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Emergence of *Proteus mirabilis* Harboring bla_{KPC-2} and *qnrD* in a Chinese Hospital

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Nineteen carbapenem-nonsusceptible *Proteus mirabilis* isolates were recovered from intensive care units in the Second Affiliated Hospital of Zhejiang University during a 3-month period. The isolates showed a high level of resistance against ciprofloxacin, in addition to their resistance against the carbapenems. Pulsed-field gel electrophoresis (PFGE) analysis showed that these isolates belonged to three clonal strains. PCRs and DNA sequence analysis of the carbapenemase and other β-lactamase genes indicated that all the isolates harbored the bla_{KPC-2} gene. Twelve of 19 isolates harbored the plasmid-mediated quinolone resistance (PMQR) genes, both the *qnrD* and *aac(6′)-Ib-cr* genes. Eight representative isolates with high levels of quinolone resistance carried the similar mutation profiles of S83I in *gyrA*, E466D in *gyrB*, and S80I in *parC*. Reduced carbapenem susceptibility was transferred to *Escherichia coli* (EC600) in a conjugation experiment, while the quinolone resistance was not. DNA hybridization showed that *qnrD* was located on a plasmid of approximately 4.5 kb. In summary, large clonally related isolates of KPC-2-producing *P. mirabilis* emerged in a Chinese hospital, and *qnrD* was detected in KPC-producing *P. mirabilis* for the first time.

Proteus mirabilis is an opportunistic pathogen that can cause diarrhea, septicemia, meningitis, and urinary tract and respiratory system infections. *P. mirabilis* has now become one of the most important nosocomial infection pathogens in China. According to antimicrobial resistance surveillance of bacteria from China in 2009 (CHINET) (16), *P. mirabilis* was ranked fourth among the *Enterobacteriaceae*. However, infections caused by *P. mirabilis*, especially isolates recovered from sputum, might be neglected when they are recovered together with multiresistant Gram-negative bacilli such as *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*.

Since the first report of *Klebsiella pneumoniae* carbapenemase (KPC) in North Carolina in 2001, bla_{KPC} has spread worldwide (10). In our hospital, bla_{KPC} was found in most *Enterobacteriaceae*, including *K. pneumoniae* (2), *Escherichia coli* (2), *Serratia marcescens* (22), *Enterobacter cloacae* (1), and *Citrobacter freundii* (21). However, there are only a few reports regarding KPC-2 in *P. mirabilis* (13, 15). Quinolone resistance in the *Enterobacteriaceae* is usually mediated by point mutations of the gyrase and topoisomerase IV genes, leading to a target modification, and other resistance mechanisms include plasmid-mediated quinolone resistance (PMQR) determinants, which have also been found to contribute to quinolone resistance. The PMQR determinant was first described for a ciprofloxacin-resistant strain of *K. pneumoniae* in 1998 (7), and the latest PMQR determinant, named *qnrD*, was found in *Salmonella enterica* in 2009 (3) and has not been reported in other bacteria, until now.

From August to October 2010, a total of 19 *P. mirabilis* isolates were recovered from two intensive care units (ICUs) in our hospital. The isolates showed reduced carbapenem susceptibility and a much higher rate of resistance against the quinolones. To understand the mechanisms of carbapenem and quinolone resistance, carbapenem resistance genes and the quinolone resistance-associated factors, both on the chromosome and on plasmids, were investigated.

MATERIALS AND METHODS

Bacterial strains. Nineteen nonduplicate clinical isolates of *P. mirabilis* were collected from two ICUs in the Second Affiliated Hospital of Zhejiang University from August to October 2010 (16 were from the neurology ICU and the other 3 were from the central ICU). Nineteen isolates were recovered from various kinds of specimens, including sputum ($n = 15$), central vein pipe ($n = 1$), urine ($n = 1$), drainage fluid ($n = 1$), and feces (1). Six *P. mirabilis* isolates from outpatients were used as carbapenem-susceptible control strains in pulsed-field gel electrophoresis (PFGE) analysis and antimicrobial susceptibility testing.

