isolates failed to transfer *qnrD* to *E. coli* C600 or *E. coli* J53 through either liquid or mating-out assays despite several attempts. Only the *aac(6′)-Ib-cr* gene of two isolates (M72 and B43) were successfully transferred to *E. coli* C600. The transconjugant exhibited a phenotype of reduced susceptibility to ciprofloxacin and was PCR confirmed to carry the *aac(6′)-Ib-cr* gene similar to that of the original isolates.

Plasmid harboring *qnrD* was successfully transferred by transduction. *qnrD* was detected in all transformants, which showed 62.5- to 300-fold increases in the MICs of quinolones compared with JM109 (Table 3).

Sequence of plasmid

The length of the whole sequences of pM60, pMB5, pMB18, and pMSL46 was 2683 bp, 2683 bp, 5201 bp, and 2683 bp, respectively. pM60, pMB5, and pMSL46 exhibited 99.9% identity with each other and they had 2187-bp bases that exhibited 99% identify with pMB18. All the plasmids were aligned with p2007057, which was a *qnrD*-carrying plasmid first described in a human clinical *S. enterica* isolate. Only 54.3% of the whole sequences of pM60, pMB5, and pMSL46, and 30.0% of pMB18 exhibited ~98% identity with p2007057, respectively. By contrast pM60, pMB5, and pMSL46 exhibited 100% identity with pCGP248 (a *qnrD*-carrying plasmid in *P. mirabilis*), and 57.0% and 61.0% of the pMB18 sequences exhibited 99% identity with pCGP248 and p3M-2B (a *qnrD*-carrying plasmid in *Proteus* spp.), respectively.

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Table 1 The resistance determinants and susceptibilities of *qnrD*-positive isolates.

Strain	Minimum inhibitory concentrations (μg/mL)				Plasmid-mediated quinolone resistance genes
	NAL	CIP	LEV	NOR	
M36	>256	32	16	>64	*qnrD*
M46	>256	16	2	8	*qnrD*, *aac(6′)-Ib-cr*, *qepA*
M51	>256	8	8	8	*qnrD*
M56	4	0.125	0.125	0.125	*qnrD*
M59	>256	4	4	8	*qnrD*
M60	>256	32	8	>64	*qnrD*
M65	>256	8	2	4	*qnrD*, *qepA*
M69	>256	1	2	4	*qnrD*, *qepA*
M72	>256	64	4	32	*qnrD*, *aac(6′)-Ib-cr*, *oqxAB*
M74	>256	2	4	4	*qnrD*
M78	>256	1	1	0.25	*qnrD*
M82	>256	32	8	8	*qnrD*
M86	>256	1	1	0.5	*qnrD*
M90	>256	4	4	8	*qnrD*, *aac(6′)-Ib-cr*
M91	8	0.125	0.25	4	*qnrD*, *aac(6′)-Ib-cr*, *oqxAB*
M103	2	0.5	0.5	0.25	*qnrD*
M110	>256	16	4	32	*qnrD*, *aac(6′)-Ib-cr*
B2	8	0.25	0.5	1	*qnrD*
B3	>256	0.125	0.5	1	*qnrD*
B5	>256	8	8	16	*qnrD*, *aac(6′)-Ib-cr*
B8	>256	4	8	16	*qnrD*, *aac(6′)-Ib-cr*
B15	>256	0.25	0.5	1	*qnrD*
B16	>256	4	8	4	*qnrD*
B18	>256	16	8	16	*qnrD*
B24	>256	2	4	4	*qnrD*
B26	4	0.03	0.125	0.5	*qnrD*
B27	>256	8	8	8	*qnrD*
B30	>256	1	2	2	*qnrD*, *oqxAB*
B33	>256	0.5	0.5	0.25	*qnrD*
B46	>256	0.25	0.5	0.5	*qnrD*
B48	>256	4	2	4	*qnrD*, *oqxAB*
B57	>256	0.5	0.25	1	*qnrD*, *qnrB*
B59	>256	0.25	0.5	0.125	*qnrD*
B65	>256	0.06	0.125	0.25	*qnrD*
B76	>256	8	0.25	4	*qnrD*
B81	8	0.25	0.5	0.5	*qnrD*
B84	>256	16	8	16	*qnrD*
B89	>256	16	16	16	*qnrD*, *qnrB*
B95	>256	0.25	1	1	*qnrD*, *aac(6′)-Ib-cr*, *oqxAB*
SL46	>256	4	16	32	*qnrD*

CIP = ciprofloxacin; LVX = levofloxacin; NAL = nalidixic acid; NOR = norfloxacin.

