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Characteristics of *Klebsiella pneumoniae* harboring QnrB32, Aac(6')-Ib-cr, GyrA and CTX-M-22 genes

Dongguo Wang[1]*, Haibao Wang[1], Yongxiao Qi[2]*, Yong Liang[2], Jin Zhang[1], Lianhua Yu[1]

[1]Department of Clinical Lab Medicine, Taizhou Municipal Hospital Affiliated to Medical College of Taizhou University, Taizhou, China

[2]Department of Lab Medicine, Medical College of Taizhou University, Taizhou, China

*Equal contribution of both authors

Abstract: Quinolone resistance in members of the *Enterobacteriaceae* family is mostly due to mutations in the quinolone resistance-determining regions of topoisomerase genes. CTX-M-22 is a member of the CTX-M family which can reduce extended-spectrum β-lactamase (ESBL) production and modulate antibiotic resistance, resulting in low ceftazidime minimum inhibitory concentrations (MICs). There are four different genes in *Klebsiella pneumoniae* (KP4707) including qnrB32 (novel qnr allele gene, HQ704413), aac(6')-Ib-cr (novel aac(6')-Ib allele gene, HQ680690), gyrA (novel gyrA allele gene, HQ680691) and CTX-M-22 gene. Five point amino acid mutations Arn(N)27 → Leu(L), Val(V)129 → Ala(A), Iie(I)142 → Met(M), Gly(G)188 → Arg(R), Val(V)212 → Iie(I) were observed in the qnr32 gene when compared to qnrB1. Of all qnrB alleles, a novel variant of the qnrB32 gene, with qnrB31, had the highest amino acid homology. Three point amino acid mutations including Trp(W)105 → Arg(R), Asp(D)182 → Tyr(Y) and Val(V)201 → Asp(D) were observed in aac(6')-Ib-cr gene, when compared to GenBank number AF479774. New variants of qnr32, aac(6')--Ib-cr, gyrA and CTX-M-22 or other genotype determinants continuously appear in different genomic sites and also outside the *Enterobacteriaceae* family. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 1, 68–74)*

Key words: *Klebsiella pneumoniae*, qnrB32, aac(6')-Ib-cr, gyrA, CTX-M-22

Introduction

Quinolone resistance in the *Enterobacteriaceae* family usually results from mutations in genes carried by chromosomally encoded type II topoisomerases, efflux pumps, or porin-related proteins. Recently, plasmid-mediated quinolone resistances (PMQR) have been reported worldwide in unrelated *Enterobacterial* species and are usually associated with mobile elements [1]. Quinolones and beta-lactams are among the three most commonly used antimicrobials in human therapeutics. PMQR determinants confer low-level resistance, but their presence could potentially facilitate evolution of the bacterial host toward higher levels of resistance by mutational alterations in type II topoisomerases. PMQR determinants are often combined with extended-spectrum beta-lactamases (ESBLs). It has been suggested that there is potential for selection of PMQR by the use of beta-lactams and of beta-lactam resistance by the use of quinolones [2].

Correspondence address: D. Wang, Department of Clinical Lab Medicine, Taizhou Municipal Hospital Affiliated to Medical College of Taizhou University of China, 381 Zhongshan Dong Rd., Taizhou, China; tel.: + 86 0576 88 85 82 62, fax: + 86 0576 88 85 80 23; e-mail: wdgtzs@163.com

Y. Qui, Department of Lab Medicine, Medical College of Taizhou University of China, 1139 Shifu Rd., Taizhou, China; tel.: + 86 0576 88 66 51 97, fax: + 86 0576 88 66 51 91; e-mail: qyxzlt@163.com

The qnr proteins protect DNA from quinolone binding to gyrase and topoisomerase IV [3, 4]. aac(6')--Ib-cr is a variant of aac(6')-Ib and is responsible for reduced susceptibility to ciprofloxacin or norfloxacin by N acetylation of a piperazinyl amine. aac(6')-Ib-cr differs from aac(6')-Ib by the following two amino acid substitutions: Trp102Arg and Asp179Tyr [5]. DNA gyrase A subunit (gyrA) gene contains a quinolone resistance determining region (QRDR) [6]. Given that *Enterobacteriaceae* with low-level quinolone resistance has an increase in minimum inhibitory concentration (MIC) of ciprofloxacin, an increase of MIC appearing resistant to quinolone is due to mutations in the QRDR.

