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Detection and genotype analysis of AmpC β-lactamase in *Klebsiella pneumoniae* from tertiary hospitals

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Abstract. The aim of the present study was to investigate the phenotype and genotype of plasmid-mediated AmpC (pAmpC) β-lactamase in *Klebsiella pneumoniae* and its antibiotic resistance. A total of 130 non-repetitive clinical isolates of *Klebsiella pneumoniae,* obtained from tertiary hospitals, were phenotypically screened for pAmpC β-lactamase production with the cefoxitin disk diffusion test. β-lactamase genes in the screened isolates were detected using multiplex polymerase chain reaction (PCR); carbapenemase genes in pAmpC β-lactamase-producing isolates that were resistant to imipenem were detected using PCR. Out of the 130 isolates of *Klebsiella pneumoniae*, 62 strains (47.7%) were resistant to cefoxitin, including 14 strains (10.8%) positive for pAmpC β-lactamase (DHA type), among which 12 strains (85.7%) were susceptible to imipenem, and 2 strains, which were carrying *Klebsiella pneumoniae* carbapenemase (KPC)-2 gene, were resistant to imipenem. The pAmpC β-lactamase-producing *Klebsiella pneumoniae* isolates from the tertiary hospitals were mainly of DHA-1 genotype, and the majority were susceptible to carbapenems; drug-resistant strains were associated with KPC-2 expression.

Introduction

AmpC β-lactamase, which is a type of cephalosporinase (1), is known to be responsible for antimicrobial resistance in gram-negative bacilli (2). The knowledge of potential risk factors for that resistance may help to limit its impact by enabling the implementation of effective control measures and judicious antimicrobial therapy (3). AmpC β-lactamases are either plasmid- or chromosomal-mediated (4). With regard to plasmid-mediated AmpC (pAmpC), no single phenotypic method is satisfactory for its detection; the combined application of phenotypic tests is necessary for the screening and confirmation of the presence of pAmpC-mediated resistance (5). Chromosomal-mediated AmpC hyperproducing *Escherichia coli* (*E. coli*) continues to be mostly susceptible to third-generation cephalosporins (6). Polymerase chain reaction (PCR) remains the gold standard for the detection of AmpC β-lactamases (7). As β-lactam antibiotics are widely used, the production of AmpC β-lactamases by bacteria results in considerable antibiotic resistance (8). Resistance to broad-spectrum β-lactams mediated by extended spectrum broad-spectrum β-lactamases (ESBLs) and AmpC β-lactamase enzymes is an increasing problem worldwide (9). In Portugal, 104/124 (83.9%) of *Enterobacteriaceae* isolates that were resistant to third generation cephalosporins were also resistant to fourth generation cephalosporins (10). In comparison with ESBL-producing gram-negative bacilli, AmpC β-lactamase-producing strains show more extensive antibiotic resistance; 29% of *Acinetobacter* strains, for example, were found to produce both ESBL and AmpC enzymes (11). To be more specific, the majority of *Acinetobacter baumannii* isolates were producers of carbapenemase and metallo-β-lactamase (12). In a study of isolates collected between 2002 and 2008, DHA-1 was the most prevalent acquired AmpC (94%), which was first identified in 2003 and was detected throughout the studied period in different institutions (13). *Klebsiella pneumoniae* is an important cause of nosocomial infection, and ESBLs and carbapenemases are a cause of emergency in multidrug-resistant (MDR) *Klebsiella pneumoniae* treatment (14). In a study of *Enterobacter cloacae*, a major nosocomial bacterium causing severe infections, the majority of the strains produced CTX-M type ESBLs, while a limited number produced SHV-type ESBLs, leading to the conclusion that the emergence of resistance genes is a public health problem (15). In a study conducted in a children's hospital, the proportion of *Enterobacteriaceae* isolates with broad-spectrum β-lactam resistance increased over a 3-year period, mainly due to the emergence of a plasmid-mediated β-lactamase gene, bla_{CMY-2} (16). Therefore, the detection of pAmpC β-lactamases is important for the improvement of the clinical use of antibiotics. In the present study, the genotype of pAmpC β-lactamase from clinical samples of *Klebsiella pneumoniae* obtained from tertiary hospitals in Xuzhou was investigated, along with their antibiotic resistance.

Materials and methods

Strain source. A total of 130 non-repetitive clinical isolates of *Klebsiella pneumoniae* were obtained from patients in

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Key words: Klebsiella pneumoniae, plasmid, AmpC β-lactamase, carbapenemase

Table I. Primer sequences for the polymerase chain reaction amplification of AmpC β-lactamase target genes.

