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The bla_{CTX-M} gene independently enhances drug resistance level to ampicillin in clinical isolates of *Klebsiella pneumoniae*

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Most *Klebsiella pneumoniae* carry a chromosomally encoded SHV-1 β-lactamase gene. Consequently, they exhibit a low-level of ampicillin resistance with a minimum inhibitory concentration (MIC) of 8–64 μg ml^{-1}.[1] Our previous studies have shown that low or no expression of the bla_{SHV} gene in *K. pneumoniae* isolates resulted in reduced ampicillin resistance levels.[2] Rice *et al.*[3] and Corvec *et al.*[4] have reported that the C→A mutation in –10 region of the promoter of the bla_{SHV} gene or deletion of a downstream region of the promoter promotes high expression of the bla_{SHV} gene, resulting in a significant elevation in the MIC of *K. pneumoniae* to ampicillin or co-amoxiclav, cefalothin and cefoxitin. These data indicate that the resistance level of *K. pneumoniae* to antibiotics is associated with the expression of the bla_{SHV} gene. However, because of variation in the upstream promoter region of the bla_{SHV} gene that lead to enhanced drug resistance levels are rare, they cannot fully explain the mechanism of enhancement of *K. pneumoniae* resistance level to ampicillin.

Drug resistance in *K. pneumoniae* is primarily determined by the presence of the extended-spectrum β-lactamases (ESBLs). The genes encoding the various β-lactamases include the bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes.[5,6] In this study, we analyzed the distribution of the β-lactamase genes in clinical isolates of *K. pneumoniae* from the Harbin area of China and determined the effects of these genes on the levels of antibiotic resistance. The aim of this study was to clarify the mechanism of how drug resistance level is enhanced in *K. pneumoniae*. Our results should provide new targets for controlling drug resistance.

Between June and October 2007, isolates of *K. pneumoniae* were collected from patients at two hospitals in Harbin, the capital city of the Heilongjiang Province. The strains were identified using routine biochemical methods and the API20E system. Fifty-six isolates were then randomly selected for this study. The MICs of each agent were determined by the micro-dilution method according to the protocols recommended by CLSI.[7] In brief, 100 μl of appropriate bacterial suspensions ($\sim 10^6$ CFU ml^{-1}) were inoculated to 100 μl of antibiotic-containing micro-plates and incubated for 18–20 h at 37 °C. MIC determinations were performed four times for each strain to ensure the reproducibility of the MICs by using quality control strain *Escherichia coli* ATCC25922. Strains with MIC values $\geq MIC_{90}$ were considered to have high levels of antibiotic resistance.

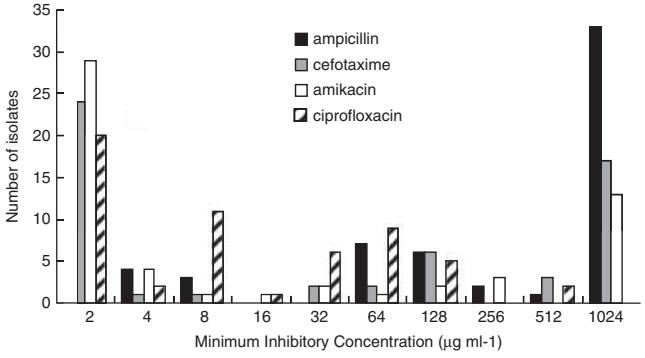

Figure 1 Distribution of MICs of ampicillin, cefotaxime, amikacin and ciprofloxacin ($n = 56$). MICs were determined by the micro-dilution method. The break point of ampicillin, cefotaxime, amikacin and ciprofloxacin were 32, 64, 64 and 4 μg ml^{-1} (standard based on M100-S19, CLSI, Wayne, PA, USA, 2009), respectively. The resistance rates to ampicillin, cefotaxime, amikacin and ciprofloxacin were 49 (87.50%), 28 (50.00%), 19 (33.93%) and 36 (64.29%), respectively.

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Ampicillin, cefotaxime, amikacin and ciprofloxacin were used for testing. The MIC value of ampicillin was generally high (Figure 1). The high-level antibiotic resistance rates to ampicillin (MIC_{90} $>1024\,\mu g\,ml^{-1}$), cefotaxime (MIC_{90} $>1024\,\mu g\,ml^{-1}$), amikacin (MIC_{90} $>1024\,\mu g\,ml^{-1}$) and ciprofloxacin (MIC_{90} $>128\,\mu g\,ml^{-1}$) were 58.92%, 30.35%, 23.21% and 12.5%, respectively.

