Eur J Clin Microbiol Infect Dis (2012) 31:1585–1591
DOI 10.1007/s10096-011-1481-x

ARTICLE

bla$_{KPC}$ and *rmtB* on a single plasmid in *Enterobacter amnigenus* and *Klebsiella pneumoniae* isolates from the same patient

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Received: 24 April 2011 / Accepted: 28 October 2011 / Published online: 12 January 2012

Abstract *Enterobacter amnigenus* (EA76) and *Klebsiella pneumoniae* (KP76) isolates with multidrug-resistant (MDR) patterns were identified from the same patient in the neurosurgery department of our hospital. An outbreak of MDR *K. pneumoniae* had also occurred in this department. To characterize the resistance mechanism and molecular epidemiology of these isolates, sequential experiments including antimicrobial susceptibility testing, polymerase chain reaction (PCR), plasmid analysis, pulsed field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) were performed. EA76 and KP76 were resistant to all of the antibiotics tested, except colistin and tigecycline. *bla*$_{KPC-2}$, *bla*$_{TEM-1}$, *bla*$_{SHV-12}$, *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-14}$, and *rmtB* genes were identified in both isolates, with *bla*$_{KPC-2}$, *bla*$_{TEM-1}$, *bla*$_{CTX-M-14}$, and *rmtB* being co-carried on one plasmid in each isolate. Further analysis showed different restriction patterns between the two KPC-carrying plasmids. Of the 11 carbapenem-resistant isolates found in the outbreak, all were resistant to all of the β-lactams tested, with 63.64% (7/11) also exhibiting resistance to aminoglycosides and 72.73% (8/11) exhibiting resistance to quinolones. PCR analysis and molecular typing of the 11 *K. pneumoniae* strains revealed that the seven aminoglycoside-resistant isolates shared the same antibiotic-resistant gene pattern and identical or one-band-difference PFGE profiles relative to KP76. In addition, all of the eight aminoglycoside-resistant isolates, including KP76, belonged to the national epidemic clone ST11. The overall results indicate the emergence of *E. amnigenus* and outbreak of ST11 *K. pneumoniae*, with both co-harboring *bla*$_{KPC}$ and *rmtB* genes on a single plasmid in our neurosurgery wards.

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Introduction

Carbapenems are considered to be the first-line therapy in the treatment of serious infections caused by extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae [1]. Therefore, the emergence and prevalent dissemination of carbapenem-resistant Enterobacteriaceae presented a serious therapeutic challenge. *Klebsiella pneumoniae* carbapenemase (KPC), a member of the class A carbapenemase family, can hydrolyze all β-lactam molecules. Since it was first reported in a *K. pneumoniae* isolate in 2001 [2], KPC has disseminated widely among nosocomial pathogens, especially Enterobacteriaceae, and has become the most frequent class A carbapenemase worldwide [1]. Fortunately, most KPC-producing isolates are still susceptible to one or more aminoglycoside antibiotics [3, 4].

Since 2003, the production of 16S rRNA methylase has been reported to be a new mechanism of aminoglycoside

resistance. So far, seven types of methylases have been identified (ArmA, RmtA, RmtB, RmtC, RmtD, RmtE, and NpmA) [5–7]. Their presence could confer a pan-resistance pattern to clinically useful aminoglycosides. The co-existence of 16S rRNA methylases and various ESBLs in Enterobacteriaceae has been reported from time to time [5, 7]. Recently, the co-existence of 16S rRNA methylase, such as ArmA and RmtB, has also been reported in KPC-producing pathogens [8, 9]. This co-existence is quite alarming because additional high-level resistance to aminoglycosides in KPC-producing isolates and their potential spread would strongly limit the therapeutic options.

In previous publications, methylase genes and bla_{KPC} were reported to be mostly located on different plasmids [8, 9]. Only Jiang et al. have reported a *K. pneumoniae* plasmid co-carrying bla_{KPC-2} and *armA* to date [10]. Moreover, when their coexistence is observed, it is mainly confined to a single isolate, and there has been no epidemic reported to date.