Antimicrobial susceptibility testing. The MICs of ampicillin, cefoperazone-sulbactam, piperacillin-tazobactam, ceftazidime, cefotaxime, meropenem, and ertapenem were determined by the agar dilution method as recommended by the standards of the Clinical and Laboratory Standards Institute (CLSI; 2011) (4). The MICs of amikacin and ciprofloxacin were determined by Etest (AB Biodisk, Solna, Sweden), according to the manufacturer's instructions. The results were interpreted in accordance with CLSI 2011 guidelines (4). *E. coli* ATCC 25922 was used for quality control.

PFGE analysis. PFGE typing of *P. mirabilis* was performed as described previously (11). Chromosome DNAs were digested by ApaI for 2 h at 37°C, and electrophoresis was carried out in a Rotaphor System 6.0 instrument (Whatman Biometra, Goettingen, Germany) through a 1.0% agarose gel in 0.5× Tris-borate-EDTA buffer under the following conditions: temperature, 14°C; constant voltage, 200 V; and switch angle, 120°, with a linear switch ramp of 5 to 35 s for 19 h. The PFGE patterns were analyzed and interpreted according to the criteria of Tenover et al. (14).

PCR amplification. Screening for the following genes was carried out by PCR amplification using specific primers: PMQR genes, including *qepA* (6), *qnrA* (9), *qnrB* (9), *qnrC* (17), *qnrD* (3), *qnrS* (9), and *aac(6′)-Ib* (5); quinolone resistance-determining region (QRDR) genes *gyrA* (18), *gyrB* (18), and *parC* (18); carbapenemase gene bla_{KPC} (19); and other β-lactamase genes, including bla_{TEM} (20), bla_{SHV} (20), bla_{CTX-M} (20), and AmpC (8). PCR amplification was performed by a TPersonal cycler (Biometra, Germany). The PCR products were sequenced using an ABI 3730

Received 12 August 2011 Returned for modification 27 November 2011
Accepted 15 January 2012
Published ahead of print 21 February 2012
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doi:10.1128/AAC.05519-11

TABLE 1 Antimicrobial susceptibility patterns of 19 KPC-producing *P. mirabilis* isolates

Antimicrobial agent	MIC (μg/ml)						
	Clone A ($n = 14$)	Clone B		Clone C ($n = 1$)	50%	90%	Other 6 isolates
		B1 ($n = 2$)	B2 ($n = 2$)				
Meropenem	0.25–2	1–2	0.5–1	0.25	1	1	≤0.125
Ertapenem	0.125–0.5	0.25	0.125–0.25	0.125	0.125	0.25	≤0.125
Ceftazidime	0.5–128	1–8	1–2	0.5	1	16	≤0.25
Cefotaxime	0.25–16	2–8	1–2	1	1	8	≤0.25
Ciprofloxacin	0.012–>32	12–24	8–>32	12	>32	>32	0.012–0.19
Amikacin	0.75–>256	1	0.75–>256	2	128	>128	0.15–2
Ampicillin	8–>128	128	128	128	2	>256	1–16
Cefoperazone-sulbactam	2–16	16	8–16	8	8	16	≤0.25–1
Piperacillin-tazobactam	0.5–32	32	8	32	32	32	≤0.25–1

sequencer (Applied Biosystems, Foster City, CA), and the sequences were then compared with the reported sequences from GenBank. Primers *qnrD*-F (5′-CGAGATCAATTTACGGGGAATA-3′) and *qnrD*-R (5′-AACAAGCTGAAGCGCCTG-3′), amplifying a 565-bp internal fragment of the *qnrD* gene, were used to generate a probe for Southern blot analysis.

Genetic environment analysis of bla_{KPC} gene. PCR mapping of the bla_{KPC} gene in *P. mirabilis* was performed, and the map was compared with that found in plasmid pKP048 (12). PCR primers and amplification conditions for bla_{KPC}-surrounding sequences were as described previously (12). The PCR products were then sequenced.