In the process of conducting experiments to detect 45 isolates of *Klebsiella pneumoniae*, we discovered an isolate KP4047 of *Klebsiella pneumoniae* emerging multi-drug resistant to antibiotics; it produced ESBL, expressed resistance to β-lactam drugs and was highly resistant to quinolones, so we decided to detect the concerned resistant genes and study them.

Four different genes were found in the isolate KP4047. These genes were CTX-M-22, qnrB32 (a novel qnr allele gene, GenBank accession number HQ704413), aac(6')-Ib-cr (a novel aac(6')-Ib allele gene, GenBank accession number HQ680690), and gyrA (a novel gyrA allele gene, GenBank accession number HQ680691). Thus, the concurrence of qnr, aac(6')-Ib, gyrA, and beta-lactamase genes (CTX-M-22) may express high-level resistance to quinolone and cephalosporin (Table 1). Figure 1 shows the bands in agarose gel electrophoresis of the isolate KP4047 of *Klebsiella pneumoniae*.

To gain further insight into the characteristics of *Klebsiella pneumoniae* of the plasmid-mediated CTX-M-22, qnrB32, aac(6')-Ib-cr, gyrA genes determinants, we performed and analyzed conjugation and a gene mapping test; the results showed significant similarities with corresponding alleles (Table 2, Figures 2–4), and there were three different length plasmids in isolates of KP4707. The CTX-M-22, aac(6')-Ib-cr, gyrA and qnrB32 genes were located in about 23.1 kb length plasmid.

Material and methods

Strain. A total of 45 isolates of *Klebsiella pneumoniae* were detected and the results will be reported in detail in future studies. One of these isolates was KP4047 which appeared resistant to quinolone and cephalosporin. It was isolated in 2008 from the pus of a patient who suffered from appendicitis with perforation in our hospital. The clonal isolate was investigated for the mechanisms responsible for resistance to quinolone (Table 3) and cephalosporin (Table 1). The MICs were determined by broth dilution methods [7].

Table 1. MICs of the isolate KP4707 of *Klebsiella pneumonae* producing ESBLs

Antibiotics	MIC [μg/ml]
Cefepine	8
Cefoxitin	4
Cefotaxime	64
Ceftazidime	8
Ceftriaxone	32
Cefazolin	128
Cefuroxime	64
Cefoperazone/sulbactam	0.05
Piperacillin/tazobactam	1
Ampicillin	> 256
Imipenem	0.025
Amikacin	64
Gentamicin	> 256
Nalidixic acid	> 256
Ofloxacin	32
Levofloxacin	16
Ciprofloxacin	8

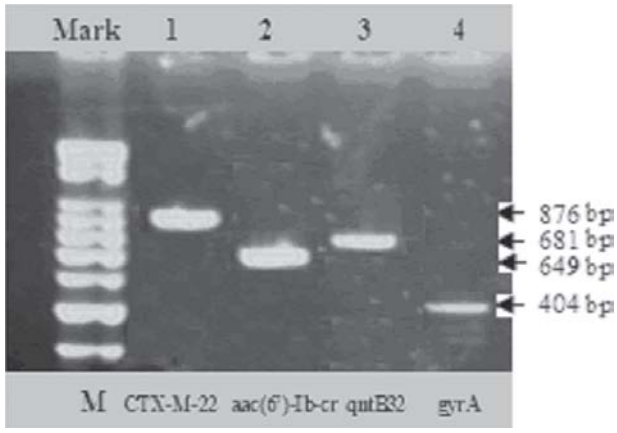

Figure 1. Agarose gel electrophoresis of the isolate KP4047 of *Klebsiella pneumoniae*. Lane 1: CTX-M-22, the bases of which were identical to those of GenBank accession number AY080894, HM470254, GU125665, EU376966, AY575772, and DQ309026); Lane 2: a novel aac(6')-Ib-cr gene (GenBank accession number HQ680690); Lane 3: a novel qnrB allele gene, qnrB32 (GenBank accession number HQ704413); Lane 4: a novel gyrA gene (GenBank accession number HQ680691). These results were determined by sequencing

Conjugation. According to a previously reported protocol [8], conjugation experiments were carried out in Luria broth (LB) with *E. coli* J53 Az^R (resistance to sodium azide) as the recipient and PCR positive strain as the donor strain. Donor and recipient cells in logarithmic phase (0.5 ml of

Table 2. Amino acid substitutions in qnrB1 to qnrB32[a]