In the present study, we describe a multiresistant *Enterobacter amnigenus* isolate and a *K. pneumoniae* isolate from the same patient, both co-carrying bla_{KPC-2} and *rmtB* genes on a single plasmid. Further studies revealed that the multidrug-resistant (MDR) *K. pneumoniae* co-harboring bla_{KPC-2} and *rmtB* had disseminated clonally in our neurosurgery department. To our knowledge, this is the first report of the detection of bla_{KPC} and *rmtB* in *E. amnigenus*, and also the first documenting of the emergence and outbreak of MDR *K. pneumoniae* co-carrying bla_{KPC} and *rmtB* on a single plasmid.

Materials and methods

Bacterial strains

Carbapenem-resistant *E. amnigenus* (EA76) and *K. pneumoniae* (KP76) and an additional 11 non-duplicate carbapenem-resistant *K. pneumoniae* isolates were collected from the neurosurgery department of The First Affiliated Hospital of the School of Medicine, Zhejiang University, China, from April 2010 to December 2010. EA76 and KP76 were isolated from the same patient simultaneously, with EA76 from blood samples and KP76 from fecal specimens. The other 11 carbapenem-resistant *K. pneumoniae* strains were obtained from the following specimens: lower respiratory tract (LRT) (n=7), upper respiratory tract (URT) (n=1), blood (n=1), cerebrospinal fluid (CSF) (n=1), and secretion (n=1). Azide-resistant *Escherichia coli* J53 and *E. coli* DH5α cells (TAKARA, China) were used as recipients in conjunction and transformation experiments, respectively.

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) of various antibiotics for KP76, EA76, and their *E. coli* transformants were determined with E-test strips according to the manufacturer's instructions (AB Biodisk, Solna, Sweden). The MICs for the other 11 carbapenem-resistant *K. pneumoniae* isolates were performed by the agar dilution method, as described in the guidelines from the Clinical and Laboratory Standards Institute (CLSI) [11]. The susceptibility results were interpreted according to the CLSI criteria [11]. For tigecycline, the US Food and Drug Administration (FDA) breakpoint values for Enterobacteriaceae were used (susceptible: ≤2 mg/L; resistant: ≥8 mg/L), and a concentration of 4 mg/L was used as the breakpoint of resistance for colistin [12]. *E. coli* ATCC 25922 was used as a quality control strain.

Conjugation, transformation, and plasmid analysis

Repeated attempts to transfer carbapenem resistance from EA76 and KP76 to sodium azide-resistant *E. coli* strain J53 by a mixed-broth mating procedure were performed. Mueller–Hinton agar (MHA) containing 0.5 mg/L meropenem and 200 mg/L sodium azide was used to select the transconjugants. Plasmids of EA76 and KP76 were extracted with the QIAGEN Plasmid Midi Kit (Qiagen, Germany) and transformed into competent *E. coli* DH5α (TAKARA, China). MHA plates containing 1 mg/L meropenem were used to select transformants. KP76-T and EA76-T, confirmed as bla_{KPC}-positive by polymerase chain reaction (PCR) analysis, were designated as *E. coli* transformants of KP76 and EA76, respectively. The plasmids in EA76-T and KP76-T were extracted with the QIAGEN Plasmid Midi Kit and digested by *Bam*HI, *Sma*I, and *Eco*RI (TAKARA, China). Plasmid DNAs and the digestion products were separated by gel electrophoresis in 0.8% agarose at 140 V for 2 h.

PCR screening and DNA sequencing

Total DNAs were prepared with the boiling method as described previously [13], and used as templates for PCR amplification. Amplification primers for bla_{KPC}, bla_{TEM}, bla_{SHV}, bla_{CTX}, bla_{IMP}, bla_{VIM}, *armA*, *rmtA*, *rmtB*, *qnrA*, *qnrB*, and *qnrS* were used as described previously [7, 14–16]. All PCR products were sequenced by the dideoxynucleotide chain-termination method by ABI 377 (ABI, USA) and the sequences were compared with the nucleotide sequences from GenBank (http://www.ncbi.nlm.nih.gov/blast/).

Pulsed field gel electrophoresis (PFGE)

Genomic DNAs prepared from KP76 and the other 11 *K. pneumoniae* strains were embedded in agarose gel plugs.

After digestion with restricted enzyme *Xba*I (TAKARA, China), DNA fragments were separated using a CHEF-Mapper XA PFGE system (Bio-Rad, USA) for 23 h at 6 V/cm and 14°C, with a pulse angle of 120° and pulse times from 5 s to 35 s. PFGE profiles were analyzed visually [17].

Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) with seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) was carried out for all *K. pneumoniae* isolates, including KP76, according to protocols provided on the MLST website for *K. pneumoniae* (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

Results

Antimicrobial susceptibility testing

EA76 and KP76 were resistant to almost all of the antimicrobial agents tested, including cephalosporins, imipenem, meropenem, amikacin, gentamicin, and ciprofloxacin, but they were susceptible to colistin and tigecycline (Table 1). Their *E. coli* transformants gained a β-lactam resistance pattern with elevated MICs of carbapenems. The MIC of ertapenem for EA76-T was as high as 16 mg/L. In addition, aminoglycoside resistance was also transferred to EA76-T and KP76-T.

The other 11 carbapenem-resistant *K. pneumoniae* isolates showed complete resistance to all of the β-lactams tested as well. Of them, eight were also resistant to ciprofloxacin and seven were highly resistant to amikacin. Moreover, all of the seven amikacin-resistant isolates were classified into the ciprofloxacin-resistant group. All of the 11 isolates were susceptible to colistin and tigecycline (Table 1).

Conjugation, transformation, and plasmid analysis

Repeated attempts to transfer carbapenem resistance from EA76 and KP76 to sodium azide-resistant *E. coli* strain J53 were unsuccessful. Two plasmids were extracted from EA76 and KP76, respectively. The larger plasmid extracted from each strain (both ~54 kb) was transformed into *E. coli* DH5α (Fig. 1a). Restriction analysis of plasmid DNAs showed different patterns between the two transformants, EA76-T and KP76-T (Fig. 1b).

PCR screening and DNA sequencing

EA76 and KP76 were positive for $bla_{\text{KPC-2}}$, $bla_{\text{TEM-1}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-14}}$, and *rmtB* genes, whereas EA76-T and KP76-T were positive for $bla_{\text{KPC-2}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-14}}$, and *rmtB* genes. This indicates that $bla_{\text{KPC-2}}$ co-existed with $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-14}}$, and *rmtB* on a single plasmid in both KP76 and EA76 strains. Of the other 11 *K. pneumoniae* isolates, all seven amikacin-resistant isolates shared an identical resistance gene profile as KP76, whereas two of the four amikacin-susceptible isolates harbored all of the genes found in KP76, with the exception of *rmtB*. The remaining two amikacin-susceptible isolates (KP45 and KP54) harbored $bla_{\text{VIM-1}}$ carbapenemase gene rather than bla_{KPC}. KP45 also carried $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-10}}$, while KP54 carried $bla_{\text{SHV-12}}$ and $bla_{\text{CTX-M-56}}$. No *qnr* genes were detected in any of the screened isolates.

PFGE

PFGE profile analysis revealed that 9 of the 11 *K. pneumoniae* isolates had identical or one-band-difference patterns relative to KP76. The remaining two isolates (KP45 and KP54), which were positive for $bla_{\text{VIM-1}}$, had distinct PFGE patterns (Fig. 2).

MLST

MLST showed three sequence types (STs) based on the analysis of the seven housekeeping genes in the 12 *K. pneumoniae* isolates. Of the three sequence types, ST11 was the dominant type and was detected in ten isolates, including KP76. The remaining two isolates, KP45 and KP54, were found to belong to ST494 and ST23, respectively. These MLST results were in accordance with our PFGE findings. The nine ST11 isolates belonged to one PFGE type, and the ST494 and ST23 isolates belonged to two distinct PFGE types.

Discussion

To our knowledge, this is the first report of an *E. amnigenus* isolate that is resistant to both carbapenems and aminoglycosides. *E. amnigenus* is a Gram-negative aerobic bacillus of the family Enterobacteriaceae. They are ubiquitous in nature and have been occasionally isolated from clinical specimens, such as sputum, wound, blood, and feces [18]. In 2006, an epidemic of post-cataract surgery endophthalmitis was reported to be caused by this pathogen in India [19]. *E. amnigenus* has been reported to be fully susceptible to almost all antimicrobial agents that are generally active against Enterobacteriaceae, including aminoglycosides, most β-lactams, fluoroquinolones, chloramphenicol, and nitrofurantoin [20]. Here, we reported an *E. amnigenus* isolate co-carrying $bla_{\text{KPC-2}}$, $bla_{\text{TEM-1}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-14}}$, and *rmtB* genes, with

