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Analysis of the drug-resistant characteristics of *Klebsiella pneumoniae* isolated from the respiratory tract and *CTX-M ESBL* genes

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ABSTRACT. The main aim of this study was to understand the relationship between the drug-resistant characteristics of *Klebsiella pneumoniae* and CTX-M-type extended spectrum β-lactamases (ESBLs), and to detect the distributions of CTX-M-type ESBLs in clinically isolated strains. *CTX-M ESBL* genes isolated from the clinical samples were amplified by polymerase chain reaction and identified by sequence analysis; the antibiotic susceptibility of the samples was determined using the Kirby-Bauer disc-diffusion method. One hundred and five strains among the 246 isolated strains of *K. pneumoniae* tested positive for ESBL production (42.68%); 92 of these produced CTX-M ESBLs. Of the 92 CTX-M ESBL strains, 81 produced CTX-M-1 ESBLs and 11 produced CTX-M-25 ESBLs. Fifty-seven of the CTX-M-1 ESBL- and six of the CTX-M-25 ESBL-producing bacteria had *CTX-M ESBL* genes that coexisted in the plasmid and chromosome. The Kirby-Bauer antibiotic susceptibility method revealed that CTX-M ESBL-positive strains showed a higher rate of resistance to cefazolin, cefoxitin, cefuroxime, ceftazidime, cefotaxime, aztreonam, levofloxacin, and cotrimoxazole, compared to the CTX-M ESBL-negative strains ($P < 0.05$). The *CTX-M ESBL* genes were commonly observed in the *K. pneumoniae* isolated from respiratory tract samples; these were significantly associated with the drug-resistant characteristics of *K. pneumoniae* to β-lactam antibiotics.

Key words: *Klebsiella pneumonia*; CTX-M ESBLs; Drug resistance

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen correlated with both community-acquired and nosocomial infections, such as pneumonia, urinary tract infections, septicemia, and wound infections; the increasing frequency of multidrug-resistant *K. pneumoniae* has led to it being classified as a major public health concern (Cao et al., 2014). Previous studies have reported TEM-type extended spectrum β-lactamases (ESBLs) and SHV-type ESBLs to have originated from CTX-M-type ESBLs. There are five subgroups in TEM-type and SHV-type ESBLs: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25; these are mainly responsible for imparting drug resistance to β-lactam antibiotics (Liu et al., 2014; Lahlaoui et al., 2014). However, CTX-M ESBLs show different carrying rates, types, and locations, in different places. In this study, we attempted to elucidate the relationship between the drug-resistant characteristics of *K. pneumoniae* and CTX-M-type ESBLs, and detected the distributions of CTX-M-type ESBLs in clinically isolated strains.

MATERIAL AND METHODS

Experimental strains

A total of 246 *K. pneumoniae* strains were isolated from sputum samples collected at the Sun Yat-sen Memorial Hospital, affiliated to the Sun Yat-sen University, between October 2013 and October 2014. The *Escherichia coli* ATCC25922 quality control strain and different types of CTX-M-type ESBLs were also provided by the Sun Yat-sen Memorial Hospital.

Preparation of chromosome and plasmid DNA

K. pneumoniae-produced ESBLs were detected using a commercial ESBL Assay Kit (Hangzhou Binhe Microorganism Reagent Co., Ltd., Hangzhou, China), according to the manufacturer protocols. The variable temperature-sodium dodecyl sulfate elimination method was used to isolate the plasmids from ESBLs; bacterial DNA was extracted using a DNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China), and the plasmid DNA extracted using a plasmid extraction kit (Tiangen Biotech Co., Ltd.). The concentration and purity of DNA was determined using a nucleic acid detector of BeckMan Coulter DU730 (Beckman Coulter, Inc., Brea, CA, USA); the DNA was stored at -20°C until further use.

Detection of the *CTX-M* gene

Primers specific for the CTX-M-type ESBLs, including CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25, were designed using the Assay Design 3.1 software (Sequenom Inc., San Diego, CA, USA), according to the manufacturer protocol. The CTX-M-type ESBLs were amplified by polymerase chain reaction (PCR), using the following designed primers: CTX-M-1, 5'-TAGGAAGTGTGCCGCTGTAT-3' and 5'-GAATCAGCGGCGCACGATCT-3'; CTX-M-2, 5'-AAAGTTCGGGAGGTCGGCTTG-3' and 5'-ACTACCCATGATTTCGGCAGA-3'; CTX-M-9, 5'-GGTTGGGAGTGGCGCTGATT-3' and 5'-ATCGAGCCGGAAGGTGTTAT-3'; CTX-M-8, 5'-ATACCCGAGGCGCGACAGA-3' and 5'-CCAGCGTCATTGTGCCGTTGA-3'; and CTX-M-25, 5'-TTGTTGAGTCAGCGG GTTGA-3' and 5'-GCGCGACCTTCCGGCCAAAT-3'. Each PCR mix was composed of 50 ng genomic DNA, 200 μM dNTP, 2.5 U Taq DNA polymerase (Promega, Madison, WI, USA), and 200 μM primers, in a total volume of 20 μL. The PCR conditions were set as follows: preliminary denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 25 s and annealing at 53°C for 40 s, with a final extension at 72°C for 6 min.