Allele	Amino acid change at position:																																			
	2	7	11	18	20	21	22	27	35	36	55	60	69	74	79	80	87	94	118	129	142	144	147	151	162	163	168	171	186	188	198	202	204	205	212	213
qnrB1	A	G	D	E	I	E	N	N	L	S	N	M	C	A	S	S	R	A	N	V	I	A	L	F	S	T	A	F	I	G	N	S	L	M	V	I
qnrB2			N												A						M									R					I	
qnrB3											K										M															
qnrB4	T				V							N			I	N		S			M	T				S		V			S			L		M
qnrB5	I				V										V						M	I									S				I	
qnrB6															A						M															
qnrB7															A						M				I										I	
qnrB8	I				V							I			V					A	M	I		L		S	I					A				
qnrB9															A						M														I	
qnrB10	I				V										V						M	I														
qnrB11	T			A	V							I			V			S			M	T				S			V		S		I	L		M
qnrB12	T			A	V							I			V			S			M	T				S			V		S		I	L		
qnrB13															A						M									R					I	
qnrB14						D									A						M													T	I	
qnrB15							S								A	N					M														I	
qnrB16															A						M	T													I	
qnrB17																					M															
qnrB18						D									A						M															
qnrB19	T				V										V						M	T									S					
qnrB20			N												A						M									R						
qnrB21	T				V							I			V					A	M	T		L		S	T				S	A				
qnrB22	T				V					C		N			I	N		S			M	T				S		V		V	S			L		M
qnrB23								Y							A						M														I	
qnrB24									M					V	A						M															
qnrB25					V							I			V				S	A	M	T		L		S	T				S	A			I	
qnrB27	I	S			V										A			S			M	T	A			A					S	A				
qnrB28	I	S			V										V			S			M	T	A			A					S	A				
qnrB29														V	A						M															
qnrB30													S		A						M															
qnrB31								L							A		S				M									R					I	
qnrB32															A					A	M									R					I	

[a]Variations from the qnrB1 sequence numbered from the second potential ATG initiation codon are shown

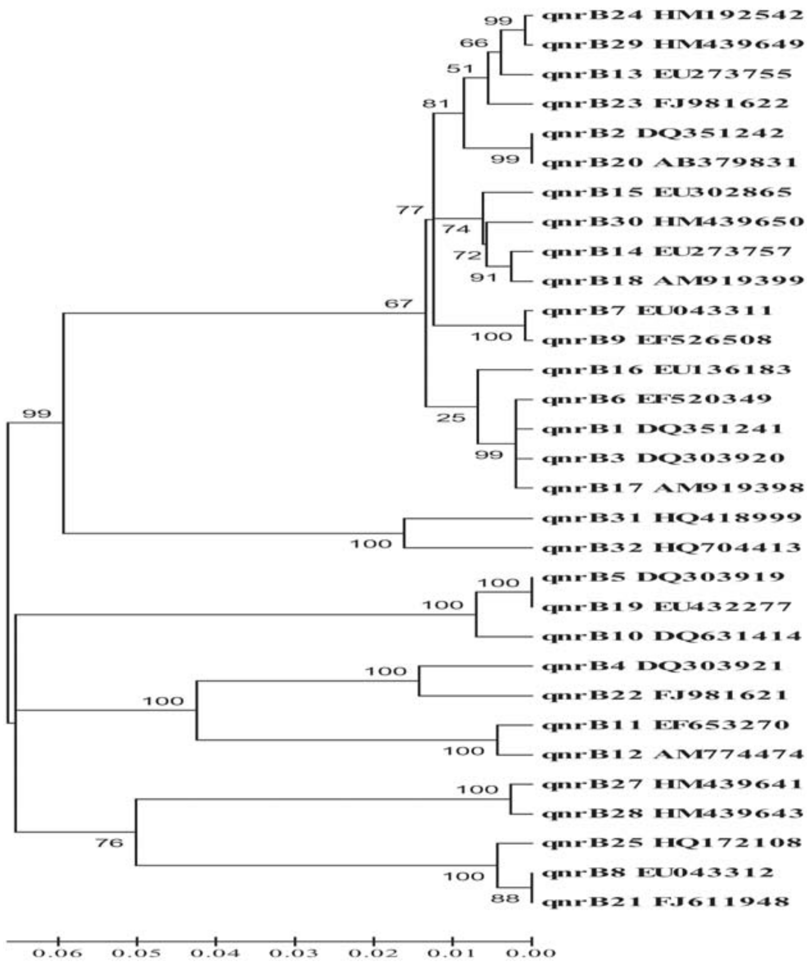

Figure 2. Phylogenetic analysis of the base sequence for qnrB allele variant. Novel variant of qnrB32 (HQ704413) in the isolate KP4707 of *Klebsiella pneumonae* had very high homology to qnrB31 gene (DQ418999)