Table 1 Clinical and in vitro characteristics of the clinical isolates and transformants of KP76 and EA76

Isolate[a]	Sex/age[b]	Origin[c]	PFGE	MLST	KPC/*rmtB*	MIC (mg/L)[d]														
						IP	MP	ETP	PP	PTC	CT	TZ	PM	AT	GM	AK	CL	CO	TGC	CTL	TZL
KP76	M/41	Stool	1	11	+/+	32	32	32	≥256	≥256	≥32	≥32	16	≥256	≥256	≥256	≥32	0.38	1	≥1	≥4
KP76-T			UD[e]	UD	+/+	1.5	0.75	2	≥256	128	≥32	≥32	3	≥256	≥256	≥256	0.012	0.13	0.75	≥1	≥4
EA76	M/41	Blood	UD	UD	+/+	16	16	32	≥256	≥256	≥32	≥32	16	≥256	≥256	≥256	≥32	1.5	1	≥1	≥4
EA76-T			UD	UD	+/+	1.5	1	32	≥256	≥256	≥32	≥32	8	≥256	≥256	≥256	0.012	0.13	0.75	≥1	≥4
E. coli DH5α			UD	UD	UD	0.125	0.016	0.125	2	2	0.125	0.25	0.02	0.02	0.38	0.75	0.012	0.02	0.19	0.13	0.25
KP31	M/25	CSF	1	11	+/+	128	64	128	≥512	≥512	≥512	256	512	≥512	≥512	≥512	≥32	1	2	UD	UD
KP34	F/47	URT	1	11	+/+	128	64	128	≥512	≥512	≥512	512	256	≥512	≥512	≥512	≥32	1	2	UD	UD
KP35	M/82	LRT	1	11	+/+	128	64	128	≥512	≥512	≥512	1024	256	≥512	≥512	≥512	≥32	0.5	2	UD	UD
KP45	F/67	LRT	2	494	−/−	4	2	4	512	512	512	128	64	≥512	8	2	16	1	2	UD	UD
KP48	M/65	LRT	1	11	+/+	128	64	128	≥512	≥512	≥512	256	256	≥512	512	≥512	≥32	0.5	0.5	UD	UD
KP54	M/72	Blood	3	23	−/−	64	64	128	≥512	512	128	256	128	≥512	4	2	1	1	1	UD	UD
KP86	M/61	LRT	1	11	+/−	256	128	256	≥512	≥512	128	128	128	≥512	0.5	8	1	1	2	UD	UD
KP98	F/46	LRT	1	11	+/−	256	64	256	≥512	512	256	512	128	≥512	2	1	0.5	1	2	UD	UD
KP104	M/46	LRT	1	11	+/+	128	64	128	≥512	≥512	≥512	128	128	≥512	≥512	≥512	≥32	2	2	UD	UD
KP111	M/62	Secretion	1	11	+/+	128	64	128	≥512	≥512	≥512	256	256	≥512	≥512	≥512	≥32	1	2	UD	UD
KP215	M/43	LRT	1	11	+/+	256	64	128	≥512	≥512	128	128	64	≥512	512	≥512	≥32	1	2	UD	UD

[a] KP76-T, *E. coli* transformant of KP76; KP76-T, *E. coli* transformant of EA76

[b] M, male; F, female; age given in years

[c] CSF, cerebrospinal fluid; URT, upper respiratory tract; LRT, lower respiratory tract

[d] IP, imipenem; MP, meropenem; ETP, ertapenem; PP, piperacillin; PTC, piperacillin–tazobactam; CT, cefotaxime; TZ, ceftazidime; PM, cefepime; AT, aztreonam; GM, gentamicin; AK, amikacin; CL, ciprofloxacin; CO, colistin; TGC, tigecycline; CTL, cefotaxime–clavulanic acid; TZL, ceftazidime-clavulanic acid