Target gene	Primer sequence (5'→3')	Fragment length (bp)
bla_{MOX}	F: GCTGCTCAAGGAGCACAGGAT R: CACATTGACATAGGTGTGGTGC	520
bla_{CIT}	F: TGGCCAGAACTGACAGGCAAA R: TTTCTCCTGAACGTGGCTGGC	462
bla_{DHA}	F: AACTTTCACAGGTGTGCTGGGT R: CCGTACGCATACTGGCTTTGC	405
bla_{ACC}	F: AACAGCCTCAGCAGCCGGTTA R: TTCGCCGCAATCATCCCTAGC	346
bla_{EBC}	F: TCGGTAAAGCCGATGTTGCGG R: CTTCCACTGCGGCTGCCAGTT	302
bla_{FOX}	F: AACATGGGGTATCAGGGAGATG R: CAAAGCGCGTAACCGGATTGG	190
bla_{DHA} (full-length primer)	F: GGAATTCCACGGAAGGTTAATTCTGATG R: GCAAGCTTTTATTCCAGTGCACTCA	1140

F, forward; R, reverse.

the Xuzhou First People's Hospital, the Affiliated Hospital of Xuzhou Medical College and Xuzhou Central Hospital (Xuzhou, China), between August and October 2012. The present study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Xuzhou Medical College. Written informed consent was obtained from all participants.

Standard strains. *E. coli* strain ATCC 25922 (American Type Culture Collection, Manassas, VA, USA) and *Enterobacter cloacae* 029 M (National Center for Medical Culture Collections, Beijing, China) served as negative and positive controls, respectively, for the production of AmpC β-lactamase. *E. coli* strain ATCC 25922 and *Klebsiella pneumoniae* strain ATCC 13883 (National Center for Medical Culture Collections) served as negative and positive controls, respectively, for the production of carbapenemases.

Identification of bacteria. The clinical isolates were inoculated on blood agar medium (Oxoid, London, UK) and incubated at 35˚C for 18-24 h. A Vitek 32 automatic microbial analyzer (BioMerieux, Lyon, France) was then used for the identification and susceptibility testing of bacteria.

Screening for AmpC β-lactamase-producing strains. Strains were screened using a Kirby-Bauer disk diffusion test (17), in which cefoxitin (30 μg; Oxoid) was used. According to the CLSI Antimicrobial Susceptibility Testing (AST) Standards (www.clsi.org/standards/micro/sub-ast/), isolates with an inhibitory zone diameter measuring ≤18 mm were suspected of being AmpC β-lactamase producers.

Multiplex PCR. AmpC β-lactamase genes were extracted using a Bacterial DNA Isolation kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China). The screened clinical isolates were employed as templates for multiplex PCR (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., Shanghai, China) (18). Bi-directional DNA sequencing of the PCR products was performed by Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China), using Basic Local Alignment Search Tool software (www.blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the genotype. The sequencing results were identified using the GenBank database (www.ncbi.nlm.nih.gov/genbank/). The primer sequences are shown in Table I.

Detection of carbapenemase genes. In accordance with a previously reported method (19), PCR (CFX96 Touch Real-Time PCR system; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to detect carbapenemase genes in pAmpC β-lactamase-producing isolates that were resistant to imipenem. The bacterial plasmid DNA extraction kit was provided by Tiangen Biochemical Technology Co., Ltd., as above. The primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China), the sequences of which are reported in Table II. SYBR Green I (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used as fluorophore and ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) was used to visualize the DNA ladder (Sangon Biotech Co., Ltd., Shanghai, China). The results were captured and evaluated using a Gel Doc 2000 agarose gel imaging system (Bio-Rad Laboratories, Inc.).

Results

Preliminary screening. Out of the 130 *Klebsiella pneumoniae* isolates that were tested, 62 strains intermediate or resistant to cefoxitin were preliminarily screened as AmpC β-lactamase-producers, corresponding to a positive rate of 47.7% (62/130).

Table II. Primer sequences for the polymerase chain reaction amplification of carbapenemase target genes.

Primer	Primer sequence (5'→3')	Fragment length (bp)
bla_{KPC}	F: GCTACACCTAGCTCCACCTTC R: ACAGTGGTTGGTAATCCATGC	920
bla_{IMP}	F: CTACCGCAGCAGAGTCTTTG R: AACCAGTTTTGCCTTACCAT	587
bla_{VIM}	F: AGTGGTGAGTATCCGACAG R: ATGAAAGTGCGTGGAGAC	261
bla_{SME}	F: AACGGCTTCATTTTTGTTTAG R: GCTTCCGCAATAGTTTTATCA	830

F, forward; R, reverse.