Culturing of strains

The PCR products were purified using the PCR product recovery kit (Hangzhou Binhe Microorganism Reagent Co., Ltd.). Competent cells (DH5α *E. coli*) were prepared using the super-competent bacterial preparation kit (Beyotime Institute of Biotechnology). PMDTM18-T (Hangzhou Binhe Microorganism Reagent Co., Ltd.) was used as the carrier, according to the manufacturer instructions. Chromas were taken to determine the resistance genotypes, by comparing against the genotypes submitted to the GenBank database.

Antimicrobial susceptibility test

The samples were subjected to the Kirby-Bauer antibiotic susceptibility test to determine the sensitivity of the *K. pneumoniae* to the commonly used clinical antibiotics. The results were analyzed based on the criteria set by the Clinical and Laboratory Standards Institute (CLSI).

Statistical analysis

The distribution of categorical variables was described by the frequency, and the median and interquartile range was used to describe the continuous variables. The association between CTX-M-type ESBLs and the drug-resistant characteristics of *K. pneumoniae* was analyzed by the χ^2 test. All P values were two-sided, and a P value <0.05 was considered to be statistically significant.

RESULTS

Detection of CTX-M ESBLs

One hundred and five of the 246 strains of *K. pneumonia* tested positive for ESBL production (42.68%); 92 of these were determined to be CTX-M ESBLs (87.62%). Of these latter 92 strains, 81 and 11 strains were determined to be positive for CTX-M-1 and CTX-M-25 ESBLs, respectively (Figures 1 and 2). Fifty-seven and six strains of CTX-M-1 and CTX-M-25 ESBLs coexisted in the plasmid and chromosome.

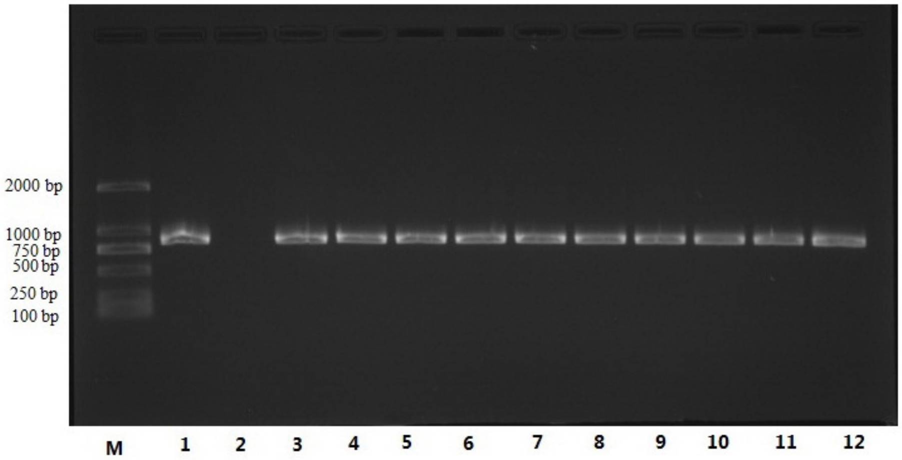

Figure 1. Detection of genes encoding CTX-M-1 extended spectrum beta-lactamases (ESBLs). *Lane M* = marker (100-2000 bp); *lane 1* = positive control; *lane 2* = negative control; *lanes 3-12* = experimental strains.

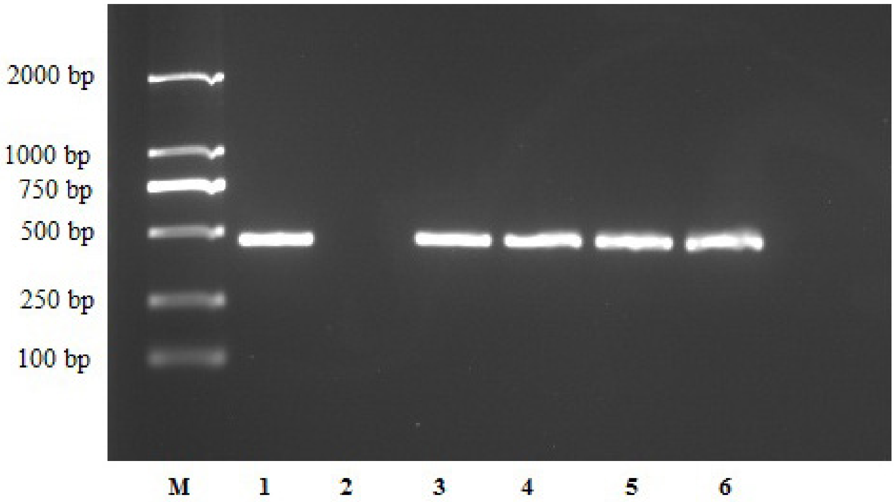

Figure 2. Detection of genes encoding CTX-M-25 extended spectrum beta-lactamases (ESBLs). *Lane M* = marker (100-2000 bp); *lane 1* = positive control; *lane 2* = negative control; *lanes 3-6* = experimental strains.