[e] UD, undetermined

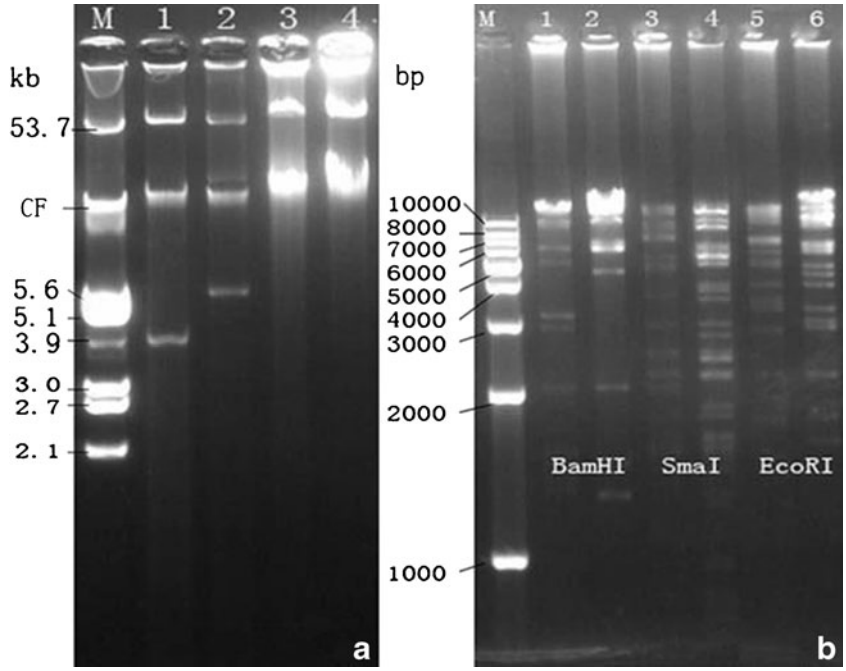

Fig. 1 **a** Plasmid DNAs profiles of EA76, KP76, EA76-T, and KP76-T. *M*, *E. coli* V517 (used as a standard for plasmid size); *lane 1*, EA76; *lane 2*, KP76; *lane 3*, EA76-T; and *lane 4*, KP76-T. **b** Restriction patterns of *Klebsiella pneumoniae* carbapenemase (KPC)-producing plasmid DNAs from EA76-T and KP76-T. *M*, 1-kb DNA ladder; *lanes 1, 3, and 5*, EA76-T; *lanes 2, 4, and 6*, KP76-T. Plasmid DNAs were digested with *Bam*HI, *Sma*I, and *Eco*RI

bla_{KPC-2}, bla_{TEM-1}, $bla_{CTX-M-14}$, and *rmtB* on the same plasmid. This isolate was resistant to almost all of the antibiotics tested, except tigecycline and colistin. Therefore, antibiotic regimens for infections involving this pathogen would become rather limited if this multiresistant isolate were to become disseminated. In addition, due to their presence in the environment, the acquisition and accumulation of resistance determinants could make *E. amnigenus* a potential natural reservoir for these determinants.

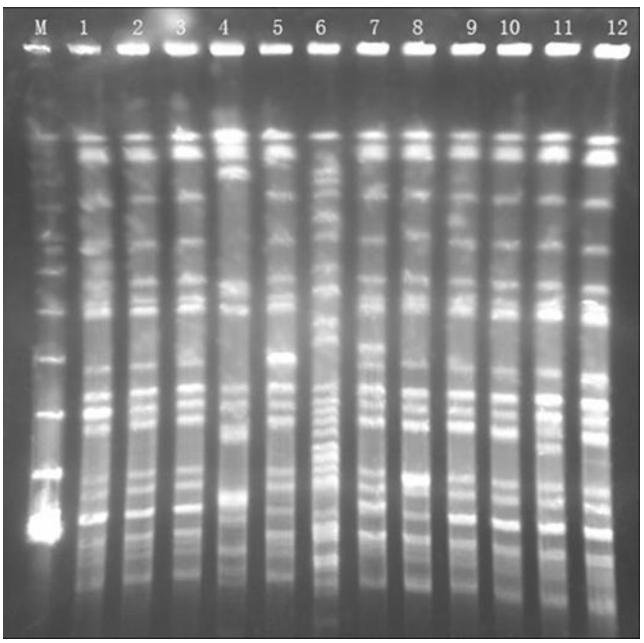

Fig. 2 Pulsed field gel electrophoresis (PFGE) of *Xba*I-digested DNAs of *K. pneumoniae* isolates from our neurosurgery wards. *M*, λ DNA ladder; *lane 1*, KP31; *lane 2*, KP34; *lane 3*, KP35; *lane 4*, KP45; *lane 5*, KP48; *lane 6*, KP54; *lane 7*, KP86; *lane 8*, KP98; *lane 9*, KP104; *lane 10*, KP111; *lane 11*, KP215; and *lane 12*, KP76