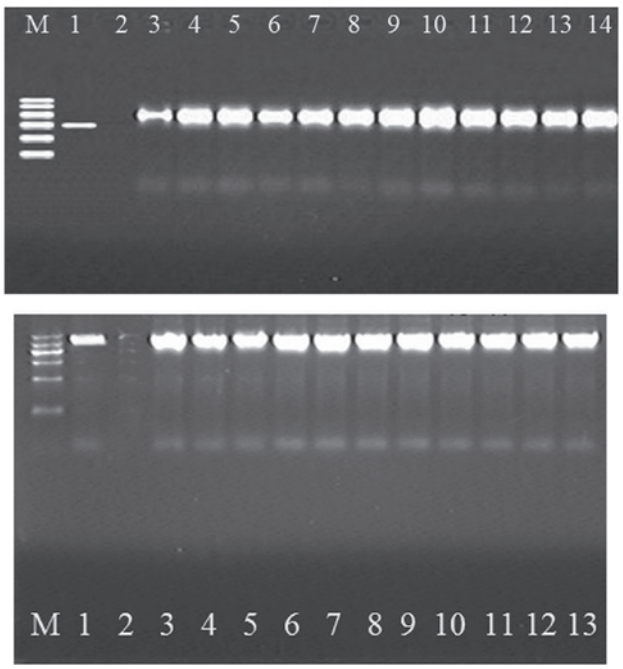

Figure 1. (A) Electrophoretogram of multiplex polymerase chain reaction (PCR) products of plasmid-mediated AmpC β-lactamase. M, DNA Marker (from top to bottom: 600, 500, 400, 300, 200 and 100 bp); 1, positive control; 2, negative control; 3-14, clinical isolates of *Klebsiella pneumoniae* carrying the bla_{DHA} gene. (B) Electrophoretogram of multiplex PCR products amplified with full-length primer. M, DNA marker (from top to bottom: 1,200, 900, 700, 500, 300 and 100 bp); 1, positive control; 2, negative control; 3-13, clinical isolates of PCR products amplified with full-length primer.

Multiplex PCR. Multiplex PCR showed the amplification of a 405-bp fragment from 14 strains, which were thus identified as DHA type. The positive rate was 10.8% (14/130; Fig. 1A).

PCR amplification. The 14 bacterial strains that were identified by multiplex PCR screening were amplified with a full-length primer for the DHA type of AmpC β-lactamase, encoding a fragment of 1,140 bp (Fig. 1B).

Sequencing results. The PCR products obtained were purified, sequenced and identified to be of the DHA-1 type according to the GenBank database.

Susceptibility test. pAmpC β-lactamases (DHA-1)-producing *Klebsiella pneumoniae* were resistant to cefoxitin, ceftriaxone, ceftazidime, cefepime, ciprofloxacin, aztreonam, piperacillin-tazobactam, amikacin, levofloxacin and sulfamethoxazole, but susceptible to imipenem (Table III).

PCR amplification of carbapenemase. PCR revealed the amplification of an ~920-bp fragment from two strains, which were thus identified as *Klebsiella pneumoniae* carbapenemase (KPC; Fig. 2). The PCR products obtained were purified, sequenced and verified to be of the KPC-2 type according to the GenBank database.

Discussion

Antibiotic resistance is a global concern (20), and the resistance to broad-spectrum β-lactams mediated by ESBLs and AmpC β-lactamase enzymes is an increasing problem worldwide (21). *Klebsiella pneumoniae* (22) is a common pathogen, from which pAmpC β-lactamase was first identified in South Korea in 1989. Since then, one or two new pAmpC β-lactamases have been reported annually (23), which are highly expressed (24) and can be transferred to other bacteria through conjugation and transformation, either by plasmid or chromosome, to cause antibiotic resistance (25). pAmpC β-lactamase-producing *Klebsiella pneumoniae* can cause serious infections (26), which makes anti-infective therapy more challenging. In Karnataka, a state in India, ESBL production was found to be 46.2% among clinical isolates of *Klebsiella pneumoniae* (27). In Islamabad, Pakistan, 54.55% of clinical isolates of *Klebsiella pneumoniae*, which were resistant to cefoxitin, were found to be pAmpC β-lactamase producers. Due to this high prevalence (28), further research into the distribution of pAmpC β-lactamases in *Klebsiella pneumoniae* and their drug susceptibility is required to improve the effectiveness of clinical antibiotic use.

In the present study, the cefoxitin disk diffusion test was used to preliminarily screen 130 *Klebsiella pneumoniae* isolates, out of which 62 strains were suspected of being AmpC β-lactamase-producers. Multiplex PCR was performed to detect the genotype of the screened strains and a 405-bp fragment was amplified from 14 strains, which were identified as being of DHA type according

Table III. Susceptibility of 14 AmpC β-lactamase-producing *Klebsiella pneumoniae* strains.