The whole sequences of pM60 and pMB18 were deposited at GenBank (accession numbers KF813021.1 and KM577619.1). pM60, pMB5, and pMSL46 contained four putative ORFs with *qnrD*, and the plasmid pMB18 contained six ORFs. It is necessary to point out that none of these three plasmids carried any other resistance gene or any mob-like element. The G+C contents of *qnrD*, pM60, and pMB18 (38.14%, 40.43%, and 41.74%, respectively) are close to those of *Providencia* spp. (38–40%) and *Proteus* spp. (38–39%) but not close to those of *S. enterica* (48%). Furthermore, several segments of these plasmids' genomes showed partial homology with the genome of *Proteeae*. For detailed information, please refer to Figure 2.

Topoisomerase mutations

We could not obtain the positive amplification products of QRDRs in *M. morganii* despite many attempts. PCR sequencing of PCR products of QRDRs in *P. mirabilis*, *P. vulgaris*, and *P. rettgeri* showed no mutations.

PFGE typing

PFGE analysis of the *qnrD*-positive strains belonging to the same species was distinct because these strains shared less than 70% (*M. morganii*) or 60% (*Proteus*) similarity

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Table 2 MIC_{50} and MIC_{90} for *qnrD*-positive strains and *qnrD*-negative strains.

Drug	MIC_{50} (μg/mL)		MIC_{90} (μg/mL)	
	qnrD+	*qnrD*−	*qnrD*+	*qnrD*−
NAL	>256	>256	>256	>256
CIP	2	0.5	16	4
LVX	2	1	8	4
NOR	4	0.25	32	16

CIP = ciprofloxacin; LVX = levofloxacin; MIC = minimum inhibitory concentration; NAL = nalidixic acid; NOR = norfloxacin.

(Figure 3), which revealed that they were divergent and not clone related, suggesting that the *qnrD* gene had emerged independently.

Discussion

PMQR determinants were widely distributed in Enterobacteriaceae.[12] To our knowledge, there is no report on the high prevalence of the *qnrD* gene in clinical isolates of *Proteeae*. In this study, the distribution characteristics of the *qnrD* gene and other related resistance determinants among 203 *Proteeae* isolates in a Chinese teaching hospital were characterized. The positive rate of the *qnrD* gene reached up to 19.7%, which was much higher than the previous study.[7]

The *qnrD* gene is the latest PMQR determinant. Since it was first described in 2009 in human clinical *S. enterica* isolates from China,[13] there have been only a few reports regarding the *qnrD* determinant in human clinical isolates. The isolates found to be positive for this determinant are as follows: two *P. mirabilis* isolates and one *Pseudomonas aeruginosa* isolate in Nigeria in 2010[14]; four *P. mirabilis* isolates, one *M. morganii* isolate in Italy, and two *P. rettgeri* isolates in France in 2011[15]; and 12 *P. mirabilis* isolates and two *M. morganii* isolates[15] in China in 2012.[16] In this study, a higher positive rate (24.6%, 50/203) of the *qnrD* determinant was detected in our *Proteeae* isolates than in the previous study. The *qnr* genes have rarely been observed in *Proteeae*, but interestingly, *qnrD* plasmids seem to be more common in *Proteeae*.[17] In addition, the difference in quinolones susceptibility between the *qnrD*-positive strains and the *qnrD*-negative strains proved that *qnrD* contributed to quinolone resistance primarily.

A total of 14 strains were found to co-produce other PMQR genes, and it was worth mentioning that *aac(6′)-Ib-cr* and *oqxAB*, two other plasmid-mediated resistance mechanisms, were also identified in our investigation. One important point is that although these mechanisms cause only low-level resistance, they favor and complement the