Conjugal transfer experiment and analysis of plasmid. Twelve *qnrD*-positive *P. mirabilis* isolates were all used as donors for the *qnrD* conjugation experiment, while the *P. mirabilis* zr61, zr28, zr69, zr55, and zr62 isolates, representing clone A with TEM, clone A without TEM, subclone B1, subclone B2, and clone C isolates, respectively, were chosen for the bla_{KPC} conjugation experiment. Rifampin-resistant *E. coli* EC600 was used as the recipient strain. The *E. coli* transconjugants were selected on Mueller-Hinton agar medium containing 600 μg/ml rifampin plus 0.2 μg/ml ciprofloxacin for the *qnrD* conjugation experiments and 600 μg/ml rifampin plus 0.18 μg/ml meropenem for the bla_{KPC} conjugation experiments. The detailed experimental method was as described previously (17). Colonies that grew on the selective medium were picked for identification by a Vitek system. Plasmid DNA preparations were obtained by an alkaline lysis technique using an AxyPrep plasmid miniprep kit (Axygen Scientific). Plasmids were separated by electrophoresis in a 0.8% agarose gel containing ethidium bromide at a constant voltage of 100 V for 1 h.

Southern blot analysis. Plasmid DNAs from *P. mirabilis* isolates were separated by gel electrophoresis and then transferred to a nylon membrane (Hybond N^+; Amersham Pharmacia Biotech, Orsay, France). Southern hybridization was performed using standard protocols with the *qnrD*-specific probe labeled by use of a DIG High Prime DNA labeling kit (Roche, Sant Cugat del Vallès, Spain) following the manufacturer's instructions.

RESULTS

Antimicrobial susceptibility. Antimicrobial susceptibility results for the 19 *P. mirabilis* isolates are listed in Table 1. The 19 *P. mirabilis* isolates exhibited resistance or reduced susceptibility to meropenem and ertapenem. Cefoperazone-sulbactam showed the best activity against *P. mirabilis*, with a susceptibility rate of 100.0%, followed by ertapenem and meropenem (89.5%), ceftazidime (84.2%), and cefotaxime (73.4%). Remarkably, the highest rate of resistance was to ciprofloxacin (89.5%), other than that for resistance to ampicillin (94.7%). All six *P. mirabilis* isolates from outpatients were susceptible to all tested antibiotics.

PFGE typing and gene detection. Nineteen carbapenem-nonsusceptible *P. mirabilis* isolates belonged to three clones, named clone A (14 isolates), clone B (2 isolates of subclone B1 and 2 isolates of subclone B2), and clone C (1 isolate). All six isolates from outpatients were distinguishable (Fig. 1). PCRs and DNA sequence analysis indicated that all 19 isolates produced KPC-2 and only 3 isolates produced TEM-1. Genetic environment analysis showed that three open reading frames (ORFs) with the order of IS*Kpn8*, bla_{KPC-2}, and IS*Kpn6*-like were identified in a 3,241-bp nucleotide segment completely identical to that in previously described plasmid pKP048 (12).

The resistance profiles of the isolates showed high levels of quinolone resistance (Table 2). The DNA and derived amino acid sequences of the QRDR genes *gyrA*, *gyrB*, and *parC* for 8 clinical isolates with similar profiles of resistance to ciprofloxacin (MICs, 4 to >32 mg/liter), including 3 of clone A, 4 of clone B, and 1 of clone C, were compared with GenBank sequences assigned accession numbers AF397169 (*gyrA*), AF503506 (*gyrB*), and AF363611(*parC*). All the 8 isolates showed the same mutations that resulted in amino acid changes of S83I in *gyrA*, E466D in *gyrB*, and S80I in *parC*, which have been clarified to be associated with resistance to quinolones (18). Screening of PMQR determinants in 19 *P. mirabilis* isolates revealed that 12 isolates harbored both *qnrD* and *aac(6′)-Ib-cr*. All the PMQR determinant-positive isolates belonged to clone A, except one which belonged to subclone B2.