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          30        40        50        60        70        80        90        100
AF479774 MTEHDLAMLY EWLNRSHIVE WWGGEEARPT LADVQEQYLP SVLAQESVTP YIAMLNGEPI
         GYAQSYVALG SGDGWWEEET
DQ303918 ---------- ---------- ---------- ---------- ---------- ---------- ----R-----
EU543272 ---------- ---------- ---------- ---------- ---------- ---------- ----R-----
EU675686 ---------- G--------- ---------- ---------- ---------- ---------- ----R-----
EF636461 ---------- ---------- ---------- ---------- ---------- ---------- ----R-----
EF100892 ---------- ---------- ---------- ---------- ---------- ---------- ----R-----
AY458016 ---------- ---------- ---------- ---------- ---------- ---------- ----R-----
EF465463 ---------- ---------- ---------- ---------- ---------- ---------- ----R-----
EUA6A636 ---------- ---------- ---------- ---------- ---------- ---------- ----R-----
EU195449 ---------- ---------- ---------- ---------- ---------- ---------- ----R-----
HQ680690 ---------- ---------- ---------- ---------- ---------- ---------- ----R-----
         110       120       130       140       150       160       170       180
AF479774 DPGVRGIDQL LANASQLGKG LGTKLVRALV ELLFNDPEVT KIQTDPSPSN LRAIRCYEKA
         GFERQGTVTT PDGPAVYMVQ
DQ303918 ---------- ---------- ---------- ---------- ---------- ---------- -Y--------
EU543272 ---------- ---------- ---------- ---------- ---------- ---------- -Y--------
EU675686 ---------- ---------- ---------- ---------- ---------- ---------- -Y--------
EF636461 ---------- ---------- ---------- ---------- ---------- ---------- -Y--------
EF100892 ---------- ---------- ---------- ---------- ---------- ---------- -Y--------
AY458016 ---------- ---------- ---------- ---------- ---------- ---------- -Y--------
EF465463 ---------- ---------- ---------- ---------- ---------- ---------- -Y--------
EUA6A636 ---------- ---------- ---------- ---------- ---------- ---------- -Y--------
EU195449 ---------- ---------- ---------- ---------- ---------- ---------- -Y--------
HQ680690 ---------- ---------- ---------- ---------- ---------- ---------- -Y--------
         190       200
```

Figure 3. Partial alignment of the nucleotide sequences of aac(6')-Ib-cr gene in GenBank. Accession numbers are indicated on the left. The boldface number represents a novel aac(6')-Ib-cr (HQ680690) gene which was found in *Klebsiella pneumoniae* in our hospital

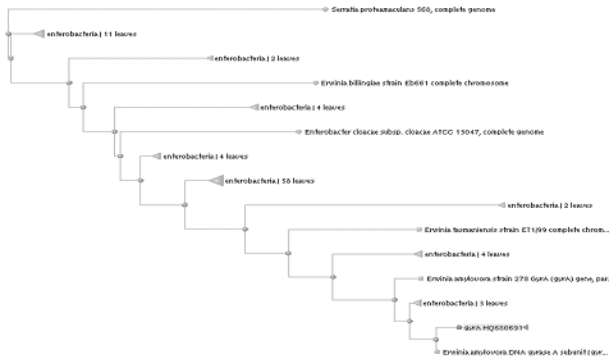

Figure 4. Distance tree of gyrA gene (HQ680691) of *Klebsiella pneumoniae* when compared with other *Enterobacteriaceae*

each) were added to 4 ml of fresh LB, followed by incubation overnight without shaking. The transconjugants were selected on Trypticase soy agar (TSA) plates containing sodium azide (300 mg/L) and ciprofloxacin (0.03 mg/L) following incubation for 18–24 h at 35°C. The results of qnrB32 are shown in Table 2. Unfortunately, the transconjugation of aac(6')-Ib-cr gene, gyrA gene and CTX-M-22 gene was not achieved. qnrB32 gene is shown in Figure 5.

PCR-amplified and sequencing. To investigate the genetic characteristics of the qnrB32 gene of *Klebsiella pneumonae* and *E. coli* J53 Az^R (conjugated) isolates, PCR was performed for the amplification of qnrB32 gene in *Klebsiella pneumonae* using the following primers: F 5'-ATGTTGTCATTACTGTATA-3', R 5'-CTAACCAATCACAGCGATG-3', and sequencing amplification with sequencing primers F 5'-ATGACGCCATTACTGTATAAAAAA-3', R 5'-CTA GCCAATAATCGCGATGCCA-3'. PCR was also done for amplification of qnrB32 in *E. coli* J53 Az^R (conjugated) using the following primers: F 5'-ATGACTCTGGCGTTAGTTGG-3', R 5'-TTAACCCATGACAGCG ATACCAA-3', then assembled sequencing results, obtained qnrB32 in the isolate KP4707 of *Klebsiella pneumonae* (GenBank accession number HQ704413) and KP4707- *E. coli* J53Az^R(conjugated) at full-length nucleotides.