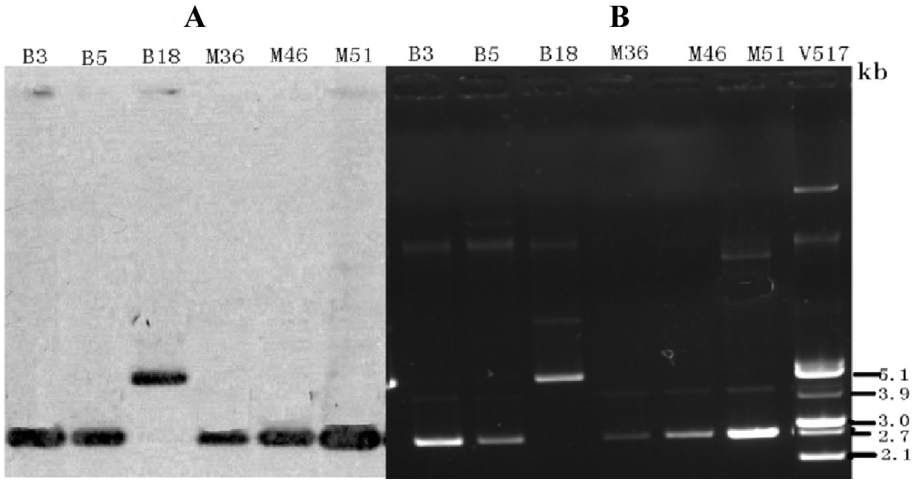

Figure 1. (A) Southern blot of the gel shown in Panel (B) after hybridization with the *qnrD* probe. (B) Plasmid profiles of part of *qnrD*-positive strains and V517 (2.1 kb, 2.7 kb, 3.0 kb, 3.9 kb, and 5.1 kb) on 0.8% agarose gel.

Table 3 Antibiotic susceptibility of JM109 and transformants.

Strain	MIC or MIC_{90} (μg/mL)			
	NAL (range)	CIP (range)	NOR (range)	LVX (range)
JM109	1	0.002	0.015	0.002
Transformants	8 (1—8)	0.5 (0.125—1)	2 (0.125—2)	0.5 (0.125—1)

CIP = ciprofloxacin; LVX = levofloxacin; MIC = minimum inhibitory concentration; NAL = nalidixic acid; NOR = norfloxacin.

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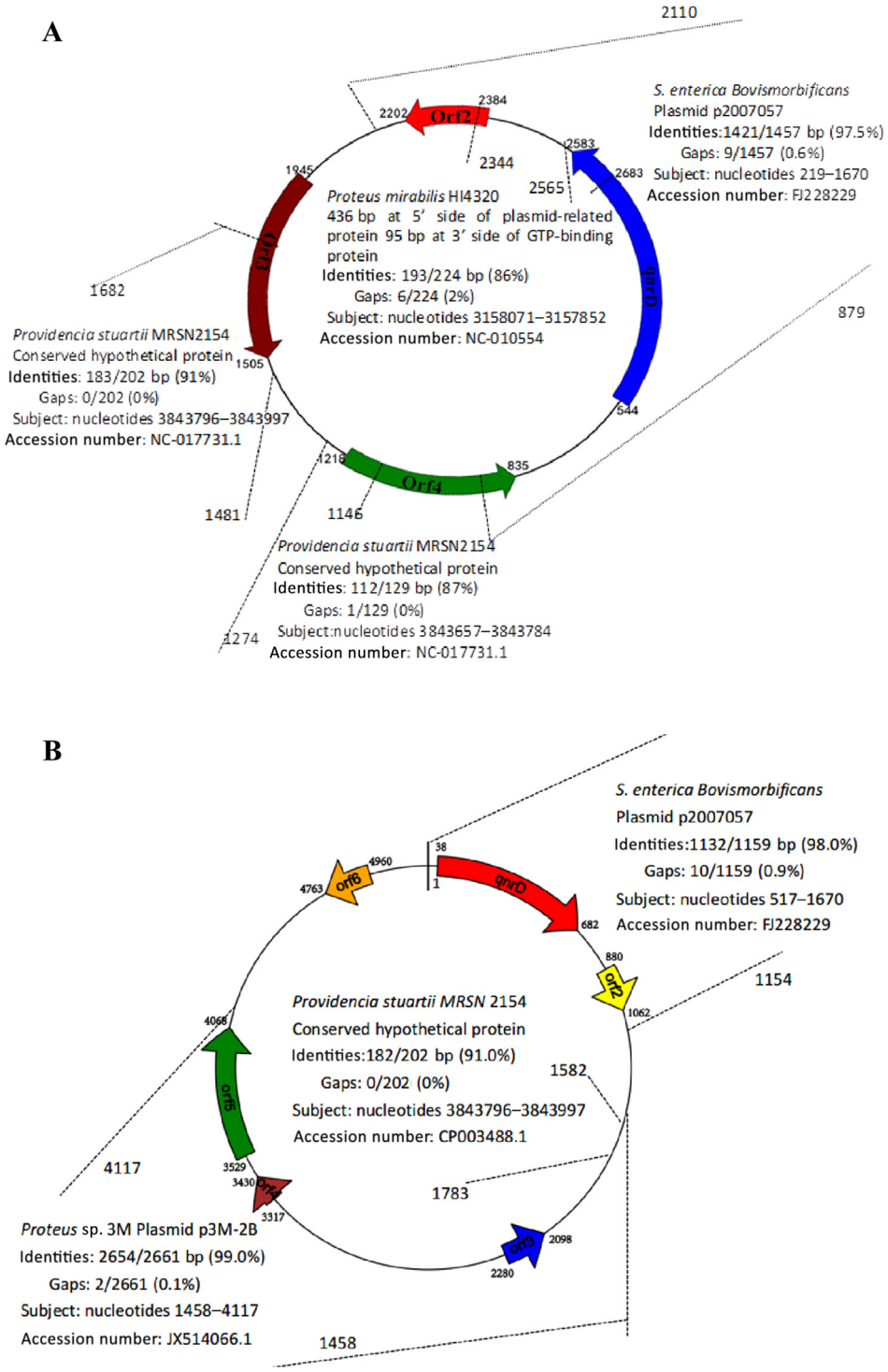