The PCR primers for the bla_{SHV},[3] bla_{TEM},[8] bla_{CTX-M}[9,10] and bla_{OXA} genes[11] were designed according to the sequences in GenBank. The PCR products were sequenced directly after the purification with a QIAquick PCR purification kit (Qiagen, Beijing, China). The SHV amino-acid sequences were aligned with those of known bla_{SHV} gene (GenBank accession no. AF124984) and homology analysis of the sequencing results in GenBank was conducted using BLAST. The SHV genotypes were determined according to the standards found at www.lahey.org/studies/webt.stm.

In the 56 *K. pneumoniae* isolates, the ESBL genes of local *K. pneumoniae* isolates consisted mainly of the bla_{SHV} gene (69.64%, 39/56) followed by bla_{CTX-M} (30.36%, 17/56) and bla_{TEM} (37.50%, 21/56) genes, and the bla_{OXA} and other genes were not detected (data not shown). Table 1 demonstrates the interrelationship among β-lactamase genes and the MIC for representative antibiotics. The strains that carried bla_{CTX-M} gene exhibited higher MIC_{50} values for ampicillin ($\geq 1024\,\mu g\,ml^{-1}$), cefotaxime ($\geq 320\,\mu g\,ml^{-1}$) and amikacin ($\geq 128\,\mu g\,ml^{-1}$) than those without the bla_{CTX-M} gene. The results suggested that different types of β-lactamase gene might affect the antibiotic resistance levels. Table 2 shows that the high-level drug resistance rate of isolates carrying the bla_{CTX-M} gene (ampicillin: 90.48%; cefotaxime: 57.14%) were significantly higher than that for strains without the bla_{CTX-M} gene (ampicillin: 40.40%; cefotaxime: 14.29%; $P<0.01$). However, the resistance level of isolates to amikacin and ciprofloxacin with and without bla_{CTX-M} gene was not significantly different ($P>0.05$). The results obtained through multivariate analysis also indicated that the bla_{CTX-M} gene was independently associated with the level of ampicillin (OR: 19.522; 95% CI: 3.307–115.259, $P<0.01$) and cefotaxime (OR: 10.193; 95% CI: 2.341–44.382, $P<0.01$; data not shown). Additionally, the bla_{SHV} and bla_{TEM} genes did not have significant effects on the high-level drug resistance to ampicillin, cefotaxime, amikacin and ciprofloxacin. These results demonstrated

Table 1 Relationship between β-lactamase genes and antibiotics susceptibility of clinical isolates

		Antibiotics susceptibility of isolates ($\mu g\,ml^{-1}$)[a]							
		Ampicillin		*Cefotaxime*		*Amikacin*		*Ciprofloxacin*	
β-Lactamase gene	*No. of isolates*	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
TEM	6	6	1024	2	1024	2	8	20	512
SHV	22	384	1024	2	870.4	2	1024	8	54.4
CTX-M	5	1024	1024	1024	1024	4	1024	4	128
SHV/TEM	5	128	1024	2	1024	2	1024	2	128
CTX-M/TEM	4	1024	1024	1024	1024	128	1024	96	512
SHV/CTX-M	10	>1024	>1024	320	1024	514	1024	3	128
SHV/CTX-M/TEM[b]	2	ND	ND	ND	ND	ND	ND	ND	ND
Non-*SHV/CTX-M/TEM*[c]	2	ND	ND	ND	ND	ND	ND	ND	ND

[a]MIC_{50}: MIC for 50% of organisms; MIC_{90}: MIC for 90% of organisms; ND: not determined.
[b]For one of the strains, the MIC values for ampicillin, cefotaxime, amikacin and ciprofloxacin are >1024, >1024, 2 and $32\,\mu g\,ml^{-1}$, whereas the MIC values of other are >1024, >1024, >1024 and $64\,\mu g\,ml^{-1}$, respectively.
[c]For one of the strains, the MIC values for ampicillin, cefotaxime, amikacin and ciprofloxacin are 64, 64, 4 and $16\,\mu g\,ml^{-1}$, whereas the MIC values of other are 128, 2, 2 and $2\,\mu g\,ml^{-1}$, respectively.