To investigate the genetic characterization of the genes of CTX-M-22, aac(6')-Ib-cr and gyrA in *Klebsiella pneumo-*

Table 3. Characteristics of one *Klebsiella pneumonae* isolate and the *E. coli* J53 Az^R clone harboring the qnrB32 gene

Strains	Point mutations of amino acid[a]	Qnr gene	MIC [μg/ml][b]			
			NAL	OFL	LVN	CIP
Isolate KP4707 of *K. pneumonae*	Arn(N)27 → Leu(L),Val(V)129 → Ala(A), Iie(I)142 → Met(M),Gly(G)188 → Arg(R), Val(V)212 → Iie(I)	qnr32	> 256	32	16	8
E. coli J53 Az^R			2	0.0039	0.00195	0.00195
KP4707- *E. coli*						
J53Az^R(conjugated)		qnr32	32	8	4	2

[a]When compared with qnrB1; [b]NAL, nalidixic acid; OFX, ofloxacin; LVX, levofloxacin; CIP, ciprofloxacin

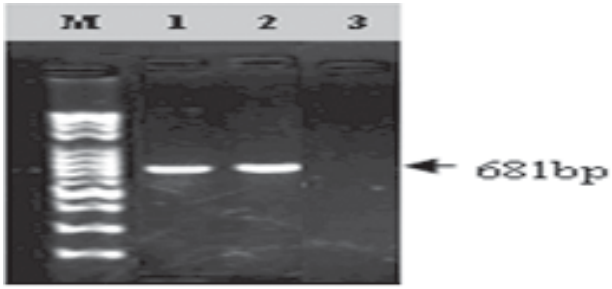

Figure 5. Agarose gel electrophoresis of qnrB32 gene. Lane 1: qnrB32 gene of the isolate KP4047 of *Klebsiella pneumoniae*; Lane 2: qnrB32 gene of the isolate KP4707- *E. coli* J53Az^R (conjugated); Lane 3: the isolate of *E. coli* J53Az^R (unconjugated)

nae, CTX-M-22 gene was amplified by PCR using the following primers: F 5'-TGGACTGCCTGCTTCCTGGGTT-3', R 5'-CAGATTCGGTTCGCTTTCACTTTT-3'; aac(6')-Ib-cr gene was amplified using the following primers: F 5'-ATGACTGAGCATGACCTTGC-3', R 5'-TTAGGCATCACTGCGTGTTC-3'; gyrA gene was amplified using: F: 5'-AACTGCTGCGTATTCTGG-3', R: 5'-TAACCGCCACTAAAGACA-3'. The bands in agarose gel electrophoresis are shown in Figure 1. Then sequencing results were assembled, and CTX-M-22, aac(6')-Ib-cr and gyrA in *Klebsiella pneumonae* at 876 bp, 649 bp and 404 bp length nucleotides were obtained.

Gene mapping. To study the plasmids carrying CTX-M-22, aac(6')-Ib-cr, gyrA and qnrB32 genes, the DNA was extracted from the plasmids (Axygen kit, USA) and separated by 0.6% agarose gel electrophoresis (60 V, 90 min) (Promega, USA). The gel was cut and the plasmid DNA fragments of different sizes were recycled. These plasmid DNA fragments served as templates for the amplification of CTX-M-22, aac(6')-Ib-cr, gyrA and qnrB genes by PCR, where the initial position of CTX-M-22, aac(6')-Ib-cr, gyrA qnrB gene plasmid was determined. The estimated size of plasmid DNA reference [8]. There were three different length plasmids in isolates of KP4707. The CTX-M-22, aac(6')-Ib-cr, gyrA and qnrB32 genes located in about 23.1 kb length plasmid.

Results

Susceptibility testing

The minimum inhibitory concentrations (MICs) were determined by broth dilution methods [7]. The MICs of the isolate KP4707 are shown in Table 1.

PCR-amplification and sequencing

Four different genes were found in isolate KP4047 of *Klebsiella pneumoniae*. These genes were CTX-M-22 gene, the sequence of which was identical to that of the gene in GenBank accession number AY080894/ /HM470254/GU125665/EU376966/AY575772/ /DQ309026, novel aac(6')-Ib-cr gene (GenBank accession number HQ680690), novel qnrB32 gene (GenBank accession number HQ704413), and novel gyrA gene (GenBank accession number HQ680691) (Figure 1).

Point mutations and phylogenetic analysis

Five point mutations of amino acid were observed in qnr32 gene: Arn(N)27 → Leu(L), Val(V)129 → Ala(A), Iie(I)142 → Met(M),Gly(G)188 → Arg(R), Val(V)212 → Iie(I), when compared with qnrB1 (Table 2) [4, 9–15, 16]. Of all qnrB alleles, novel variant of the qnrB32 gene, with qnrB31, had the highest amino acid homology (Figure 2) [4, 9–16]. Three point amino acid mutations including Trp(W)105 → Arg(R), Asp(D)182 → Tyr(Y) and Val(V)201 → Asp(D) were observed in aac(6')-Ib-cr gene, when compared with GenBank number AF479774(6) (Figure 3). The distance tree about *Klebsiella pneumoniae* gyrA gene HQ680691 is shown in Figure 4, when compared with other *Enterobacteriaceae*.