	Susceptible		Intermediate		Resistant	
Antibiotic	Strains	Rate (%)	Strains	Rate (%)	Strains	Rate (%)
FOX	0	0	4	28.6	10	71.4
CRO	3	21.4	0	0	11	78.6
CAZ	2	14.3	1	7.1	11	78.6
FEP	3	21.4	0	0	11	78.6
ATM	3	21.4	0	0	11	78.6
TZP	7	50	2	14.3	5	35.7
AMK	4	28.6	0	0	10	71.4
CIP	1	7.1	1	7.1	12	85.7
LVX	3	21.4	0	0	11	78.6
IMP	12	85.7	0	0	2	14.3
SMZ	2	14.3	0	0	12	85.7

FOX, cefoxitin; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; TZP, piperacillin-tazobactam; AMK, amikacin; CIP, ciprofloxacin; LVX, levofloxacin; IMP, imipenem; SMZ, sulfamethoxazole.

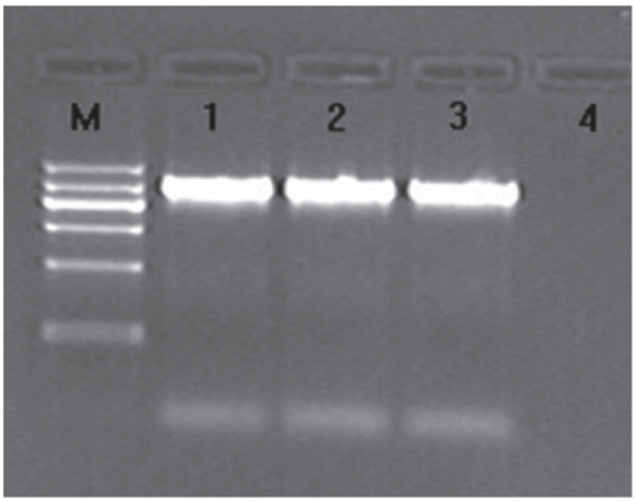

Figure 2. Electrophoretogram of PCR products of *Klebsiella pneumoniae*. carbapenemase. M, DNA Marker (from top to bottom: 1,200, 900, 700, 500, 300 and 100 bp); 1 and 2, two clinical isolates of bacteria; 3, positive control; 4, negative control.

to the fragment size. These strains were amplified with full-length primer encoding a fragment of 1,140 bp, and the PCR products obtained were purified, sequenced and identified as being the DHA-1 type. The positive rate of pAmpC β-lactamase-producing *Klebsiella pneumoniae* in the samples from Xuzhou tertiary hospitals was 10.8%, which was consistent with the report by Ding *et al* (29), but higher than the rate reported by Alvarez *et al* (30). Although CMY is the most common type of AmpC β-lactamase in the world, the DHA-1 and ACT types predominate in China (31). In the present study, the DHA-1 type was verified through genotype analysis of AmpC β-lactamase in *Klebsiella pneumoniae*, which was in accordance with a previous study (32); therefore, DHA-1 AmpC β-lactamase is speculated to be the main genotype in China.

The susceptibility test showed that AmpC-producing *Klebsiella pneumoniae* was resistant to multiple drugs, including third and fourth generation cephalosporins, such as ceftriaxone, ceftazidime and cefepime. The fourth generation cephalosporins can quickly pass through the outer membrane and are generally used to treat infections due to AmpC-producing bacteria (33). These bacteria, however, were found in the present study to be highly resistant to cefepime (78.6%), and therefore the use of fourth generation cephalosporins is not recommended. AmpC-producing *Klebsiella pneumoniae* was also found to be highly resistant to quinolone and aminoglycoside antibiotics, including ciprofloxacin, levofloxacin and amikacin, but susceptible to carbapenem antibiotics since carbapenems are stable against β-lactamases and have high affinity for penicillin-binding proteins (34). Carbapenem antibiotics are therefore recommended for the treatment of infections caused by AmpC-producing strains of *Klebsiella pneumoniae*.

In the present study, there were two isolates of AmpC β-lactamase-producing *Klebsiella pneumoniae* that were found to be resistant to imipenem, which might indicate the production of KPC (34) or other antibiotic resistance mechanisms; therefore, PCR was applied to amplify carbapenemase genes, and confirmed that the strains were KPC-producing bacteria. KPC was first discovered in *Klebsiella pneumoniae* and named KPC-1 by Yigit *et al* (35), and is highly resistant to imipenem and meropenem. KPC has been reported worldwide, as well as in Zhejiang, Anhui, Tianjin and Shanghai in China (36). The present study has also identified KPC in Xuzhou, confirming that KPC is not limited by geographical boundaries. *Klebsiella pneumoniae* carrying two drug-resistance genes makes anti-infective therapy more difficult; therefore, it is recommended that tertiary hospitals in Xuzhou should strengthen the monitoring of AmpC β-lactamase in *Klebsiella pneumoniae*, and consider using carbapenems instead of fourth generation cephalosporins for the treatment of infections caused by AmpC-producing bacteria.

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