Table 2 Comparison of the effects of β-lactamase genes on the antibiotics-resistance level of *K. pneumoniae*

		No. of antibiotic-resistant isolates with high or low MIC ($\mu g\,ml^{-1}$) values (%)[b]							
		Ampicillin		*Cefotaxime*		*Amikacin*		*Ciprofloxacin*	
β-Lactamase gene[a]		*High MIC (MIC≥1024)*	*Low MIC (MIC≤512)*	*High MIC (MIC≥1024)*	*Low MIC (MIC≤512)*	*High MIC (MIC≥1024)*	*Low MIC (MIC≤512)*	*High MIC (MIC≥128)*	*Low MIC (MIC≤64)*
SHV	+	23/39 (58.97)	16/39 (41.03)	9/39 (23.08)	30/39 (76.92)	11/39 (28.21)	28/39 (71.79)	3/39 (7.69)	36/39 (92.31)
	−	10/17 (58.82)	7/17 (41.18)	8/17 (47.06)	9/17 (52.94)	2/17 (11.76)	15/17 (88.24)	4/17 (23.53)	13/17 (76.47)
TEM	+	10/17 (58.82)	7/17 (41.18)	8/17 (47.06)	9/17 (52.94)	4/17 (23.53)	13/17 (76.47)	4/17 (23.53)	13/17 (76.47)
	−	23/39 (58.97)	16/39 (41.03)	9/39 (23.08)	30/39 (76.92)	9/39 (23.08)	30/39 (76.92)	3/39 (7.69)	36/39 (92.31)
CTX-M	+	**19/21 (90.48)****	2/21 (9.52)	**12/21 (57.14)****	9/21 (42.86)	8/21 (38.10)	13/21 (61.90)	5/21 (23.81)	16/21 (76.19)
	−	14/35 (40.00)	21/35 (60.00)	5/35 (14.29)	30/35 (85.71)	5/35 (14.29)	30/35 (85.71)	2/35 (5.71)	33/35 (94.29)

Abbreviation: MIC, minimum inhibitory concentration.
Significant indicated by bold. **$P<0.01$ (χ^2 test).
[a]+, Positive for the β-lactamase gene; −, negative for the β-lactamase gene.
[b]High MIC: high-level antibiotic resistance, dependent on MIC_{90} values (MIC for antibiotics for 90%); low MIC: low-level antibiotic resistance, between break point and MIC_{90} values; MIC_{50} and MIC_{90} were determined using SPSS 13.0 software (Stats Data Mining Co., Ltd., Beijing, China).

that the bla_{CTX-M} gene independently enhances the drug resistance levels of *K. pneumoniae* clinical isolates to ampicillin and cefotaxime.

Currently, the most common penicillin antibiotics, such as ampicillin, are rarely used to treat *K. pneumoniae* infections and the strains generally exhibit low-level resistance to ampicillin.[1] However, there were significant differences in the ampicillin MIC values in the *K. pneumoniae* isolates we collected. High-level ampicillin-resistant strains (MIC $\geq 1024\,\mu g\,ml^{-1}$) accounted for 58.93% of the isolates, suggesting that many *K. pneumoniae* strains have a high level (high value of MICs) of ampicillin resistance. Retrospective studies on the penicillin resistance of *Streptococcus pneumoniae* have also shown that although the frequency of penicillin use has decreased in European countries, the level of penicillin resistance has not decreased.[12,13] Thus, the strategies for controlling antibiotic use do not affect the antibiotic resistance levels of bacteria. Instead, the persistence of drug-resistant genes explains the continued resistance. This study took a new perspective and investigated whether the ESBL-related genes affected the antibiotic resistance levels of bacteria. The results showed that the bla_{CTX-M} gene was closely associated with the high-level drug resistance to ampicillin and cefotaxime (Table 2).

Previous studies have shown that the enhanced antibiotic resistance of *K. pneumoniae* is associated with mutations in the upstream region of the bacterial bla_{SHV} gene promoter. For example, the C→A mutation in the upstream −10 region of the bla_{SHV} gene promoter results in high levels of SHV expression, thus enhancing bacterial resistance to drugs, such as ampicillin, ceftazidime and piperacilline/tazobactam.[3] However, our study did not find any mutation in the upstream promoter region of the *K. pneumoniae* bla_{SHV} gene or other plasmid-encoded SHV mutants; the bla_{SHV} genes (SHV-1, SHV-11 and SHV-33) were all chromosomally encoded.[14,15] Thus, our results suggest that mutations in the upstream promoter region of the *K. pneumoniae* bla_{SHV} gene that appear to enhance antibiotic resistance may be an accidental phenomenon.