Conjugation and analysis of plasmid. *P. mirabilis* isolates failed to transfer quinolone resistance to *E. coli* EC600 by conjugation. Transfer of reduced carbapenem susceptibility from *P. mirabilis* to *E. coli* EC600 was successful for all isolates except *P. mirabilis* zr62, which belonged to clone C. The bacteria that grew on medium containing rifampin and meropenem were named *E. coli* transconjugants zr61, zr28, zr69, and zr55, respectively. The plasmid profiles indicated that bla_{KPC} was located on an approximately 45-kb plasmid in clone A and subclone B2 isolates, while in subclone B1, it was located on an approximately 54-kb plasmid. All *qnrD*-positive isolates of clone A and subclone B2 harbored two other plasmids with sizes of approximately 4.5 kb and 2.6 kb, respectively (Fig. 2a). A plasmid was not observed in *E. coli* transconjugant zr61, which may be due to the low plasmid copy number.

DNA hybridization. Southern blot analysis of *P. mirabilis* zr61 (clone A) and zr55 (subclone B2) hybridized with a *qnrD*-specific probe confirmed that *qnrD* was encoded on the 4.5-kb plasmid (Fig. 2b).

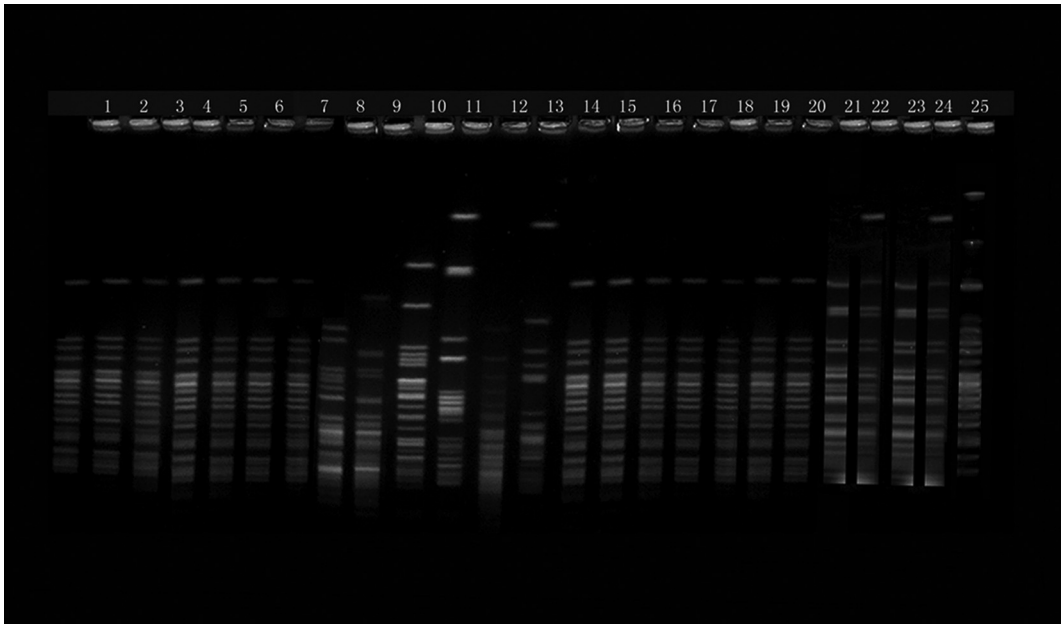

FIG 1 PFGE patterns of chromosomal DNA restriction fragments from 25 nonduplicate clinical *P. mirabilis* isolates. Lanes: 1 to 7 and 14 to 20, clinical isolates of *P. mirabilis* that belonged to clone A; 21 and 23, clinical isolates of *P. mirabilis* that belonged to subclone B1; 22 and 24, clinical isolates of *P. mirabilis* that belonged to subclone B2;, 25, clinical isolates of *P. mirabilis* that belonged to clone C; and 8 to 13, PFGE typing of six normal *P. mirabilis* isolates from intestinal tract.