Antimicrobial susceptibility test

By Kirby-Bauer method, positive strains of CTX-M ESBLs showed higher rate of drug resistant to cefazolin, cefoxitin, cefuroxime, ceftazidime, cefotaxime, aztreonam, levofloxacin, and cotrimoxazole when compared with negative strains of CTX-M ESBLs ($P < 0.05$; Table 1). However, there were no significant difference in the drug-resistant rate between two groups in terms of cefepime, piperacillin/tazobactam, cefoperazone/sulbactam, imipenem, and amikacin ($P > 0.05$).

Table 1. Resistance of different types of CTX-M extended spectrum beta-lactamase (ESBL)-encoding *Klebsiella pneumonia* to various antimicrobials.

Antibiotics	CTX-M ESBLs				χ^2	P value
	Positive strains (N = 105)	%	Negative strains (N = 141)	%		
Ampicillin	105	100.00	141	100.00	-	-
Cefazolin	83	79.05	74	52.48	18.39	<0.05
Cefoxitin	76	72.38	82	58.16	5.30	<0.05
Cefuroxime	79	75.24	60	42.55	26.16	<0.05
Ceftazidime	55	52.38	44	31.21	11.22	<0.05
Cefotaxime	83	79.05	71	50.35	21.16	<0.05
Cefepime	51	48.57	72	51.06	0.15	>0.05
Aztreonam	61	58.10	59	41.84	6.36	<0.05
Piperacillin/Tazobactam	28	26.67	30	21.28	0.97	>0.05
Cefoperazone/Sulbactam	15	14.29	15	10.64	0.75	>0.05
Imipenem	2	1.90	0	0.00	2.71	>0.05
Levofloxacin	47	44.76	29	20.57	16.50	<0.05
Amikacin	7	6.67	6	4.26	0.70	>0.05
Cotrimoxazole	80	76.19	67	47.52	20.57	<0.05

DISCUSSION

Multi-drug resistant *K. pneumoniae* has recently emerged as a troublesome nosocomial pathogen worldwide. Positive ESBLs are mainly responsible for drug resistance to β-lactam antibiotics (Liu et al., 2014; Lahlaoui et al., 2014). The rate of detection of ESBLs in *K. pneumoniae* was calculated to be 42.68%; this was lower than the rate observed in Iran (59.20%), and higher than that seen in Beijing (32.21%). However, the rate of detection of CTX-M ESBLs in ESBL-positive *K. pneumoniae* (87.62%) was similar to that seen in Beijing (84.80%), and higher than that in Iran (23.90%) (Feizabadi et al., 2010; Ghafourian et al., 2011; Zou et al., 2011; An et al., 2012). Therefore, we concluded that the production of ESBLs and CTX-M ESBLs differed regionally; this may be attributed to the increasing habit of antibiotic use, or the choice of detection method. Our study identified CTX-M-1 ESBL as the major subtype of CTX-M ESBLs; this was similar to the results reported by Li et al. (2014) in Hubei, China, and Mohamudha Parveen et al. (2012) in the south of India.

In this study, we discovered that the *CTX-M* genes in 57 and 6 strains of CTX-M-1 and CTX-M-25 ESBL-producing *K. pneumoniae* coexisted in the plasmid and chromosome DNA. The *CTX-M-1 ESBL* genes present in both plasmids and chromosomes propagated with greater ease than those present only in the plasmid or chromosome; this was because the *CTX-M ESBL* genes coexisting in the plasmids and chromosomes could spread in the plasmid or chromosome through the function of ISEcpI (Chouchani et al., 2012). The coexistence of *CTX-M-1 ESBL* genes in the plasmid and chromosome may be attributed to the widespread use of antibiotics in clinics, and the frequent use of antibiotic stock farming; cumulatively, this has resulted in the increase in drug-resistant genes in bacteria.

CTX-M ESBL-positive *K. pneumoniae* showed higher drug resistance to β-lactam antibiotics, compared to CTX-M ESBL-negative bacteria ($P < 0.05$), which suggested a significant association between the drug resistance to β-lactam antibiotics and CTX-M ESBLs. However, the rate of resistance to cefepime and cefoperazone/sulbactam was not significantly different between CTX-M-positive and -negative strains ($P > 0.05$); this may be because fourth-generation cephalosporins are generally more stable under the effect of β-lactamase. In addition, CTX-M ESBL activity could be restrained by the activity of a β-lactamase inhibitor (Perez et al., 2007). Further studies are required to confirm these results.

In conclusion, the *CTX-M ESBL* genes were commonly observed in *K. pneumoniae* isolated from the respiratory tract; these were significantly associated with the increased resistance of *K. pneumoniae* to β-lactam antibiotics. Therefore, both phenotypic and genotypic methods are required to detect the presence of CTX-M ESBL production in *K. pneumoniae* isolated from the respiratory tract.

Conflicts of interest

The authors declare no conflict of interest.

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