In summary, this study indicates that the bla_{CTX-M} gene promoted the high-level ampicillin resistance of *K. pneumoniae* (MIC $\geq 1024\,\mu g\,ml^{-1}$). Our results also indicate a novel and decisive role for the bla_{CTX-M} gene in bacterial drug-resistance levels, a finding that would further elucidate the modes of action of bacterial drug-resistant genes. Owing to the increase in selective pressures for antibiotic resistance and the movement and spread of drug-resistant genes, the epidemiology of drug resistance is constantly changing. Our data demonstrate that there is a relationship between the bacterial bla_{CTX-M} gene and the resistance of *K. pneumoniae* isolates to ampicillin. Further studies are required to determine if this relationship is unique to *K. pneumoniae* or is characteristic of other Enterobacteriaceae.

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1 Haeggman, S., Lofdahl, S., Paauw, A., Verhoef, J. & Brisse, S. Diversity and evolution of the class A chromosomal beta-lactamase gene in *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **48,** 2400–2408 (2004).
2 Fu, Y. *et al.* Differential expression of bla(SHV) related to susceptibility to ampicillin in Klebsiella pneumoniae. *Int. J. Antimicrob. Agents* **29,** 344–347 (2007).
3 Rice, L. B. *et al.* High-level expression of chromosomally encoded SHV-1 beta-lactamase and an outer membrane protein change confer resistance to ceftazidime and piperacillin-tazobactam in a clinical isolate of *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **44,** 362–367 (2000).
4 Corvec, S. *et al.* Increased resistance to beta-lactams in a *Klebsiella pneumoniae* strain: role of a deletion downstream of the Pribnow box in the blaSHV-1 promoter. *Int. J. Antimicrob. Agents* **28,** 308–312 (2006).
5 Livermore, D. M. Beta-lactamases in laboratory and clinical resistance. *Clin. Microbiol. Rev.* **8,** 557–584 (1995).
6 Bradford, P. A. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* **14,** 933–951, table of contents (2001).
7 CLSI, Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing: 19th Informational Supplement M100-S19* (Wayne, PA, USA, 2009).
8 Arlet, G. & Philippon, A. Construction by polymerase chain reaction and use of intragenic DNA probes for three main types of transferable beta-lactamases (TEM, SHV, CARB) [corrected]. *FEMS Microbiol. Lett.* **66,** 19–25 (1991).
9 Gniadkowski, M. *et al.* Cefotaxime-resistant Enterobacteriaceae isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-hydrolyzing beta-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. *Antimicrob. Agents Chemother.* **42,** 827–832 (1998).
10 Sabate, M. *et al.* Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing beta-lactamase (CTX-M-9) from *Escherichia coli* in Spain. *Antimicrob. Agents Chemother.* **44,** 1970–1973 (2000).
11 Steward, C. D. *et al.* Characterization of clinical isolates of *Klebsiella pneumoniae* from 19 laboratories using the National Committee for Clinical Laboratory Standards extended-spectrum beta-lactamase detection methods. *J. Clin. Microbiol.* **39,** 2864–2872 (2001).
12 Barkai, G. *et al.* Community prescribing and resistant *Streptococcus pneumoniae*. *Emerg. Infect. Dis.* **11,** 829–837 (2005).
13 Hennessy, T. W. *et al.* Changes in antibiotic-prescribing practices and carriage of penicillin-resistant *Streptococcus pneumoniae*: a controlled intervention trial in rural Alaska. *Clin. Infect. Dis.* **34,** 1543–1550 (2002).
14 Chaves, J. *et al.* SHV-1 beta-lactamase is mainly a chromosomally encoded species-specific enzyme in *Klebsiella pneumoniae*. Antimicrob. *Agents Chemother.* **45,** 2856–2861 (2001).
15 Lee, Y. H., Cho, B., Bae, I. K., Chang, C. L. & Jeong, S. H. *Klebsiella pneumoniae* strains carrying the chromosomal SHV-11 beta-lactamase gene produce the plasmid-mediated SHV-12 extended-spectrum beta-lactamase more frequently than those carrying the chromosomal SHV-1 beta-lactamase gene. *J. Antimicrob. Chemother.* **57,** 1259–1261 (2006).