DISCUSSION

KPCs have largely been detected in many genera from many countries. However, reports about KPC-producing *Proteus* spp. have been rare. Tibbetts et al. (15) first reported a single isolate of *P. mirabilis* harboring bla_{KPC-2} in 2008. In the current study, we first reported the clonal dissemination of KPC-2-producing *P. mirabilis* in ICUs. The 3,241-bp structure sequenced in this study was identified in *Enterobacteriaceae* isolates from our hospital, indicating probable transmission of bla_{KPC-2} from other common species of *Enterobacteriaceae* to *P. mirabilis*. It also indicated that interhospital clonal spread has happened because the sequence of the 3,241-bp nucleotide segment was completely identical to that of plasmid pKP048 (12), which was from a transconjugant of *K. pneumoniae* strain KP048 isolated from another hospital in the same city.

As is known, KPCs are capable of hydrolyzing β-lactams, including carbapenems, cephalosporins, penicillins, and aztreonam. Nevertheless, many KPC-producing *P. mirabilis* isolates in the current study were susceptible to carbapenems and even cephalosporins. The conjugation study indicated that plasmid-mediated KPC-2 contributed to carbapenem resistance or reduced carbapenem susceptibility in *P. mirabilis*. Interestingly, the MICs of carbapenems and cephalosporins for some *E. coli* transconjugants were higher than those for *P. mirabilis* (Table 2). For most of our previous studies, we found that the resistance level in the *E. coli* transconjugants is always lower than or similar to that in their parent isolates, as observed for *S. marcescens*, *K. pneumoniae*, *E. coli*, and *C. freundii* (2, 21, 22). However, in our recent study, we did observe higher MICs for the *E. coli* transconjugant than its parent isolate, the KPC-producing *Morganella morganii* (unpublished data). We suspect that the expression level or the stability of the KPC enzyme depends on the genetic background of the carrier of this gene rather than the KPC gene and the plasmid itself. However, further studies are required to elucidate the detailed mechanism of the phenomenon.

Antimicrobial susceptibility results showed a very high rate of resistance to ciprofloxacin (89.5%). The screening and identification of mutations in the *gyrA*, *gyrB*, and *parC* genes from the 8 typical isolates which had higher resistance levels (MICs, 4 to >32 mg/liter for ciprofloxacin) indicated that all the 8 isolates had the

TABLE 2 Antimicrobial susceptibility patterns of *P. mirabilis* isolates that belonged to various clones and their *E. coli* transconjugants

Antimicrobial agent	MIC (μg/ml)								
	P. mirabilis zr61	*E. coli* transconjugant zr61	*P. mirabilis* zr28	*E. coli* transconjugant zr28	*P. mirabilis* zr69	*E. coli* transconjugant zr69	*P. mirabilis* zr55	*E. coli* transconjugant zr55	*E. coli* EC600
Meropenem	0.5	2	0.25	2	1	8	1	1	0.0625
Ertapenem	0.25	4	0.25	8	0.5	16	0.5	4	0.125
Ceftazidime	128	16	0.5	32	8	128	2	32	0.25
Cefotaxime	32	16	0.5	32	8	128	1	8	0.5
Ciprofloxacin	>32	0.064	>32	0.19	24	0.125	>32	0.19	0.25