Discussion

Plasmid-mediated genes, such as qnr and aac(6')-Ib--cr may facilitate the spread and increase the prevalence of quinolone-resistant strains. To date, qnr genes have been widely detected in southern and eastern Asia, North and South America and Europe. Four different genes from the isolate of *Klebsiella pneumoniae* (KP4707) were found in our lab: qnrB32, aac(6')-Ib-cr, gyrA and CTX-M-22, all of which had a very high MIC of quinolone and a relatively low MIC of ceftazidime, showing them to be multi-resistant to quinolone and cephalosporin (Table 1).

QnrB, like qnrA and qnrS [13], provides low-level resistance to quinolones and belongs to the pentapeptide repeat family, one member of which has recently been shown to have a DNA-like structure allowing it to mimic DNA as a substrate for DNA gyrase [17, 18]. For a protein in which overall structure is important rather than catalytic activity, considerable amino acid variability may be permissible. Qnr, the gene product, is a member of the pentapeptide repeat family of proteins and has been shown to block the action of ciprofloxacin on purified DNA gyrase and topoisomerase IV [4, 16, 19]. The analysis of point mutations of amino acid of QRDR and phylogenetic analysis showed two point mutations were observed in the qnr32 gene: Val(V)129 → Ala(A) and Gly(G)188 → Arg(R) (Table 3), when compared with other qnrB alleles (Table 2). Of all qnrB alleles, novel variant of the qnrB32 gene, with qnrB31, had the highest amino acid homology (Figure 2); although there were several amino acid differences in qnrB32 gene and other qnr alleles, a certain amount of nucleotides sequence differences were found. The qnrB32 gene shared 97.1% nucleotides identity with qnrB31 (we reported a novel gene in another isolate of *K. pneumoniae*, and logged it in GenBank, the number is HQ418999), and shared 91.6% nucleotides identity with qnrB1. QnrB alleles variation sites were relatively fixed. Although the base composition of the variable sites were subject to change, and the expression of amino acid composition was largely identical although with minor differences, it has been suggested that there would exist a certain number of 'silent' nucleotide mutations in qnrB32; these are worthy of further study.

Aac(6')-Ib-cr, a novel PMQR protein, was first reported in 2003, but is now recognized to be widely disseminated. This variant of the aminoglycoside acetyltransferase gene aac(6')-Ib confers resistance to certain quinolones and to aminoglycosides [17]. Recently, two other PMQR mechanisms have been described. Aac(6')--Ib-cr, a variant aminoglycoside acetyltransferase capable of modifying ciprofloxacin and reducing its activity, is now recognized to be widely prevalent and circulated together with qnr genes [6]. The other PMQR determinant is the quinolone efflux pump gene, qepA, and is mediated by a probable transposable element flanked by two copies of IS26 [20]. Three point amino acid mutations including Trp(W)105 → Arg(R), Asp(D)182 → Tyr(Y) and Val(V)201 → Asp(D) were observed in aac(6')-Ib-cr gene, when compared with GenBank number AF479774(6) (Figure 3).

The concurrence of aac(6')-Ib-Cr and qnrB genes existed in KP4707, aac(6')-Ib-Cr gene had modification to aminoglycoside and also had modification to quinolone, expressed resistance to aminoglycoside such as gentamicin and amikacin, and quinolones such as nalidixic acid, ofloxacin, levofloxacin and ciprofloxacin. The Aac (6')-Ib-Cr gene did not only separate function to quinolone, but also expressed co-ordination function with multiple genes such as qnrB and gyrA in our studies. The existence of the aac(6')-Ib-Cr gene increased the MICs of quinolone resistance level.