The EA76 and KP76 isolates that were co-carrying bla_{KPC} and *rmtB* were from the same patient. Their KPC-producing plasmids were of the same size and shared an identical resistance determinant profile. Nevertheless, restriction analysis of the two KPC-producing plasmids showed different patterns. Further screening of carbapenem-susceptible *E. amnigenus* isolates from fecal specimens of the same patient were unsuccessful. These results together argue against our initial expectation that EA76 obtained its bla_{KPC} gene directly from KP76 via the in vivo transmission of a co-harboring plasmid as described previously [21]. It is possible that EA76 acquired the bla_{KPC} gene via the mobility of transposon. Since bla_{KPC} genes were reported to be located within a roughly 10-kb Tn-3-type transposon, Tn*4401*, which can facilitate their mobility between different clones of the same species or even between clones of different genera [1]. Or, perhaps most likely, EA76 could have acquired the KPC-producing plasmid elsewhere, given that KPC-producing *K. pneumoniae* were known to be disseminated widely in our hospital before the isolation of EA76 (data unpublished).

We found that 7 (63.64%) of the 11 other carbapenem-resistant *K. pneumoniae* isolates from our neurosurgery wards were also highly resistant to aminoglycosides. PCR analysis revealed that these seven isolates had identical

antimicrobial gene profiles as KP76. PFGE revealed that six of these seven isolates shared a common identical PFGE profile with KP76, whereas the remaining isolate showed a one-band-difference profile (Fig. 2). MLST analysis showed that all seven of these isolates together with KP76 belonged to ST11, which is also the dominant clone of KPC-producing *K. pneumoniae* in China [22]. These observations strongly suggest that the ST11 *K. pneumoniae* isolates containing bla_{KPC-2} and *rmtB* on a single plasmid had disseminated clonally in our neurosurgery wards. Two of the remaining four *K. pneumoniae* isolates fell into the same PFGE type as KP76, and belonged to the ST11 clone as well. They carried the same resistance genes as KP76, except *rmtB*. This could partly indicate that *rmtB* could have disseminated via the mobilization of transposon or other transposable elements between *K. pneumoniae* isolates as well, since *rmtB* has been reported to be located adjacent to mobile genetic elements [5, 13]. The other two remaining *K. pneumoniae* isolates carried totally different resistance genes and belonged to distinct PFGE and MLST types.

In our study, 9/12 (75%) carbapenem-resistant *K. pneumoniae* isolates showed ciprofloxacin resistance, including all eight KPC–RmtB co-producing isolates. However, no *qnr* genes were identified in any of the *K. pneumoniae* isolates. Quinolone resistance in these strains could have been mediated by mutations of the gyrase and topoisomerase IV genes or by efflux pump mechanisms.

In conclusion, here, we have reported an *E. amnigenus* strain and a *K. pneumoniae* strain that each co-carried bla_{KPC-2} and *rmtB* on a single plasmid; nevertheless, the two plasmids were different. Further studies revealed that the MDR ST11 *K. pneumoniae* co-carrying bla_{KPC-2} and *rmtB* had disseminated widely in our neurosurgery wards. The co-carriage of various antibiotic resistance determinants on one plasmid can provide survival advantages and evolutionary benefits to bacteria in an antibiotic-rich environment, and facilitate their wide spread. In addition, it can also facilitate the co-transmission of various competent resistance determinants among different pathogens. These observations, together with the fact that few therapeutic options are available for these infections, compel us to take urgent actions to contain this outbreak and the persistent spread of KPC–RmtB co-producing *K. pneumoniae* in the hospital setting.

Acknowledgments This work was supported by the National Basic Research Program of China (no. 2007CB513004). We thank the platform Genotyping of Pathogens and Public Health (Institut Pasteur) for coding the MLST alleles and profiles.

Disclosure statement No competing financial interests exist.

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