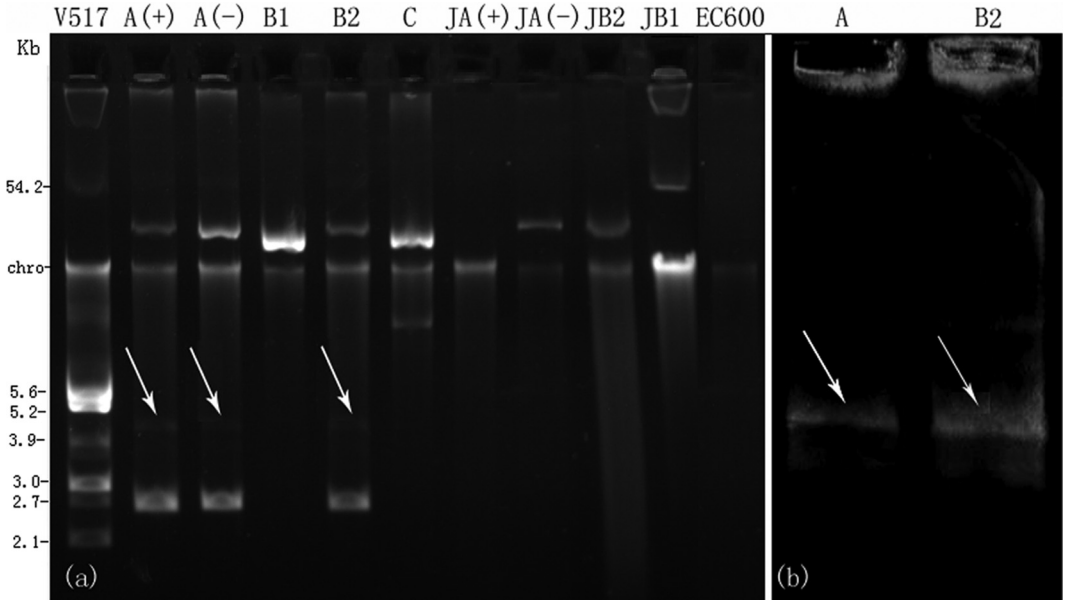

FIG 2 (a) Plasmid profiles of *P. mirabilis* and *E. coli* transconjugants. Lanes show *E. coli* V517 (lane V517); *P. mirabilis* clone A with bla_{TEM-1} [lane A(+)], *P. mirabilis* clone A without bla_{TEM-1} [lane A(−)], subclone B1 (lane B1), subclone B2 (lane B2), clone C (lane C), *E. coli* transconjugant of TEM-producing clone A isolate [lane JA(+)], *E. coli* transconjugant of TEM-negative clone A isolate [lane JA(−)], *E. coli* transconjugant of the subclone B2 isolate (lane JB2), *E. coli* transconjugant of the subclone B1 isolate (lane JB1), and *E. coli* EC600 (lane EC600; negative control). (b) Hybridization with *qnrD* (lane A, *P. mirabilis* isolate belonging to clone A; lane B2, isolate belonging to subclone B2). Arrows indicate location of the *qnrD* plasmid.

same mutation profiles of S83I in *gyrA*, E466D in *gyrB*, and S80I in *parC*, indicating that those mutations do contribute to the higher level of quinolone resistance. On the other hand, screening of PMQR determinants identified that the predominant genes among KPC-producing *P. mirabilis* isolates are *qnrD* (63.2%, 12/19), a novel PMQR determinant first found and only found in an *S. enterica* isolate from China, and *aac(6′)-Ib-cr* (63.2%, 12/19). This was the first detection of the *qnrD* gene in KPC-producing *P. mirabilis* isolates.

Conjugal studies proved that *P. mirabilis* isolates failed to transfer quinolone resistance to *E. coli* EC600 by conjugation, indicating that the quinolone resistance of *P. mirabilis* is mainly referred to the chromosomal QRDRs rather than plasmid-mediated factors. These data further supported the view that QRDRs are responsible for the higher level of quinolone resistance, while the plasmid-mediated factors play only a minor role in resistance or a lower level of resistance.

In summary, we have identified by clonal typing that the carbapenem-resistant *P. mirabilis* isolates from the intensive care units are very closely related and that they carried both the plasmid-mediated bla_{KPC} gene and the *qnrD* gene on separate plasmids and also found that the QRDRs on the chromosomes responsible for the higher level of quinolone resistance had similar mutation profiles. It is highly possible that KPC genes are transmitted even among different species to create multiple-drug-resistant pathogens. Care has to be taken to prevent the spread of such factors conferring high-level resistance.

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