Figure 2. Structure of (A) pM60 and (B) pMB18 and main alignment results (dotted lines). pM60, pMB5, and pMSL46 exhibited 99.9% identity with each other, and therefore, we selected pM60 as the representative here. GTP = guanosine-5′-triphosphate; *S. enterica* = *Salmonella enterica*.

selection of other resistance mechanisms.[18] This was the first detection of the *oqxAB* genes in the clinical isolates of *M. morganii*. Antimicrobial-susceptibility results showed decreased fluoroquinolone susceptibility or low-level fluoroquinolone resistance was exhibited by most isolates with *qnrD* and other PMQRs, reflecting the importance of PMQRs determinants in fluoroquinolone-resistance mechanisms. However, the isolate M91 co-carrying *qnrD*, *aac(6′)-Ib-cr*, and *oqxAB* was found to be sensitive to ciprofloxacin and levofloxacin. Therefore, *qnr* determinants alone may not confer resistance to quinolones, but they can supplement other quinolone-resistance mechanisms.[19]

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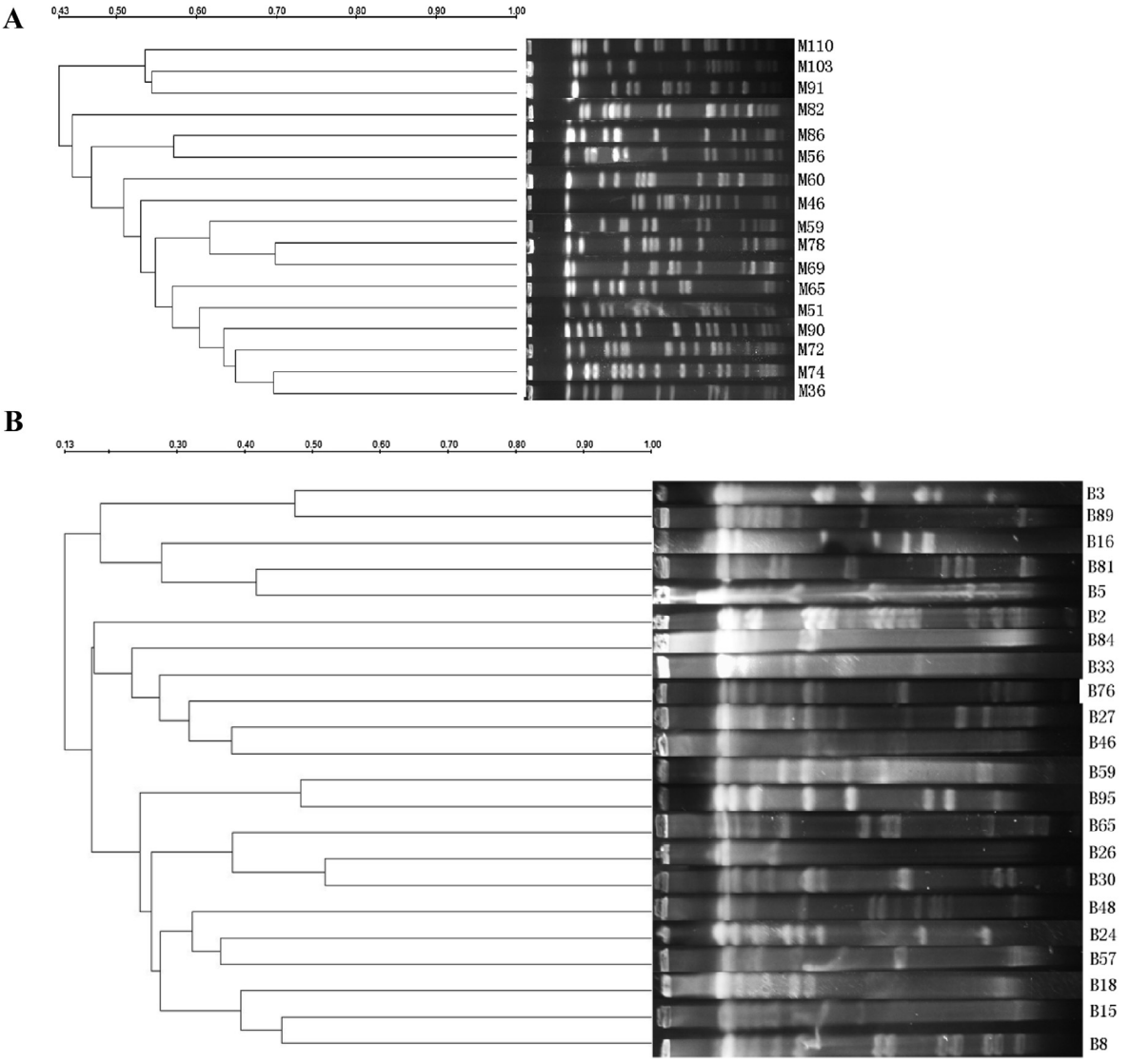