The subunits of DNA gyrase are encoded by gyrA and gyrB, and the corresponding subunits of topoisomerase IV are encoded by parC and parE [20, 21]. In gram-negative bacteria, DNA gyrase is a prime target of quinolones rather than topoisomerase IV [20]. Mutations within the QRDR of gyrA have been observed as a major mechanism of quinolone resistance in *Escherichia coli* [21]. The gyrA gene was more complex in *Enterobacteriaceae*; there are 1,024 amino acids full length in gyrA, the quinolone resistance determining region (DRQR) is located in sites of 67–106 amino acids, the regions of our detection contained the range of amino acids. Through Blastn comparison to gyrA bases (novel GenBank accession number HQ680691), we found it shared more than 99% nucleotides identity with *Erwinia amylovora* AY209013; *Erwinia amylovora,* which can cause fire blight, leading to devastating diseases to pear, apple and other Rosaceae, the disease mainly mediated by plasmid PEA29, can be spread with seedlings, fruit and packaging materials, etc. The isolate KP4047 of the gyrA gene was likely to come from the plasmid of *Erwinia amylovora*. The results of the isolate KP4047 suggested that most human infections are relevant to the nature plant and animal pathogens.

CTX-M-22 is a member of the CTX-M family which can reduce the ESBL production, and modulate the antibiotic resistance resulting in low ceftazidime MICs. These structures may complicate the detection of these ceftazidime-hydrolyzing CTX-M--type enzymes [22]. The isolate KP4707 producing ESBLs had lower MIC of ceftazidime (8 μg/ml) than to other cephalosporin (Table 1). The nucleotides of the *CTX-M-22* gene were identical to those of the gene

in GenBank accession number AY080894/HM470254/ /GU125665/EU376966/AY575772/DQ309026. Because CTX-M gene usually co-existed with qnrB gene and the isolate KP4707 produced ESBL and expressed resistance to quinolone, it exacerbated multi-drug resistance to bacteria and posed significantly more difficulty in choosing antibiotics. To avoid multiple drug resistance to strains in clinic, there should be strict controls on the abuse of antibiotics.

We also identified three novel genes including qnrB32 (HQ704413), aac(6')-Ib-cr (HQ680690) and gyrA (HQ680691) which may confer the resistance to quinolone, and they existed in a plasmid of about 23.1 kb length in *Klebsiella pneumonae.* The isolate KP4707 was obtained from the pus of a patient suffering appendicitis with perforation in our hospital. Resistance to quinolone was demonstrated by the MICs, determined by broth dilution methods. Results showed the qnrB32, aac(6')-Ib-cr and gyrA genes in *Klebsiella pneumonae* were attributed to the quinolone resistance, and the characterization was similar to that of other qnrB, aac(6')-Ib-cr and gyrA alleles. The MICs were far higher than those of the wild strain demonstrated by conjugation experiment. Unfortunately, the transconjugation of aac(6')-Ib-cr gene, gyrA gene and CTX-M-22 gene was not achieved.

As new variants of qnr, aac(6')-Ib-cr, gyrA and CTX-M or other genotype determinants continuously appear in different genomic sites and also outside the *Enterobacteriaceae* family, further studies are required to determine the extent of their distribution and to assess their clinical implications as contributors to quinolone and cephalosporin resistance.

Acknowledgments

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