Figure 3. Pulsed-field gel electrophoresis analysis of (A) 17 *qnrD*-positive *Morganella morganii* and (B) 22 *qnrD*-positive *Proteus*.

Until now two *qnrD*-carrying plasmids, around 4.2 kb and 2.7 kb in length, have been identified in human clinical isolates.[13,16,20] In our research, DNA hybridization showed that *qnrD* in *M. morganii*, *P. rettgeri*, and most *Proteus* isolates was located on a ~2.7-kb plasmid, with the exception of three *P. mirabilis* isolates (B18, B84, and B89), in which it was on a ~5.2-kb plasmid, which is the first report of a new plasmid containing *qnrD*. In addition, it is worth pointing out that all the three aforementioned strains showed higher fluoroquinolone resistance than the other *qnrD*-carrying strains.

Previous studies have reported that the *qnr* genes were related to integron structures[13,19]; however, none of our plasmids carried further resistance determinants or mob-like element except *qnrD*, such as integron structures and transposons. Conjugal studies proved that all the isolates failed to transfer the *qnrD* gene to *E. coli* C600 or *E. coli* J53 by liquid conjugation and filter mating, further supporting the notion that the *qnrD*-carrying plasmids are untransferable. In addition, the *qnrD* gene had emerged independently among our strains according to the PFGE analysis. In other words, the *qnrD* gene did not have the characteristic of horizontal transmission and clonal transmission. Thus, the distribution of *qnrD* could be explained from the perspective of bacterial evolution. Chromosomal elements could transfer on their own or be mobilized by transferable plasmids and research has shown that large chromosomal elements could transfer via the mobilization of segments of the chromosome by conjugative plasmids through co-integration across identical insertion sequences located on both replicons.[21] It is possible that the *qnrD* gene could be a product of co-integration in the bacterial evolution process, involving only a segment of the chromosome and attributed to acquired resistance through genetic mutation under antimicrobial selective pressures. In addition, the plasmid carrying *qnrD* showed a high similarity with *Providencia* spp. and *Proteus* spp., which provided evidence for the frequent association of *qnrD* with the *Proteeae*. All the aforementioned information further supports the conjecture that *qnrD* could be more closely

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related to the *Proteeae* bacteria than the other *qnr* genes and that *Proteeae* were an essential source or carrier of the *qnrD*-harboring plasmids. By contrast, the plasmids carrying *qnrD* were successfully transferred by transduction, and transformants showed 62.5—300-fold increases in the MICs of quinolones compared with recipient, which directly proved that *qnrD* contributed to quinolone resistance and suggested that the *qnrD*-carrying plasmids could spread via other ways unlike conjugation, such as natural transformation and transduction.

With regard to the mutation data of the QRDRs for all *qnrD*-positive strains, we could not amplify the QRDRs in *M. morganii*, which is likely because the primers used to amplify the QRDRs for Enterobacteriaceae were not available for *M. morganii* and this is due to its unknown genome according to the study of Mazzariol et al.[22] There were no mutations in the QRDR among other strains. This is similar to previous studies, where topoisomerase mutations have not always been investigated in *qnrD*-positive clinical strains to evaluate how both mechanisms interact.[17]

In conclusion, *qnrD* genes are highly prevalent in *Proteeae* clinical isolates in our hospital and the study results suggest that *qnrD* could originate from *Proteeae* or could be present in these bacteria as a reservoir; moreover, *qnrD* could transfer and spread within the same or across different bacterial species if the plasmid acquires mobile elements under antimicrobial selective pressures.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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